Published in final edited form as:

Biotechnol J. 2012 March ; 7(3): 397–408. doi:10.1002/biot.201100188.

Biochemical and Mechanical Extracellular Matrix Properties Dictate Mammary Epithelial Cell Assembly

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Abstract

Understanding cell migration and cell-cell interactions are key to understanding cell invasion, a critical step in the progression of breast cancer. Biochemical and mechanical cues of the extracellular matrix have been shown to play important roles in cell-matrix and cell-cell interactions. We have experimentally tested the combined influence of these cues to better understand cell motility, force generation, cell-cell interaction and assembly in an ‘in vitro’ breast cancer model. MCF-10A non-tumorigenic mammary epithelial cells were observed on surfaces with varying fibronectin ligand concentration and polyacrylamide gel rigidity. Our data shows that cell velocity is biphasic in both matrix rigidity and adhesiveness. The maximum in cell migration velocity occurs only at specific combination of substrate stiffness and ligand density. We found cell-cell interactions reduce migration velocity. However the traction forces cells exert onto the substrate increase linearly with both cues, with cell in pairs exerting higher maximum tractions observed over single cells. A relationship between force and motility shows a maximum in single cell velocity not observed in cell pairs. Cell-cell adhesion becomes strongly favored on softer gels with elasticity 1250 Pascals (Pa), implying an existence of a compliance threshold that promotes cell-cell over cell-matrix adhesion. Finally on gels with stiffness similar to pre-malignant breast tissue, 400Pa, cells undergo multi-cellular assembly and division into three-dimensional spherical aggregates on a two-dimensional surface.

Keywords

Polyacrylamide gel; substrate rigidity; cell assembly; three-dimensional aggregates; breast epithelial metastasis

Introduction

Understanding cell migration and cell-cell interactions are key to understanding cell invasion, a critical step in the progression of breast cancer. Events of tissue destabilization, loss of cell-cell adhesiveness and increased cell-matrix interaction ultimately result in cell invasion and metastasis. Both genetic events and extracellular matrix (ECM) changes play important roles in supporting invasion [1-3]; however the role that the ECM plays is still unclear. The quantitative relationship between adhesiveness and compliance of the ECM

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Conflict of Interest: The authors declare no conflict of interest.

leading to disruption of multicellular structures and cell invasion are the primary focus of this research.

Research on mammary epithelial cells has shown progression of breast cancer is associated with tissue stiffening in vivo and in three-dimensional culture [1, 4, 5]. Experiments in three-dimensional basement membrane gels found the breast epithelial cells to form ordered multicellular aggregates called acini – a concentric spherical shell of cells with a hollow lumen [5-7]. At higher substrate stiffness such as coincidentally observed in cancerous tissue, the acini tend to be disordered and display an invasive cancerous phenotype [5]. Furthermore progression and invasiveness of breast cancer in vivo is also associated with increasing ligand density, such as fibronectin (FN) and collagen [8, 9]. The ECM expression levels of FN are also found significantly elevated in sites of breast cancer metastases [10, 11].

The question then arises whether this tissue disassembly and cell invasiveness is regulated by differential cell-cell interaction modulated by cell-cell communication or altered cell motility due to cell adhesion and substrate stimuli. Previously Steinberg and Foty showed that cell assembly into multicellular structures could be controlled by differential cell-cell interaction. However, these investigations were carried out solely through manipulating the level and specificity of cadherin expression [12, 13]. But it is now apparent that it is not only intrinsic cell properties but also extracellular substrate stiffness and adhesiveness that play significant roles in cell-cell interaction and multicellular structure formation as well [1, 5-7, 14-16].

Further motivation to better understand the cell-cell cohesion and tissue disassembly stems from 2D in vitro experiments that have shown ECM properties to affect individual cell behavior. Mechanical properties of substrates, such as can be engineered in polyacrylamide gels, have shown to affect cell speed, persistence and direction of migration [17-20]. Recently we showed that endothelial cells display reduced motility on compliant gels due to communication through the substrate [21]. The mechanical properties of the ECM thus affect signaling pathways within the cell through mechanically responsive sensors [22, 23], such as decrease of FAK phosphorylation on compliant substrates [5] and regulation of actomyosin contractility [22, 24]. Changes in biochemical extracellular environment, such as increasing surface ligand density also affects individual cell spreading and force generation [25, 26]. Further computational predictions of the impact of the mechanical and biochemical cues on migration in 3D have been developed [27-29]. However the effect of both biochemical and mechanical ECM properties on migratory and interactive cell behavior have yet to be experimentally tested or quantified.

