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Mechanical forces and tumor cells: insight into the biophysical aspects of cancer progression

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MECHANICAL FORCES AND TUMOR CELLS: INSIGHT INTO THE BIOPHYSICAL ASPECTS OF CANCER PROGRESSION

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DEDICATION

I would like to dedicate this dissertation to my parents and beloved wife Payal Indra who have always stood by me in each and every aspects of my personal and professional life. I would also like to dedicate this work to my graduate advisor Dr. Karen A. Beningo who has been my guide and philosopher in every step of my graduate career at Wayne State University.

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CHAPTER 1

INTRODUCTION

Mechanical forces play a vital role in controlling several cellular and physiological processes. (Vial *et al.*, 2003; Czirok *et al.*, 2004; Ewald *et al.*, 2008; Gieni and Hendzel, 2008). For example, the migration of cultured cells have been shown to be guided by the strength of adhesion between integrins and extracellular ligands, the amount of traction stress generated by the cell onto the substrate (Munevar *et al.*, 2001), and the rigidity of the substrate (Lo *et al.*, 2000b). Similarly, during tissue repair the isometric tension generated by contractile myofibroblast cells helps to close the wound edges (Desmouliere *et al.*, 2005). Likewise, the stiffening of a tumor mass due to extracellular protein deposition is predicted to influence cancer progression (Desmouliere *et al.*, 2005). These examples suggest that mechanical factors are as important as biochemical factors to the regulation of cellular and tissue behavior. Likewise, any changes in mechanical factors could result in disease progression such as, tumor formation and metastasis.

Cellular changes in biochemical, genetic and biological properties have been well documented for tumor cell invasion and metastasis. In contrast, changes in mechanical properties of tumor cells and their substrate during metastatic progression are currently ill defined. To understand the role of mechanical forces in the process of metastasis new conceptual approaches are necessary pertaining to tumor cell dissemination.

Cancer can be defined as uncontrolled proliferation, invasion and inappropriate migration of cells and is recognized as the leading cause of death worldwide (Hanahan and Weinberg, 2000). When tumor cells break through the basement membrane and

invade the surrounding stromal tissues, metastasis is defined. The metastatic cascade is a sequential process in which cells from the primary tumor invade the neighboring tissues, travel through the blood stream and colonize in distant tissues (Liotta *et al.*, 1991; Felding-Habermann *et al.*, 2001; McDonald and Baluk, 2002). To successfully migrate and colonize, the cancer cells must adhere to the surrounding matrix in the stromal compartment and subsequently within a distant tissue, where they will form a secondary tumor. Foreign tissue microenvironments may differ in extracellular matrix (ECM) rigidity and tissue rheology from the primary tumor tissue (Paszek *et al.*, 2005). In order to colonize in the new microenvironment, cancer cells must adapt themselves to respond not only to different biochemical signals, but also to changes in the biophysical cues of the environment (Joyce and Pollard, 2009; Kumar and Weaver, 2009a). Indeed, a tumor cell makes several adaptations in response to biophysical cues including the loss of cell-cell adhesion, alteration of integrin expression, rearrangement of cytoskeleton structure, and increased mobility (Guo and Giancotti, 2004b; Bissell *et al.*, 2005).

Epithelial to Mesenchymal Transition

Epithelial to Mesenchymal Transition (EMT) plays a vital role in tissue morphogenesis, body patterning, wound healing and development (Martin and Parkhurst, 2004; Ewald *et al.*, 2008). EMT is described by partial or complete loss of epithelial cell-type characteristics and gain of mesenchymal cell-like properties. At the cellular level the process of EMT results in the loss of apico-basal polarity and disassembly of adherens junctions of epithelial cells (Peinado *et al.*, 2004; Townsend *et al.*, 2008). These changes are due to the replacement of E-cadherin with N-cadherin

and intermediate filaments with vimentin and are considered as the molecular hall marks of EMT (Ikenouchi *et al.*, 2003). Together these changes alter the epithelial cytoskeleton structure and transition the cell to the spindle shaped mesenchymal phenotype (Micalizzi *et al.*, 2010). During this transition process, epithelial cells are also known to acquire highly invasive and migratory properties (Guarino *et al.*, 2007). A large number of studies suggest that this epithelial plasticity is also involved in pathological conditions including fibrosis and tumor cell metastasis (Lopez-Novoa and Nieto, 2009). Nonetheless, despite extensive reports in the literature, the importance of EMT to cancer metastasis remains controversial, basically due to the lack of pathological evidence at the site of the secondary tumor.

The Role of Mechanical Forces in Tissue Integrity and Disease Progression

Individual cells in multicellular organisms are subjected to different types of forces including tensile and fluid shear stresses. In response to extracellular forces, the cell exerts reciprocal forces through a phenomenon referred to as mechanoreciprocity (DuFort *et al.*, 2011). At least two kinds of forces are produced by the cell at the cell-substrate interface. The first are actin-myosin generated contractile forces, known as traction force (Lauffenburger and Horwitz, 1996; Sheetz *et al.*, 1998). Traction forces are produced by the cell during the process of active migration. The second cell-substrate force is generated by the cytoskeleton which helps to maintain tensional homeostasis (Butcher *et al.*, 2009). A proper balance between extracellular and cell generated forces are necessary to maintain adult tissue homeostasis. For example, the growth of skeletal muscle tissue depends on mechanical loading (Bird *et al.*, 2000), similarly shear stress of blood flow maintains the health of vascular tissues (Takahashi

et al., 1997). However, each tissue has its unique characteristic range of mechanical stiffness which changes to an optimum level over time either during development or during a change of function (Butcher *et al.*, 2009). In addition to these naturally occurring changes, drastic changes or loss of tissue homeostasis occurs during the onset of disease as seen in fibrosis or tumor formation (Paszek *et al.*, 2005; Wells, 2005).

Tumor growth is associated with excessive ECM deposition and thereby palpable stiffening of the tissue (Garra, 2007). Tumor tissue also encounters excessive compressive stress due to uncontrolled cell proliferation which leads to increase in interstitial pressure (Paszek and Weaver, 2004b). Disruptions of tensional homeostasis in the tumor microenvironment is predicted to influence cellular biophysical properties including cellular morphology, cell motility, cytoskeleton organization, traction force production and cell-substrate adhesion (Paszek *et al.*, 2005). Moreover, it has been shown that malignant transformation of normal fibroblasts are associated with changes in traction force production (Munevar *et al.*, 2001).

Cellular Mechanotransduction Machinery

The mechanotransduction machinery of cells includes mechanosensors and force generating molecules that enable cells to counteract forces in the *in-vivo* environment. For example, calcium gated ion channels (Parker and Ingber, 2007), cadherin complexes (Tzima *et al.*, 2005) and transmembrane protein integrin (van der Flier and Sonnenberg, 2001; Helmke *et al.*, 2003) have been shown to act as cellular mechanosensors whereas the actin cytoskeleton is known to be the cellular force producing tool (Sanger *et al.*, 1983; Bereiter-Hahn, 2005). When mechanical cues are

received at the cell-cell or cell-substrate interface the signal is transmitted to the cell interior by sequential activation of signaling molecules a process known as outside-in mechanotransduction (Qin *et al.*, 2004). At the cell-substrate interface the outside-in mechanotransduction pathway is initiated when specialized anchoring junctions, known as focal complexes are established (Partridge and Marcantonio, 2006a). The assembly of a focal complex begins with the formation of adhesive contact between the extracellular ligand and the integrin (Figure 1.1). Integrin-ligand interaction leads to the conformational changes in the integrin resulting in activation of the molecule (Campbell and Humphries, 2011). The active form of integrin favors integrin clustering, recruits a large number of signaling molecules such as, talin, vinculin, src and focal adhesion kinases (FAK) (Margadant *et al.*, 2011) and establishes a link between the actin cytoskeleton and the extracellular ligand (Paszek *et al.*, 2009). Once the cytoskeleton link is established, actomyosin contractility at the adhesion junction promotes the formation of focal adhesions, increased tyrosine phosphorylation of focal adhesion kinase and interactions between multiple signaling complexes to promote growth, migration and differentiation (Miranti and Brugge, 2002; Berrier and Yamada, 2007). Given the importance of outside-in signaling, it is obvious that mechanical changes in extracellular environment lead to changes in focal adhesion parameters and altered mechano-signaling cascades.

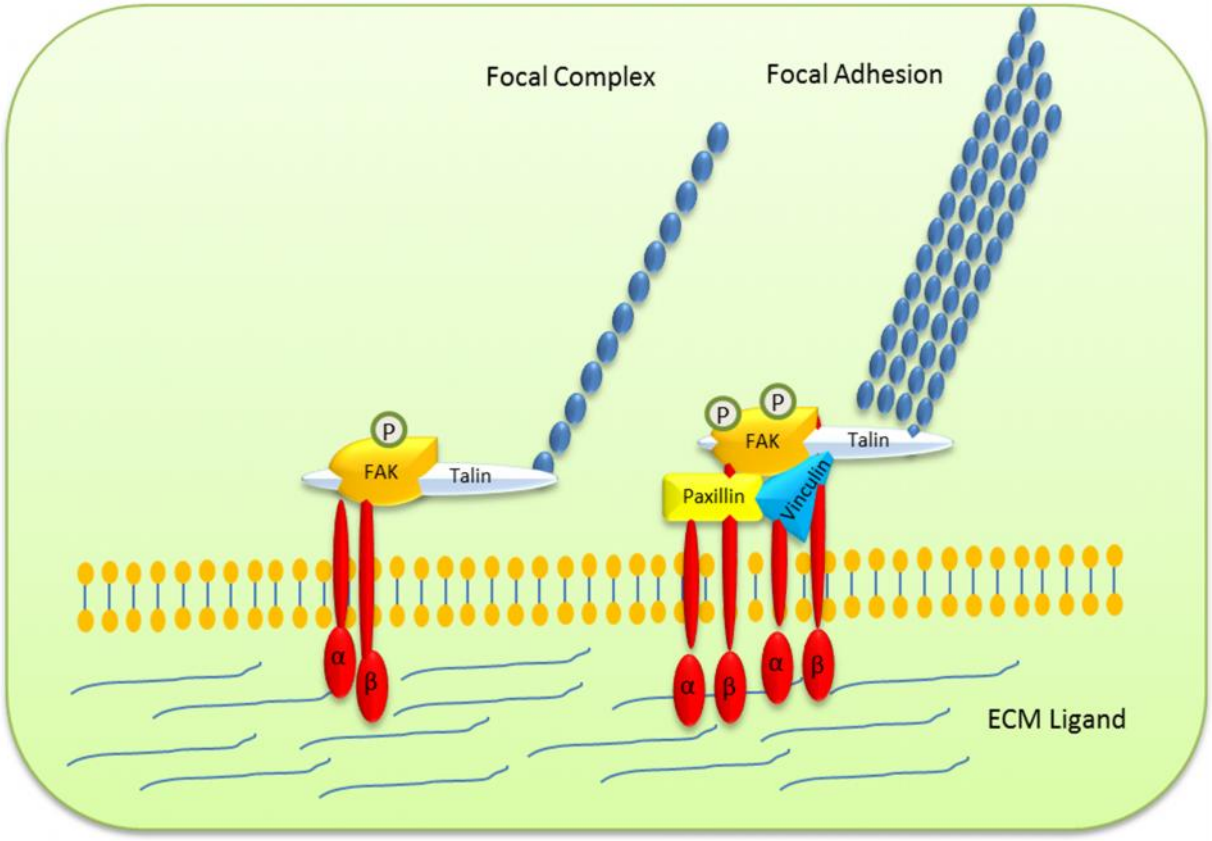

Figure 1.1. *Components of cellular mechanotransduction. Integrin mediated cell adhesion establishes a linkage between ECM and actin cytoskeleton. Contractile force generated by actin cytoskeleton is resisted by the stiffness of the ECM and enable cells to probe the matrix elasticity at the resolution of cell-matrix adhesion. Stiffer matrix produces higher reciprocal forces which facilitates integrin oligomerization, formation and recruitment of focal adhesion proteins, and FAK phosphorylation.*

Integrin in Mechanotransmission

Among the wide array of membrane receptor proteins present at the cell-substrate interface, integrin has been one of the best studied of these transmembrane receptor proteins. Integrins are a large family of heterodimeric glycoproteins composed

of α and β subunits responsible for linking the actin cytoskeleton to the ECM. The integrin family includes at least 18 α and 8 β subunits forming more than 24 different functional receptors (Calderwood, 2004). Many studies suggest that integrins serve as a bi-directional mechanotransducers (Hynes, 1992; Lewis and Schwartz, 1995; Ingber, 2003). Integrins permit the cells to detect the extracellular force at the resolution of individual adhesion sites (Felsenfeld, 2005). Conversely, it transmits the actin-myosin generated traction force back out into the surrounding environment through the focal adhesions (Bershadsky *et al.*, 2003a). In recent years, it was found that integrin αvβ3, acts as a rigidity sensor at the leading edge of a fibroblast (Jiang *et al.*, 2006b). Thus the structure and functions of this important molecule including its affinity to extracellular ligand, activation and clustering are tightly regulated by cell-ECM interaction (Sims *et al.*, 1991; Woodside *et al.*, 2001). The active form of integrin turns on several signaling pathways important for adhesion, migration, proliferation and assembly of extracellular matrix (Huttenlocher *et al.*, 1996; Martin-Bermudo and Brown, 2000; Assoian and Klein, 2008).

Integrins in Cancer

Differential expression of various integrin receptors on cancer cells is thought to be associated with differences in metastatic behavior (Shaw et al., 1999) and malignancy (Friedrichs *et al.*, 1995; Mukhopadhyay *et al.*, 1999). For example, during breast cancer metastasis several integrins such as α6β4, αvβ3 are up-regulated whereas several other integrins such as, α5β1, αvβ1, α1β1, α2β1 and α3β1 have been shown to be down regulated (Bartsch *et al.*, 2003). Based on this observations, it is logical to predict that integrin expression changes with the alteration of cellular and

extracellular forces. Furthermore, given the diversity of mechanical environments they must traverse, it reasonable to predict that as tumor cells become more metastatic, they likely suppress their normal mechanotransduction ability.

Changes in Cell Migration during Metastasis

Migration of a cell is a tightly regulated sequential event that begins with the polymerization of actin cytoskeleton and protrusion of the leading edge. The protruding edge establishes a new adhesion sites to the underlying substrate by integrin and focal adhesion components (Balaban *et al.*, 2001). The newly established adhesion sites not only stabilize the actin filaments but also serve as the site for producing traction stress (Tan *et al.*, 2003). In addition to adhesion at the cell front, cell-ECM engagement disassembles at the rear allowing cell to propel forward (Iwanicki *et al.*, 2008). Thus cell adhesions, dehadhesions and actin generated contractile forces are the critical steps for cell motility (Figure 1.2). Any changes in adhesion or defects in cytoskeleton contractility can lead to abnormal cell motility, and potentially- invasion and metastasis. The higher migratory capacity of cancer cells presumably comes from their ability to use different modes of migration in a context dependent manner (Poincloux *et al.*, 2011). For example, a number of studies elucidated that tumor cells follow the mesenchymal mode of adhesion dependent migration for invading the surrounding tissues (Sahai and Marshall, 2003). However, other studies showed that tumor cells follow less adhesive, amoeboid mode of migration (Wolf *et al.*, 2003). From these, it is imperative that the major cause of cancer related death results from altered cell adhesion and motility.

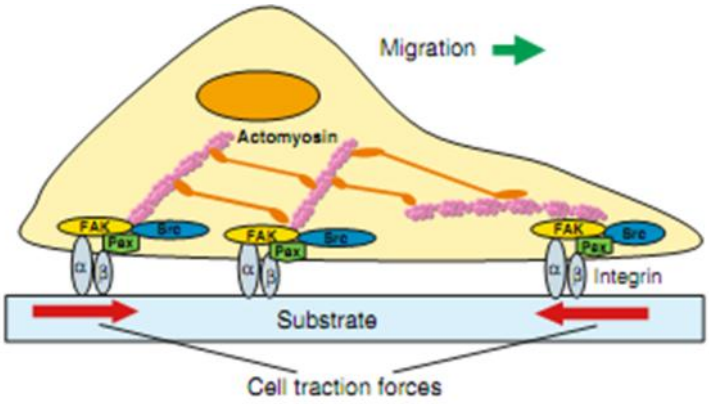

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Figure 1.2. *Illustration of cell migration. Actin polymerization results in extension of the leading edge and the establishment a contact with the underlying matrix. Cytoskeleton generated traction stress at the site of adhesion drags the cell body forward while release of cell-substrate contact at the rear causes the forward locomotion of cell.*

Cellular Response to Changes in Extracellular Compliance and Composition

The extracellular matrix is a complex mixture of proteins with a large variety of biochemical and biophysical properties. It has been shown that a number of cellular activities including migration, proliferation and apoptosis are guided by this insoluble protein component (Boudreau and Jones, 1999). Based on the structural localization the extracellular matrix can be divided into two broad categories - basement membrane and interstitial matrix. As the name suggests the basement membrane is found under the basal side of polarized epithelial cells and separates the epithelial cells from connective tissue enriched stromal environment. This thin layer of ECM proteins is primarily composed of laminin and type IV collagen. The second category, interstitial matrix, primarily consists of type I collagen and is present within the epithelial cells to provide

mechanical stability (Guo and Giancotti, 2004b). Each of these categories of ECM has its unique characteristics of protein composition and biophysical properties. These properties are not static and can change dramatically through remodeling steps during wound healing and development. Structural changes in the ECM include degradation, assembly and deposition which lead to changes in rigidity and ligand concentration (Butcher *et al.*, 2009) ultimately affecting cellular behavior. Cells are known to respond to changes in substrate stiffness by migrating towards a more rigid location, this is a phenomenon known as durotaxis (Lo *et al.*, 2000b). Due to mechanoreciprocity, the magnitude of traction forces is also dependent on rigidity. For example, on stiffer substrates cells generate more traction force and develop a flatter morphology than they do on softer substrate (Lo *et al.*, 2000b). However, this compliance dependent response is known to be cell type specific and cells are thought to prefer their host tissue rigidity (Georges and Janmey, 2005). For example, spinal cord derived motor neurons that have originated from softer tissue microenvironments extend neuritis only on softer substrate (Flanagan *et al.*, 2002). In contrast smooth muscle cells and fibroblasts exist within relatively rigid environments and thus extend processes more avidly on hard substrate (Engler *et al.*, 2004b; Yeung *et al.*, 2005a). However, extracellular stiffness also changes during the progression of disease. During tumor progression for example, the rigidity changes within a tumor and within its stroma (Paszek and Weaver, 2004b). However, how these changes affect metastatic cancer cells and multiple stages of the metastatic cascade is poorly understood.

