CANCER METASTASIS AND ELASTICITY OF MICROENVIRONMENT

BY

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DISSERTATION

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ABSTRACT

Cancer deaths are primarily caused by metastases, not by the parent tumor. During metastasis, malignant cells detach from the parent tumor, and spread through circulatory system to invade new tissues. Due to the intrinsic difficulties of predicting and monitoring in vivo metastasis, the physical-chemical mechanisms and parameters within the cellular microenvironment that initiate the onset of metastasis remain largely unknown (Chapter 1). Such a gap in the understanding and prediction of the onset of metastasis is of particular concern in colon cancer. We discovered that HCT-8 colon cancer cells can be induced to undergo a phenotypic transition similar to the early stage of metastasis (MLP, i.e. metastasis-like phenotype) simply by growing them on a substrate with appropriate mechanical stiffness (Chapter 2). This metastasis-like transition is observed as a change from a flattened, epithelial cell (E cell) to a rounded, dissociated cell morphology (R cell).

We have carried out a comprehensive biophysical, biochemical and animal metastasis study to explore this E-R transition (Chapter 3). We found, R cells express a remarkable number of in vitro biophysical and biochemical metastasis hallmarks, such as loss of cell-cell adhesion and cell-substrate interaction, gain of anchorage-independence growth, decrease of cell elasticity, alteration of migration patterns in blood capillary, mimicking micro-channels and increase of stem cell markers expression (Chapter 4). RNAseq analyses indicate metastasis-enhancing gene expression pattern was activated in R cells. The results of both in vitro invasion assays and in vivo animal model metastasis experiments verified that R cells are significantly more invasive and tumorigenic than the original E cells that were never exposed to soft substrates (Chapter 5). Furthermore, we demonstrated additional cancer cell lines (SW480, HCT116 colon cancer cells and DU145 prostatic cancer cells) also exhibit a similar E-R transition following culture on the
appropriate mechanical microenvironment. This *in vitro* model may provide unique opportunities to enable the dissection of not only the early mechanical and molecular events responsible for the ability of colon cancer cells to sense and respond their tumor mechanical microenvironment but also the downstream molecular mechanisms leading to the onset of metastasis (Chapter 6). We provided in-depth discussion and proposed a mechanosensing model to explain this E-to-R transition (Chapter 6). Our findings may also help to identify new molecular markers of the early stages of metastasis and for the design of anti-metastatic therapeutics.

Apart from the major focus of this dissertation, i.e. an *in vitro*, mechanics-induced metastasis-like phenotype (MLP), we also investigated the influence of the mechanical microenvironment on cell behavior in three additional projects: (1) the investigation of whether cardiac cells can interact with one another mechanically, and if so, how does the interaction depend on cell-cell separation, and the stiffness of the medium (Chapter 7); (2) development of a simple, novel and general method to pattern a variety of cell adhesion molecules, i.e. Fibronectin (FN), Laminin (LN) and Collagen I (CN), etc. and living cells on PA gels (Chapter 8); and (3) development of a finite-element-method-based cell traction force microscopy (TFM) technique to estimate the traction forces produced by multiple isolated living cells as well as cell clusters (Chapter 9).
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CHAPTER 1. INTRODUCTION

1.1 Overview of Cancer Metastasis

Metastasis, the spread of cancer cells from the primary tumor and invasion to new sites, is responsible for 90% of cancer mortality [1-3]. During metastasis, malignant cancer cells escape from the tumor by detaching from one another or from other stroma cells and the extracellular matrix [1, 4-6]. The escaped cells actively up-regulate proteinases and alter their adhesion ligands to degrade and modify their surrounding ECM [1, 2, 7-9]. Concurrently, they up-regulate their motility and the resistance to apoptosis for successful vascular spread and invasion of distant healthy tissues/organs.

Successful identification of metastasis-triggering signals is critical for the design of novel anti-metastasis therapeutics. Unfortunately, the signals and associated molecular mechanisms regulating metastasis remain enigmatic to date [3, 10, 11]. It has been long believed that, in addition to intrinsic genomic alterations of tumor cells, the progress of malignancy also can be driven by extrinsic microenvironment cues, such as MMP proteases released by activated stromal cells [5, 12], persistent inflammation associated with tissue wounding [13-16], and the loss of apicobasal polarity in surrounding epithelial cells [17, 18]. The relative contribution of these extrinsic and intrinsic cues; however, as well as the influence of the mechanical microenvironment on the regulation of tumor disassociation and metastasis, is not known.

1.2 Mechanical Microenvironment and Cancer Metastasis

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Increasing evidence indicates the mechanical microenvironment plays a critical role in regulating tumor cell responses [19, 20]. Tumor cells sense, and respond to external mechanical cues by coordinated reorganization of the actin network, and cell-cell and cell-substrate adhesion pattern/molecules [11, 21]. For example, mammary epithelial cells form normal acinar parenchyma when cultured on substrates of physiological stiffness but display the structural and transcriptional hallmarks of a developing tumor when cultured on ECMs of stiffness resembling tumor stroma [22]. When *in vivo* dormant breast cancer cells are cultured on 2D *in vitro* plastic dishes, they readily proliferate regardless of their *in vivo* behavior. Surprisingly, when these same cells are grown in a 3D culture matrix, they show distinct growth properties that correlate with their dormant or proliferative behavior at metastatic sites *in vivo* [23]. Furthermore, an appropriately soft fibrin gel microenvironment produces a metastatic variant of murine B16-F1 melanoma cells that are highly tumorigenic in animal models [24]. There is no evidence; however, showing that a metastasis-like phenotype can be triggered by mechanical cues when cancer cells are cultured on a 2D substrate *in vitro*.

In this dissertation, for the first time, we report experimental evidence indicating human colon carcinoma (HCT-8) cells can exhibit a metastasis-like phenotype (MLP) *in vitro* when cultured in the presence of an appropriate 2D mechanical microenvironment. We used the term, MLP, since the cells exhibit several *in vivo* metastatic characteristics. These include dissociation from parent colonies, sustained proliferation and increased motility, down-regulation of E-cadherin expression, reduction of cell adhesion (both specific and non-specific), alteration of cell elasticity, up-regulation of MMP2, expression of a metastasis-enhancing gene pattern, and a stable cell-state-transition [1-3, 5, 8, 25-27]. This *in vitro* MLP raises the possibility that the *in
\textit{vivo} mechanical force balance between cellular structures and external microenvironment may serve as a signal to trigger the onset of metastasis.
CHAPTER 2. MECHANICS-INDUCED METASTASIS-LIKE TRANSITION

2.1 Introduction

We examined human colon carcinoma (HCT-8) cells cultured on four substrates of different mechanical stiffness to resemble a wide range of physiological tissue mechanical microenvironments [28, 29]. Polyacrylamide (PA) hydrogels were fabricated to have varying mechanical stiffness: 1.05 ± 0.17 kPa, 20.73 ± 1.03 kPa and 47.05 ± 1.86 kPa. 1.05 ± 0.17 kPa were used to mimic mammary gland, lymph node, brain and breast tissues, because they have stiffness ranging from 0.1 to 2 kPa [22, 29-31]. 20.73 ± 1.03 kPa were prepared to mimic embryonic myocardium, muscle, lung, normal and fibrotic human liver, which possess stiffness ranging from 9 to 25 kPa [32-36]. 47.05 ± 1.86 kPa were used to mimic in vivo cartilage [37, 38]. Since most routine cell culture is performed on polystyrene tissue culture petri dishes (Elastic modulus: ~ 3.6 GPa), polystyrene served as the fourth substrate. The stiffness of all PA gels and living cells was measured by Asylum Atomic Force Microscopy [39] (Fig. 2.1). To avoid the bias from an ECM effect, substrates with same mechanical stiffness were functionalized with fibronectin, laminin and collagen, respectively. We used two distinct cell densities in culture, 150,000 cells / cm², and 50,000 cells / cm².

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Figure 2.1: The micromechanical properties of PA gels and HCT-8 cell monolayers were determined by atomic force microscopy (AFM). (a) The plots of AFM tip indentation force
versus the tip indentation (δ) into the substrates or cell surface. All experiments were carried out in aqueous environment at 37°C. We used conical-shaped tip model (20 different lateral indentation positions for each substrate). (b) The histogram of microscopic Young’s modulus (E) of substrates and HCT-8 cell monolayers. The Young’s moduli were obtained by fitting the force versus indentation plots with the appropriate indentation model (see Section 2.2).

2.2. Experimental Setup and Working Principle

Human colon adenocarcinoma HCT-8 cells (ATCC catalog No.: CCL-244) were cultured in the cell media consisting of RPMI 1640 (Gibco No.: 23400-062) supplemented with 2 grams sodium bicarbonate per liter, 10% horse serum (Gibco No.: 26050-088), 1× antibiotic-antimycotic (Gibco No.: 15240-062) and 1mM sodium pyruvate (Gibco No.: 11360). Ma104 epithelial cells (embryonic African green monkey kidney; from M.A. Bioproducts) were cultured in the cell media consisting of MEM (Gibco No.: 41500-018) supplemented with 2 grams HEPES per liter, 2.2 grams sodium bicarbonate per liter, 1× antibiotic-antimycotic (Gibco No.: 15240-062), and 5% fetal bovine serum. The PA gels substrates were made with varied relative concentrations of acrylamide (Bio-Rad) and N, N’- methylene bis-acrylamide (Bio-Rad) to obtain different density of cross-link. The density of cross-link controlled the elastic moduli of hydrogels. The relative concentration of acrylamide and N, N’- methylene bis-acrylamide and corresponding elastic moduli of PA gels are shown in Table 2.1. To enable cell adhesion, the surface of PA gels was covalently coated with human fibronectin, laminin or collagen (BD Science) with concentration varied from 4 µg/mL to 25 µg/mL.
Table 2.1: The varied relative concentrations (mol. /v) of acrylamide and N, N’- Methylene bis-acrylamide solutions used to fabricate polyacrylamide (PA) gels with different Elastic modulus. The stiffness of all PA gels was determined by Atomic Force Microscopy (AFM).