Furthermore, cell motility has been connected on intracellular level to cell-cell interaction through key common intracellular players in integrin and cadherin molecular pathways [5, 30-33]. However, this cross-talk between the two molecular mechanisms has yet to be translated to cellular phenotype and behavior. It is still unclear how the interplay of both adhesion and compliance collectively affect cell phenotype and behavior, such as motility and cell-cell interaction. Here we further investigate effects of substrate adhesiveness and compliance on cell motility, spreading, cell-cell interaction and assembly. We publish a novel correlation between cell motility and traction force cells exert onto the underlying substrate. The maximum traction forces cells exert are significantly higher for cell pairs in contact than individual cells. We thus examine how cell-cell adhesivity modulates cell motility and invasiveness in breast epithelial cells as a model of oncogenesis.

To effectively isolate and understand the ECM influence on the invasive phenotype, our experimental system connects the behavior of an individual cell to that of multi-cellular

structure. This connection between the single cell, two cells and tissue-like aggregates is the key in learning about the fundamental factors controlling tumorigenic and metastatic development. Thus our research focuses on individual and interacting cell behavior in a strictly controlled environment. This controlled in vitro extracellular environment is designed such that adhesion and elasticity can be varied simultaneously and independently. The mechanical properties are reproduced with polyacrylamide gels [34, 35]. In this work, we vary the stiffness of the gels from an elastic modulus of 400 Pascals (Pa), which is close to pre-malignant tissue, to 1250 Pa, which is the elastic modulus of stroma adjacent to the tumor, to 2500 and 7900 Pa, which represents the range of stiffness of tumors [5, 22]. A wide range in FN ligand coating density is tested as well. To decouple the genetic predisposition for tumorigenesis from cell behavior we studied normal non-tumorigenic human breast epithelial cells [6]. Furthermore the cells are not stimulated by any additional growth or soluble factors beyond the growth medium requirement, so not to change, enhance or diminish the intracellular signaling cascades elicited by the ECM. Plating these cells on substrates of mechanical and biochemical properties similar to those in vivo allows us to understanding the key factors that control cell behavior.

In this paper we report the effect of the complex relationship of ECM adhesiveness and compliance on cell migration and cell-cell cohesion. We find there is an optimum where cells are highly motile and invasive and cell-cell cohesion is rarely observed. This optimum is a function of both ligand concentration and matrix stiffness. Further we show that cell-matrix and cell-cell adhesion have complex, overlapping effects on cell behavior. Increasing substrate adhesivity induces an increase in cell motility and thus cell dispersion, but also promotes cell-cell adhesivity. The increase in cell-cell contact duration then in turn decreases cell motility. The effects on cell assembly are modulated by the mechanical properties of the ECM, but not the chemical properties. When substrate elasticity is decreased to that of pre-malignant tissue, cell-cell cohesion is maximized which leads to the formation of three-dimensional aggregates. Our results thus show there exists a delicate balance between cell-matrix and cell-cell adhesion that is controlled through mechano-chemical alteration of the extracellular environment.

Materials

Cell Culture

MCF10A human breast epithelial cells were purchased from American Type Culture Collection and maintained at 37°C and 5% CO₂ in growth media published by Debnath et al [7]. The final media recipe contained DMEM/F12 supplemented with 5% Donor Horse Serum (Invitrogen, Carlsbad, CA), 20 ng/ml EGF (Peprotech, Rocky Hill, NJ), 0.5 ug/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 ug/ml insulin (Sigma-Aldrich, St. Louis, MO). Experimental media was supplemented 1/1000 parts Penn/Strep, Fungizone and Gentamicin (Invitrogen). Experiments were conducted on cells between passages 3 and 8. Cells were synchronized prior to plating. Cells were allowed to fully spread on the functionalized glass for 4 hours and overnight on gel substrates, prior to experimenting.