In addition to rigidity, cellular response has been shown to be modulated by the combination integrin-ligand interaction (Chan *et al.*, 1992). Several studies showed that

both extracellular ligands and their receptor integrin exhibit redundancy in their specificity for binding with each other. For example integrin αvβ3 shows strong affinity for both vitronectin and fibronectin. Similarly fibronectin can bind with multiple numbers of integrin receptors including α5β1, α3β1 and αvβ3. (Boudreau and Jones, 1999). This overlapping integrin-ligand interaction suggests that each combination of integrin-ligand adhesion has specialized function (Boudreau and Jones, 1999). Because integrin expression is known to be modulated during development and disease, it is necessary to understand the particular combination of integrin-ligand interaction involved in sensing the mechanical rigidity and how that interaction changes with metastatic progression.

Cellular Response to Localized Stimulation

In addition to sensing changes in rigidity, a second type of mechanical signal could result from pulling on ECM fibers by highly contractile neighboring cells (Menon and Beningo, 2011). Almost every cell of our body encounters this localized pulling force during a functional change and during development. For example, the contractile forces generated by myofibroblast cells help to bring the wound edges together during tissue repair (Hinz, 2007). Another example is, in the mammary gland, localized contractile forces generated by myoepithelial cells play a critical role during lactation (Raymond *et al.*, 2011). Furthermore, mammary gland associated stroma is enriched with highly contractile cells of fibroblastic origin (Kunz-Schughart and Knuechel, 2002; Schor *et al.*, 2003). Stromal fibroblasts are known to modulate the ECM architecture by matrix remodeling and contraction during mammary gland development and tumor formation (Kammertoens *et al.*, 2005; Shieh *et al.*, 2011). Not surprisingly, tumor

formation simulates many of same responses seen in wound healing (Kalluri and Zeisberg, 2006). Despite the knowledge of stromal interaction in mammary gland morphogenesis and tumor development the mechanical role of these stromal cells is poorly defined. The role of these contractile cells in modifying the ECM architecture (Paszek and Weaver, 2004b), secretion of growth factors (Powell *et al.*, 1999) and prevention of immune response to cancer cells (Lieubeau *et al.*, 1999) have already been studied, however the influence of tugging and pulling produced by these cells has been overlooked. Because cells within in-vivo environment are likely to encounter dynamic environmental rigidity and localized applied stimulation, it is important to understand the cellular response when both the mechanical forces are present within the cellular microenvironment.

CHAPTER 2

AN *IN-VITRO* CORRELATION OF MECHANICAL FORCES AND METASTATIC CAPACITY

This chapter has been published

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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 of 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.*, 2003a; Li and Wang, 2009). 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 behaviors (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 have done so far to show how traction stress change 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 is unknown .

Inappropriate migration of a tumor cell from the primary tumor is an early step in the process of metastasis. Subsequent cascade of events include, invasion of tumor cells into the stroma, intravasion 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 decrease 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 adhesion and the level of active beta-1 integrin as the metastatic capacity increased. We interpreted this as a gradual shift towards 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 con-current 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 was purchased from ATCC. Cultures were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Hyclone, UT, USA), and supplemented with100 U/ml penicillin, 2 mM L-glutamine, and 100µg/ml streptomycin. Cells were grown in a standard culture incubator at 37°C with 5% CO_2. Adhesion assays and Traction Force Microscopy (TFM) were performed as described previously (Beningo *et al.*, 2002a; Undyala *et al.*, 2008). The hydrogels were covalently coated with 5 µg/cm^2 bovine plasma fibronectin (Sigma, Saint Louis, USA). A concentration of 5% acrylamide, and 0.08% *N,N*methylene-bis-acrylamide were used to prepare the substrates for this study.

Microscopy

An Olympus IX81 ZDC inverted microscope was used to acquire both phase contrast and fluorescence images. The objective lenses used for these studies were the 10X/0.25 NA CP-Achromat lens for phase contrast images and 40X/0.75 NA Plan-Neofluar lens for fluorescence images. Temperature control and CO_2 were maintained by a custom built stage incubator when live cell imaging was performed.

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), coated with extracellular matrix protein fibronectin for cell adhesion and embedded with fluorescent microbeads for tracking the deformation on the substrate. The elasticity of the substrate was determined to be $2.4X10^4$ N/m^2 by indentation and the Hertz equation as described (Lo *et al.*, 2000b). Prior to taking the traction force measurements, cells were cultured for 24 hours on the substrates. Stressed and null images were obtained by first imaging the beads in the presence of the cell, followed by removal of the cell with a microneedle. Analysis of the data was carried out using LIBtrc custom software to calculate the average integrated traction stress as described (Dembo and Wang, 1999; Marganski *et al.*, 2003).

Cell Adhesion Assay

A centrifugation assay was performed to measure the strength of cell-substrate adhesion as described earlier with slight modification (Guo *et al.*, 2006a). Briefly, approximately $2.5x10^4$ cells were seeded on to fibronectin-coated polyacrylamide

substrate (5% acrylamide and 0.08% bis-acrylamide) in specifically designed chambers. The cells were then allowed to adhere for 30 minutes at 37°C. The chambers were centrifuged in a Beckman (Fullerton, CA) TJ-6 centrifuge at 1800xg for 5 minutes. 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 for each cell type.

Cell Migration Assays

The migrations of breast cancer and control cell lines were monitored by time lapse video microscopy with 40X 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 37ºC. 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, $1x10^5$cells/ml were suspended in buffered liquid collagen type-I at a final concentration of 1.65mg/ml (pH 7.4; 3mg/ml; Pure Col, Advanced Biomatrix, USA). Self-constructed glass chambers were filled with 400µl of suspension, sealed and incubated at 37ºC. 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, $1x10^6$ cell were suspended in 350µl of DME (25mM HEPES) and mixed with 200µl platelet poor human plasma collected in sodium Citrate (Innovative Research, Novi, MI, USA). The suspension was placed into 7.25x55mm glass tubes (Bio Data corporation, USA). 200µl of HEPES DMEM

containing 28mM $CaCl_2$ and 5U/ml human thrombin (Innovative Research, Novi, MI, USA) was then added to the tube, mixed and incubated at 37ºC for 3hrs 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 (microns/minutes) and persistence (minutes) were calculated by quantifying the centroid displacement every 2 minutes over a 120 minute period. Migration speed is shown as +/- 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 (5µg/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 200µl pipette tip to create a uniform, cell free area with a width of approximately 1mm. Cell debris was removed by washing with fresh 37ºC culture medium and incubated for 30 minutes at 37ºC. Wound closure was monitored by time lapse microscopy for a period of 24 hours at 20 minutes intervals. At least three different wounds for each cell line were monitored. The percentage of the wound filled in micrometers was determined using Image J software (NIH) as the average distance from the wound origin covered by cells 24 hours after wounding.

Immunofluorescence

Cells adhered to no.1 coverglass coated with fibronectin (5μg/cm^2) were washed with warm cytoskeleton buffer (137mM NaCl, 5mM KCl, 1.1 mM Na2HPO4, 0.4mM KH2PO4, 2mM MgCl2, 2mM EGTA, 5mM PIPES and 5.5 mM glucose, pH 6.1) and fixed for immunofluorescence in 4% paraformaldehyde and 0.1% Triton X-100 for 10 minutes, followed by blocking 1 hour with 5% Bovine serum albumin (BSA). Fixed cells were incubated with 1:50 Alexa Flour 546 phalloidin (Invitrogen, CA) for 1 hour, 1: 1000 anti-paxilin antibody (Clone 349, Chemicon International, USA) for 4 hours and 1:1000 Alexa Fluor 488 anti-mouse secondary (Invitrogen, CA) for 1 hour. All incubations with antibody 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-localized with actin fibers at the leading edge were 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, San Jose, CA, USA) or 1:1000 anti-N-Cadherin antibody (BD Biosciences, San Jose, CA, USA) or 1:200 anti-fibronectin antibody (Abcam Inc, Cambridge, MA, USA) overnight at 4°C followed by 1hour incubation with Alexa Fluor 488/546 anti-mouse secondary (Invitrogen, CA). Collected images were pseudo-colored and overlaid to identify areas of co-localization where necessary.

Western Blot Analysis

Cells cultured to 70% confluency on standard 100mm culture plates were rinsed with ice cold 1X Phosphate Buffered Saline (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 manufacturer's instructions. Samples (50 μg of protein, unless otherwise specified) were subjected to SDS-PAGE on 10% mini gels and transblotted onto PVDF membrane (Millipore). The membrane was blocked in 5% Non-fat Dry milk, 0.1% Tween-20 in PBS for 2 hours at room temperature before exposure to the primary antibody (except for vimentin which was blocked in 0.1% Tween-20 in Tris Buffered Saline (TBS)). Antibody source and concentration were as follows [1: 4000 mouse monoclonal E-Cadherin (Clone 36, BD Biosciences, San Jose, CA, USA), 1:5000 mouse monoclonal N-Cadherin (Clone 32, BD Biosciences, San Jose, CA, USA), 1:300 mouse monoclonal fibronectin (Clone IST-9, Abcam Inc, MA, USA), 1:2500 mouse monoclonal vimentin (Clone RV202, BD Pharmingen, San Jose, 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 vimentin antibody was performed for 1 hour 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 [BD Biosciences, San Jose, 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).

To determine the level of expression of active β1 integrin, polystyrene cell culture plates were coated with either, 5μg/cm^2, 0.05μg/cm^2 or no fibronectin (Sigma, USA) and incubated overnight at 4°C. The plates were rinsed with sterile 1X PBS, blocked with 1% bovine serum albumin (BSA) and incubated overnight at 4°C. After blocking the plates were rinsed with sterile 1X PBS and cells were seeded, harvested and lysed as described above. Blots were probed with 1:500 rat anti-mouse monoclonal active β1-integrin antibody (Clone 9EG7, BD Pharmingen, San Jose, 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 Aslaskon and Miller (1992) and summarized in *Figure 2.1*. Briefly, the first cell line (67NR) is non-metastatic and forms only primary tumors, the second (168FARN) can invade and enter the circulation, but cannot re-colonize; the third (4T07) completes all steps of the metastatic cascade, but only forms micro-metastases, 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).

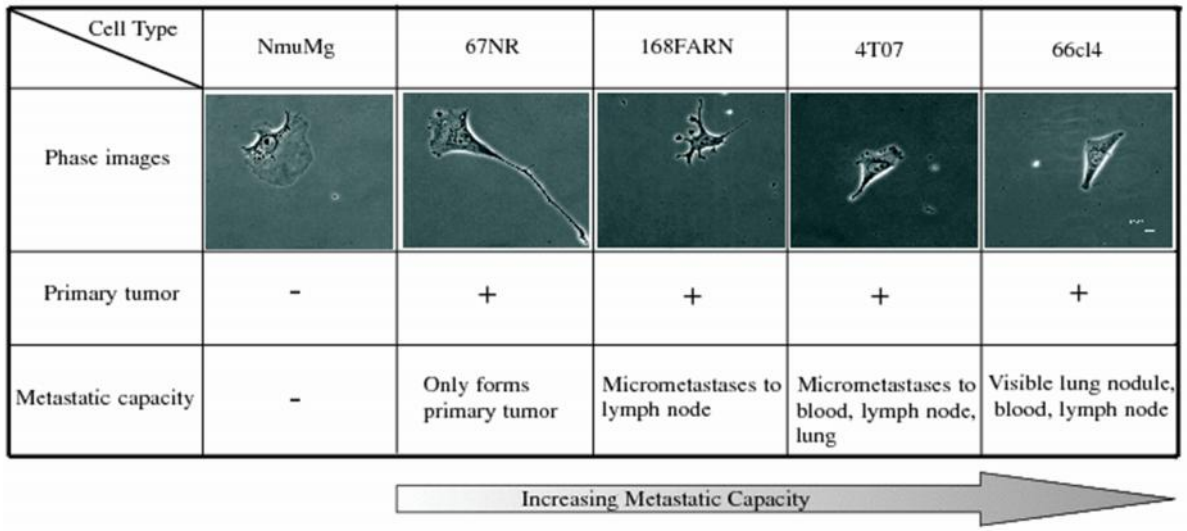


Figure 2.1. *Characterization of the murine breast cancer cell lines. Phase images of the normal mammary gland cell line and four mice breast cancer cell lines, along with their metastatic potential and capacity to form the primary tumor when injected into mice.*

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 they initially derive from the same genetic background (Dexter *et al.*, 1978; Aslakson and Miller, 1992; Giancotti and Ruoslahti, 1999; 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 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 immuno-fluorescence the epithelial marker E-cadherin was expressed only at the cell-cell junction of normal murine mammary gland cells (Figure 1A). Surprisingly, the expression of the mesenchymal marker N-Cadherin, was observed only in 67NR and 66cl4 (Figure 2.2(A)). Two intermediate cell lines, 168FARN and 4T07, did not express either of these two proteins (Figure 2.2(A)). These observations were further confirmed by western blot (Figure 2.2(B)).

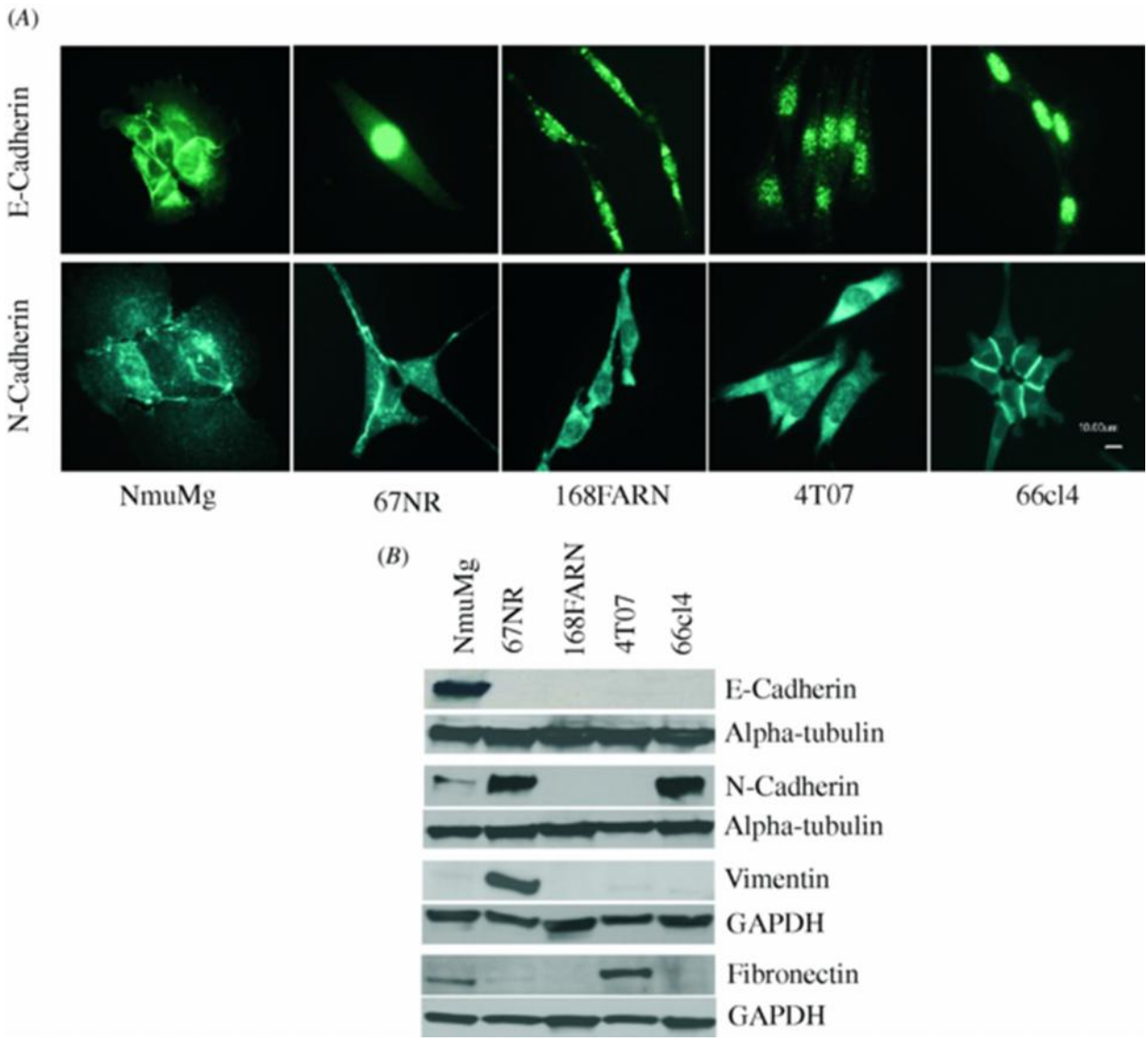


Figure 2.2. *The epithelial to mesenchymal transition is incomplete in the panel of breast cancer cells. (A) Immunofluorescent images of E-cadherin and N-cadherin at the cell–cell junction of Nmu Mg, 67NR and 66cl4. (B) Whole cell lysates of NmuMg (lane 1), 67NR (lane 2), 168FARN (lane 3), 4T07 (lane 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).