<table>
<thead>
<tr>
<th></th>
<th>Soft PA gels</th>
<th>Intermediate-stiff PA gels</th>
<th>Stiff PA gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (mol./v)</td>
<td>8%</td>
<td>8%</td>
<td>8%</td>
</tr>
<tr>
<td>Bis-acrylamide (mol./v)</td>
<td>0.01%</td>
<td>0.13%</td>
<td>0.48%</td>
</tr>
<tr>
<td>Stiffness (kPa)</td>
<td>1.05 ± 0.17</td>
<td>20.73 ± 1.03</td>
<td>47.05 ± 1.86</td>
</tr>
</tbody>
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Atomic force microscopy with silicon-nitride cantilever was used to determine the stiffness of the PA gels as well as HCT-8 cell monolayer. Following indentation experiments using contact mode, we fitted the applied force vs. indentation displacement curves by the conical-tip approximation (Eqn. 2.1) and extract the substrates elastic modulus [39]:

$$z - z_0 = (d - d_0) + \sqrt{\frac{k(d - d_0)}{2\pi E(1 - \nu^2)\tan(\alpha)}}$$  \hspace{1cm} (2.1)

In Eqn. 2.1, z and d are cantilever’s base PZT displacement and the cantilever tip deflection, respectively. $z_0$ is the vertical position of piezo-controller as it drives the AFM tip to contact the substrate surface, and $d_0$ is the initial cantilever deflection prior to bending. $\nu$ is the Possion’s ratio of to-be-characterized hydrated substrates ($\nu = 0.45$ to 0.5 in present study). $\alpha = 35^\circ$ is the half open-angle of cantilever tip. The spring constant of silicon-nitride cantilever, k, is pre-calibrated as 148.14 pN/nm using standard thermal method.
We used the live-dead assay (kit No. L7012, Molecular Probes, Invitrogen) to investigate cell viability. A working solution was prepared with 1 part of kit component dye A (Syto 9) and 1 part kit component dye B (propidium iodide, “DEAD”) in 100 parts PBS. DAPI was used for staining cell nuclei. Rhodamine phalloidin (520/650, red) was used as fluorescent conjugate to bind specifically to F-actin filaments (not G-actin). We used an inverted optical microscopy (Olympus IX81, Olympus America) and a high-speed SPOT camera to record phase-contrast video of cell behavior. To minimize any change in the environment during continuous imaging, a temperature controller equipped environment chamber (PrecisionControl LLC) was used to maintain controlled condition (appropriate humidity, 5% CO2, and 37°C) throughout the time-lapse video recording. A Leica SP2 confocal microscopy (Leica SP2, Heidelberg, Germany) with Amira (Advanced3DVisualization and Volume Modeling) software was used to investigate cell nuclei, actin structures, Vinculin, MMP2-9 and E-Cadherin.

2.3 Metastasis-like Phenotype (MLP) on 21-47 kPa Gels

On 21 kPa and 47 kPa PA gels, HCT-8 cells first adhered to substrates, divided, and attached to each other. They then formed cell colonies with well-defined boundaries (Fig. 2.2a) in 2-4 days. Depending on initial seeding density, each colony consisted of 100s-1000s of well-attached cells. For identical seeding density, the colony size on 47 kPa gels was consistently larger than that on 21 kPa gels. On 1 kPa gels, cells did not spread sufficiently but remained rounded, possibly due to a lack of cell traction [40, 41], and occasionally formed small 3D colonies (Fig. 2.3a and 2.3b). On hard polystyrene substrates with mechanical stiffness of 3 GPa, the cells spread completely and reached 100 % confluence by the end of 2 days (Fig. 2.3c and 2.3d). After seven days, we found on 21 kPa and 47 kPa gels, some mitotically-competent and
motile single cells began to disassociate from colony boundaries and migrated away from the cell colonies (Fig. 2.2b). The dissociated cells lost their epithelial phenotype and display spherical shapes with a shape factor of $0.92 \pm 0.03$ (Fig. 2.2 c).
Figure 2.2: Human colon carcinoma (HCT-8) cells show metastatic-like phenotype (MLP) after 7-day culture on intermediate stiffness gels (E=21 kPa, with fibronectin
**functionalization**). (a) HCT-8 cells formed cell colonies on intermediate stiffness gels in 2-4 culture days. (b) Some single cells, as indicated by arrow, began to dissociate from colonies on the 7\textsuperscript{th} day. (c) An entire colony disassociated into individual cells after 11 days. (d) Same original HCT-8 cells plated on hard polystyrene substrate (regular tissue culture Petri dish, $E = 3.6$ GPa) under the same culture condition as in (a)-(c) formed a confluent layer and did not show MLP. Scale bar: 100 µm. (e) 2D migration trajectories of 5 randomly selected disassociated cells on 21 kPa PA gels’ planar surface. Z-axis is temporal coordinate. (f) Plot of absolute distance of the same 5 randomly selected disassociated cells from the origin versus time. The black dots indicate the times when cells were undergoing mitosis. (g) Selected frames from a real-time video recording a representative cell disassociation cascade from a cell cluster on 21 kPa PA gel. This disassociation process was finished within 5 hours and disassociated R cells extruded filopodia and migrated away from the parent E island. The time scale is Hours: Minutes.

Confirmed by dye exclusion live-dead assays, these dissociated cells retained full viability. Within 2 weeks of culture on 21 kPa gels, 70% ~ 90% of the cell colonies completely disassociated into numerous single cells (Fig. 2.2c) and the population of dissociated cells increased dramatically. No cell disassociation was found on very soft 1 kPa gels (Fig. 2.3a-b) or hard polystyrene substrates (Fig. 2.2d and 2.3c-d) under the same culture conditions. The cell dissociation phenotype was independent of substrate functionalization, but the time to the onset of dissociation was different - 7 days for fibronectin and collagen functionalization, and 15 days for laminin functionalization. Furthermore, the occurrence of dissociation process and time for its initiation is independent of original HCT-8 cell passage number. However, we found the use
of lower passage HCT-8 cells resulted in a larger percentage of disassociated cells: at the 7th day of culture on 21 kPa gels, 100% of the colonies dissociated from HCT-8 cells with passage 6, whereas only 5% of the colonies dissociated for cells with passage ≥ 43. Nevertheless, after extended duration of culture, most colonies dissociated. Our time-lapse video imaging (Fig. 2.2e-g) shows that once dissociation starts, it takes 5-10 hrs for the colony to be completely dissociated, a process that was much faster than that of colony formation (2-4 days). After disassociation, these cells extruded long filopodia and migrate away in a random fashion with a maximum migration speed $0.9 \pm 0.2 \, \mu\text{m/min}$ (measured on 2D flat substrates, n=25). Normal, non-cancerous epithelial (Ma104) or Bovine endothelial cells cultured under the same conditions as described above, showed cell colony formation, but no cell dissociation (Fig. 2.4). In the following Sections, we will present results showing that this in vitro dissociation process share a number of characteristics with in vivo metastasis transition. Therefore, we titled this process as the acquisition of “metastasis-like phenotype (MLP)”. We also named the original flattened, epithelial HCT-8 cell and rounded, dissociated cell as E and R cells, respectively.
Figure 2.3: HCT-8 cells cultured on either very soft PA gels (1 kPa) or stiff polystyrene substrates (~3.6 GPa) did not show metastasis-like phenotype (MLP). (a) Phase-contrast picture of HCT-8 cells cultured on 1 kPa gels after 4 days. (b) Phase-contrast picture of HCT-8 cells cultured on 1 kPa gels after 7 days. They did not show metastasis-like phenotype (MLP). (c) HCT-8 cells cultured on stiff polystyrene substrates (~3.6 GPa) formed continuous cell layers after 2~3 culture days, but did not show the MLP cell dissociation process. Phase-contrast image shows HCT-8 cells cultured on polystyrene substrates after 4 days. (d) Phase-contrast image of HCT-8 cells cultured on polystyrene substrates after 10 days. Cells did not show the MLP. Scale bar: 100 µm.
Figure 2.4: Normal cells cultured under the same condition did not show MLP. (a) Normal MA104 cells (African Green Monkey Kidney) cultured on 21 kPa PA gels and stiff polystyrene substrates (E= 3.6 GPa) after 11 culture days. Scale bar: 100 µm. (b) Normal Bovine endothelial cells cultured on 21 kPa PA gels and stiff polystyrene substrates (E= 3.6 GPa) after 11 culture days. Scale bar: 200 µm.
2.4 Stable Metastasis-like Phenotype (MLP)

To address the question whether the HCT-8 cell transition to the MLP is reversible when cells are re-exposed to hard substrates, we harvested the disassociated HCT-8 R cells after 14 days of culture on 21 kPa PA gel substrates by two independent methods: (1) trypsin treatment of the whole PA gel surface, and (2) mechanical removal of the dissociated cells from PA gel surface by gentle fluid shear. The detached cells were carefully re-plated onto both fresh 21 kPa PA gels and hard polystyrene substrates. Surprisingly, we found the disassociated HCT-8 R cells retain their disassociated phenotype regardless of the degree of stiffness of the new substrates and the method of harvesting (Fig. 2.5). This response to the substrate stiffness is drastically opposite to that of the original HCT-8 E cells maintained on polystyrene culture dishes (Fig. 2.2a and 2.3c). Besides the loss of response to substrate stiffness, the disassociated HCT-8 cells consistently maintained the disassociated phenotype following three additional passages in culture on polystyrene dishes. Additionally, comparison of the E-Cadherin staining of both the original HCT-8 cells and harvested disassociated HCT-8 cells re-plated on polystyrene substrates showed a sustained loss of E-Cadherin (reduced 4.86 ± 1.91 times) in the re-plated HCT-8 R cells (Chapter 3).