Gel Preparation

Coverslip activation, N-6-((acryloyl)amino)hexanoic acid (N-6) synthesis and fibronectin (FN) functionalized polyacrylamide gel were all prepared and characterized in accordance with published protocols and considerations [5, 18, 20, 21, 34-36].

Briefly, glass coverslips were chemically activated with 3-aminopropyltrimethoxysilane, washed, incubated with 0.5% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min (Sigma-Aldrich), washed and then air-dried. Polyacrylamide gel solution was prepared with

acrylamide (40% w/v solution), N,N'-methylene-bis-acrylamide (2% w/v solution), TEMED, and APS all purchased from Bio-Rad Laboratories, Hercules, CA buffered with HEPES (Sigma-Aldrich). N-6 dissolved in ethanol was added to the solution. The solution pH was adjusted to 6.0 with 1.2N HCl and the solution is degassed. 10% APS in H₂O solution was added 1/2000 to the final volume, mixed and dispensed onto the activated coverslips. The gel solution was sandwiched with another coverslip and allowed to gel for 45 min with N-6 copolymerizing with acrylamide to generate a reactive gel surface. The gel was then thoroughly washed with ice cold deionized water and excess water aspirated. Fibronectin from bovine plasma (Sigma-Aldrich) dissolved to appropriate concentration in cold PBS was added to the gel surface and incubated at 4°C overnight. The functionalized gel was incubated in 1/100 ethanolamine (Sigma-Aldrich) solution in 50 mM HEPES (pH 8.0) for 30 min and washed extensively in PBS prior to cell plating.

Immunofluorescence and Imaging

Cell nuclei were stained with Hoechst 33342 FluoroPure dye and Qtracker Cell Labeling Kit was used for long term cell tracking (Invitrogen). Cells were imaged in real time with Nikon Inverted Eclipse TE300 microscope (Nikon Instruments Inc, Tokyo, Japan) and a Photometric Cool Snap HQ camera (Roper Scientific, Trenton, NJ) in a temperature controlled chamber at 37°C and 5% CO₂. Fluorescence was detected with 4900 DAPI and 51004v2 FITC/TRITC filter sets (Chroma Technology Corp, Rockingham, VT). A LabView (National Instruments, Austin, TX) program was used to control the mechanical stage and timed exposure for picture capture.

Cells were plated sparsely at 1000 cells/cm² overnight and isolated single or two interacting cells were observed for 3 to 6 hours. Images were taken at 10-30 minute intervals, to minimize long-term light exposure while accurately capturing behavior. Cells on 400 Pascal gels were imaged every hour for 8 hours. The time stamp HH:MM records the time hours:minutes. Each gel experimental condition was repeated at least twice and more than 3 cells or cell pairs were analyzed per experiment. Cell area and two cell interaction duration and distance were quantified manually with NIH ImageJ software (<http://rsbweb.nih.gov/ij/>). Cell velocity was quantified with macros code written in ImageJ software that tracked the fluorescently tagged nucleus. All data was then compiled and analyzed separately for single cells and cell pairs that maintained contact, with code written in Matlab 7.0 software (Mathworks Inc., Natick, MA). Statistical significance was determined with ANOVA regression analysis and student's t-test for pair-wise comparison and p-values reported. Contact fraction is calculated by dividing time two cells are in visible contact divided by time of the entire observation period.

Traction Force Microscopy

Fluorescent bead displacements at the cell-substrate adhesion surface were calculated with respect to the no-cell containing unstressed trypsinized surface. Displacement vectors were used to calculate substrate strain and the corresponding cell traction stresses with LIBTRC 2.x code developed by Prof. Micah Dembo, Boston University [37-39].

Results

Biochemical and mechanical cues of ECM dictate cell migration velocity

Biochemical and mechanical properties and gradients in extracellular environment have been individually shown to affect cell spreading and migration [17, 19, 20, 25]. Cells have been shown to exhibit biphasic velocity profile as a function of compliance [18]. Our further investigation of the combined effect of both cues on cell motility report novel findings that cell speed is a shifting biphasic function of both mechanical and biochemical cues, Figure 1.