The expression 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 2.2(B)). On the other hand, fibronectin was strongly expressed by 4T07 (Figure 2.2(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 2.2(B)). Our results, consistent with recent observation, show that the EM transition is incomplete in all cells of the panel and suggests 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 verses normal cells (Munevar *et al.*, 2001; Ghosh *et al.*, 2008). However, to our knowledge, this relationship has not been defined with regard to 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 2.3(A)). Furthermore, as a general observation, the spatial organization of the stress vectors were highly disorganized in 66cl4 and forces were not concentrated at the most leading edge as with control cells (Figure 2.3(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 towards 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 minutes before being subjected to centrifugal forces of 1800 x g for 5 minutes (Figure 2.3(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 this reduced adhesion in the most metastatic cells results in lower traction stress.

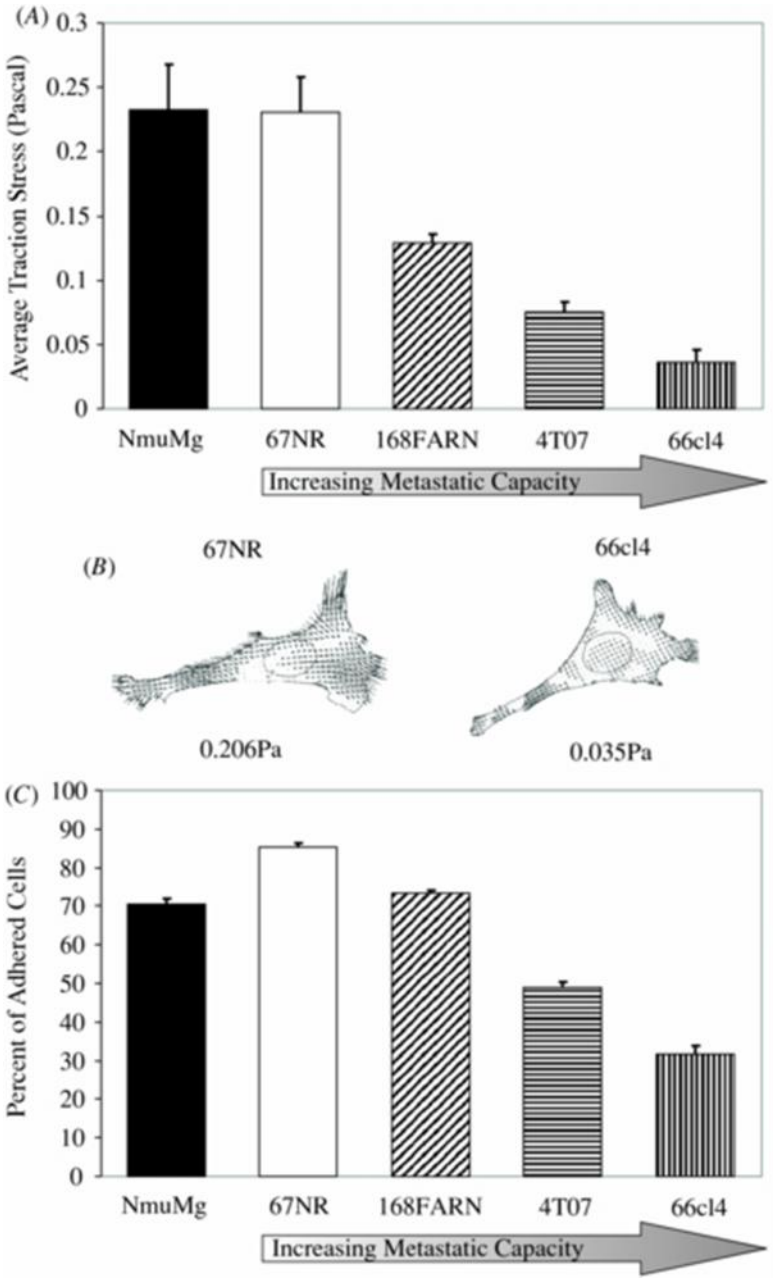


Figure 2.3. *The productions of stress in two-dimension and adhesion strength are inversely related to the metastatic capacity. (A) Average integrated traction stress (Pascal) produced by the normal murine mammary gland cell line and the panel of mice breast cancer cell lines on a polyacrylamide substrate coated with 5 μg* 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 ± s.e.m. from three separate experiments. The data is expressed as a percentage of control as defined by a wild-type mammary epithelial cell.

The Number of Focal Adhesions Decrease with Increasing Metastatic Capacity

Efficient migration and traction result from cell appropriate adhesion and contraction. 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 2.4(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 (Figure 2.4(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 per cell in the most metastatic line (Figure 2.4(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.

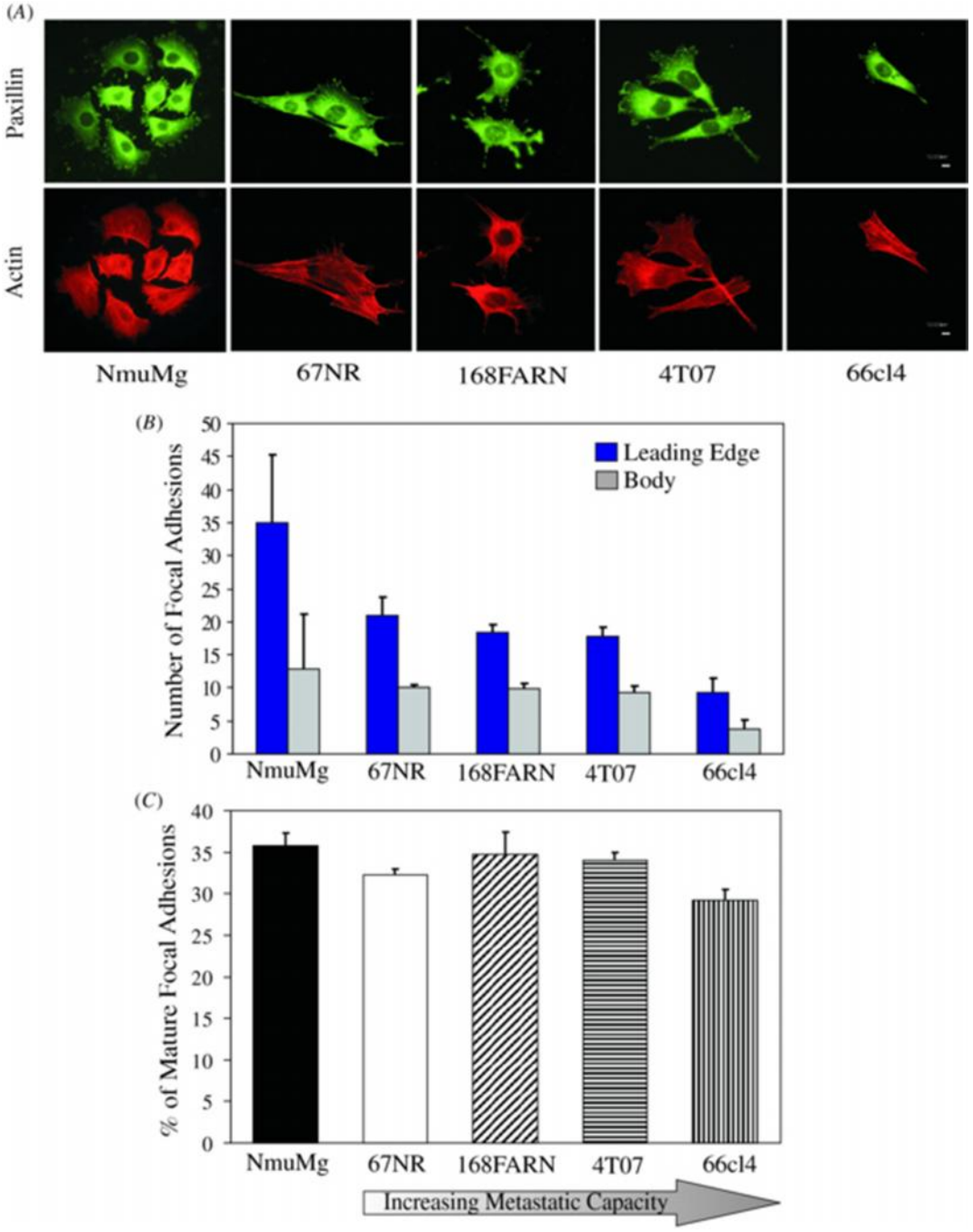


Figure 2.4*. Focal adhesion number, but not their maturation, is decreased during metastatic progression. (A) Immunofluorescence analysis for a focal adhesion protein*

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 total number of paxillin-containing adhesions relative to those co-localized with actin stress fibers at the leading edge and inside the cell body. (C) The mean ± s.e.m. of the number of mature focal adhesions is represented in each bar, expressed as a percentage calculated from the number of focal adhesions at the leading edge and inside the cell body (n = 15 cells).

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.*, 2004a). 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 (0.05, 0.26 and 5 μg/cm^2), the higher being the concentration used for the TFM and adhesion studies (Figure 2.5(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 2.5(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.

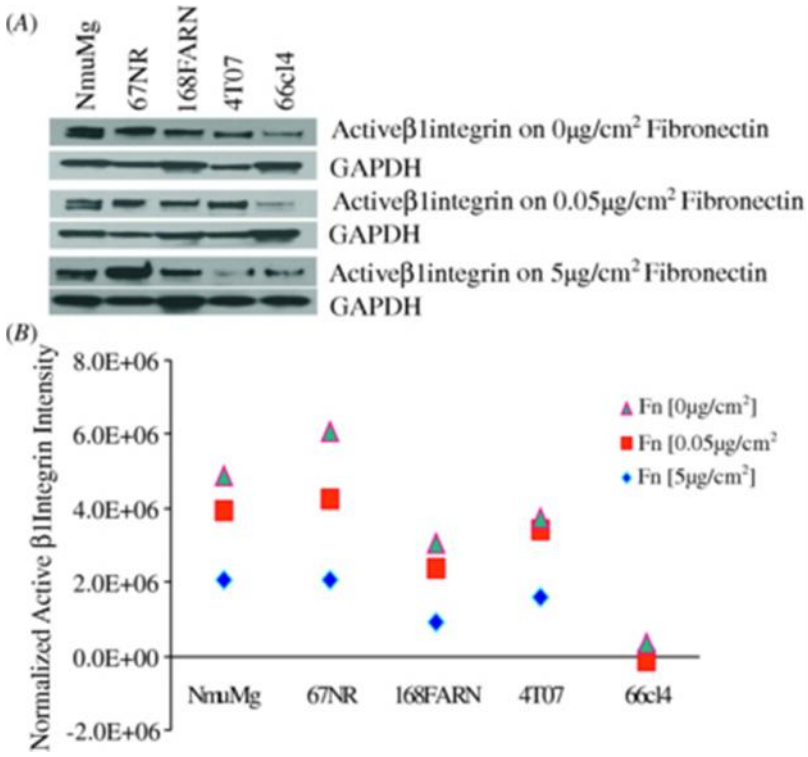


Figure 2.5*. Active β1 integrin at various concentrations of fibronectin. (A) Active β1 integrin was detected by western blot from lysates of cells cultured on 0.05 μg cm^{-2}, 5 μg cm^{-2} fibronectin or without fibronectin. Blots were probed with an antibody against mouse active β1 integrin. The level of GAPDH served as a load control. (B) Line graph represents the normalized intensity of active β1 integrin bands (Y-axis) and cell type (X-axis).*

Collective Migration Decreases with Metastatic Ability

During migration cells move not only as single entities, but 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 hour period (Figure 2.6(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 2.6(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 2.6(B)), 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 hours.

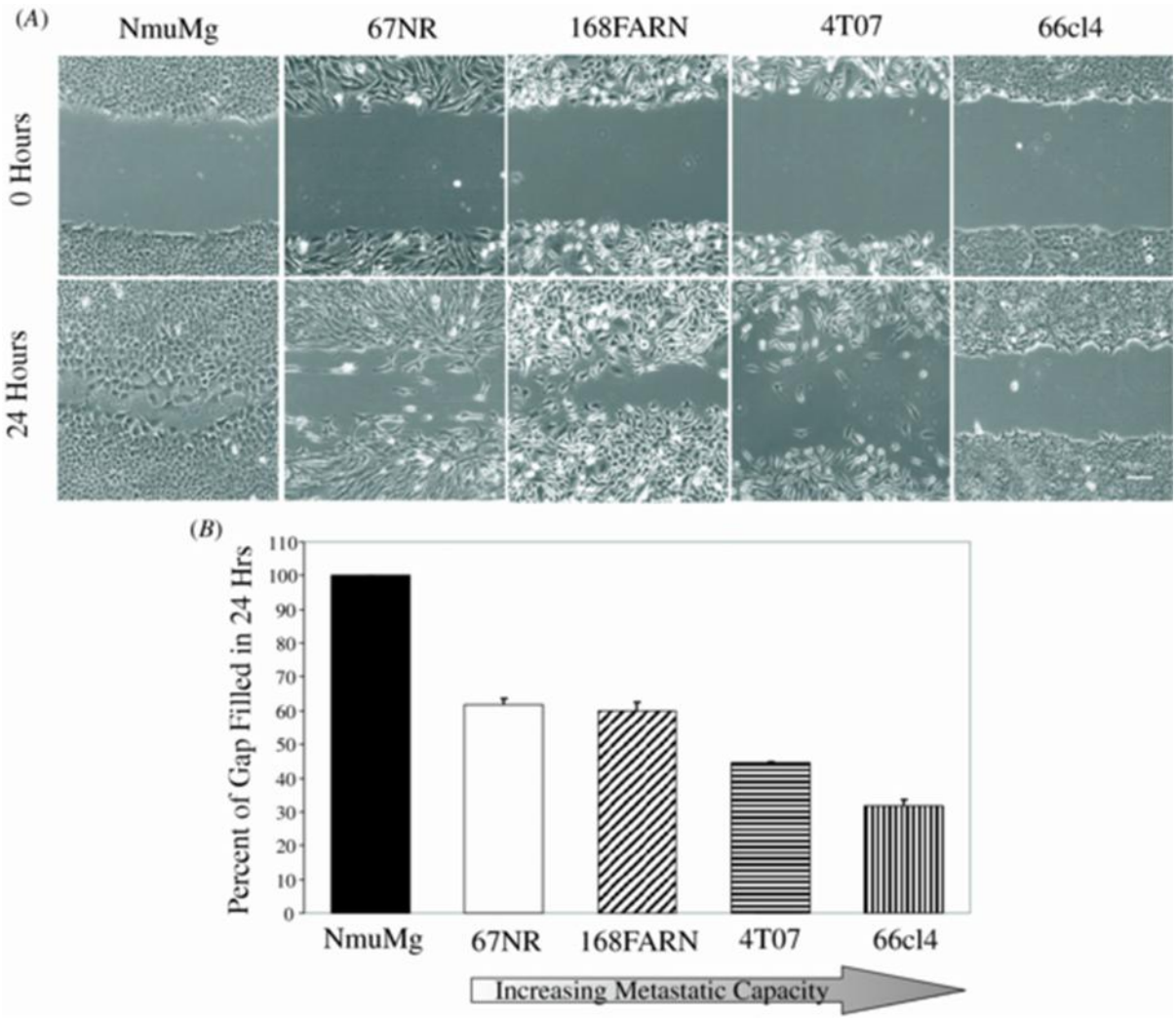


Figure 2.6*. The rate of collective migration decreases with increasing metastatic capacity. (A) Motility of a confluent monolayer of mouse breast cancer cell lines in in-vitro wound healing assay. Wound closure was monitored by phase contrast microscopy at the indicated time points. Original magnification, 10×. Scale bar = 100 μm. (B) Percent of the wound gap filled was determined as the distance from the wound origin, in μm, covered by cells in 24 h following initiation of the wound. Three independent experiments are displayed in each bar, representing their mean value± s.e.m.*

Single Cell Migration Rates Decrease with Metastatic Capacity in 2-D but Increase in 3-D

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-dimensions (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 coverslips 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 microns/minute to 23 microns/minute as the metastatic capacity increased, a change of 45% (Figure 2.7(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 2.7(B); see movies 6-9, available at 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 (Figure 2.7 (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 microns/minute 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 3-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 microns/minute to 50 microns/minute, while the change from two-dimensional collagen to three-dimensional collagen jumped from 30 microns/minute to 50 microns/minute, respectively.