Our results suggest that exposure of HCT-8 E cells to the intermediate stiff mechanical microenvironment (21 ~ 47 kPa) has stably, perhaps irreversibly, locked them into a MLP that is more characteristic of a dissociative rather than associative (monolayer) or anchorage-dependent cell growth. While culture on hard substrates (polystyrene) preserved the original E cells’ associative cell growth phenotype typical of in vitro epithelial monolayers for more than 50 passages, a single exposure to intermediate soft PA substrate triggered these cells to transition to a new, dissociative, MLP cell state in only 7 days. The results of these harvest-and-re-culture
experiments also imply that HCT-8 cells became disassociated in response to specific mechanical signals, rather than non-specific degradation of local microenvironments during long-term in vitro culture.

**Figure 2.5: The overview of stable in vitro MLP transition.** HCT-8 E cells were first cultured on 21 kPa PA gel with surface functionalized by fibronectin. By 14 days of cultured, most of the cell colonies disassociated. These disassociated R cells were harvested by trypsinizing the whole PA gel surface and re-cultured on fresh fibronectin-coated PA gel (E= 21 kPa), and hard polystyrene substrates (E = 3.6 GPa). (a1-a3) The re-cultured disassociated R cells on 21 kPa PA gels on 2nd, 5th and 8th culture days. (b1-b3) The re-cultured R cells on hard polystyrene substrate (E = 3.6 GPa) on 2nd, 5th and 8th culture days, respectively. In both cases, the cells persistently retained their disassociated phenotype (MLP) regardless of the degree of stiffness of the new substrates, in contrast to forming colonies on soft gels and monolayer on hard substrates.
(c1-c3) and (d1-d3) show the original HCT-8 cells cultured under same condition on PA gel and polystyrene substrates. Note on 8th day, dissociation of colonies happened and cells showed MLP.

2.5 Fate Decision of E-to-R Transition

To examine when the cell fate for HCT-8 E-R transition is decided on 20 kPa gels, we carried out cell re-plating experiments by switching the culture substrates from 20 kPa PA gels to 3 GPa polystyrene substrates (~3 GPa) for different periods, i.e. 5 hrs, 1 day, 2 days, 3 days, 4 days, and 5 days. On the 6th culture day, the R cells percentages in all conditions were quantified (Fig. 2.6). HCT-8 cells having been cultured on PA gels for less than 2 days showed 100 % E phenotype on 6th day on polystyrene substrates (Fig. 2.6a). However, after being cultured on PA gels for 3 days, 2.18 ± 0.01 % among all HCT-8 cell populations on polystyrene substrates show R cell phenotype on 6th day (Fig. 2.6b).

Furthermore, after being cultured on gels for 4 and 5 days, respectively, 0.94 ± 1.10 % and 6.03 ± 3.52 % of cells on polystyrene substrates showed R cell phenotype (Fig 2.6c and d). The quantitative T-test characterization of R cell percentage showed that, the R cell percentages were significantly more pronounced after 4 - 5 days culture on gel than those after 1-3 days culture with p-value < 0.0001 (Fig. 2.6e). Also, the cells exposed to soft environment for more than 4-5 days showed larger numbers of thick 3D cell spheroid structures, which were spatially closer to emergent R cells (Fig 2.6d). In contrast, the cells exposed to soft environment for less than 3 days showed only cell monolayer, and did not possess spheroid structures (Fig. 2.6a). These observations indicate, though the dissociation phenotype on gels doesn’t happen until 7th
day, the fate of E-R transformation under the mechanical microenvironment influences has been decided and starts at much earlier time, i.e. on 3rd day.
Figure 2.6: E-R transition requires 3-4 days of continuous culture on soft substrates. To determine the R cells percentage after varied culture days on gels, we harvested and re-plated
cells to stiff polystyrene substrates, and quantifies R cells numbers at same time point: 6\textsuperscript{th} day. (a) HCT-8 cells having been cultured on gels for 2 days were cultured on polystyrene substrates for 4 days. Pure E-phenotype were observed. (a1-a2) Enlarged yellow zones in (a) and show the representative cell phenotype. (b) HCT-8 cells having been cultured on gels for 3 days were cultured on polystyrene substrates for 3 days. Note there were some dissociated R cells among larger E cells populations; (b1-b2) Enlarged yellow zones in (b) and show the representative MLP cell phenotype. (c) HCT-8 cells having been cultured on gels for 4 days were cultured on polystyrene substrates for 2 days. Note R cells at the E-cell colonies edges. (c1-c2) Enlarged yellow zones in (c) and show the representative MLP cell phenotype. (d) HCT-8 cells having been cultured on gels for 5 days were cultured on polystyrene substrates for 1 day. Note the appearance of both R cells and thick cell domes (indicated by blue arrows) among E cells. (d1-d2) Enlarged yellow zones in (d) and show the representative MLP cell phenotype. (e) Quantitative summary of R cells percentage of all above 4 cases.

To investigate the continuity of E-R transition, we maintained the same overall culture durations of HCT-8 cells on PA gel of intermediate stiffness, 21 kPa (4 days) and stiff polystyrene substrate (4 days), but strategically varied their starting culture time points on soft and hard substrates (Fig. 2.7a). The HCT-8 cells, which were harvested from PA gels at different time points (D0: 5 hrs; D1: 1 day; D2: 2 day; and D3: 3 day), were exposed to polystyrene for the same amount of time (4 days), and re-plated back on soft gels. The exposure duration of these cells on 21 kPa and polystyrene are the same, but the time points for harvesting are different (Fig. 2.7a). The percentages of MLP for all cases were examined daily after 7\textsuperscript{th} culture day to probe the characteristic of E-R transition. We found, the E cells cultured on PA gels for 3
days show MLP transition on PS substrate after 7 days, while those cultured less than 3 days on PA gels show comparative amount of MLP transition after they are re-exposed to PA gels for another 6-7 days (Fig. 2.7b). The results indicate, after only 3 days culture on PA gel, HCT-8 cells can propagate towards R stage despite the change of mechanical microenvironment. Furthermore, even the overall exposure on PA gels are the same 3 days, our results indicate that only the continuous and non-interrupted 3-day exposure can induce the E-R transition. For cells cultured on PA gels with several continuous but less-than-3-day exposures, E-R transition was not carried out. In another word, the E-to-R transition for cells is not accumulated continuously (Fig. 2.7b).
Figure 2.7: Investigation of accumulatbility of the E-to-R transition. The cells grown on soft gels substrates were transferred to rigid substrates after different culturing times. After letting the cells being cultured on the rigid substrates for four days, they were transferred back to the soft substrates. (a) By maintaining HCT-8 cells the same overall culture durations 3 days on soft
microenvironment and 4 days on stiff microenvironment, we strategically vary their starting culture time points on soft and hard substrates, respectively (D0, D1, D2, and D3). (b) We examine the percentage of MLP for all cases at the same time point (from 7th day to 14th day) to explore the characteristic of E-to-R transition. The statistical analysis of cell islands showing R dissociated cells on PS substrates with 4 different interrupting scenarios for culture. The quantitative observation time is from 7th day to 14th day.

2.6 Cellular Microstructures

To study the influence of mechanical microenvironment on cell microanatomy and functionality, we visualized the three dimensional structure of actin cytoskeleton of HCT-8 cells using laser-scanning confocal microscopy. For this purpose, the cells were fixed and the actin network was visualized by staining with phalloidin-rhodamine labeled antibodies (Fig. 2.8a-c). We found that, prior to dissociation, cell colonies on 21 kPa PA gels show well-defined actin networks near the substrate. The network spans the entire colony (Fig. 2.8a1). HCT-8 cells in the uppermost layer of the cell colonies display cortical actin around the cell membrane, but no intracellular stress fibers (Fig. 2.8a2 and a3). In contrast, cells cultured on hard polystyrene substrates (which do not dissociate) show well-aligned actin stress bundles within individual cells (indicated by arrows in Fig. 2.8c), implying large intracellular tension forces. This is expected from earlier studies which showed a correlation between substrate stiffness and intracellular forces as well as actin polymerization [42]. After disassociation, HCT-8 R cells on PA gels show only cortical actin structures, and no actin stress bundles (Fig. 2.8b), implying low intracellular and cell-substrate forces. These observations suggest that low intracellular forces exist in cells on 21 ~ 47 kPa substrates before dissociation compared to those experienced by
monolayer cells in contact with a hard polystyrene or glass substrate. Surprisingly, disassociated HCT-8 R cells that re-cultured on hard polystyrene substrates also show less filamentous actin (by $1.8 \pm 0.1$ times) than the original HCT-8 cultured under the same condition.

Since the cell nucleus and actin filaments are physically-linked [43], and the cell nucleus can be stretched by actin filament tension [44, 45], we investigated nuclear stretching using DAPI staining of HCT-8 cell nuclei in cells grown on both 21 kPa gels and stiff polystyrene substrates (Fig. 2.8d). To quantify the extent of nucleus stretch, we defined a nucleus-stretching factor as the length ratio of elliptical major axis to minor axis. We found that the nuclei in HCT-8 cells on stiff polystyrene substrates are stretched (Fig. 2.8d1-d4) with a nucleus stretching factor (major to minor axis ratio) of $2.82 \pm 0.86$ (Day 1), $2.51 \pm 0.33$ (Day 3), $2.42 \pm 0.65$ (Day 5) and $2.40 \pm 0.36$ (Day 7). While on 21 kPa gels (Fig. 2.8d5-d9), HCT-8 cells display less stretched and more circular-shaped nuclei, with a nucleus stretching factor of $1.26 \pm 0.17$ (Day 1), $1.39 \pm 0.26$ (Day 3), $1.31 \pm 0.30$ (Day 5) and $1.37 \pm 0.24$ (Day 7). Our results indicate the intracellular forces on 21 kPa gels are lower than those on stiff polystyrene substrates.
Figure 2.8: Actin cytoskeleton and nuclear deformation of HCT-8 cells grown on 21 kPa gels and stiff polystyrene substrates were visualized and quantified using confocal microscopy. HCT-8 E cells cultured on 21 kPa PA substrates show diffuse and cross-cellular actin network, whereas cells cultured on hard substrates (E= 3.6 GPa) display well-defined and
intracellular actin stress bundles. The cells on stiff polystyrene substrates show highly stretched nuclei compared to those on 21 kPa gels. The nucleus stretching is consistent with the actin cytoskeleton organizations and indicates that the intracellular tension force in HCT-8 cells on 21 kPa gels are lower than those in cells on stiff polystyrene substrates. (a) HCT-8 E cells colonies on 21 kPa gel substrates on 5th culture day. Intense cross-cellular F-actin filaments (indicated by white arrowheads in Fig. a1) are found at the interface of cell colony bottom and gel substrate top (Fig. a1). In contrast, R cells show only cortex actin as altitude increases (white arrowheads, Fig. a2, a3 show different upper layers with altitude as y-axis). Scale bar: 25 µm. (b) Disassociated HCT-8 R cells on 21 kPa substrates on 7th culture day. R cells only show cortex actin (white arrowheads) throughout the cell body, without any detectable stress bundles. Fig. b1 and b2 show cellular actin organization at different altitudes. Scale bar: 15 µm. (c) Cell monolayer on polystyrene substrates (E = 3.6 GPa) on 7th culture day showing intracellular well-aligned actin stress bundles (white arrowheads, Fig. c1), implying large intracellular tension forces. Fig. c2 shows the actin cytoskeleton of the same cell layer at higher elevation. Scale bar: 20 µm. (d) d1-d8 show DAPI staining of nuclei of HCT-8 E cells on 21 kPa gels and stiff polystyrene substrates on 1st, 3rd, 5th and 7th culture day. d9 shows the dissociated cells with DAPI appearing on 7th day. Scale bars: 10 µm. The nucleus-stretching factor is defined as the length ratio of elliptical nucleus major to minor axis (d10 inset, n = 30).