Cell migration curve has a maximum as a function of increasing ligand concentration at each compliance (Figure 1A). Regression analysis with ANOVA on 1250, 2500 and 7900 Pa gels yields p-values 0.0001, 0.005 and 0.03 respectively. The maximal peak cell speeds in pair-wise comparison to the respective minimum speed are statistically with p-values < 0.003 at each compliance. There is a shift in curve peaks as the substrate stiffness decreases. Thus to achieve maximum velocity, a higher concentration of ligand is required to compensate for the decrease in matrix stiffness. The inverse relationship between substrate stiffness and ligand density supports the hypothesis that both mechanical and biochemical cues play important compensating roles in cell migration.

On softer 2500 and 1250 Pa gels at FN concentrations below 0.01 mg/ml, cells are not motile, as compared to high motility observed on stiffer 7900 Pa gel or a glass substrates. Also note the biphasic behavior is not observed on 1250 Pa gels. Therefore, very compliant gels reduce the ability of ligand density to modulate motility.

Further comparison of cell motility on gels of different stiffnesses was performed at three FN ligand concentrations, 0.01, 0.05 and 0.1 mg/ml (Figure 1B). The cell speed goes through a maximum as the substrate stiffness increases and the maximum shifts to higher stiffness with decreasing ligand concentration. At lower FN concentration the stiffness of the matrix must be higher to achieve the same speed as at higher FN concentration and lower gel rigidity. Comparatively the effect of FN surface density and stiffness is not the same on cell spread area, which is a monotonically increasing function of both cues (Supplemental Figure 1). Figure 1C is a three-dimensional plot of cell migration velocity as a function of both FN concentration and substrate compliance, combining the results from Figure 1A and B. The plot shows the maximum in migration velocity is reached at an intermediate value of both adhesion and compliance.

ECM mediated cell-cell adhesion slows migration

To explore if cell-cell adhesion hinders cell migration, we compared the motility of individual cells and cells in contact (Figure 2). It is observed that interacting cell pairs have lower migration velocity over individual cells across the majority of surface combinations. The difference is especially prominent at peak migration velocities and becomes statistically significant on more compliant gels, at stiffnesses of 2500 and 1250 Pa (Figure 2A). This difference is observed at higher FN concentrations at and above 0.1 mg/ml.

In Figure 2B, single cell motility and the motility of cells in pairs are compared for three FN concentrations as a function of gel compliance. Slower motility is observed in cell pairs at peak migration velocities, with statistically significant differences at a high FN density, 0.1 mg/ml, on a 2500 Pa gel. Video micrographs of cell pairs and their interactions visually support these quantitative results of the role of matrix adhesiveness and compliance in the velocity of cell motion of cells in pairs (Supplemental Figure 2). Softer compliance and higher ligand density result in similar decreases in the velocity of cells in pairs.

Further quantification of the effect of cell-cell interaction shows novel findings that cell migration depends on duration of cell-cell contact, Figure 3A. Cell-cell interaction is represented by fraction of time over the entire observation period the cells spend in contact. The migration velocity of cell pairs decreases linearly as a function of cell contact duration on FN coated gels. ANOVA regression analysis of the linear fit at each stiffness yields p-values < 0.002 . Best fit analysis of the data also shows the magnitude of the negative slope relating velocity to stiffness is increasing with increasing gel stiffness. Thus cell-cell adhesion has a more pronounced effect and decreases cell motility more acutely on softer 1250 Pa substrate than on stiffer substrates.

Further effect of cell-cell adhesion on migration is illustrated in Figure 3B. The relationship between migration velocity and cell spreading area is quantified for isolated single cells (i) and cells in pairs (ii). For both, the migration velocity is a biphasic function of cell area, although area is a linearly increasing function of substrate adhesion and compliance (Supplemental Figure 1). The maximum velocity is observed for isolated cells at spread area of 750 to 1000 μm^2 . Cells outside this range of spread area are not able to migrate as fast, no matter the ECM stiffness or ligand density. Cell pair contact prevents cells from reaching the same maximum velocity as single cells at that same spread area (Figure 3B).