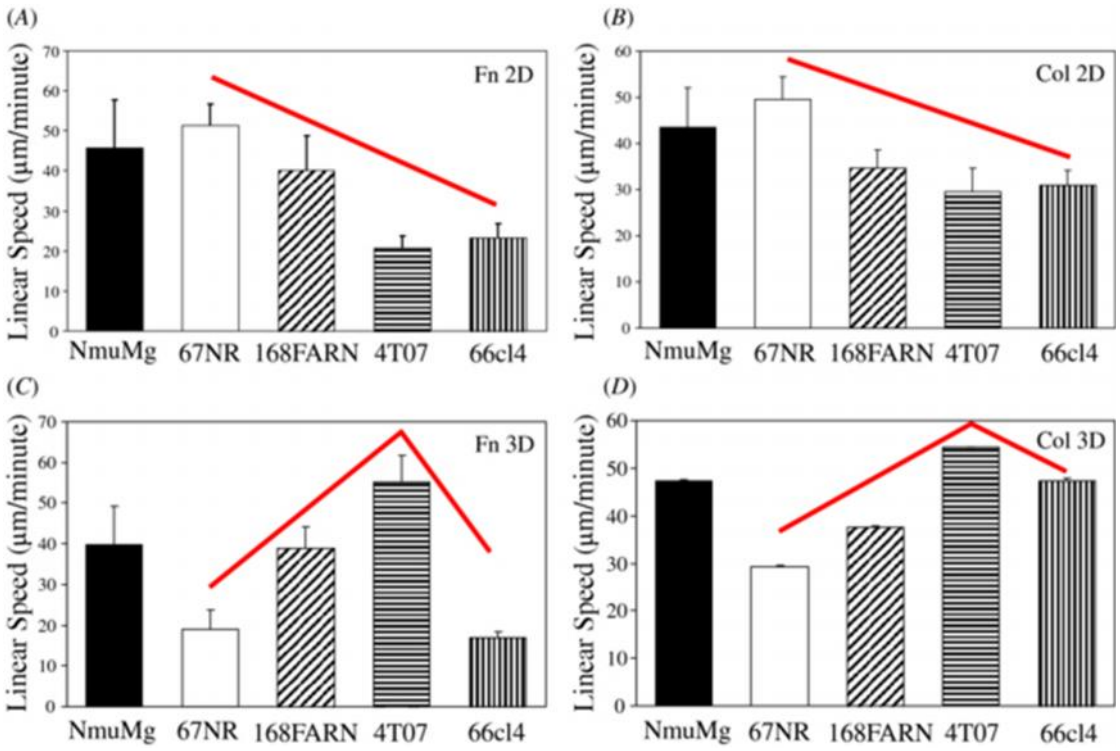


***Figure 2.7**. Highly metastatic cells migrate efficiently in three dimensions but not in two dimensions. Linear migration speed of the panel of murine breast cancer cell lines on two-dimensional (A) fibronectin- and (B) collagen-coated coverslips and in three dimensions within (C) fibrin–fibronectin clot and (D) collagen gel using time-lapse video microscopy. The average rate of migration expressed in µm min^{-1} for a period of 2 h. The mean value ± s.e.m. from ten sets of images is represented by each bar.*

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 2.8). However, a tremendous reduction in persistence was observed in all cells in

three-dimensional culture systems, producing a 4 to 5 fold decrease and no evidence of ECM selectivity (Figure 2.8).

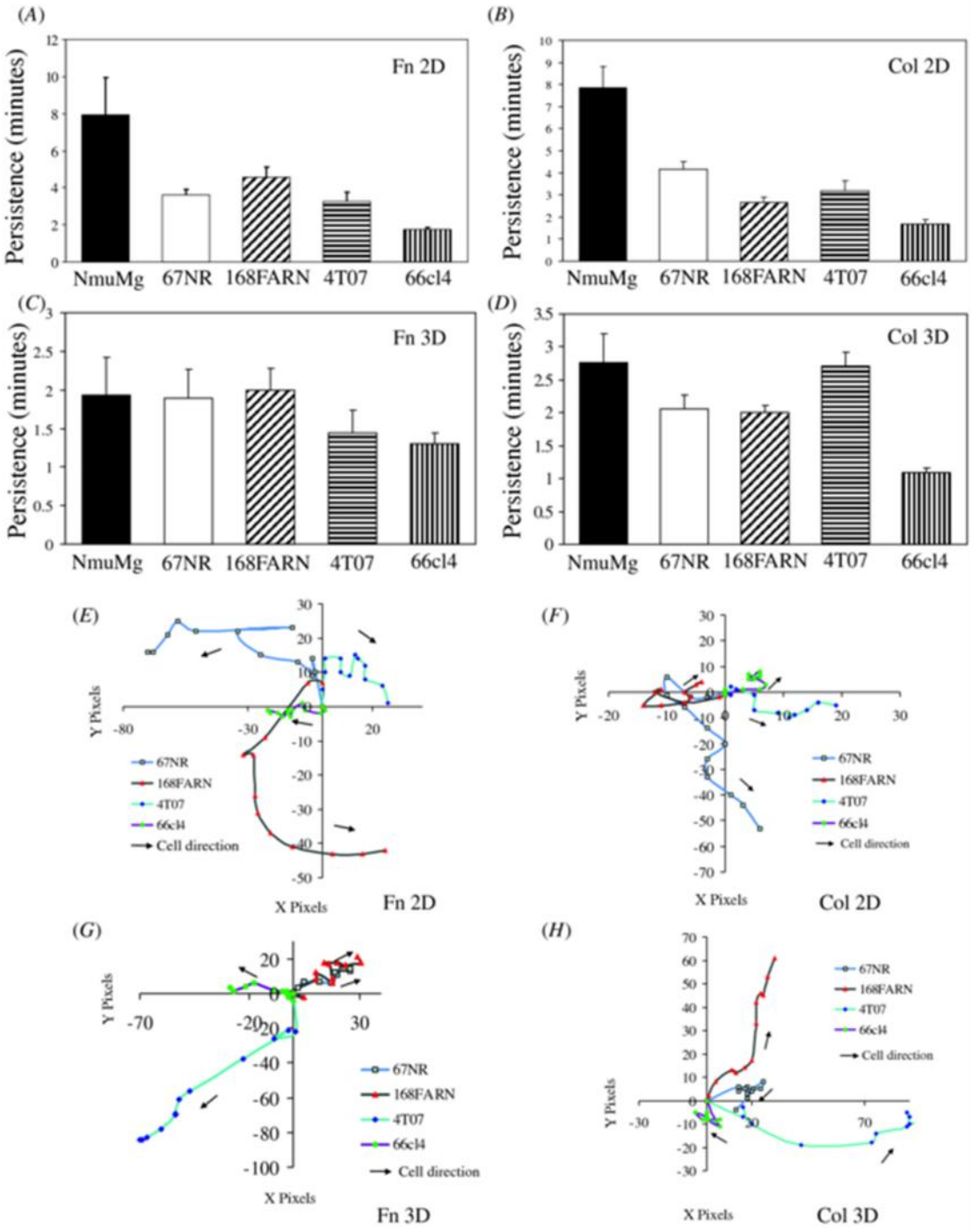


Figure 2.8*. Directional persistence is reduced as metastatic capacity increases. The average persistence of migration in two-dimensional (A) fibronectin- and (B) collagen-coated plates and within three-dimensional (C) fibrin–fibrinogen clot and (D) collagen gels. Persistence is expressed in min; each bar represents the mean value ± 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.*

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 amoeboid 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 tumorogenic and subsequently develops metastatic properties, it incorporates several biochemical and biophysical changes (Guo and Giancotti, 2004b; Lopez *et al.*, 2008; Mierke, 2008; Kumar and Weaver, 2009a). 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 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 a moderately aggressive metastatic cell line (Giancotti and Ruoslahti, 1999).

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 step-wise

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 step-wise decline in the traction.

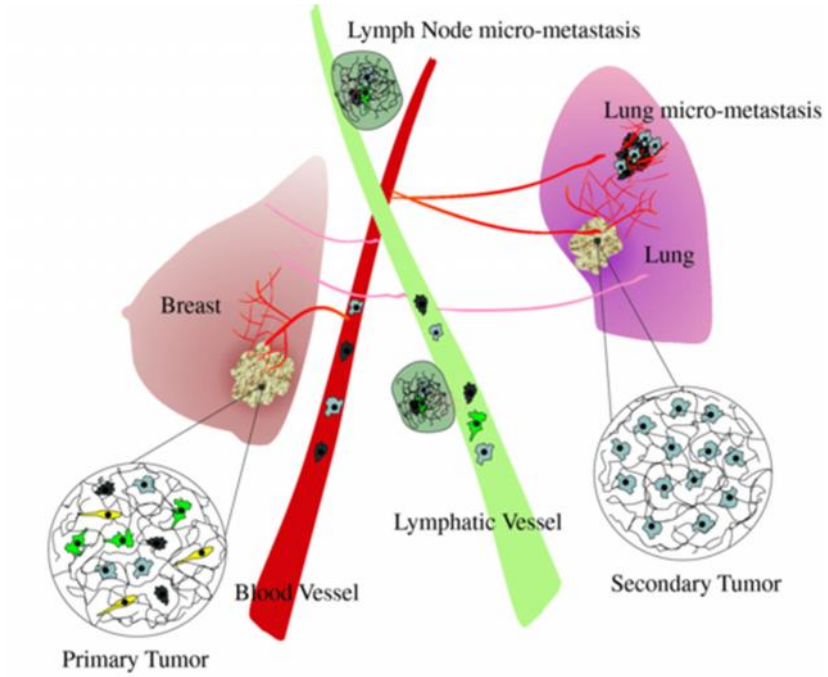
The measurement of traction stress is essentially a read-out of the health of the contractile system of the cell in a two-dimensional environment. Across the panel, adhesions and stress fibers appeared morphologically normal. At a functional level, the adhesions matured normally though the total number 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 become 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 was 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 secrete more MMP compared to the least metastatic cell 67NR (Giancotti and Ruoslahti, 1999).

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 marker are likely to vary as well.



	Traction Stress	2D Migration Rate	3D Migration Rate
67NR	++++	++++	+
168FARN	+++	+++	++
4T07	++	++	++++
66cl4	+	+	+++ [Ligand Dependent]

Figure 2.9*. 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.*

In summary, we have found an inverse correlation between traction stress and metastatic capacity within a panel of breast cancer cell lines (see figure 2.9). 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*.

ACKNOWLEDGEMENTS

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CHAPTER 3

AN *IN-VITRO* CORRELATION OF METASTATIC CAPACITY, SUBSTRATE RIGIDITY AND ECM COMPOSITION

This chapter has been published

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ABSTRACT

The process of metastasis requires a metastatic cancer cell to invade a variety of micro-environments of variable stiffnesses. Unlike metastatic cells, normal cell function and viability is dependent on the stiffness of the environment and used as a cue to maintain cell health and proper tissue organization. In this study we have asked if metastatic cells can ignore the parameter of stiffness and if this ability is gradually acquired and if so, through what mechanism. Using a panel of mouse mammary tumor cells derived from the same parental tumor, but possessing different metastatic abilities, we cultured the cells on hard and soft substrates conjugated with collagen or fibronectin. Normal and non-metastatic tumor cells responded to changes in stiffness on fibronectin, but not collagen. However the more metastatic cells ignored the change in stiffness on fibronectin coated substrates. This lack of response on fibronectin correlated with a change in the expression level of the α3 integrin subunit, activation of the β1 subunit and phosphorylation of FAK at Y397. We conclude that through fibronectin, changes in the activation and tethering of the beta-1 integrin provides a mechanism for metastatic cells to disregard changes in compliance to survive and navigate in environments of different stiffness.

INTRODUCTION

The complex mixture of extracellular matrix proteins found in connective tissues can create variability in the compliance of the extracellular matrix (ECM). Changes in compliance are known to regulate cell adhesion (Juliano, 2002), migration (Lo *et al.*, 2000a), tumor invasion (Paszek *et al.*, 2005), phagocytosis (Beningo *et al.*, 2002b) and development (Jiang *et al.*, 2006a). Cells sense and respond to changes in matrix compliance through a proposed feedback loop involving the internal contractile mechanisms of the cell. Multiple cell-types are known to respond to substrate rigidity including epithelial (Kostic *et al.*, 2009), fibroblasts (Kostic and Sheetz, 2006), neurons (Kostic *et al.*, 2007), and muscle cells (Isenberg *et al.*, 2009). The rigidity for optimum function for a normal cell is thought to be dependent on its host tissue stiffness (Engler *et al.*, 2008) but this correlation is likely lost or modulated in highly migratory cells like neutrophils (Yeung *et al.*, 2005b), and in metastatic cells (Paszek and Weaver, 2004a) which come in contact with various tissue rigidities. Previously it was shown that oncogenic transformation resulted in rigidity-independent spreading and proliferation of fibroblast and epithelial cells (Paszek *et al.*, 2005). However, metastatic progression is a complex multi-step process and single oncogenic transformation provides an inadequate picture of how cells at different stages of this process might alter their cellular behavior in response to changes in environmental rigidity. In addition, while the protein composition of the ECM and matching cellular receptors are known to dictate the cellular response to substrate rigidity (Rowlands *et al.*, 2008), it is unclear how this substrate-ligand specificity correlates with the rigidity sensing mechanism during metastatic progression.

Cell-ECM interactions are primarily mediated by the αβ heterodimeric, transmembrane protein, integrin (Wegener and Campbell, 2008). Integrins act as a conduit between extracellular ligands and the cytoskeleton (Janmey and McCulloch, 2007) and respond to the external substrate rigidity through a counter-response exerted by the actomyosin network (Friedland *et al.*, 2009). Integrins are mechanosensors and undergo conformational changes in response to mechanical force. These conformational changes lead to enhanced cell-ECM adhesion, focal adhesion formation, cell spreading (Friedland *et al.*, 2009) and FAKpY397 phosphorylation (Shi and Boettiger, 2003). Among the integrin family, the β1 subunit of integrin has been implicated in several key processes of malignant progression and metastasis (Park *et al.*, 2006) and known to interact with a repertoire of ECM ligands including collagen, fibronectin, laminin and vitronectin (Wiesner *et al.*, 2005). Integrin specificity arises from its modular structure and the β1 subunit can heterodimerize with nine different alpha subunits (Gong *et al.*, 1997) to form the largest subfamily of integrins.

Integrin mediated extracellular cues are transduced internally through focal adhesion components (Schwartz, 2001). Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, is a central signaling component of focal adhesions. Phosphorylation of FAK relays integrin mediated signals to signaling pathways involved in modulating cell adhesion (Avizienyte and Frame, 2005), migration (Gilmore and Romer, 1996), shape (Martin *et al.*, 2002), growth, proliferation (Pirone *et al.*, 2006) and apoptosis (Frisch *et al.*, 1996). One of the tyrosine sites of FAK at 397 is auto-phosphorylated immediately following integrin clustering (Wei *et al.*, 2008). Most importantly, phosphorylation of FAK

at Y397 is involved in rigidity sensing and fibroblasts that have a deletion of this residue are unable to discriminate between hard and soft substrates (Wang *et al.*, 2001b).

In this study, we have asked if cancer cells at increasingly aggressive stages of the metastatic cascade, respond differently to changes in environmental rigidity and if the response is ECM and integrin specific. Using a panel of murine breast cancer cell lines derived from a single parental tumor, but possessing different metastatic potential, we evaluated the area of cell spreading, and the expression levels of β1, α3 and α5 integrins and the level of FAKpY397 phosphorylation on substrates of different rigidities coated with either collagen or fibronectin. We have found that metastatic progression results in changes in mechanosensory behavior in a fibronectin dependent manner, such that as the cells become more metastatic, their ability to differentiate between soft and rigid substrates is lost on fibronectin. Furthermore, we have found that this ability to ignore changes in compliance correlates with the activation of β1 integrin, phosphorylation of FAK and upregulation in the expression of α3 integrin in the more metastatic cells. Our results could suggest that as cancer cells progress in metastatic potential, one of the parameters they optimize is the ability to ignore the changes in compliance that would be encountered on their metastatic journey, a parameter that a normal cell uses to maintain tissue organization.

MATERIALS AND METHODS

Cell Culture and 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), (Fig. 3.1) and a normal murine mammary gland cell

line was purchased from ATCC. Cultures were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Hyclone, UT, USA), and supplemented with100 U/ml penicillin, 2 mM L-glutamine, and 100µg/ml streptomycin. Cells were grown in a standard culture incubator at 37°C with 5% CO_2. Polyacrylamide hydrogels were conjugated with bovine collagen type I (BD Biosciences, MA, USA) or bovine plasma fibronectin (Sigma, Saint Louis, USA) at 5µg/cm^2 as described previously (Beningo *et al.*, 2002b). Laminin (BD Biosciences, MA, USA) at 5µg/cm^2 conjugation was carried out as described earlier (Kaverina *et al.*, 2002). Substrate compliance was controlled by varying the concentration of *N,N*methylene-bis-acrylamide. All substrates used in this study were either, 5% acrylamide and 0.1% bis-acrylamide (7.69 ± 2.85 kPa) designated the hard substrate or 5% acrylamide and 0.04% (1.30 ± 2.85 kPa) referred to as soft substrate (Guo *et al.*, 2006b).