2.7 Metastasis-like Phenotype (MLP) Inhibited by Blebbistatin

To test whether intracellular force serves as the mechanical cue for MLP transition, we used blebbistatin, a potent inhibitor of non-muscle myosin II ATPase [46, 47], to abolish the intracellular force generated by myosin II in HCT-8 cells on 21 kPa gels. We adjusted the
intracellular force levels by varying the dosages (0 µM, 2 µM, 5 µM and 10 µM) of blebbistatin. It is shown that blebbistatin can disrupt directed cell migration, but it does not block cell movement [48, 49]. We found, without any blebbistatin or with the lowest dosage of blebbistatin (2 µM), the disassociated R cells emerge from the boundaries of the cell colonies after 6~7 days of culture (Fig. 2.9). The daily percentages of cell clusters dissociating in dishes without blebbistatin were 5.7 ± 2.3 % (6th day), 13.3 ± 4.9 % (7th day), 17.3 ± 8.2 % (8th day), 31.2 ± 7.2 % (9th day). With 2 µM blebbistatin, the percentages were 4.1 ± 0.7 % (6th day), 9.3 ± 7.3 % (7th day), 6.0 ± 5.9 % (8th day) and 4.6 ± 4.0 % (9th day). However, for dishes with 5 µM and 10 µM of blebbistatin treatment, no dissociation could be identified throughout the entire 30-day culture time (Fig. 2.9). Together, our results suggest the appropriate intracellular force generated by myosin II contributes to triggering the MLP cell state transition.
Figure 2.9: Blebbistatin inhibits cell dissociation process by inactivating Myosin II. (a) Various dosages of blebbistatin, 0 µM, 2 µM, 5 µM, and 10 µM, are used to inhibit the myosin II activities of HCT-8 cells cultured on 21 kPa gels and reduce intracellular force. The HCT-8 E
cells without blebbistatin or with the least amount of blebbistatin (2 µM) treatment initiate the
cell dissociation process after 6-7 days. The cells with higher blebbistatin concentrations (5 µM
and 10 µM) do not show any cell dissociation. The red arrows point to the dissociated cells.
Scale bar: 100 µm. (b) Percent of cell clusters dissociated following 6-9 days in culture on PA
substrates in the presence and absence of blebbistatin. These data suggest appropriate
intracellular forces may contribute to triggering of the cell dissociation process.

2.8 Discussion and Conclusion

The studies reported in the present Chapter describe a remarkable influence of the
mechanical microenvironment on the behavior of HCT-8 colon cancer cells in vitro. Exposure of
HCT-8 cells to PA gel substrates of appropriate mechanical stiffness triggers a dramatic
transition, from an epithelial phenotype to a metastasis-like phenotype (MLP). The dissociated
HCT-8 cells display a number of in vivo metastatic hallmarks.

A number of reports have shown that normal tissue cells sense, adjust, and change their
function in response to their mechanical microenvironment as much as they do in response to
soluble chemical messengers [41, 50-54]. In particular, a mechanical-signal-mediated normal
cell dispersal phenotype was reported in [48]. Here, it is reported that clusters of normal cells in
contact with very soft (4.41 ± 0.57 kPa) PA gel substrates remain as stable clusters. However, the
clusters disperse and the cells dissociate from the clusters when they are in contact with stiffer
substrates (12.40 ± 1.61 kPa PA gel or polystyrene substrates). This substrate stiffness-driven
dispersal and durotaxis [55] have been hypothesized to originate from the competition between
cell-ECM versus cell-cell signals. As the substrate stiffness increases, cell-substrate adhesion
dominates over cell-cell adhesion leading to cell dispersal. Durotaxis of normal cells towards the
stiffer substrates has widely been observed and reported extensively by various groups [50, 55-58]. The dissociation of cancer cells from the cell clusters reported here is distinct from these earlier studies. In our studies, the HCT-8 cells first formed clusters, and then dispersed on the same soft substrate. Once dissociated, HCT-8 R cells did not form clusters anymore after re-plating on fresh soft or hard substrates, i.e. they lost their ability to sense substrate stiffness. This type of mechanosensing phenomenon leading to a stable change in the MLP has not previously been reported in the literature.
CHAPTER 3. CELL ADHESION

3.1 Motivation

During metastasis, malignant cancer cells turn off cell adhesion activity, de-adhere from their neighbors or the extracellular matrix (ECM), enter the lymphatic system or the blood stream as cell suspensions, invade new host tissues and organs, regain adhesive activity and form new tumor colonies[1, 5, 11]. Thus, adhesion plays a central role in management of successful metastasis.

In spite of the central role that adhesion plays during metastasis, measurement of adhesion at the single cell scale for cancer cells is not widely studied, primary due to limitation of instrumentation and appropriate theory applicable for living cells. Furthermore, a quantitative study of the adhesion properties of cancer cells during the early phases of metastasis is lacking, largely because of the challenges in timely identification of the onset of in vivo metastasis and the heterogeneity in biochemical and cellular properties of individual tumor cells. In this chapter, we first show the altered HCT-8 cells behaviors during malignant E-to-R transition with the focus on the alterations in their cell-cell adhesion molecule, E-Cadherin. Second, we present the results of both specific and non-specific cell-cell vs. cell-probe interaction during in vitro cancer metastasis in the context of the changes in adhesive cellular properties. At the single cell scale, we used a novel micro-scale Bio-MEMS instrumentation for the sensitive measurement of adhesive forces and quantified the non-specific cell adhesion in the framework of fracture mechanics. Finally, we show that full recovery of the E-Cadherin expression and cell-cell

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adhesive activity on dissociated R cells can only partially restore their E phenotype. Our data suggests that E-Cadherin may not play a significant role in the upstream regulation of the mechanosensing cascade and the complete restoration of the E cell phenotype likely requires multiple components in addition to E-Cadherin.

Our quantitative data on cancer cell adhesion at various stages of metastasis may be used to ultimately correlate adhesive strength with cell adhesion molecule (CAM) gene expression. Quantitative measurements of cancer cell adhesion offers the potential of discovering molecular mechanisms that cancer cells employ to regulate adhesion and malignant transition during metastasis.

3.2 Design, Materials, and Methods

To stain E-Cadherin, we fixed cultures at appropriate time points with paraformaldehyde (4%) at 37°C for 30 minutes followed by permeabilization in 0.1% Triton ×100 for 15 minutes. Mouse primary antibody anti-E-Cadherin (Invitrogen, SKU No.: 18-0223) and Alexa Fluor® 488 goat anti-mouse IgG (H+L, 495/519, green) (Invitrogen, SKU No.: A-11029) were used to conjugate with E-cadherin molecules. Following immunofluorescent staining, HCT-8 cells were imaged with Z step size of 200 nm using Yokogawa Spinning-disc confocal microscopy with appropriate fluorescent filters and Andor IQ software (Andor technology). The E-cadherin density per unit membrane area was calculated for colony-associated E cells and dissociated R cells from two sectional confocal images at 2.5 µm and 3.2 µm heights above the substrate.

Coulter counter assay was used to quantify the reduction rate and extent of single cell number as cell aggregates form in suspension as a function of culture time. HCT-8 E and R cells (MA-104 cells as control) were harvested and individualized by trypsin/EDTA treatment and
were restored in complete culture medium containing serum to neutralize residual trypsin. The cell suspensions were placed in 17×100 mm capped polypropylene tubes (Falcon No.: 352059) and were rotated end over end at 7-8 revolutions per minute in a Labquake shaker (Barnstead/Thermolyne Model No.: 41510) for 1 hour at 37 °C to allow recovery of any surface cell adhesion molecules (CAMs) or other proteins. The recovery of CAMs following trypsinization was guaranteed by identifying the increase in cell aggregate number as incubation duration prolongs. The pre-incubation time is chosen as 1 hour other than longer to avoid over-aggregation and ensure differentiation of the precise adhesion rate kinetic. Portions of the pre-incubated cells (0.3 ml, approximately 5×10^5 cells) were placed in flat bottom 3 dram shell vials (Fisher catalog No.: 0333926D) and rotated in a gyratory water bath shaker (G-76, New Brunswick) at 12 rpm at 37 °C for 5, 10, 20, 40, 60, 80, 100 and 120 minutes. At the end of each time period, cells were diluted with 8 mL 0.9% saline and placed on ice to stop further cell aggregation.