Cells in contact exert higher maximum tractions

In understanding role of the underlying substrate on cell motility and behavior it is intuitive to measure the forces the cells exert onto the substrate as well. Although traction forces have been measured and reported with a diversity of techniques and in numerous cell types, we found no data published connecting cell forces to cell motility. Here we report new data linking cell motility and average force profiles as well as significant differences between single and connected cells with traction force microscopy methods developed by Dembo et al [37-39].

Figure 4A compares average cell force magnitudes cells exert onto the underlying substrate, while in contact with another cell (empty bars) or alone (black bars). We find that average forces are not statistically different for single cells or cells in pairs. The force magnitudes are linearly increasing with increasing substrate stiffness and ligand density, as expected and published previously [5, 40, 41]. However the maximum traction stresses the cells are able to exert onto the substrate are found to be significantly higher for cells that are in contact than their single cell counterparts, Figure 4B. ANOVA linear regression analysis was performed for Average Force and Maximum Traction as a function of the increasing substrate stiffness and ligand density conditions. For both single cells and cells in contact the linear increase in Average Force and Maximum Traction is statistically significant with all p-values < 0.0001. Maximum traction stresses plotted as a function of cell spread area are consistently greater for cells in cell pairs (Δ) over a range of spread area, Figure D. The maximum stress cells exert is enhanced through a cell-cell connection. A cell-cell adhesion junction activates cell-matrix adhesion sites and the cytoskeleton enough to compensate for the smaller spread area and allows cells in pairs to exert higher stresses onto the ECM.

Plot of cell velocity as a function of average force cells exert on the five different substrates unveils a biphasic relationship for single ($\blacksquare$) cell migration, Figure 4C. This relationship has not been reported previously and confirms the idea that there is an intermediate amount of force and thus cell-substrate adhesivity that elicits maximum migration. However this relationship is observed to be linear for cells that are in contact (Δ). The velocity does not reach the maximum observed in single cells and furthermore continues to increase as it exerts more force onto the substrate. A plausible explanation could be that the maximum peak is shifted. Higher forces measured on higher stiffness gels would answer this hypothesis, but the scope of our experiments was to remain within physiological range of ECM stiffness.

ECM compliance promotes formation of 3D multicellular structures on 2D surface

Cell-cell adhesion's significant effect on cell behavior on soft gels leads us to hypothesize that the reduction in substrate compliance would further promote cell-cell adhesiveness and increase cell assembly. Cells plated on 400 Pa gels coated with 0.1 mg/ml FN were barely motile compared to on stiffer substrates. Cell assembly was observed after 24 hours of incubation on polyacrylamide gel, Figure 5A. As illustrated, it took the cell in the image 7 hours to approach its neighbor. The behavior on stiffer substrates at the same concentration

(see Figure 1B) was much more rapid, with a time for translocation on order of minutes. Cell division and re-arrangement into more spherical or ellipsoid aggregates was observed after 48 hours of incubation on these PA gels, as illustrated in Figure 5B and C.

After 72 hours of incubation on 1250 and 400 Pa gels, close packed cell aggregates were found on the two-dimensional surfaces as illustrated in Figure 6. Similar structures were not observed on stiffer substrates (Supplemental Figure 3). On 1250 Pa gels, the cells formed islands of two-dimensional aggregates, as illustrated in Figure 6A-B. However on 400 Pa gels the aggregates were three-dimensional, as confirmed with fluorescent nuclear stain showing nuclei residing on top of other nuclei, Figure 6C-D. Phase microscopy z-axis focus also confirmed cells at multiple heights of the aggregate, Figure 6E-F.

