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An In vitro Correlation of Metastatic Capacity, Substrate Rigidity, and ECM Composition

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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 FAKpY397. 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. J. Cell. Biochem. 112: 3151–3158, 2011.

KEY WORDS: MECHANOSENSING; FOCAL ADHESION KINASE; INTEGRIN; EXTRACELLULAR MATRIX; METASTASIS

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., 2000], tumor invasion [Paszek et al., 2005], phagocytosis [Beningo et al., 2002], and development [Jiang et al., 2006]. 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., 2005], and in metastatic cells [Paszek and Weaver, 2004], 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

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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 Y397 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., 2001].

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, phopshorylation 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. 1) and a normal murine mammary gland cell line (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C under 5% humidified CO_2. Polyacrylamide hydrogels were conjugated with bovine collagen type I (BD Biosciences, MA) or bovine plasma fibronectin (Sigma, Saint Louis) at 5 $\mu g/cm^2$ as described previously [Beningo et al., 2002]. Laminin (BD Biosciences, MA) at 5 $\mu g/cm^2$ 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., 2006] unless otherwise specified.

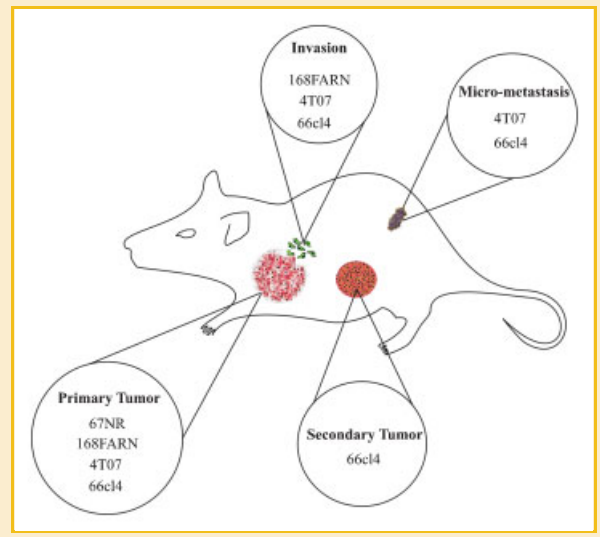

Fig. 1. Metastatic properties of the murine breast cancer cell lines. 67NR is non-metastatic line and does not come out from the primary tumor. 168FARN line is invasive and enters into the lymphatic vessel but unable to extravasate. 4T07 line can complete all the steps of metastatic cascade but unable to form the secondary tumor. 66cl4 line completes all the steps of metastasis and forms the secondary tumor.

MICROSCOPY

Images were acquired on an Olympus IX81 ZDC inverted microscope, equipped with a 40X/0.75 NA Plan-Neofluor lens for phase-contrast images. Live cells were imaged at 37°C under 5% CO_2 on a microscope equipped with a custom stage incubator. All images were acquired with a Boost EM-CCD-BT2000 camera (Diagnostic Instruments, Sterling Heights, MI) driven by IPLab software (BD Biosciences, MA).

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 1×10^4 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., 2002]. The cultures were incubated overnight at 37°C/5%CO_2 in a culture incubator. After 24 h, images of cells were captured at 40× 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 (10 × 8 cm) was activated as previously described [Beningo et al., 2002] 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 1× 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). The protein content was determined by the DC protein assay (Bio Rad) according to manufacturer's instructions. Samples (25 μg of protein, unless otherwise specified) were subjected to SDS–PAGE on 4–20% mini gels and trans-blotted onto PVDF membrane (Millipore, CA). The membrane was blocked for 2 h 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 h at 4°C [1:5,000 mouse monoclonal actin (BD Pharmingen, CA); 1:500 mouse monoclonal active anti-β1 integrin (BD Pharmingen, CA; Clone 9EG7); 1:2,500 Rabbit polyclonal anti-α5 integrin (Millipore, CA); 1:500 mouse monoclonal anti-α3 integrin (BD Pharmingen, CA); 1:1,000 Rabbit polyclonal Anti-FAK[pY^{397}] (Invitrogen, CA). The membranes were washed and incubated with the species appropriate horseradish-conjugated secondary antibody (Abcam, Cambridge, MA; BD Pharmingen, CA; 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. 1) and normal murine mammary gland cell line (NmuMg) were seeded onto the substrates at equal concentrations. After culturing cells for 24 h 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. 2A 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. 2B).

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. 3A 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. 3B). 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. 3C). Similar results were also observed on laminin coated hard and soft substrates (Supplementary Fig. 1). Together these results suggest an ECM dependent suppression of the rigidity sensing process with metastatic progression.

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. 4B) and remains the same on collagen-coated substrates (Fig. 4A). However, on the rigid substrates, less activated β1 subunit was observed in the metastatic cells on both collagen and fibronectin (Fig. 4A 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.