The surface non-specific adhesions of HCT-8 E and R cells are studied using a micro-fabricated bio-MEMS force sensor (Fig. 3.1) [59-65]. The calibrated sensor consists of a micro cantilever beam with spring constant k of 3.48 nN/m attached to a flat probe (Fig. 3.1a). Following immersion into aqueous cell culture environment at 37 °C, the flat probe is brought in contact with cell islands’ lateral convex surface at the boundary (Fig. 3.1b-c). After a 2-minute contact, force sensor is pulled away horizontally from the cell island at a constant quasi-static speed of 2.1 ± 0.4 µm/s (Fig. 3.1c). Due to the cell-probe adhesion, the sensor beam deforms during retraction. The short contact duration between the cell and the probe prevents the activation of cell integrins and the formation of any focal adhesion on the probe (takes > 30
minutes to form [66, 67]). Therefore, only non-specific adhesive interactions can be formed between the cell surface and the SiO$_2$-coated probe.

**Figure 3.1: The surface non-specific adhesion of HCT-8 E and R cell islands.** (a) Phase-contrast image and schematics drawing demonstrate that the nonfunctionalized micro-fabricated Si force sensor with a flat probe and with known force-deflection relation is manipulated by a high-resolution x-y-z Piezo-stage to contact cell islands’ lateral convex surface (on x-y plane). (b) Confocal microscopy of a typical E cell islands show the height of islands is on the order of 30 ~ 50 µm. The vertical height of bio-MEMS probe is 5 ~ 10 µm. (c) After a 2-minute contact, force sensor is horizontally pulled away at a constant speed of 2.1 ± 0.4 µm/s. The sensor beams deform by d and give the force F, while the cell adhesion between the probe and cell surface hinders retraction of the sensor. Here the probe is not functionalized.

Lentiviral infection for E-Cadherin up-regulation and Western Blotting were carried out using following protocol. Human E-Cadherin cDNA was cloned into the 2K7/Neo lentivector
[68], which was used to package virus with Viralpower Lentivirus Packaging System (Invitrogen) as described in [68]. For infection, lentiviruses (MOI, 10–200) were directly added into culture medium with polybrene (6 μg/mL, Sigma) and incubated with cells for ~20 h. Cells were analyzed 48 h after infection. Cells (5×10⁶) were lysed directly with Laemmli sample buffer (Bio-Rad). 25 μl of each sample were separated by SDS-PAGE and analyzed by western blot. E-cadherin antibody (Cell Signaling) was used at the dilution of 1:1,000. The Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

### 3.3 Patterns and Expression of E-Cadherin

E-Cadherin is an essential cell-cell adhesion molecule and its expression has been consistently found to show varying degree of decrease in metastatic cells [5, 25]. Hence, it has been regarded as a clinically useful tumor malignancy marker. We examined the pattern of E-Cadherin immunofluorescent staining in colony-associated and disassociated HCT-8 cells. We found, E-Cadherin appears as continuous and dense lines along cell-cell contact borders in colony-associated cells (Fig. 3.2a1). In contrast, E-Cadherin in disassociated HCT-8 R cells shows a weak pattern on the cell membrane (Fig. 3.2a2). Following the quantification of E-cadherin density per unit membrane area using Confocal microscopy, we found the disassociated R cells down-regulate their surface E-Cadherin expression by 4.73 ± 1.43 times compared to their E cell counterparts in colonies (Fig. 3.2a). We also examined expression of E-Cadherin on R cells which were harvested from 21 kPa gels and re-cultured on hard polystyrene substrates (Fig. 3.2b), and found they retained their down-regulated expression level (by 4.86 ± 1.91 times) compared to the non-disassociated cells (Fig. 3.2b1, 3.2b2). Our data imply that E-cadherin
expression in the disassociated cells cannot be restored by re-culturing back on a stiff substrate alone.

**Figure 3.2:** HCT-8 R cells show significantly lower E-Cadherin expression compared to E cells. (a) Histograms of E-Cadherin stain density per unit cell membrane area for HCT-8 cells on 21 kPa substrates on the 7th day of culture. The two histograms are for cells before (red) and after dissociation (blue), respectively. n=35 for each histogram. Fig. 3.2a1 and 3.2a2 show confocal images of E-Cadherin stain of corresponding cells before and after dissociation. The white arrowheads in Fig. 3.2a1 indicate the continuous and dense E-Cadherin distribution along the cell-cell contact borders. The white arrowheads in Fig. 3.2a2 indicate a weak E-Cadherin staining pattern on the disassociated cells’ membrane and the cell-cell contact regions. Scale
bars: 10 μm. Fig. 3.2a1’ and 3.2a2’ are the magnified views of the grey squared areas in Fig. 3.2a1 and 3.2a2, respectively. (b) Histograms of E-Cadherin staining density per unit cell membrane area for original HCT-8 E cells (not exposed to 21 kPa PA gels) and re-cultured disassociated HCT-8 R cells (that had been cultured on 21 kPa PA gels for 14 days) on stiff polystyrene substrates (3.6 GPa) on 4th culture day. n=35 for each histogram. The white arrowheads in Fig. 3.2b1 indicate the rich E-Cadherin presence along the cell-cell contact borders. The white arrowheads in Fig. 3.2b2 indicate a weak E-Cadherin pattern on the re-cultured disassociated cells’ membrane. Scale bars: 10 μm. Fig. 3.2b1’ and 3.2b2’ are magnified views of the grey squared areas in Fig. b1 and b2, respectively.

3.4 HCT-8 Cells Display Decreased Homotypic Cell-Cell Adhesion

Specific homotypic cell-cell adhesion rates for HCT-8 cells before and after plating on 21-47 kPa substrates (before and after disassociation), and normal Ma104 cells were quantified and compared using a Coulter counter assay as described in Section 3.2. In all cases, the cells were harvested from either polystyrene or PA surfaces, individualized by trypsin treatment, and allowed to recover from trypsinization under the same incubation conditions prior to measuring intercellular adhesion rates. Interestingly, disassociated HCT-8 cells (harvested from PA substrates) displayed a markedly lower extent and rate of cell-cell adhesion as compared to the original HCT-8 cells cultured on hard polystyrene substrates (Fig. 3.3). After 120 minutes of incubation, 84.8 ± 4.0 % of the disassociated HCT-8 cells remained as single cells, in contrast to 37.6 ± 6.1 % of original HCT-8 cells and 6.1 ± 0.4 % of normal Ma104 cells (Fig. 3.3a). Furthermore, this reduced cell-cell adhesion is independent of passage number. Note: cells in suspension regain 40% of adhesion within 1 hr and 80% within 3 hrs, and the rest of the
adhesion takes about 18 hrs [69]. The rate of adhesion is best measured within the first 3 hrs of suspension. It is this rate that we are interested at here. This rather remarkable result, showing the cell-cell adhesion capacity of HCT-8 cells is nearly absent after they disassociate from cell colonies, is also consistent with our finding of a pattern of reduced E-Cadherin expression (Fig. 3.2a). Taken together, these results strongly support the hypothesis that HCT-8 cells down-regulate cell adhesion molecules (CAMs) in response to mechanical cues they experience while growing on intermediate stiff but not very stiff substrates.

![Diagram showing Coulter counter adhesion test](image)

**Figure 3.3:** Comparison of Cell-Cell adhesion rates of original (not exposed to 21 kPa PA gels) HCT-8, disassociated HCT-8 cells harvested from 21 kPa PA gels, and normal epithelial Ma104 cells. (a) Data suggests that disassociated HCT-8 R cells have low cell-cell and non-specific adhesion compared to original HCT-8 E cells and normal MA-104 cells. (b)
Photomicrographs of dissociated and original HCT-8 cells taken after 2 hrs of gyration followed by Coulter counter measurements. The relative absence of cell aggregates in the dissociated HCT-8 R population is consistent with their low cell-cell adhesion rate shown in (a).

3.5 Cell Adhesion Energy Studied by Bio-MEMS Force Sensor

The surface non-specific adhesions of HCT-8 E and R cells are measured using a micro-fabricated bio-MEMS force sensor (Fig. 3.2) [59-65]. We found that, during retraction of the bio-MEMS sensor, E-cell islands stretch locally by 15-20 µm resulting in a conical shape (Fig. 3.4). Note that this stretch is different from that due solely to membrane tether, which consists of stretching only the phospholipid bilayer [70, 71]. During probe retraction, the cone is continuously stretched and the cell contact length with the probe drops in a stepwise fashion. The increase of force between cell and probe is reflected in the progressive increase of the gap between a fixed reference and the bio-MEMS probe (from D₀ to D₁). At critical value of force, Pₐ, the cone suddenly detaches from probe (Fig. 3.4e). The entire force vs. cell stretching characteristics (P vs. Δ, Fig. 3.4a) and the cell-probe separation are optically recorded by video camera (Fig. 3.4b-d). Throughout the pulling process, force-displacement curve increases monotonically until final cell/probe separation, as shown in Fig 3.4a. In the following, we quantify the adhesion energy between the cell island and the probe.
Figure 3.4: Surface non-specific adhesion of E cell islands. (a) The non-functionalized micro-fabricated Si force sensor with a flat probe and with known force-deflection relation is manipulated by a high-resolution x-y-z Piezo-stage to contact cell islands’ lateral convex surface (on x-y plane). (b) Confocal microscopy of cell islands show the height of islands is on the order of 30~50 µm. The vertical height of bio-MEMS probe is 5 ~10 µm. (c) After a 2-minute contact, force sensor is horizontally pulled away at a constant speed of 2.1 ± 0.4 µm/s. While the cell adhesion between the probe and cell surface hinders retraction of the sensor, the sensor beams deform by δ, giving the force F. Note that the probe is not functionalized. The 2-minute contact between the probe and cells prevents the activation of cell integrins and the formation of any cell focal adhesion, which takes >30 minutes to form.