In addition to the time-lapse phase microscopy images, we further tested whether the aggregates assembled into the three-dimensional structure or proliferated to form the structures. Prior to plating on 400 Pa gels, half of the cell population was incubated with cell tracker beads while the other half was not. The mixed final sample was plated and incubated for 72 hours. Some three-dimensional aggregates were found to have both bead and no-bead containing cells, confirming cell assembly is involved in formation of three-dimensional aggregates. In some three-dimensional aggregates, none of the cells had beads while in others all cells contained beads, implying that cells divided into the structures (Supplemental Figure 4). These results were found on 400 and 140 Pa gels, where the latter is the elasticity similar to that of normal breast tissue (Supplemental Figure 4B). Increasing the stiffness of the substrate and decreasing the ligand density did not produce similar two nor three-dimensional aggregates (Supplemental Figure 3). These cells were observed scattered throughout the gel surface, loosely or completely un-attached to other cells.

Discussion

Mechanical and biochemical properties of ECM individually have been shown to affect cell behavior, specifically cell spreading, migration and interaction [5, 19, 20, 25, 42]. However the extent of ECM dictating cell phenotype and directing cell motility, interaction and tissue formation has not been fully isolated, tested and understood. Here we explore the importance of the two ECM cues acting in concert in a breast cancer model, without the influence of oncogenic or soluble factors.

We report novel findings that cell velocity is a shifting biphasic function of substrate stiffness and also of ligand density. Breast epithelial cells show a maximum in migration velocity with ligand concentration for each substrate compliance and with increasing gel stiffness at constant ligand density, Figure 1. The maximum velocity peak shifts as a function of both stiffness and ligand concentration with an inversely dependent relationship. Therefore to achieve maximum speed, softer mechanical properties must be compensated with higher biochemical stimuli and lack of ligand density is rescued by substrate stiffness. However there is a limit to the rescue ability of the cues for each other, as seen on the 1250 Pa soft gels. Cell motility does not decrease with increasing ligand density. Importantly we also find that it takes a ten fold increase in ligand density to initiate cell motility on softer substrate.

Figure 4C data shows this maximum cell migration of single cells is a biphasic function of the force the cells exert onto the substrate. This relationship is not observed when measuring the motility of cells in pairs over the tested range. Optimum cell migration is also a function of the cell spread area, evoked at multiple specific combinations of the two ECM conditions. Figure 3B(i) shows cell speed is a biphasic function of the cell spread area and that maximal velocity is only achieved by single cells over a narrow range of spread area. Both cues of the

ECM stimulate the cell to spread and dictate the optimum area where the cell can then achieve rates of maximum migration. Furthermore, it has been previously shown that the spread area is directly related to the cells ability to generate force [5, 43] and that maximum cell speed is related to intermediate level of force generation, for numerous integrin and ligand expression levels [44]. Thus we can elucidate the link between intermediate spread area and maximum velocity lies in the cells ability to generate optimum traction with the substrate. Our results conclude that both mechanical and biochemical factors acting together evoke optimum spread area and force generation to achieve maximum velocity.

In this work we also investigated the role of cell-cell adhesion in cell motility. Previously, Reinhart-King and co-workers showed that cell-cell interactions between bovine aortic endothelial cells on compliant surfaces can decrease cell migration and dispersion [21]. We find mammary epithelial cell-cell interaction consistently decreases the velocity of the cells in contact on all substrate configurations (Figs 2, 3 and Supplemental Figure 2). Moreover the magnitude of the reduction depends on ligand density and compliance of the ECM environment. It is the compliance of the ECM that dictates the extent of contact inhibited migration. Although prolonged cell-cell contact decreases cell motility on all substrates, the decrease becomes more appreciable as the substrate stiffness decreases, Figures 2A and 3A. It is physiologically interesting that we find statistically significant decrease in motility on gels of stiffness similar to that of pre-malignant tumor adjacent stroma [5].

We find an additional phenotypic response of higher matrix stiffnesses activated by cell-cell adhesion. Cells in pairs exhibit stronger cell-matrix adhesion than single cells, as illustrated the maximum in traction stress, illustrated in Figures 4B and D. There is also a difference in force-motility relationship caused by cell-cell interaction, Figure 4C. These findings link extracellular environment to collective cell behavior through the connection between cell-matrix and cell-cell adhesions.