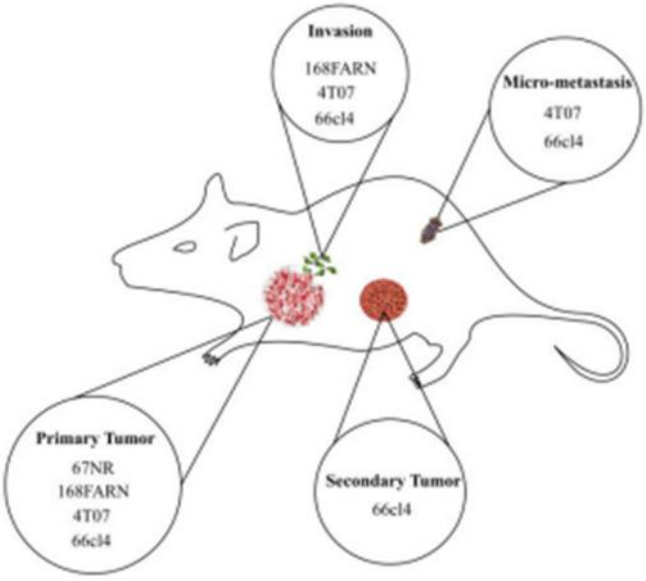

Figure 3.1. *Metastatic properties of the murine breast cancer cell lines. 67NR is non-metastatic line and does not come out from the primary tumor. 168FARN line is invasive*

and enters into the lymphatic vessel but unable to extravasate. 4T07 line can complete all the steps of metastatic cascade but unable to form the secondary tumor. 66cl4 line completes all the steps of metastasis and forms the secondary tumor.

Microscopy

An Olympus IX81 ZDC inverted microscope was used to acquire phase contrast images. The objective lens used for these studies was the 10 X/0.25 NA CP-Achromat lenses. Images of live cells were collected while the cells were maintained at 37ºC and supplied with 5% CO_2 on a custom built stage incubator.

Cellular Assay for Sensing Compliance

To test for cellular response to changes in the compliance of the polyacrylamide substrates (described above) we measured the area of cell spreading. Briefly, approximately $1X10^4$ cells were seeded on to the ECM coated polyacrylamide substrates (5% acrylamide and 0.1% or 0.04% bis-acrylamide) in previously described chambers (Beningo *et al.*, 2002b). The cultures were incubated overnight at $37^{\circ}C/5\%CO_2$ in a culture incubator. After 24 hours, images of cells were captured at 40x for each cell type, under each of the experimental conditions of substrate rigidity and ECM ligand. Image J software (NIH) was used to quantify the average cell area.

Western Blot Analysis

To acquire enough protein for western analysis, cells were cultured on larger polyacrylamide substrates prepared in an electrophoresis mini-gel casting unit. One of the gel casting plates (10x8cm) was activated as previously described (Beningo *et al.*, 2002b) and the other plate was silanized for easy removal after casting. After

polymerization the polyacrylamide gel was washed and coated with bovine plasma fibronectin, collagen or laminin as described above. Cells were cultured to 70% confluency on the gels and were rinsed with ice cold 1X 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 manufacturer's instructions. Samples (25μg of protein, unless otherwise specified) were subjected to SDS-PAGE on 4-20% mini gels and trans-blotted onto PVDF membrane (Millipore, CA, USA). The membrane was blocked for 2 hours at room temperature in 5% Non-fat Dry milk, 0.1% Tween-20 in TBS for Actin, active β1, and α5 and α3 integrin. For probing FAK phosphorylation, membranes were blocked in 5% BSA, 0.1% Tween-20 in TBS. Membranes were incubated with primary antibody for 18 hours at 4°C [1:5000 mouse monoclonal actin (BD Pharmingen, CA, USA); 1: 500 mouse monoclonal active anti-β1 integrin (BD Pharmingen, CA, USA; Clone 9EG7); 1:2500 Rabbit polyclonal anti-α5 integrin (Millipore, CA, USA); 1:500 mouse monoclonal anti-α3 integrin (BD Pharmingen, CA, USA); 1:1000 Rabbit polyclonal Anti-FAK[pY^{397}] (Invitrogen, CA, USA). The membranes were washed and incubated with the species appropriate horseradish-conjugated secondary antibody (Abcam, Cambridge, MA, USA; BD Pharmingen, CA, USA; GE Healthcare, Buckinghamshire, UK). Signals were detected with the ECL plus detection kit (GE Healthcare, Buckinghamshire, UK).

RESULTS

Changes in Compliance are Sensed Differently Dependent on the ECM and Metastatic Ability

Collagen is the main component of mammary epithelial tissue and normal epithelial cells express high levels of collagen receptor (Zutter *et al.*, 1999). It has also been shown that collagen deposition increases with the formation and development of a tumor, resulting in a change of stiffness within the tumor and its stroma (Kass *et al.*, 2007). Based on this, we used hard and soft substrates coated with collagen type I to test the cellular response to changes in compliance at various stages of metastatic progression. Four murine breast cancer cell lines with different metastatic capacity (Fig.3.1) and normal murine mammary gland cell line (NmuMg) were seeded onto the substrates at equal concentrations. After culturing cells for 24 hours the average total area the cells had spread under each of the conditions was calculated. We were surprised to find that both normal and metastatic cell types showed no statistically significant difference in cell area, spreading equally on soft and hard substrates (Fig 3.2 (A) and (B)). On collagen, the cell area was found to be approximately 0.003 mm^2 for all the cell lines on both hard and soft substrates, with the exception of the most metastatic line 66cl4 whose area is smaller at 0.002 mm^2 (Fig 3.2(B)).

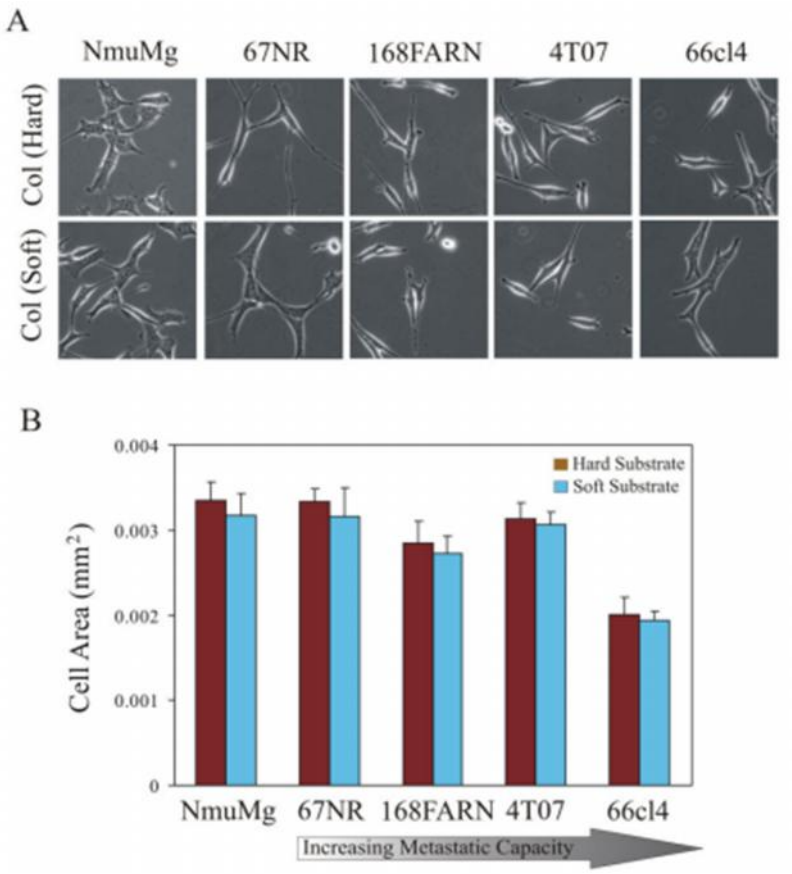

Figure 3.2. *Normal and Metastatic cells did not detect changes in stiffness on collagen. (A) Phase images of normal mammary gland cell line and entire panel of mice breast cancer cell lines showing the morphology and spread area on collagen coated hard and soft substrate. (B) Bar graph represents the quantified cell areas (mm^2) of the entire panel on soft (blue bars) and hard (red bars) collagen coated substrates from three independent experiments. Each bar represents the mean value +/- s.e.m. of thirteen independent fields of images. (n=13).*

This led us to test the rigidity response to other extracellular matrix components, including fibronectin and laminin. We found, as previously described, the cell area of normal cells was significantly reduced on fibronectin coated soft substrates compared to

hard substrates (Fig. 3.3 (A) and (B)) indicating a fibronectin mediated response to rigidity. On the contrary, as the cell types increased in metastatic potential, we observed a statistically significant indifference of the cells to the hard or soft substrate (Fig. 3.3 (B)). A plot of the percent change between cell area of cells grown on fibronectin coated hard and soft substrates reveals a 50% change in the normal cells, a 30% change in the non-metastatic line (67NR) and less than a 5% change in area in the most metastatic cell lines (Fig. 3.3 (C)). Similar results were also observed on laminin coated hard and soft substrates (Fig.3.4 (A)-(C)). Together these results suggest an ECM dependent suppression of the rigidity sensing process with metastatic progression.

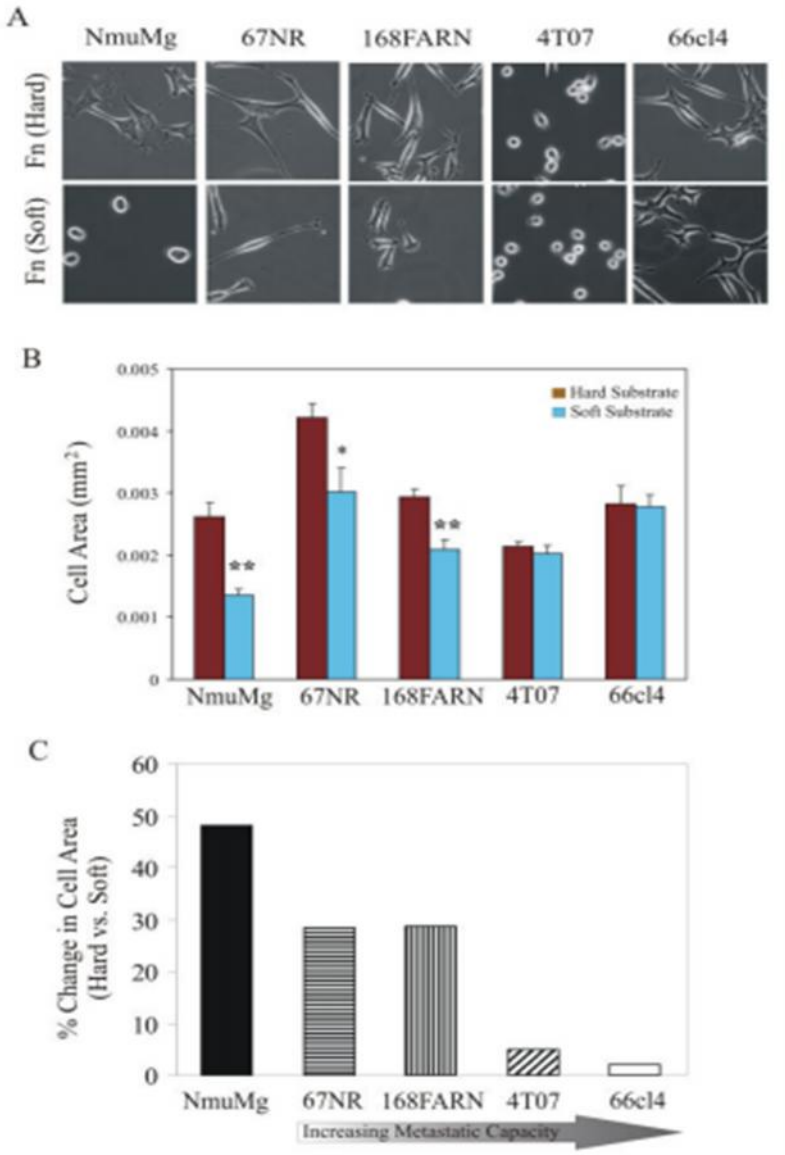

Figure 3.3. *Normal cells, but not metastatic cells, detect changes in stiffness on fibronectin coated hydrogels. (A) Phase images normal mammary gland cell line and the entire panel of mice breast cancer cell lines showing the morphology and spread area of cells on fibronectin coated hard and soft substrate. (B) Bar graph represents the quantified cell areas (mm^2) of the entire panel on soft (blue bars) and hard (red bars) fibronectin coated substrates from three independent experiments. Each bar represents*

*the mean value +/- s.e.m. of thirteen independent fields of images. (n=13). ** P<0.0004 and * P<0.014. (C) Bar graph represents the percentage change in cell area from hard to soft substrate while cultured on fibronectin coated substrate.*

Increased Activation of β1 Integrin by Metastatic Cells in Response to Fibronectin Coated Soft Substrates

Our data suggest the ability of the more metastatic cells to ignore the sensing mechanism is ECM dependent. An obvious mechanistic target is β1 integrin as it binds to both collagen and fibronectin, but more importantly, multiple studies have found an up-regulation of β1 integrin during metastasis (Park *et al.*, 2006). However, it is not clear if upregulation of β1 subunit in metastatic cells is substrate rigidity and ECM composition dependent. In addition, β1 integrin is known to be a mechanosensors (Litzenberger *et al.*, 2010). Testing for expression of total β1 integrin can be misleading as it does not reflect the amount of activated receptor, thus we used an antibody specific to the activated β1 subunit to look for a change coherent with the cell spreading response observed when the cell panel was cultured on collagen or fibronectin substrates. These results were consistent with the trend observed in the cell area on fibronectin and collagen coated soft substrates. In response to soft substrate, the amount of active β1 integrin in metastatic cells increased as compared to normal and non-metastatic cells on fibronectin (Fig.3.5.(B)) and remains the same on collagen coated substrates (Fig. 3.5.(A)). However, on the rigid substrates, less activated β1 subunit was observed in the metastatic cells on both collagen and fibronectin (Fig. 3.5 (A) and B)) indicating that cell spreading in metastatic cells on rigid substrate could be achieved by less ECM engagement. Together, our results suggest that as metastatic

capacity increases, the more metastatic cells alter their ECM engagement to compensate for changes in rigidity, something normal cells do not do.

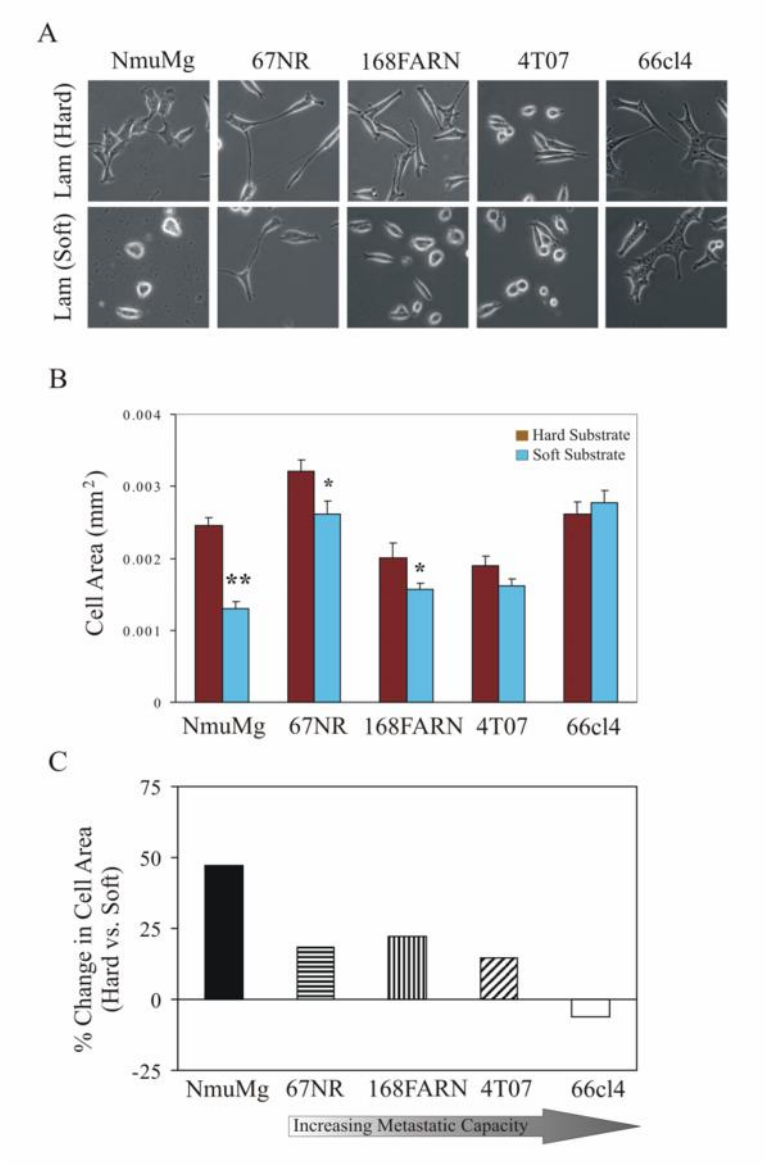

Figure 3.4. *Metastatic cells do not sense compliance on laminin coated hydrogels. (A) Phase images of normal mammary gland cell line and the entire panel of mice breast cancer cell lines showing the morphology and spread area of cells on laminin coated hard and soft substrate. (B) Bar graph represents the quantified cell areas of the entire panel on soft (blue bars) and hard (red bars) laminin coated substrates from three*

*independent experiments. Each bar represents the mean value +/- s.e.m. of thirteen independent fields of images. (n=13). *** P<0.0001 and * P<0.014. (C) Bar graph represents the percentage change in cell area from hard to soft substrate while ECM ligand is laminin.*

FAK Phosphorylation Levels Differ in Metastatic Cells Compared to Non-Metastatic Cells in Response to Substrate Rigidity

Phosphorylation of FAK on Y397 has previously been associated with malignancy, cytoskeleton tension (Paszek *et al.*, 2005) and cell spreading (Partridge and Marcantonio, 2006b). Furthermore, this specific residue of FAK has been shown to be auto-phosphorylated upon β1 integrin activation in a rigidity dependent manner (Wei *et al.*, 2008). Most importantly, phosphorylation of FAK at Y396 is specific to integrin engagement to fibronectin, but not to integrin clustering (Shi and Boettiger, 2003). Given our observations that on fibronectin coated substrates the cell area and amount of active β1 integrin differs greatly in the metastatic versus the non-metastatic cells, dependent on the substrate rigidity, we tested for a correlation with phosphorylation levels of FAK. Western blot analysis was performed on lysates of each cell line grown on fibronectin coated hard and soft substrates. Blots were probed with antibodies specific to phosphorylated tyrosine 397 of FAK (Fig. 3.5 (B)). We found a similar trend in response to hard and soft as observed for active β1 integrin, such that as cells increased in metastatic potential, the level of Y397 phosphorylation dropped on the more rigid fibronectin coated substrates, but levels increased on the soft substrate as metastatic potential increased (Fig. 3.5 (B)). These results indicate that as these cells acquire greater metastatic abilities they may override the rigidity sensing mechanism by

manipulating the activation of a β1 integrin and consequently the levels of FAK phosphorylation.