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, 2006]. 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 Y397 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

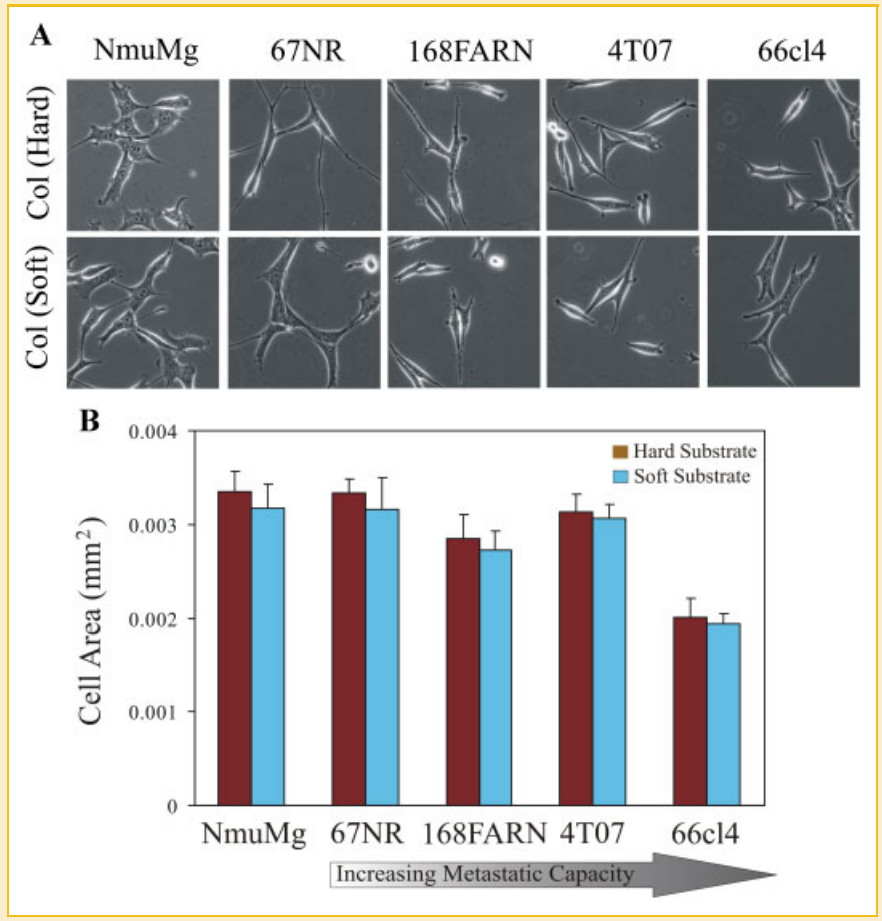

Fig. 2. Normal and Metastatic cells did not detect changes in stiffness on collagen. A: Phase images of normal mammary gland cell line and entire panel of mice breast cancer cell lines showing the morphology and spread area on collagen coated hard and soft substrate. B: Bar graph represents the quantified 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 13 independent fields of images. ($n = 13$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

to phosphorylated tyrosine 397 of FAK (Fig. 4B). 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. 4B). 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.

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. 5). Furthermore, little difference in the expression levels from the cell panel was observed on hard substrates (Fig. 5). 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

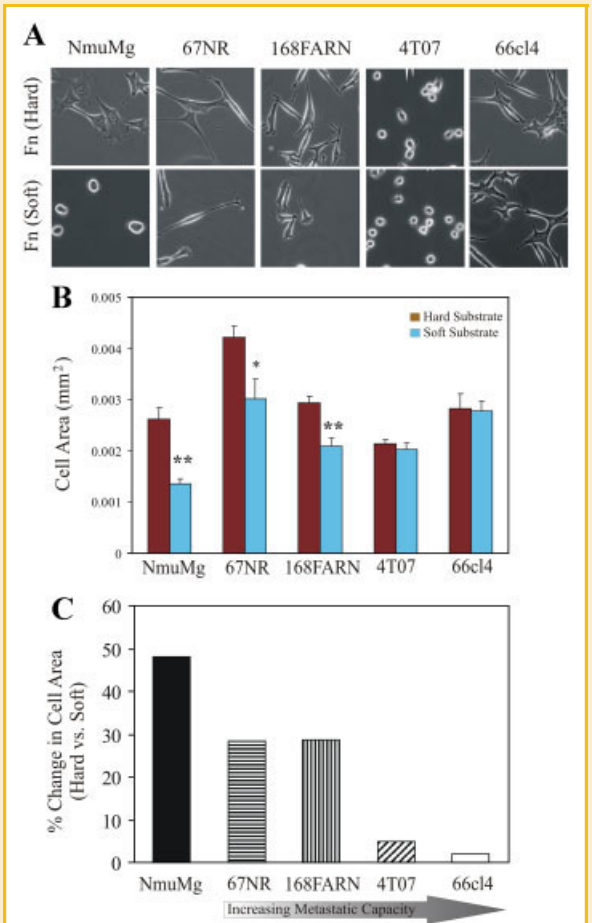

Fig. 3. Normal cells, but not metastatic cells, detect changes in stiffness on fibronectin-coated hydrogels. A: Phase images normal mammary gland cell line and the entire panel of mice breast cancer cell lines showing the morphology and spread area of cells on fibronectin-coated hard and soft substrate. 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 13 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. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