Represented in schematic Figure 7, our data implies increasing biochemical stimuli on gel surface activates stronger cell-cell adhesion through a cascade of intracellular molecular events. These activated cell adhesion junctions then maintain cells in continuous contact. Once adherent to each other, the cells' migration is then inhibited. On stiffer substrates, increase in surface ligand concentration beyond the optimum amount continues to strengthen cell-matrix adhesion and decrease cell motility for individual cells and cell pairs alike.

Integrin-cadherin crosstalk and connection has been previously explored [33, 40, 45] and our results suggest it plays a role in collective cell behavior. One possible interpretation of our findings is that focal adhesions are activated by biochemical and mechanical properties of the ECM, whereas adherens junctions (AJs) are activated by biochemical cues and inhibited by mechanical cues. Cell-cell interaction is activated and favored at high ligand density, but is reduced by the rigidity of the ECM. At high stiffness, the adhesion between two cells is not able to override the strength of cell-matrix adhesion due to increasing mechanical cues of the ECM. But on softer gels cell-cell interaction is stronger and thus cell migration is limited and cell-cell assembly is promoted, summarized in Figure 7.

The mechanical conditions of the environment increase the affinity and strength of cell-cell adhesion to such an extent that mammary epithelial cells organize into three-dimensional structures. Mechanical cues on elastic gels of compliance similar to pre-malignant tissue, 400 Pa, shows cells unable to spread nor significantly migrate. However, when in close proximity, cells come together and remain attached. With time they divide and rearrange themselves into spherical three-dimensional structures even on two dimensional gels (Figs 5 and 6). The cells do not need a three-dimensional environment, as they are mechanosensing the softness of the substrate which drives the formation of an energetically optimal spherical

three-dimensional aggregate. These aggregates may be the two-dimensional analogues of the energetically favorable spherical acini structures these cells form in three-dimensional gels [5]. Comparatively, cells on 1250 Pa gels cluster and stick together and form an aggregate of cells, but do not protrude in 3rd dimension (Figure 6). Recently Califano and Reinhart-King showed that bovine aortic endothelial cells assemble into tubular structures on low compliance gels (at elasticity below 1250 Pa) [14].

Thus it may be that cells of all types are encoded with a mechanical sensitivity to substrate elasticity that drives them into multi-cellular structures appropriate to their phenotype. It is interesting to explore the universality of this compliance associated switch further. Thus the flexible substrate induces tissue-like aggregation of MCF10A cells, similarly as the 3T3 cells studied [43] and bovine endothelial cells [14]. These extensive results beg to question other cell types to understand if all cells experience a universal compliance threshold.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by Graduate Research Fellowship from the National Science Foundation and NIH grant HL085303. In addition we are thankful to Eric Johnston for programming and technical assistance.

Abbreviations

ECM	Extracellular Matrix
FN	Fibronectin
Pa	Pascals

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Figure 1.

MCF10A human breast epithelial single cell migration velocity is plotted as a function of both biochemical and mechanical cues of the extracellular matrix (ECM). Single cell velocity is observed on substrates with increasing fibronectin (FN) ligand concentration (A) on (■) glass, and polyacrylamide gels of (◆) 7900 Pa, (▲) 2500 Pa, (●) 1250 Pa Young's elastic modulus. Velocity profile displays a shifting biphasic function of FN concentration. For each gel stiffness, the maximum velocity is statistically higher than corresponding curve minimum, p -values < 0.003 . (B) Gel compliance also dictates single cell motility in a shifting biphasic manner, as seen on (■) 0.01 mg/ml, (●) 0.05 mg/ml and (▲) 0.1 mg/ml FN bulk concentration gels with increasing stiffness. The peak velocity on 0.1 mg/ml 2500 Pa gels is statistically higher than on 7900 Pa gels with p -value < 0.002 . The statistical difference between these stiffnesses on 0.01 mg/ml gels has p -value < 0.01 . (C) 3D mesh plot and underlying 2D projection demonstrate the shifting maximum velocity peak as a function of FN concentration and gel compliance ECM parameters. Each experimental condition was repeated twice. $\pm$ SEM

Figure 2.