Energy is required to separate the cell island from the probe due to adhesion between them. We want to estimate cell-probe adhesion energy from the above experiments and the concepts of non-linear fracture mechanics [72, 73]. As the probe stretches the island, strain
energy of the island increases. As the stretch or the corresponding force exceeds a threshold (Fig. 3.5a), part of the cell island detaches from the probe as an interfacial crack (Fig. 3.5b). The crack then stops growing. The island can be stretched further with increase in force. Note that during the crack growth, force decreases due to the stretch of the island, but the sensor is moved further away from the cell island until the sensor detects an increase in force. Thus, in the force-stretch curve (where time is not shown), force remains constant (as a hanging weight) when crack is advancing. The energy necessary to drive this crack growth can be estimated from the change in the potential energy of the force and the cell island before and after the growth, assuming that the strain energy of the cell island has no dissipation.

Figure 3.5: Analysis of adhesion energy between HCT-8 E cells and force sensor. (a) Force-displacement measurement for a representative HCT-8 E cell island (black square symbol) and R cell (green diamond symbol). The y-axis on left shows the adhesion force measured by the bio-
MEMS force sensor as a function of the cell stretch. The y-axis on right shows the concurrent cell-probe contact length as the same cell stretches. From frame 37 to frame 38, the crack-probe attachment decreases (i.e., cell separates from the probe) by 1.07 µm, at a force $P_1 = 36.51$ nN. Adhesion force is negligible for R cells. (b) Phase-contrast images of cell-probe contact. The contact length decreases from 14.72 µm to 13.65 µm.

Let $a$ be the length of the contact between the cell island and the probe (Fig. 3.6). Since the depth of the probe is only 5 µm, the island remains in contact over the full depth of the probe during crack growth. Let $P$ be the force applied by the probe on the cell island, $\Delta$ be the corresponding stretch. Let $P_1$ be the force at which the crack advances by a distance $d\alpha_1$. For a fixed contact length, $a$, the strain energy of the cell island due to stretch $\Delta_1$ is $\int_0^{\Delta_1} Pd\Delta$, and the potential energy, $PE$, is

$$PE = \int_0^{\Delta_1} Pd\Delta - P_1\Delta_1 = P_1\Delta_1 - \int_0^{\Delta_1} \frac{\partial P(\Delta,a)}{\partial \Delta} \Delta d\Delta - P_1\Delta_1 = -\int_0^{\Delta_1} \Delta(P,a)dP,$$

where integration by parts is used [72]. If the cell-probe contact decreases by $d\alpha_1$, then the potential energy, $W_1d\alpha_1$, released to drive a separation $d\alpha_1$ is as follows.

$$W_1d\alpha_1 = d\alpha_1 \frac{\partial PE}{\partial a} = -d\alpha_1 \int_0^{\Delta_1} \frac{\partial P(\Delta,a)}{\partial a} dP$$

(3.1)

The integral on the right can be interpreted as follows. Consider the P-$\Delta$ relation from a cell stretch experiment in Fig 3.6. Here, force increases to $P_1$ (point A on the P-$\Delta$ curve) when the cell-probe contact decreases from $a$ to $a-d\alpha_1$, and the cell stretch increases by $\Delta_B - \Delta_A$. If the cell stretch experiment were carried out with the cell-probe contact of $a-d\alpha_1$, then P-$\Delta$ path would
follow the dotted line OB. At fixed \( P \), \( da_1 \frac{\partial \Delta}{\partial a} \bigg|_{P_1} \) is the change of \( \Delta \) due to decrease in cell-probe contact by \( da_1 \), and \( dP \frac{\partial \Delta}{\partial a} \bigg|_{P_1} \) is the area of the strip shown by the shaded region (Fig. 3.6).

Thus, the integral on the right side of Eqn. (3.1) is the energy, \( W_1 \), spent in reducing the contact length by \( da_1 \) at fixed force \( P_1 \), and is given by the area bounded by the OABO. The energy of separation or the adhesion energy per unit area of the cell-probe interface is then given by \( \gamma_1 = \frac{W_1}{h \ da_1} \), where \( h \) is the height of the probe. In Fig. 4, force is increased from \( P_1 \) at B to \( P_2 \) at C along BC. At C, cell island separates from the probe by a distance \( da_2 \). Thus the new contact length is \( a-da_1-da_2 \). Let OD be the trajectory of the P-\( \Delta \) curve for this contact length. Then, following the above, \( \gamma_2 = \frac{W_2}{h \ da_2} \), where \( W_2 \) is the area within OCDO. In an experiment, one may have several \( \gamma \)s and can calculate an average \( \gamma = \frac{\gamma_1+\gamma_2+…+\gamma_n}{n} \).

Figure 3.6: (a) Schematic of the force-stretch (P-\( \Delta \)) relation and (b) the method used to estimate the adhesion energy between the probe and the cell island. The probe adheres with the cell island over a contact length, \( a \), and stretches the island by \( \Delta \) while measuring the force \( P \).
(from O to A). At the fixed force $P_1$, cell island detaches from the probe by $da_1$ and gets stretched by $\Delta_B - \Delta_A$ (from A to B). The force is farther increased along BC. The area within OABO is the energy, $da_1W_1$, consumed in separating the cell island from the probe by $da_1$. The energy of adhesion per unit area of contact is thus, $\gamma_1 = da_1W_1/(h da_1)$, where $h$ is the height of the probe normal to the paper.

We apply the above approach to calculate $\gamma$ for the cell-probe interface using the experimental $P-\Delta$ relations. Fig. 3.7 shows the close-up of the experimental $P-\Delta$ curve of Fig 3.5a. The figure shows that the cell-probe contact length decreases from $a_1 = 14.72 \, \mu m$ to $a_1-da_1=13.65 \, \mu m$ at $P_1= 36.51 \, nN$. $P-\Delta$ follows the trajectory OAB. The force was then increased along the path BC in the $P-\Delta$ curve. In order to calculate $\gamma_1$, we extrapolate CB to the origin shown by the dotted line. Thus, the area within OABO gives $W_1 da_1= 22.36 \times 10^{-15} \, J$, and $\gamma_1=W_1/h = 4.18 \, mJ/m^2$ (h=5 $\mu m$). We performed similar experiments on several HCT-8 E islands and found the non-specific adhesion energy $\gamma$ between pre-MLP HCT-8 E cell islands and the probe as $4.71 \pm 2.11 \, mJ/m^2$ (n=9). The maximum force prior to complete detachment of the cell island from the probe, $P_c$, is determined as $249.77 \pm 30.32 \, nN$ (n=9).
Measurement of non-specific adhesion was done using a MEMS probe. A part of the experimental force-stretch (P-Δ) data of Fig. 3a is shown. Force is increased to A when the cell island detached from the probe by 1.07 μm at a constant force of $P_1 = 36.51$ nN. During the cell-probe detachment, the cell island stretches from A to B. Force is then increased along the path BC. In order to measure the adhesion energy, the loading path BC is extrapolated to the origin, O. The area within the curve OABO gives the energy necessary to separate the cell island from the probe by 1.07 μm over the 5 μm depth of the probe.

The non-specific adhesion properties of dissociated post-MLP HCT-8 R cells are evaluated using same experimental methods and theory. The post-MLP HCT-8 R cells display isolated and spherical morphology, with low E-Cadherin expression[61, 62, 74]. Determined by 3D con-focal imaging technique, the R cells have height of $16.63 \pm 2.81$ μm [61, 62]. In order to
satisfy complete lateral adherence and avoid touching the substrate, a bio-MEMS sensor with a smaller probe (5 × 5 µm² square cross-section) is chosen to contact the R cells (Fig. 3.8). It is found that the detachment force between the cell and the probe is negligible, 1.14 ± 0.13 nN (n=25). Correspondingly, the cell shows no measurable stretch at detachment, and no significant progressive detachment between the probe and the cell was observed (Fig. 3.8a). Using thin-shell model described in Section 3.6, the adhesion energy is estimated as 0.07 ± 0.02 nN/µm (or mJ/m²; Fig. 3.9). They are 70-80 times lower than those of HCT8 E cells. Thus the R cells are in a “lubricated” state enabling them to be more circulatory and invasive. Our recent in vitro basement membrane invasion assay confirms this hypothesis [74].

![Figure 3.8: Surface non-specific adhesion of R cells measured by micro-fabricated bio-MEMS force sensor.](image)

(a) Adhesive force of R cells on MEMS probe as the probe is moved away from the cells after 2 min contact (n=25). (b - e). Phase-contrast images of R cells and MEMS probe when non-specific adhesion between them is measured. The maximum detachment force measured is < 2.5 nN, while the cell deformation is barely noticeable. Scale bar: 40 µm.
3.6 Non-specific Adhesion Energy Analysis using Thin-shell Theory

We also used thin-shell theory to calculate the cell-probe adhesion energy. The thin-shell model was originally derived by Brochard and de Gennes [75], similar to JKR theory [75-84], to calculate adhesion energy (or separation energy) between spherical thin-shells and a flat surface. In thin-shell theory, the interfacial energy $W_{adh}$ between a deformable hollow sphere of radii $R$ and a flat substrate is given by $\gamma = W_{adh} = P_c / (\pi R)$, where $F_c$ is the detachment force (or pull-off force). In the cell island-probe system, the probe is flat. The cell island has two principle radii of curvature at the contact: one corresponds to the in-plane radius of the island, and the other to the out of plane curvature. Thus the thin shell theory with $\gamma = W_{adh} = P_c / (\pi R)$ does not strictly apply to this case. Similarly is the case for the JKR theory, unless a modified JKR theory [85, 86] is used that accounts for cylindrical contacts. However, we use the thin-shell theory only to obtain an approximate estimate of the adhesion energy to compare with the values obtained in the previous section. The approximate energy is calculated using the out of plane radius of the island at its mid-height where the probe contacts the island. The R cells, on the other hand, are spherical in shape, and therefore the thin shell theory can be applied more appropriately. For cell islands, we found $W_{adh} = \gamma = 5.22 \pm 1.09$ nN/µm (or mJ/m²) (n=12). The summary of adhesion energies calculated by 2 methods for both HCT-8 E and R cells is shown in Fig. 3.9.