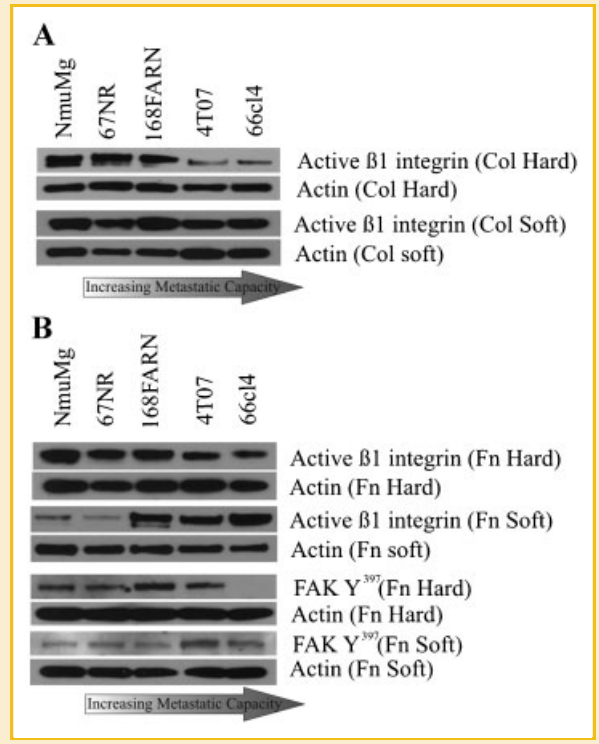

Fig. 4. Ligand bound β1 1integrin expression and phosphorylation level of FAK at Y397 is modulated in metastatic cells on fibronectin-coated hydrogels. A: Total and ligand bound β1 integrin was detected by western blot from lysates of cells cultured on hard or soft hydrogel coated with 0.05 μg/cm^2 bovine type-I collagen and fibronectin. Blots were probed with antibody against active β1 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 0.05 μg/cm^2 bovine plasma fibronectin. Blots were probed with antibody against mouse FAKpY397. The level of actin served as a load control. Data represent three independent experiments.

above, we determined that expression levels of the α3 subunit in the metastatic panel (Fig. 5). 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.

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., 2003; 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, 2009]. 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

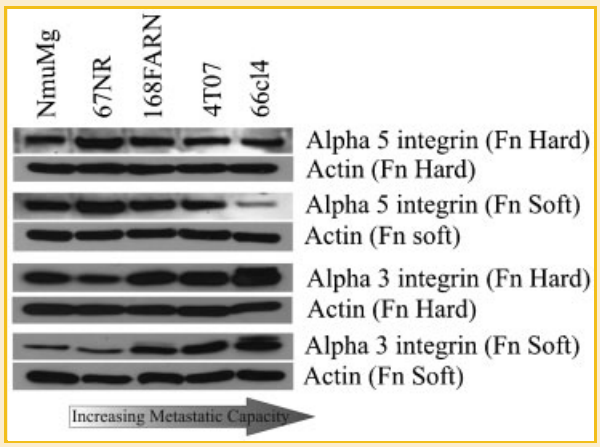

Fig. 5. Alpha-5 and alpha-3 integrin expression on fibronectin-coated hydrogels. Alpha-5 and alpha-3 integrin was detected by western blot from lysates of cells cultured on hard or soft hydrogel coated with 0.05 $\mu g/cm^2$ fibronectin. Blots were probed with antibody against mouse total $\alpha 5$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

determined that modulation of expression of $\alpha 3\beta 1$, 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. S1). 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 $\beta 1$ 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, 2004]. 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 $\beta 1$ 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 $\beta 1$ integrin signal regarding the substrate rigidity was transduced. These data suggest that, metastatic cells modulate their adhesion parameter through $\beta 1$ 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 $\beta 1$ integrin responsible for this mechanism. Surprisingly, the expression levels of the most potent and well-characterized fibronectin receptor, $\alpha 5$ subunit [Hemler, 1990], did not correlate with spreading and activation of $\beta 1$ integrin, suggesting it is not likely to pair with $\beta 1$ integrin in mechanotransduction. This result is also supported by previous finding that $\alpha 5\beta 1$ 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 $\alpha 3$ subunit of integrin was a reasonable choice, as $\alpha 3\beta 1$ 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 $\alpha 3$ subunit to be low in normal and non-metastatic cells and increase rapidly in metastatic cells on softer substrate. Although, the trend of $\alpha 3$

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.

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