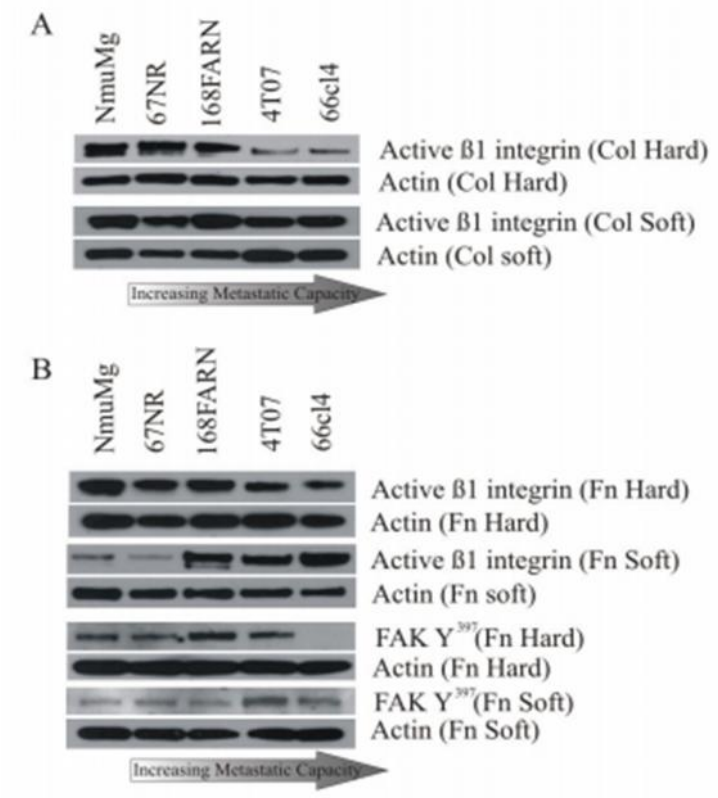

Figure 3.5. *Ligand bound β1integrin expression and phosphorylation level of FAK at Y397 is modulated in metastatic cells on fibronectin coated hydrogels, but not on collagen. (A) Total and ligand bound 1 integrin was detected by western blot from lysates of cells cultured on hard or soft hydrogel coated with 0.05μg/cm^2 bovine type-I collagen and fibronectin. Blots were probed with antibody against active 1 integrins. The level of actin served as a load control. Data represent three independent experiments. (B) FAKpY397 was detected by western blot from lysates of cells cultured on hard or soft hydrogel coated with 0.05μg/cm^2 fibronectin. Blots were probed with antibody against mouse FAKpY397. The level of actin served as a load control. Data represent three independent experiments.*

Correlation of the Expression of Alpha Subunits with Activation of β1 Integrin

Our data link the cellular response to fibronectin and substrate rigidity to metastatic progression. More specifically, this response is mediated through the regulation of β1 integrin activity. However, the β1 subunit can dimerize with various alpha subunits, although its dimerization with the α5 subunit forms the most specific integrin receptor for fibronectin (Roca-Cusachs *et al.*, 2009). These data along with previous reports (Nam *et al.*, 2010; Roman *et al.*, 2010) prompted us to determine if α5 is indeed the subunit responsible for the differential response we describe above.

We compared the total expression of the α5 integrin subunit in lysates from the panel of murine metastatic cells grown on hard and soft substrates coated with fibronectin. Western blot analysis revealed a subtle decline in the expression of α5 with increasing metastatic abilities on the soft fibronectin coated substrates (Fig. 3.6). Furthermore, little difference in the expression levels from the cell panel was observed on hard substrates (Fig.3.6). Most importantly, a difference in the expression levels between the non-metastatic and the metastatic cells was not strong, with the exception of the downregulation observed on the soft substrates for the most metastatic line 66cl4. These results did not correlate with those of the active β1 subunits profile under the same conditions.

A less selective integrin receptor that interacts with fibronectin, as well as laminin and collagen is the α3β1 integrin (Kreidberg, 2000). This integrin has been found to be frequently overexpressed in breast cancer cells (Morini *et al.*, 2000) and down regulation of this receptor has been shown to reduce invasion in breast cancer cells (Mitchell *et al.*, 2010). Using the same approach as described above, we determined

that expression levels of the α3 subunit in the metastatic panel (Fig.3.6). A subtle and gradual increase in expression of α3 subunit was found in metastatic cells cultured on rigid substrate. However, the expression of α3 integrin was found to be greatly enhanced in metastatic cells as compared to non-metastatic cells (NmuMg and 67NR) cultured on soft substrate. This data suggests that alpha3 subunit is overexpressed in metastatic cells and potentially acts as a partner of beta1 integrin in transmitting deregulated rigidity response.

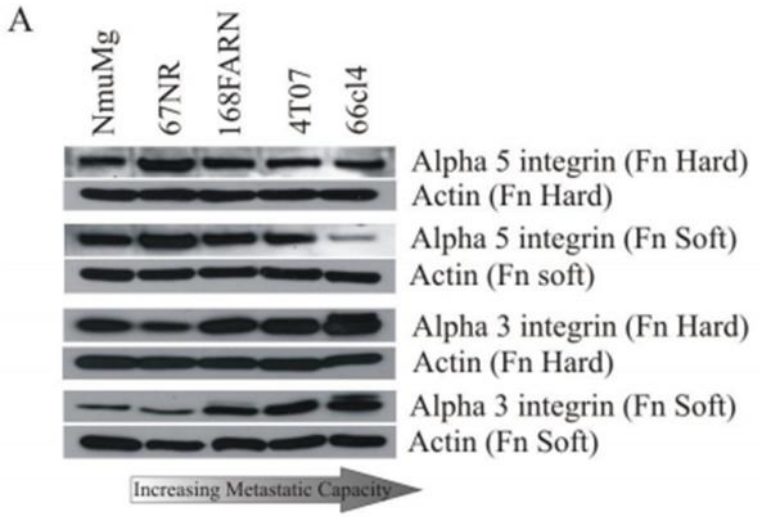

Figure 3.6. *Alpha-5 and alpha-3 integrin expression on fibronectin coated hydrogels. (A) Alpha-5 and alpha-3 integrin was detected by western blot from lysates of cells cultured on hard or soft hydrogel coated with 0.05μg/cm^2. Blots were probed with antibody against mouse total 5 or 3 integrin. The level of actin served as a load control. Data represent three independent experiments.*

Together these results suggest that as cells progress in metastatic ability a fibronectin dependent interaction involving the α3 and β1 integrin subunits provides a

potential conduit to overcome the changing parameter of mechanical compliance that a metastatic cell will encounter.

DISCUSSION

There are many cues used by a cell to maintain its proper place within a tissue, however we know the least about the physical cues. We do know that normal cellular function requires a cell to maintain a normal tensional environment to thrive (Bershadsky *et al.*, 2003b; Ingber, 2008). Unlike normal cells, a metastatic cell has obviously ignored its mechanical environmental cues and will likely need to modulate them as it encounters environments of variable mechanical properties during the multiple stages of the metastatic cascade (Kumar and Weaver, 2009b). For instance, as it leaves the tumor and enters the loose connective tissue it will move from a rigid environment to a softer environment and must compensate for these changes in compliance if it will survive (Parekh *et al.*, 2011). In this study we have asked if the response to mechanical compliance remains constant or varies as the tumor cells progress in metastatic abilities. We have found that indeed this panel of breast cancer cells has gradually evolved a mechanism to disregard compliance cues, in a fibronectin dependent manner. In addition, we have also determined that modulation of expression of α3β1, along with phosphorylation of FAK at tyrosine 397, correlates with the metastatic cells ability to ignore the compliance cues.

To support our hypothesis, it was first important to determine that a cell response, such as cell area, differed on hard and soft substrates for normal and non-metastatic cells, and that this difference was gradually lost as cells progressed in metastatic abilities. We were surprised that we did not see the anticipated response for

collagen coated substrates as has been previously reported for normal and malignant cells (Wang *et al.*, 2000). One obvious explanation for this lack of response is that epithelial cells normally interact with collagen type IV basement membrane, as well as laminin and fibronectin, and not the collagen type I known to be a prevalent component of stroma (Schedin *et al.*, 2004). On the other hand, all of the cell types could normally have exposure to fibronectin during their metastatic journey in *in-vivo* environments. While the amount of fibronectin found in the basement membrane can vary, we have previously shown that 67NR and NmuMg can produce fibronectin (Indra *et al.*, 2011). When we quantified the cell area on fibronectin substrates we observed a cellular response to the change in stiffness, such that normal cells and the least metastatic cells could sense the difference, while the most metastatic cells did not respond to the change in stiffness. A similar response was also observed with the basement membrane protein, laminin (Fig. 3.4). These data suggest that fibronectin and laminin receptors, but not a collagen type I receptor are used by this cell panel to detect substrate compliance. In addition, disregarding the compliance cues from the environment as metastatic capacity progresses could be a strategy for maximizing growth and motility.

In search of a mechanism for these abnormal responses by metastatic cells we tested for the expression levels of beta-1 integrin on collagen and fibronectin coated soft and hard substrates. This receptor was an obvious starting point because it is expressed in breast epithelial cells, binds to fibronectin, collagen and laminin, and is known to be a mechanosensor (Park *et al.*, 2006; Litzenberger *et al.*, 2010). We found that the normal and non-metastatic cells cultured on fibronectin had less activated beta-

1 integrin on the soft substrates, indicating less receptor-ligand engagement and likely weaker cytoskeletal tension consistent with their cell response. Conversely, the metastatic cells increased the amount of active beta-1 on the soft substrate, allowing them to compensate for the change in rigidity and transmit a misleading mechanical signal in order to thrive within fibronectin-rich, softer, foreign environment. On the rigid substrate less activated β1 subunit was observed in the metastatic cells on both collagen and fibronectin suggesting a less adhesion dependent spreading. Thus, highly metastatic cells are able to modulate their activity to accommodate the compliance of the environment and appear to use the fibronectin interaction through beta-1 integrin to do so.

Engagement of the ECM may not necessarily translate to internal activation, however tyrosine phosphorylation of focal adhesion kinase is known to regulate integrin mediated downstream signaling events (Guo and Giancotti, 2004a). More specifically, residue 397 on FAK is phosphorylated only when receptor-ligand tethering occurs (Paszek *et al.*, 2005; Wei *et al.*, 2008). Our result on the activation status of β1 integrin indicated greater integrin-fibronectin tethering on compliant substrate than on rigid substrates in metastatic cells 4T07 and 66cl4. Indeed antibodies specific to the phosphorylated tyrosine residue at 397 of FAK confirmed our finding that the extent of integrin-fibronectin tethering increases in metastatic cells cultured on softer substrates. Furthermore, reduced expression of phosphorylated FAK at 397 in metastatic cells on rigid substrate correlates with the reduced expression of beta1 integrin activation, suggesting less cytoskeletal tension and reduced adhesion dependent spreading. However, integrin mediated fibronectin tethering was greater in normal and non-

metastatic cells on harder substrates, suggesting that the β1 integrin signal regarding the substrate rigidity was transduced. These data suggest that, metastatic cells modulate their adhesion parameter through β1 integrin activation, FAK pY397, ECM engagement and subsequent spreading in-order to migrate through the fibronectin-rich environment in a rigidity independent manner.

Since integrin mediated adhesion is mediated not only by β subunit, we went on to determine the potential α subunit partner of the β1 integrin responsible for this mechanism. Surprisingly, the expression levels of the most potent and well characterized fibronectin receptor, α5 subunit (Hemler, 1990), did not correlate with spreading and activation of β1 integrin, suggesting it is not likely to pair with β1 integrin in mechanotransduction. This result is also supported by previous finding that α5β1 is involved in cell adhesion and not mechanotransduction (Roca-Cusachs *et al.*, 2009). Since, we have found a similar response of cell area on fibronectin and laminin coated substrate we suspected an alpha partner that was common for both the ligands. The α3 subunit of integrin was a reasonable choice, as α3β1 tethers both laminin and fibronectin and has been reported to upregulated in metastasis (Giannelli *et al.*, 2002). We have found the expression levels of the α3 subunit to be low in normal and non-metastatic cells and increase rapidly in metastatic cells on softer substrate. Although, the trend of α3 subunit expressions remain the same in rigid substrates, the changes in expression pattern with increasing metastatic capacity is not drastic. These results implicate α3 subunit as a potential partner of β1 integrin in fibronectin mediated sensing of substrate stiffness.

In summary, this panel of breast cancer cells provided a means to observe the gradual ability of cancer cells to disregard changes in compliance. Normal epithelial cells and the non-invasive cells (67NR) respect their boundaries through mechanical and biochemical cues provided by the relatively stiffer basement membrane. However as the cells become more invasive and move into the softer stroma they change their ECM interactions such that differences in mechanical compliance are ignored.

In conclusion, we have found that as cancer cells progress in metastatic potential they alter their ability to sense the rigidity of their environment and that they do so by increasing the amount of active β1 integrin and FAK phosphorylation in a fibronectin dependent manner.

CHAPTER 4

AN *IN-VITRO* CORRELATION OF METASTATIC CAPACITY AND DUAL MECHANOSTIMULATION

ABSTRACT

Physiologically, cells are under the influence of multiple forms of mechanical input. For example, a cell is subjected to mechanical forces from tissue rigidity, shear and tensile stress, and transient applied strain. Significant progress has been made in understanding the cellular mechanotransduction mechanisms in response to a single mechanical parameter. However, our knowledge of how the cell responds to multiple mechanical inputs has been limited. In this study we have tested the cellular response to the simultaneous application of two mechanical inputs, substrate compliance and transient stimulation. Our results suggest that cells will restrict their response to a single mechanical input at a time and when provided with two mechanical inputs simultaneously, one will dominate. In normal and non-metastatic mammary epithelial cells we found that the cells respond to applied stimulation and will override compliance cues in favor of the applied mechanical stimulus. Surprisingly, however, metastatic mammary epithelial cell remain nonresponsive to either of the mechanical cues. Our results suggest that within our assay system, metastatic progression may involve the down-regulation of multiple mechanotransduction pathways.

INTRODUCTION

Mechanotransduction is a mechanism that regulates cellular behaviors during development (Czirok *et al.*, 2004; Krieg *et al.*, 2008), tissue morphogenesis (Engler *et*

al., 2006), wound healing (Desmouliere *et al.*, 2005), and cancer cell invasion (Paszek *et al.*, 2005). A number of molecular players of this mechanosensitive pathway have been identified. Examples include, integrins (Katsumi *et al.*, 2004), stretch-activated ion channels, (Brakemeier *et al.*, 2002), cadherins (Muller, 2008), and focal adhesion kinases (Leucht *et al.*, 2007). With the assistance of other signal molecules working in concert, the mechanical signals are converted into molecular responses. Examples of these responses include actin polymerization, integrin activation, tyrosine phosphorylation and the secretion of signaling molecules for survival, adhesion, proliferation and cell migration (Wang *et al.*, 2001a; Paszek *et al.*, 2005; Chaturvedi *et al.*, 2007). Nonetheless these responses arise upon the application of a single mechanical stimulus. Our understanding of what occurs to these mechanotransduction responses when multiple mechanical inputs are applied simultaneously is limited. However, it has previously been reported that endothelial cell migration is positively influenced when fluid shear stress is applied to cells on compliant substrates, but not on rigid substrates (Song *et al.*, 2009). Aside from this study little else is known of how multiple mechanical cues are interpreted.