Although we have used 2 theories to analyze the cell adhesion energy, the first fracture mechanics based theory is more applicable, as it does not require any specific cell shape. However, the second theory, i.e. thin-shell model, relies on the spherical shape of tested objects. With the measurement of applied force and cell contact area dynamics, the first theory gives better estimate of the cell-probe interfacial energy than that given by the thin-shell theory. For the dissociated R cells that did not show measurable cell-probe contact area change during
detachment, we could only apply the thin-shell theory to estimate adhesion energy. For E cells, we used both theories respectively and it turned out their adhesion energy estimations are close to each other (Fig. 3.9).

![Graph showing adhesion energy comparison between HCT8 E cells and HCT8 R cells.]

**Figure 3.9: Comparison of adhesion energies of HCT-8 E cells (n=9) and R cells (n=25).** Based on the 2 models, the cell-probe non-specific adhesion energy for pre-MLP HCT-8 E cells is $4.71 \pm 2.11$ mJ/m$^2$ and $5.22 \pm 1.09$ mJ/m$^2$ (or nN/µm), respectively. The adhesion energy for post-MLP HCT-8 R cells is $0$ mJ/m$^2$ and $0.07 \pm 0.02$ mJ/m$^2$ (or nN/µm), respectively, which are 60-70 times lower than that for the E cells. The adhesion energies measured by two different methods match within the same order of magnitude.

### 3.7 Partial MLP Phenotype Restoration by E-Cadherin Induction
To further examine the role of E-Cadherin in regulating the expression of MLP, we overexpressed E-Cadherin in MLP cells to explore whether the dissociated HCT-8 cells can form colonies again, i.e. whether the MLP can be reversed. For these experiments, we employed a lentivirus infection system to restore the E-Cadherin expression in dissociated HCT-8 cells displaying the MLP [68]. The majority (90-100%) of these dissociated HCT-8 cells were successfully transduced as judged by expression of red fluorescent protein (Fig. 3.10a1-a6). Western blotting experiments show that E-Cadherin protein expression in the virus-infected, dissociated HCT-8 cells was up-regulated by ~3.58 fold (Fig. 3.10b), which is equivalent or slightly higher than the amount of E-Cadherin expressed on original HCT-8 cells (Fig. 3.10e). Approximately 60% of these cells displayed a somewhat flattened or epithelial shape (E cells) as compared to control dissociated HCT-8 displaying the MLP where >95% the cells were of the round form (R cells) when cultured on hard plastics (Fig 3.10d, 7e). The remaining 40% of these cells remain as R cells (Fig. 3.10d3). In addition, unlike the original HCT-8 cells (Fig. 3.10d3), these E-Cadherin up-regulated dissociated cells do not form the typical monolayers with merged cell boundary on hard polystyrene substrates, but remain as individual cells even as they grow to confluency (Fig. 3.10d2). On 21 kPa PA gels, they partially form some clusters (Fig. 3.10d5), but the cells within the clusters seem to be separate from one another. These clusters are clearly distinct from those formed by original HCT-8 cells (Fig. 2.2a).

To explore the effect of E-Cadherin up-regulation, we compared homotypic cell-cell adhesion kinetics of dissociated HCT-8 cells with and without E-Cadherin up-regulation. We found the rate of cell-cell adhesion increased nearly 5-fold following E-Cadherin up-regulation in dissociated HCT-8 cells, from -0.05 to -0.26 (Fig. 3.10c). The extent of cell-cell adhesion after 120 minutes of incubation, was 11.3 ± 4.4 % for dissociated HCT-8 cells as compared to 52.8 ±
6.3% following E-Cadherin up-regulation. The adhesion-rate regression analysis indicates that after E-Cadherin up-regulation, 100% recovery of adhesion in HCT-8 R cells is reached (cell adhesion rate = -0.26) compared to the original HCT-8 cells (cell adhesion rate = -0.23). A similar increase in cell adhesive rate and extent was also seen in the original, non-dissociated HCT-8 cells following E-Cadherin up-regulation (Fig. 3.10c). These results indicate that while partial recovery of the E-cell phenotype and cell-cell adhesive activity occurs following restoration of E-Cadherin expression in dissociated HCT-8 cells, complete restoration of the E-cell phenotype likely requires multiple components in addition to E-Cadherin.

Figure 3.10: Inducing E-Cadherin expression in MLP cells partially restores the non-MLP phenotype. (a) The percentage of transduced dissociated HCT-8 cells is judged by expression of
red fluorescent protein. The phase-contrast and fluorescent pictures of E-Cadherin up-regulated cells at 2nd and 3rd day after infection are shown in (a1-a2) and (a3-a4), respectively. The E-Cadherin up-regulated dissociated cells harvested for Coulter counter adhesion assay is shown in (a5-a6); (b) Western blotting data shows the E-Cadherin content present in both not-treated and E-Cadherin up-regulated dissociated HCT-8 cells with GAPDH as loading control; (c) Homotypic cell-cell adhesion rates of original HCT-8 (no treatment and E-Cadherin up-regulated), dissociated HCT-8 (no treatment and E-Cadherin up-regulated) and normal MA-104 cells (control); (d) Morphological patterns of dissociated HCT-8 (no treatment and E-Cadherin up-regulated) and original HCT-8 cells (no treatment) on 21 kPa PA gels and stiff polystyrene substrates; (e) The normalized E-cadherin expression levels on original HCT-8 (no treatment; tested by fluorescent staining) and dissociated HCT-8 cells (no treatment and E-Cadherin up-regulated; by Western blotting). The percentage of epithelial morphology cells vs. overall cell populations in these 3 cell types, respectively. It shows after E-Cadherin in dissociated HCT-8 cells is overexpressed to close to that in original HCT-8, the MLP is partially restored to the non-MLP phenotype. Error bars represent standard deviations.

3.8 Discussion and Conclusion

To our knowledge, it is the first report on cell adhesion of human colon cancer cells during in vitro metastatic transition induced solely by mechanical microenvironment. The present study shows that, when triggered by the appropriate substrate rigidity cues, the HCT-8 R cells lose their surface adhesion and acquire autonomy, which is an essential hall-marker of cancer metastasis[1, 2, 4, 9, 59, 87-89].
The physical properties of culture substrates are found to widely affect the phenotypes and gene expression of a number of normal and cancerous cells [11, 21, 22, 42, 44, 50, 51, 61, 62, 74, 90-101]. In response to stimuli from the microenvironment, cells adhere to and spread on substrates followed by sensing and processing both mechanical and chemical signals [50, 92, 94, 97, 102-112]. Our discovery on HCT-8 E-to-R transition suggests that appropriate substrate mechanical rigidity may aid in the initiation of the early events in cancer metastasis progression. After the 7th day of culture on the appropriate soft substrates, the dissociated HCT-8 R cells show remarkably diminished adhesion (both specific and non-specific [61, 71, 113-115]) compared to their E counterparts. Unlike pre-MLP HCT-8 E cells, the dissociated HCT-8 R cells show attenuated cell-cell contact and they become insensitive to the variation of substrate-stiffness. We found that the R cells’ proliferation is not impaired by weak anchorage with substrate or other cells (Fig. 1b), similarly to metastatic cells which are known for their anchorage independent features [6, 25]. It is known that E-Cadherin is down-regulated in most, if not all, epithelial tumors during the progression to tumor malignancy [5, 25], and presence of E-Cadherin can strongly suppress the invasiveness of malignant cells [6, 116]. This study reveals that colon cancer cells can attain this trait by being cultured on the appropriate soft substrate (stiffness ~ 20-47 kPa) and potentially becoming invasive. Indeed, our recent in vivo animal model tests suggest that R cells are more tumorigenic than E cells (Chapter 5).

In summary, we have presented a methodology to measure adhesion between a living cell or a cell cluster and a bio-MEMS probe. The theoretical framework for the method is developed. Our results indicate that during in vitro metastasis, metastatic HCT-8 R cells strategically lower their surface non-specific adhesion, and increase their resistance to anokisis to survive in anchorage-free conditions. The in vitro model presented in this paper allows systematic study of
the early phases of metastasis and corresponding biophysical and biochemical kinetics, i.e. cell adhesions and cancer hall marks. The model may help design possible anti-metastatic therapeutic agents as well. Also, our study of E-Cadherin immunofluorescent staining and homotypic cell-cell adhesion confirmed and suggested that the cell adhesion function is dramatically altered following E-to-R transition. Our finding that E-Cadherin up-regulation only partially restored the MLP in the dissociated HCT-8 cells suggests E-Cadherin may play a downstream rather than an initial signal transduction role in the mechanosensing cascade.
CHAPTER 4. METASTASIS-LIKE FUNCTIONALITIES

4.1 Motivations

During metastasis, malignant cancer cells escape from the tumor by detaching from one another or from other cells and the extracellular matrix (ECM) [1, 4-6]. The escaped cells actively express proteinases and alter their adhesion ligands to degrade and modify their surrounding ECM [1, 2, 7-9]. Simultaneously, they up-regulate their motility and resistance to apoptosis for successful vascular spread and invasion of distant healthy organs [4, 6, 69]. Concurrently, these cells lower their stiffness [117-120], i.e., increase their compliance to flow through small capillaries [2, 89, 121]. A quantitative study of the mechanical and biophysical properties of cancer cells during the early phases of metastasis, however, is lacking [11, 21, 87, 122], largely because of the challenges in detecting the onset of metastasis in vivo and the heterogeneity in biochemical and cellular properties of individual tumor cells [1, 11, 25, 123].

In this Chapter, we investigated the presence and dynamics of typical known cancer metastasis hallmarkers during the process of human colon carcinoma cells (HCT-8) in vitro metastasis-like phenotype (MLP) transition. We examined cell elasticity, cell-substrate traction, cellular mechanosensitivity, expression of Matrix Metallic Proteinase (MMP) and the acquisition of anoikis resistance. Our study reveals that colon cancer HCT-8 cells can attain some important metastasis characteristics solely by culture on the appropriately soft substrate.