MCF10A cell migration velocity of single cells is compared to cells in contact. Velocity is plotted as a function of (A) increasing FN ligand concentration on gels of decreasing stiffness ($\blacklozenge$) 7900 Pa, ($\blacktriangle$) 2500 Pa, ($\bullet$) 1250 Pa Young's modulus and (B) as a function of compliance on ($\blacksquare$) 0.01 mg/ml, ($\bullet$) 0.05 mg/ml and ($\blacktriangle$) 0.1 mg/ml FN bulk concentration. Cell velocity ($\mu\text{m}/\text{min}$) is plotted for single cells (filled markers) and cell pairs in contact (empty markers). Each experimental condition was repeated twice. * p -value < 0.005 , $\pm$ SEM

Figure 3.

Migration velocity is a function of cell-cell contact fraction (A) and cell spread area (B). (A) The slopes of the best fit lines of cell pair motility as a function of cell-cell adhesion duration are -1.0 , -1.9 , and -2.4 for 7900, 2500, and 1250 Pa gels respectively. ANOVA regression analysis of the linear fit at each stiffness yields p -values < 0.002 (B) Cell migration is a biphasic function of cell spread area for (i) single cells (filled markers) and (ii) cells in contact (empty markers). Maximum migration velocity is achieved by single cells in area range of 750 to $1000 \mu\text{m}^2$, which is not observed in cell pair motility. The cell velocity ($\mu\text{m}/\text{min}$) is measured on (■) glass, (◆) 7900 Pa, (▲) 2500 Pa, (●) 1250 Pa Young's modulus.. $\pm$ SEM

Figure 4.

Tractions exerted by cell increase linearly with both substrate stiffness and ligand density, with cells in contact exerting higher maximum tractions. (A) Average traction forces (nN) were calculated for single cells (white bars) and those in contact (black bars) and are plotted as a function of substrate stiffness and adhesivity on soft and rigid substrates, Elastic Modulus 1250 and 7900 Pa respectively. (B) Magnitude of the maximum traction vectors were averaged at each substrate with statistically higher tractions exerted by cell pairs, *p-value < 0.05 and **p-value < 0.002 in a pair-wise comparison. ANOVA linear regression analysis of average force and maximum tractions for both single and cells in contact yields p-values < 0.0001. (C) Migration velocity is a biphasic response to average traction force in single cells (■). This relationship is linear for cell pairs (Δ). (D) Maximum tractions increase linearly as a function of cell spread area with higher slope observed in cell pairs. Each experimental condition was repeated twice. $\pm$ SEM

Figure 5.

MCF10A cells assemble, divide and re-arrange into three-dimensional aggregates on soft gels with elastic modulus similar to pre-malignant tissue. (A) 24 hrs after plating, MCF10A cells were imaged for 8 hours (see methods) and observed to assemble. 48 hours after plating the cells were observed to divide (B) and re-arrange (C) into spherical three-dimensional aggregates on two-dimensional FN coated polyacrylamide gels. All gels are 400 Pa and coated with 0.1 mg/ml FN. * denotes a cell undergoing division. HH:MM. Scale bar = 50 μ m

Figure 6.

MCF10A cells on soft FN coated polyacrylamide gels 24 hours after plating (A and C) and the respective aggregates 72 hours after plating (B, D-F). (A-B) Two-dimensional aggregates on 1250 Pa gels coated with 0.1 mg/ml FN. (C-D) On softer 400 Pa gels coated with 0.1 mg/ml FN, three-dimensional aggregates are shown with inset fluorescent image of Hoechst dye stained nuclei in third dimension locations (D). (E-F) Bottom to top view of a three-dimensional aggregate 72 hrs after plating, with $z=0\ \mu\text{m}$ at the gel surface and slice $32\ \mu\text{m}$ above on 0.1 mg/ml FN coated 400 Pa gel. 72 hr snapshots with fluorescent beads were taken to test whether cells assemble or proliferate into three-dimensional structures via pre-incubating half of the cells with fluorescent beads and mixing equally with unlabeled cells prior to plating. * denotes a cell undergoing division. Scale bar = $50\ \mu\text{m}$

Figure 7.
Schematic summary of effects of biochemical and mechanical cues of the ECM on cell-cell and cell-matrix interactions leading to cell motility and cell-cell assembly.