Cells *in-vivo* are exposed to complex biophysical cues which play important roles in tissue patterning, development, and individual cell behavior (Deugnier *et al.*, 1995; Takahashi *et al.*, 1997; Liu *et al.*, 1999). The manner in which individual cells in a tissue context respond to these extracellular cues and maintain the tissue architecture is largely unknown. It is believed that cellular behavior changes to accommodate the differences in extracellular biophysical cues that occur during the change of purpose and development. For example, the tissue repair process is concomitant with the

stiffening of the tissue. The change in compliance results from ECM synthesis and pulling force that are exerted by the contractile myofibroblast. These factors work to bring the wounded edge together (Hinz, 2007). Similarly, mammary gland development involves the deposition of ECM and an accumulation of stromal fibroblast for the formation of the ductal tree (Schedin and Keely, 2011). However, abnormal stiffening of the tissue and excessive contractile force result in fibrosis during wound healing and tumor formation in the breast (Tomasek *et al.*, 2002; Paszek and Weaver, 2004b). Given the importance of multiple mechanical cues in maintaining tissue integrity, it is necessary to understand the cellular response when more than a single mechanical input is received in both normal and disease contexts.

Previous studies have shown that cellular response to substrate compliance (Flanagan *et al.*, 2002; Yeung *et al.*, 2005a) or tugging and pulling forces (Feneberg *et al.*, 2004; Sun *et al.*, 2008) are cell type dependent. In this study we have developed a novel two-dimensional *in-vitro* assay system to understand how cells respond to substrate compliance and transient mechanical stimulation, simultaneously. We have found that normal and non-metastatic mammary epithelial cells respond differently to dual mechanical inputs as compared to metastatic mammary epithelial cells. When both the mechanical cues are provided in a two-dimensional system, normal and non-metastatic cells preferentially responded to transiently applied mechanical cues by overriding the signal from the substrate compliance. Surprisingly, metastatic tumor cells did not respond to either of mechanical cues. We interpret this to suggest that metastatic progression could be associated with the down regulation of select mechanosensors.

MATERIALS AND METHODS

Cell Culture and Spheroid Preparation

Four sub-populations of murine breast cancer cell lines derived from the same primary tumor, but possessing variable metastatic potential (generous gift from Dr. Fred Miller, Karmanos Cancer Institute), and normal murine mammary gland cell line was purchased from ATCC. Cultures were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Hyclone, UT, USA), and supplemented with100U/ml penicillin, 2mM L-glutamine, and 100µg/ml streptomycin. Cells were grown in a standard culture incubator at 37°C with 5% CO_2 supply. Multicellular spheroids were prepared by culturing cells on agar coated 96-well plates. Briefly, 96-well plates are coated with 50 µl of sterile 2% agar and UV sterilized for 15 minutes. Trypsinized cells were resuspended in cell culture media and approximately 1X 10^4 cells/ ml were pipetted into each well. For spheroid development the plate was placed on a rotating platform rotating at 1.83 Hertz inside a humidified cell culture incubator.

Substrate Preparation

Polyacrylamide gels were prepared with modifications as described previously (Beningo *et al.*, 2002a; Menon and Beningo, 2011). The flexibility of the substrate was manipulated by maintaining the total acrylamide concentration at 5% while varying the bis-acrylamide concentration between 0.04% (1.30 ± 2.85 kPa, referred to as soft) and 0.1% (7.69 ± 2.85 kPa, referred to as hard) (Guo *et al.*, 2006b). Each substrate was embedded with 50µl of fluorescently labeled beads (0.2 µm spheres, carboxylate-modified). To create the modified culture well, a 20mm hole was drilled with 1mm thickness to the bottom of a 60mm culture dish (Nunclon). A chemically treated

coverslip (Beningo *et al.*, 2002a) was then attached via vacuum grease to the bottom of the culture dish. Approximately, 200µl of hard substrate solution treated with ammonium per sulfate (APS) and TEMED was plated into the culture well filling half of the well volume. A silanized coverslip (25 µm diameter) was placed on top of the solution leaving a small gap on the opposite side of the well. A paramagnetic bead of 800µm (Cospheric, CA, USA) was inserted through the gap formed between the top and bottom coverslip, followed by 200µl of APS and TEMED treated soft substrate. The top coverslip was gently moved over the top of the unpolymerized substrate to close the gap. Before the gel polymerized, a magnet was used to position the paramagnetic bead within the softer substrate and placing it approximately 100 µm away from the border of the two substrate compliances (Fig. 4.1 (A) and (C)). Following polymerization, the top coverslip was carefully removed. For cell adhesion, bovine plasma fibronectin (Sigma, Saint Louis, USA) at a concentration of 5µg/cm^2 was conjugated on top of the polyacrylamide substrate (Beningo *et al.*, 2002b).

Application of the Mechanical Stimulus

Mechanical stimulation was applied as described earlier (Menon and Beningo, 2011) with slight modification. Briefly, the assay plate was positioned 0.05 cm above a rare earth magnet of 12,000Gauss (25mm in diameter and 5.5mm in thickness). The magnet was rotated below the culture plate at 160 rpm (2.6 Hz) in an orbital field of 2cm on an orbital rotator (Barnstead, Roto Mix- Type 50800, USA). The distance of the assay plate and the rotational speed of the magnet were adjusted based on the data obtained from bead displacements previously observed from cultured fibroblasts (described in result section).

RESULTS

Designing an Assay for the Simultaneous Application of Two Mechanical Stimuli

The purpose of this study was to understand how cellular sensing to rigidity and applied mechanical cues changes with metastatic progression. To answer this question, we designed an *in-vitro* assay system where both the mechanical cues are provided and manipulated. Variation in substrate compliance was provided by casting polyacrylamide hydrogels of two different rigidities side by side (Fig. 4.1 (A-C)). The entire substrate was conjugated with extracellular protein fibronectin to create a uniform adhesive field for cell attachment. We chose fibronectin because we have previously shown that compliance sensing properties by this panel of cell lines is fibronectin dependent (Indra and Beningo, 2011b). To provide applied stimulation from the softer part of the substrate, an 800μm paramagnetic bead was embedded within the soft hydrogel and positioned 100 μm away from the border where the substrates of two different rigidities meet. Transient mechanical pull was created from the softer part of the substrate by placing the assay plate above a rotating rare earth magnet. The entire assay set-up was placed within a tissue culture incubator. The non-metastatic and metastatic breast cancer cell lines of the murine panel spheroids were placed at the border of the two compliances (hard and soft) and the applied stimulus was created by the rotating magnet.

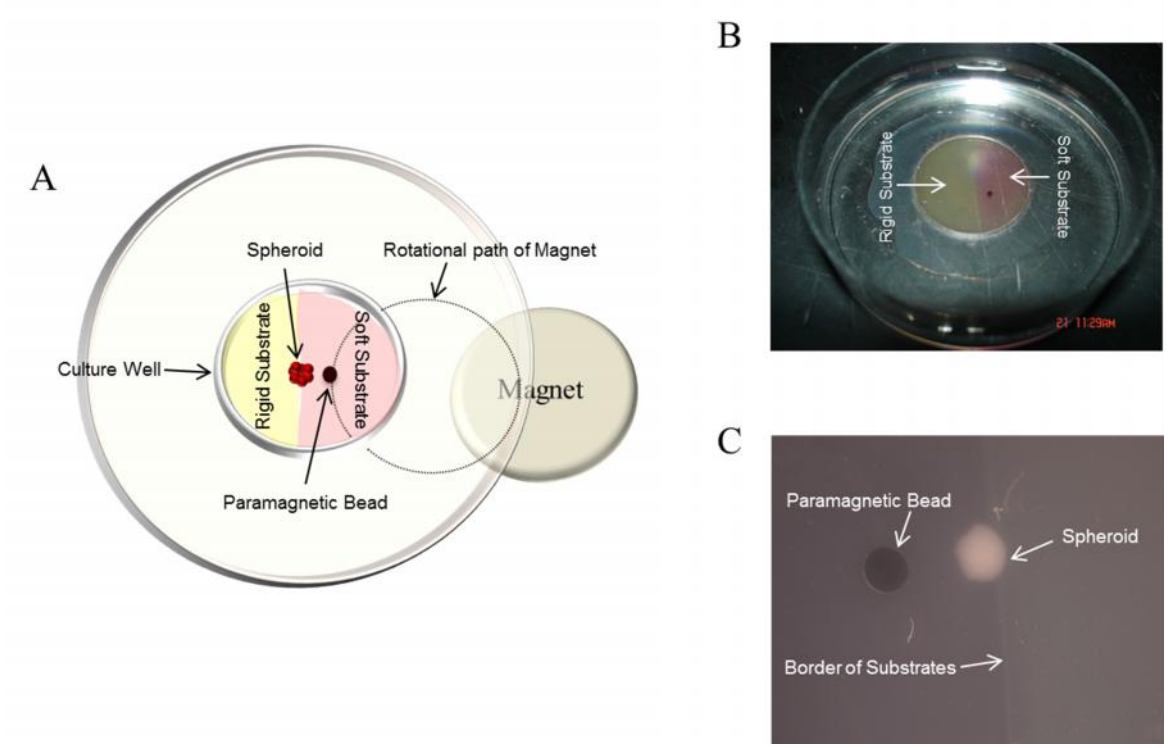

Figure 4.1. *Substrate for an in-vitro assay of dual-mechanostimulation. (A) Schematic of the culture well cast with soft and rigid polyacrylamide hydrogels side-by-side and conjugated with bovine plasma fibronectin on the surface. A paramagnetic bead was embedded within the compliant substrate and positioned approximately 100 μm away from the border of the two substrates. The multicellular spheroid was placed on the border of the two rigidities. A rare earth magnet rotated 0.5 cm below the assay plate. The rotational path of the magnet is displayed as dotted lines. (B) The assay plate before placing the spheroid on the substrate. (C) The assay plate magnified at 2X after placing the spheroid on the substrate.*

Physiological Relevance of Magnetic Stimulation

To understand the response of mammary epithelial cells to the contractile forces generated by neighboring cells, we adjusted the stimulation in our assay system to

physiologically equivalent of fibroblast cells. To do this, we first verified if the magnetic stimulation can transiently displace the paramagnetic bead embedded within the soft substrate (Fig. 4.2 (A)). We realized that a 12,000 Gauss rare earth magnet of 25mm in diameter and 5.5mm in thickness is capable of producing approximately 800 Gauss magnetic force if it rotates 0.5cm under the assay plate at 2.33 Hertz and completes a 2cm orbital diameter. When we simulated this setup under the microscope we observed 0.15-0.25μm and 0.17-0.28μm displacement of fluorescent microbeads in the x and y plane respectively due to the transient pulling force caused by the paramagnetic bead under magnetic tension. To determine the physiological significance of this pulling force, a monolayer of mouse embryonic fibroblast (MEF) cells was cultured on the assay substrate and the displacement of the embedded fluorescent microbead at the edge of cell monolayer was recorded (Fig. 4.2 (B)). The fluorescent bead displacement due to the contractile forces produced by MEF cells, was found to be in the range of 0.19-0.89μm and 0.09-0.21μm in x and y planes, indicating forces generated by the cells is comparatively higher than that of the transient mechanical pulling caused by magnetic stimulation in our assay. Furthermore, the data obtained using our assay system is predicted to be more conservative than *in-vivo* situation because highly contractile myofibroblast cells found in mammary gland associated stroma is reported to produce higher contractile forces as compared to fibroblast (MEF) cells used for this study (Wrobel *et al.*, 2002).

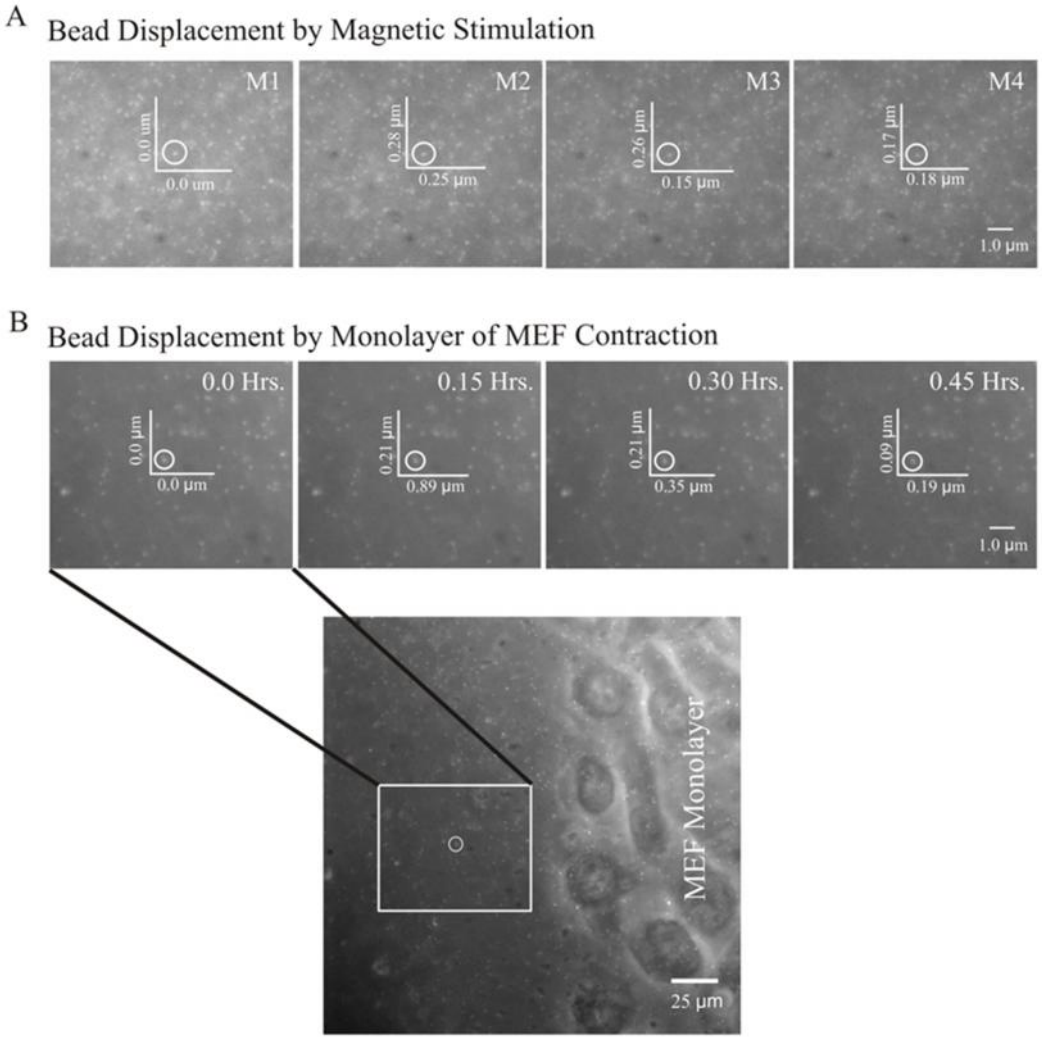

Figure 4.2. *Bead displacement upon magnetic stimulation and cellular contraction. (A) The displacement of a florescent microbead (0.2µm) embedded within the assay substrate due to the pulling forces generated by the paramagnetic bead upon magnetic stimulation. X and Y coordinates of the florescent microbead are shown while the rotating magnet under the assay plate is on four equidistant positions of its rotational path. (B) Bottom panel showing the merged images of a monolayer of mouse embryonic fibroblast (MEF) cells and fluorescent microbeads (0.2µm) embedded within the assay substrate. A bead approximately 100 µm away from the edge of the cell monolayer was*

outlined and magnified (25X). The displacement of the bead is shown in X-Y coordinates on 2-D plane due to the contractile forces generated by MEF cells at 15 minute intervals.

Transient Mechanosensing Overrides the Compliance Sensing Properties in Normal Mammary Epithelial Cells

Under normal physiological condition mammary epithelial cells are known to encounter changes in substrate compliance and transient pulling force (Schedin and Keely, 2011). Based on this, we first tested the response of normal murine mammary gland cells (NmuMg) in our assay system (Fig. 4.3 (A) and Fig.4.4 (A)). For this purpose, a multicellular spheroid of NmuMg was placed at the border of two compliant hydrogels conjugated with fibronectin. After the adhesion of the spheroid to the substrate, the assay plate was kept with or without constant magnetic stimulation for 36 hours. Images of cellular dissemination were taken before and after stimulation. The distance of the cell disseminated from the spheroid was calculated by drawing a line from the edge of the spheroid and plotted as a bar graph. Similar to our previous finding (Indra and Beningo, 2011b), the dissemination of NmuMg cells was found to be dependent on the rigidity of the substrate. When transient stimulation was not provided, the edge of the disseminating NmuMg cells extended to 174 μm on the rigid half of the substrate, in sharp contrast to a distance of 87 μm on the softer substrate ($p<0.05$) (Fig. 4.3 (B)). Surprisingly, a transition in the pattern of cellular dissemination was observed when transient stimulation was provided. The dissemination of NmuMg cells from the spheroid was now 365 μm on the soft part of the substrate containing the magnetic stimulation as compared to 206 μm on hard part of the substrate without

magnetic stimulation ($p<0.05$) (Fig. 4.4 (B)). Given that normal mammary gland cells have a low dissemination distance on softer substrate, unless the magnetic stimulation is applied, suggests that the transient pulling and tugging overrides the compliance sensing mechanism.

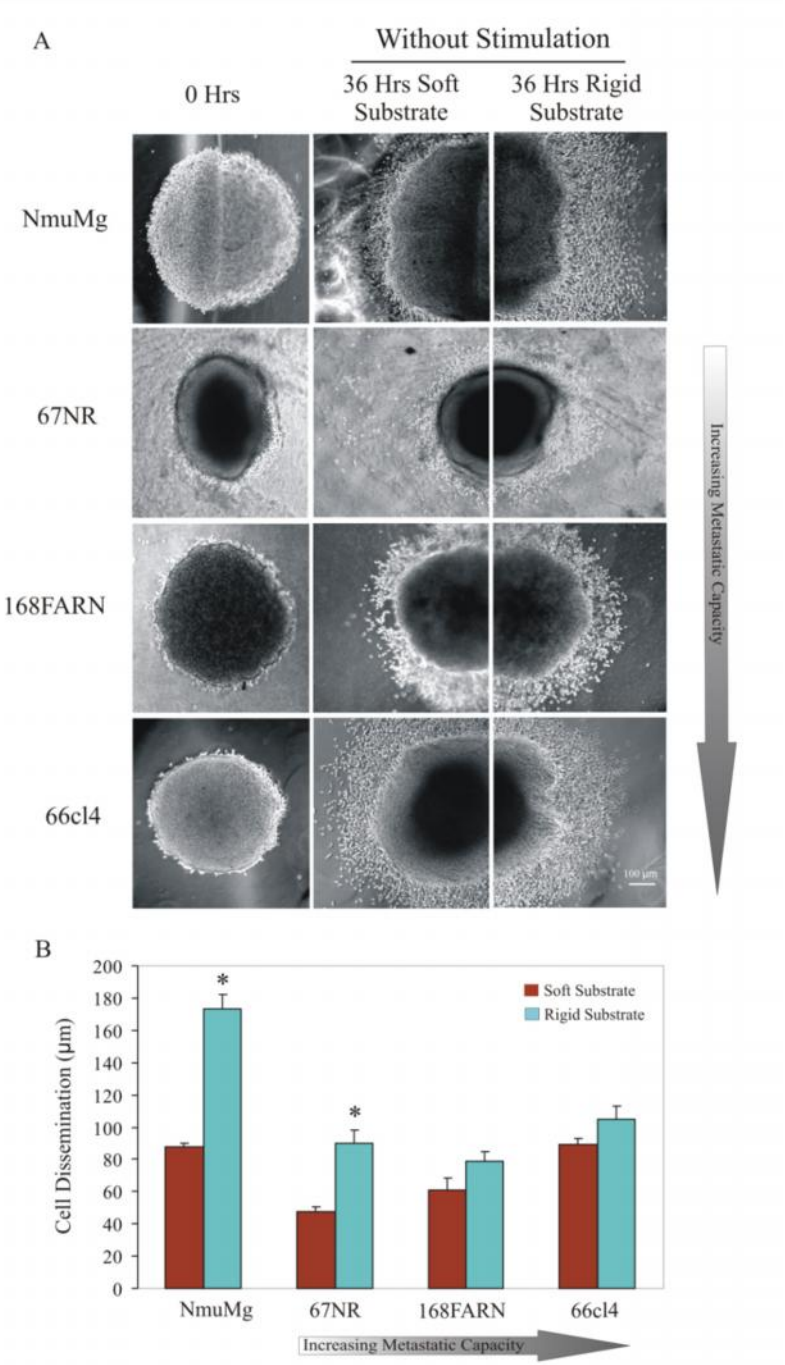

Figure 4.3. *Compliance guided cellular dissemination is lost with metastatic progression. (A) Dissemination of cells from the multicellular spheroids without transient stimulation at 0 hours (left lane) and 36 hours on soft (middle lane) and rigid (right lane) substrate are shown for NmuMg and the panel of murine breast cancer cell lines. (B) Bar graph represents the length of disseminated cells (μm) from the edge of the spheroid after 36 hours. Red and blue bars represent the distance of disseminated cells (in μm) on soft and rigid substrates, respectively. Each bar represents mean ± s.e.m., results from three separate experiments. * indicates p value <0.05.*

Metastatic Progression Correlates with an Inability to Sense both Compliance and Transient Mechanical Stimulation

We have previously shown that the ability of mammary epithelial cells to sense changes in compliance decreases with the gain of metastatic capacity (Indra and Beningo, 2011a). This led us to ask weather metastatic progression is also associated with a progressive loss in the ability to sense transient tugging and pulling forces. We tested the panel of murine breast cancer cell lines of varying metastatic potential in our assay system. Without transient stimulation, 67NR and 168FARN cells disseminated 43 and 18 μm further on rigid substrate as compared to softer substrate ($p<0.05$) (Fig. 4.3 (B)). This result demonstrates that, as with NmuMg cells, 67NR and 168FARN cells responded to substrate compliance. Furthermore, as we have reported earlier (Indra and Beningo, 2011b), a gradual decline in compliance response was seen with metastatic progression (Fig. 4.3 (A) and (B)). However, similar to our earlier finding (Indra and Beningo, 2011b), this compliance dependent cellular dissemination is lost when we tested the most metastatic cell line, 66cl4 (Fig. 4.3 (A) and (B)). When we

provided transient stimulation, like normal mammary gland cells, the non-metastatic cell lines (67NR and 168FARN) responded to the transient stimulation by overriding the substrate compliance and disseminated into the soft substrate (Fig. 4.4 (A)). 67NR and 168FAR disseminated further 125 and 333 μm respectively, on softer substrate as compared to on rigid substrate ($p<0.05$) (Fig. 4.4 (B)). However, the metastatic cell, 66cl4 neither sensed changes in compliance nor did they respond to the transient stimulation (Fig. 4.4 (B)). Together these results suggest that the cells within this panel lose their mechanosensing abilities for both compliance and transient tugging and pulling as progress in metastatic capacity.

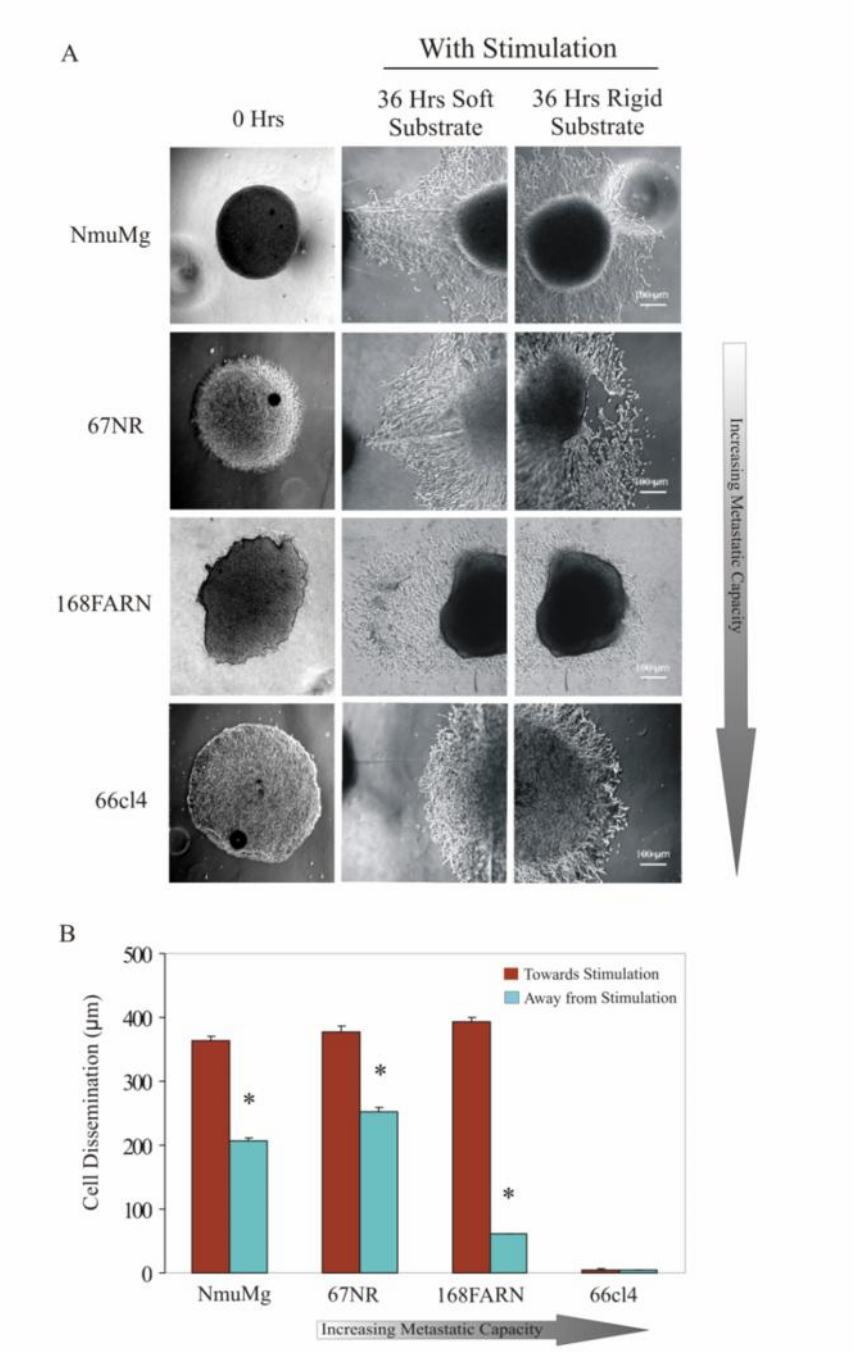

Figure 4.4. *Normal and non-metastatic cells, but not metastatic cells, sense transient stimulation and override mechanical signals of substrate compliance. (A) Dissemination of cells from the multicellular spheroids with transient stimulation at 0 hours (left lane) and 36 hours on soft (middle lane) and rigid substrates (right lane). (B) Bar graph*

*represents the length of disseminated cells (μm) from the edge of the spheroid after 36 hours of stimulation. Red and blue bars represent the distance of disseminated cells (in μm) towards (on soft substrate) and away (on rigid substrate) from the stimulation, respectively. Each bar represents mean ± s.e.m., results from three separate experiments. * indicates p value <0.05.*

DISCUSSION AND FUTURE DIRECTIONS

The importance of mechanical forces in regulating the cellular behavior has been well established (Lansman *et al.*, 1987; Vogel and Sheetz, 2006; Sniadecki, 2010). However, many of these studies are concentrated on understanding the cellular behavior in response to single mechanical stimulation. To advance our understanding of how mechanical cues affect physiological systems where multiple biophysical cues are present simultaneously, we must also study cell behavior in response to complex mechanical environments. In this study we have provided two forms of mechanical stimulation in the form of compliance and transient mechanical pull, and asked whether cells can respond to these mechanical cues when delivered simultaneously. We have correlated these mechanosensory responses with the metastatic progression.

Given the importance of microenvironmental compliance and transient stimulation in mammary gland development and tumor progression we have tested the influence of these mechanical inputs on normal murine mammary epithelial cells and a panel of murine breast cancer cells. Our assay system was designed to provide simplicity and physiological relevance to determine the cellular response to more than one mechanical cue. Compliance of the soft and rigid part of the substrate was optimized based on the physiological range of compliance reported during tumor

formation (Butcher *et al.*, 2009). The magnitude of transient stimulation provided in our assay system was also optimized based on the contractile forces generated by a monolayer of fibroblast cells. In addition, to understand the cellular response in tissue context we have tested the mechanical cues on multicellular spheroids instead of using individual cells.

We have previously shown that the ability to sense changes in compliance decreases gradually as cells become more metastatic (Indra and Beningo, 2011a). However, in this present study, when the transient mechanical cue was provided along with the compliance cue, as for normal cells, non-metastatic 67NR and 168FARN cells responded to this applied cue by ignoring the substrate compliance. Surprisingly, highly metastatic 66cl4 cells did not show any change in dissemination, as if the mechanotransduction pathway was turned off. The inability to sense these mechanical cues could result in the loss of directional migration in highly metastatic cells, as previously described for cancer cell invasion and metastasis (Byers *et al.*, 1991). From these results we surmise that transient stimulation overrides the ability to sense changes in compliance and unlike metastatic cells, tumorogenic, but non-metastatic cells, retain normal sensing behavior. In addition, we can suggest that mammary epithelial cells respond to one mechanical input at a time, but the dominance of the cue could be cell type dependent. However, our study has been limited to two forms of stimulation; it is also likely that a cell can respond to multiple mechanical inputs when other combinations of biophysical cues are provided. It is also likely that any number of biochemical factors would influence these observations, as the biophysical and biochemical systems are not independent.

Our simple assay system provides an in-vitro methodology for the application of more than one mechanical cue. In the future, it will be important to determine the cellular response to different combination of multiple mechanical cues using in-vitro and in-vivo approaches. Furthermore, future studies will be needed to identify the mechanosensors and the transduction pathways involved in sensing the transient stimulation and how this pathway is influenced in metastatic progression. We have previously shown that the status of integrin β1 activation and phosphorylation of FAK at tyrosine 397 is involved in sensing the substrate compliance (Indra and Beningo, 2011b). From increased dissemination and migration of normal and non-metastatic cells on softer substrate we would predict that in the presence of transient stimulation, the sensing of cellular compliance is turned off by over activation of β1 integrin and subsequent increased phosphorylation of FAKp397. Thus it is reasonable to test the status of the active form of integrin β1 and FAKp397 in cells disseminated on softer substrate in response to applied stimulation. It is also possible that other mechanosensory molecules could be involved in sensing the transient stimulation and activation could result in over activation of β1 integrin, increased FAKp397 and deregulated compliance sensing. In addition, the β3 subunit of integrin has been shown to be the mechanosensor (Jiang *et al.*, 2006b), hence a next logical step would be to determine the localization and activation of β3 integrin in response to transient stimulation.

CHAPTER 5

SUMMARY AND CONCLUSIONS

In this dissertation, I have investigated the correlation of metastatic progression with changes in cellular and extracellular biophysical properties. In Chapter 2, I have demonstrated that an inverse relationship exists between metastatic capacity and cellular force production ability. Using a panel of murine breast cancer cell lines I found that with the progression of metastatic capacity, a cell switches to a less adhesive mode of migration. This results in decreased traction stress, adhesion strength, migration speed, focal adhesion at the leading edge and β1 integrin activation in two-dimensional in-vitro setup. However, this less adhesive mode of migration enables the highly metastatic cells to migrate faster in an in-vitro three-dimensional environment. I concluded 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. In chapter 3, I set out to determine changes in cellular response to substrate compliance as a cell becomes metastatic. The results showed that compliance sensing properties of mammary epithelial cell decrease with metastatic progression in ECM composition dependent manner. I have shown that fibronectin but not type I collagen transmits the extracellular rigidity response inside the cell by interacting with integrin α3β1 and FAKp397 phosphorylation. I have also demonstrated that over activation of integrin α3β1 and increased phosphorylation of FAK at tyrosine 397 in metastatic cells results in a down regulation of compliance sensitive behavior. In the third part of this thesis (chapter 4), I went one step further to determine the cellular response to more than one mechanical cue- substrate compliance and transient

stimulation. My data showed that normal and non-metastatic mammary epithelial cells are responsive to one mechanical cue at a time of the cues selected and when both the cues are provided, transient cue overrides the substrate compliance. However, metastatic cells showed nonresponsiveness to both the cues.

I concluded that cellular mechanical characteristics and cellular response to variations in extracellular mechanical cues change gradually with the gain of metastatic characteristics.

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ABSTRACT

MECHANICAL FORCES AND TUMOR CELLS: INSIGHT INTO THE BIOPHYSICAL ASPECTS OF CANCER PROGRESSION

by

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Mechanical forces play an important role in the regulation of cellular behavior and physiological processes including adhesion, migration, proliferation, tissue repair, embryogenesis and development. In addition, a number of diseases including cancer, have been linked to changes in cellular and extracellular mechanical properties. However, whether a correlation exists between the progression of cancer towards metastasis and mechanical factors has not been clearly defined. Additionally, how a cell responds to changes in extracellular mechanical cues as it gains metastatic abilities is poorly understood. To address these questions, we have utilized a panel of murine breast cancer cell lines with progressive metastatic capacity. We have asked how the cell's ability to produce mechanical forces changes as the cells progress in metastatic abilities. Furthermore we have asked if metastatic progression changes the cells' ability to respond to altered extracellular mechanical cues in two-dimensional in-vitro cultures. Our results indicate that with metastatic progression, the strength of adhesion and traction stress progressively decreases. Furthermore we observe a downward trend in

the number of focal adhesions at the leading edge, and subsequent reduction in the activation of integrin β1 and migration speed. We have also found that this cell panel loses its ability to sense changes in compliance as metastatic abilities increase and that this occurs in a fibronectin dependent manner. We found that the loss in mechanical sensing is associated with a decrease in integrin (α3)β1 activation and FAKpY397. Finally, we showed that when a transient mechanical cue is provided, and coupled to changes in compliance, normal and non-metastatic cells respond preferentially to the transient cue. However, the metastatic cells neither sensed changes in compliance, nor did they respond to the transient stimulation. Together these results show that a cell's ability to produce mechanical force, and sense extracellular mechanical forces, progressively decrease with the gain of metastatic characteristics.

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