4.2 Design, Materials, and Methods

To study the cell anoikis resistance, PA gels with 20 kPa stiffness were prepared on glass-slide with grids (EMS Inc.) following the procedures described in Chapter 2, but without any surface ECM functionalization, i.e. fibronectin or laminin (BD Bioscience). These non-functionalized surfaces prevent any specific or non-specific cell anchorage. Single E and R cells were separately cultured on gels at a starting density of $1 \times 10^5$ cells per gel. Three independent repeats were carried out with 4 gels for each cell type. Daily imaging was carried out to monitor the anchorage-free survival of cells. Care was taken during gentle medium change to minimize cell loss, though a few cells in suspension were unavoidably removed. The formation of spheroid colonies from single cells, the survival of single cells, and the growth of cells in suspension were observed and quantified with the assistance of gridded glass-slides. After 1-week of culture, a 2.0 ml aliquot of the cell suspension containing both spheroids and single cells was harvested per gel following gentle shaking to ensure unbiased sample acquisition, and examined between 2 transparent, sterile glass-slides under a light microscope. A 0.4% sterile filtered Trypan Blue solution (Sigma–Aldrich, St. Louis, MO) was applied in the cell medium to quantify the percentage of viable cells. For each 2 ml sample, 40 spots were imaged and the numbers of live/dead cells were counted. Percentage of viable cells is defined as number of viable (unstained) cells vs. total number of cells. Data for single cells and spheroids are compared respectively.

4.3 R cells are mechanically softer than E cells

Cancer cells with higher metastatic potential generally display greater deformability [117-120, 124], which allows their easy transit through vasculature during metastasis. We compared deformability of HCT-8 E and R cells using the contact mode of atomic force
microscopy (AFM). HCT-8 E and R cells were both cultured on PS substrates under identical environment conditions (37 °C) throughout AFM analysis (Fig. 4.1). We found R cells are 2-3 times softer, with Elastic modulus = 0.47861 ± 0.44339 kPa (n=8), than the E cells with Elastic modulus = 1.13107 ± 0.49646 kPa (n=12; P value = 0.0077). The softened R cell elasticity agrees with the previously reported altered actin cytoskeleton architecture in R cells (Fig. 4.2). Specifically, on hard PS substrates E cells show well-organized, straight-ordered actin stress bundles within individual cells (Fig. 4.2a). In contrast, HCT-8 R cells show only cortical actin structure with no actin stress bundles (Fig. 4.2b), implying a more compliant state.

Figure 4.1: Atomic force microscopy (AFM) was used to measure mechanical stiffness of HCT-8 cells before and after E-R transition. (a) Force-indentation curves were recorded as each cell were indented and unloaded. The square-dot and circle-dot curves represent the force vs. indentation displacement characteristics of HCT-8 E and R cells, respectively. The data were best fitted (line curves) using an improved Hertz model to extract the Elastic modulus of cells as

![Graph](image-url)
described in methods. (b) Comparison of the cell elasticity of E and R cells. n=12 for E cells and n=8 for R cells.

Figure 4.2: Actin cytoskeleton imaging of HCT-8 E and R cells was visualized using the high-resolution laser scanning confocal microscopy. Cells were stained using the phalloidin-rhodamine immunocytochemical assay. (a) E cells cultured on hard PS substrates (which do not dissociate) show well-organized, straight-ordered actin stress bundles within individual cells; (b) HCT-8 R cells show only the devolved cortical actin structure with no actin stress bundles, implying a more irregular and compliant state. (c) The profiles of fluorescent light intensity
across the cell body of both types display the distinct architectures contrasts of respective actin cytoskeletons.

4.4 Cell-substrate Traction

Using the finite-element-method-based traction force microscopy (Chapter 9), we investigated the cellular traction that HCT-8 E and R cells applied on the PA gel substrates (Fig. 4.3). We found, following the transition, both single R cells and clusters applied weak cell traction on substrates (Fig. 4.3b). The maximum traction, 150-200 Pa, that R cells applied showed consistently 2-3 folded lower than that of original HCT-8 E cells, 350-400 Pa (Fig. 4.3a). Together with cell adhesion results (Chapter 3), our study suggests that post-transition HCT-8 R cells possess trait of self-autonomy, which has been reported as one of essential metastasis hallmarks [2, 89].

Figure 4.3: Quantification and visualization of cell traction were measured using finite-element-method-based traction force microscopy. (a) HCT-8 E cells cultured on 1 kPa PA
gels for 3 days. (b) The re-plated HCT-8 R cells on 1 kPa PA gels for 3 days. The cultured conditions for both cases are identical.

4.5 Attenuation of Cell Mechanosensitivity

To explore how HCT-8 R cells respond to varying physiologically-relevant substrate stiffness, harvested HCT-8 R cells were cultured on fresh stiffness-gradient PA gel substrates with stiffness varying continuously from 1 to 20 kPa (Fig. 4.4a, left to right). The stiffness-gradient substrate is coated with a uniform fibronectin concentration to allow cell attachment to the substrate [102, 125, 126]. For comparison, both HCT-8 E cells and normal Monkey Kidney Fibroblast (MKF) cells, without any prior exposure to PA gels, were plated on the same stiffness gradient substrates and surface functionalization (Fig. 4.4b and 4.4c). The normal MKF cells were chosen as control because they are known to be mechanosensitive to substrate stiffness [127]. We found, in contrast to HCT-8 E cells and normal MKF cells, HCT-8 R cells constitutively showed very limited substrate contact areas regardless of substrate stiffness. The R cells’ contact area with the substrate is about 40-60% of their apparent projected area. As measured by 3D confocal microscopic imaging, the R cell contact area with substrate is only $49.5 \pm 20.9 \, \mu m^2$ (n=34), which is $3.8 \pm 0.3$ fold smaller than E cells (n=47), suggesting that R cells have weaker adhesion with the substrate than E cells. The weak adhesion of R cells with substrate is also consistent with the observation that R cells show a smaller projected area, than E cells on the same stiffness substrate (Fig. 4.4d). The projected area of isolated cells without any neighboring cell contact, of is $1.9 \pm 0.6$ fold smaller for R cells (n=68) than E cells (n=61).

HCT-8 R cells also show a remarkable insensitivity to changing the mechanical-stiffness of their culture substrate. They retain a rounded phenotype and limited adhesion area to
substrates regardless of the substrates’ stiffness (Fig. 4.4a, indicated by white arrows; Fig. 4.4d, 4.4e and 4.4g). When the substrate stiffness varied over a 20-fold range, the spread area of single R cells increased only about 27 %, (from 156.2 ± 42.1 µm² on a 1 kPa region (n=62) to 197.9 ± 83.6 µm² (n=56) on a 20 kPa region) (Fig. 4.4d). Across the stiffness tested, the increase in R cells’ spread area is not as dramatic as that of E and MKF cells. On 5 kPa, 10 kPa and 15 kPa regions, their spread areas are 158.2 ± 40.3 µm² (n=56), 182.3 ± 32.2 µm² (n=63), and 190.9 ± 82.5 µm² (n = 57), respectively (Fig. 4.4d). Also, the R cell shape factor changed only 7% from 0.9 ± 0.2 on a 1 kPa region to 0.8 ± 0.2 on a 20 kPa region (Fig. 4.4g; The shape factor, S = $4\pi A/P^2$, where A is the area of the cell and P is the perimeter. S=1 for perfect circular shape and 0 for irregular shape), indicating constitutive rounded shape independent of the substrate stiffness. On 5 kPa, 10 kPa and 15 kPa regions, the shape factors of single R cells are 0.8 ± 0.1, 0.8 ± 0.2, and 0.9 ± 0.2, respectively (Fig. 4.4g). After prolonged culture (60 days), R cells did not show any reversal toward an epithelial morphology on all substrates, regardless of stiffness, even very rigid polystyrene (3 GPa)[61]. In addition, daily recording via video microscopy indicates that R cells show no sign of impairment of proliferative activity even after several months in culture. In contrast, both HCT-8 E cells and MKF cells cultured on the same type of stiffness gradient substrates show obvious sensitivity to the mechanical stiffness of their culture substrate. The individual isolated E cells spread area increases 2.5 fold over 20-fold substrate stiffness change, from 239.6 ± 191.9 µm² on the 1 kPa region to 578.1 ± 429.8 µm² on the 20 kPa region (Fig. 4.4b, indicated by white arrows). As substrates become rigid, the HCT-8 E cells display a greater spread area, with their spread areas 270.8 ± 201.7 µm² (n=51), 276.0 ± 104.8 µm² (n=62), and 442.7 ± 367.7 µm² (n = 55) on 5 kPa, 10 kPa and 15 kPa regions, respectively (Fig. 4.4b). Their shape factor decreased from 0.9 ± 0.2 on the 1 kPa region to 0.6 ± 0.2 on the
20 kPa region (Fig. 4.4g). Across other stiffness tested, the single E cells shape factors are 0.8 ± 0.2 (on 5 kPa region), 0.8 ± 0.1 (on 10 kPa region), and 0.7 ± 0.3 (on 15 kPa region), respectively. The mechanosensitivity of MKF is even more pronounced as compared to HCT-8 cancer cells (Fig. 4.4c). The spread area of individual isolated MKF cells (Fig. 4.4c; indicated by white arrows) increases 5 fold across the gradient substrate, from 286.4 ± 86.2 µm² (n=46) on the 1 kPa region to 1421.7 ± 845.7 µm² (n=31) on the 20 kPa region (Fig. 4.4d). As the substrate stiffness increases, their spread area increases dramatically, and are 578.1 ± 373.1 µm² (n=62), 749.9 ± 355.5 µm² (n=63), and 1218.6 ± 773.5 µm² (n = 59) on 5 kPa, 10 kPa and 15 kPa regions, respectively. Concurrently with increasing substrate stiffness, single MKF cells spread to a more irregular morphology, with their shape factor decreasing from 0.9 ± 0.1 on the 1 kPa to 0.5 ± 0.2 on the 20 kPa regions, respectively (Fig. 4.4g). On the intermediate stiffness regions, i.e. 5 kPa, 10 kPa and 15 kPa regions, the shape factors of single MKF cells are 0.7 ± 0.3, 0.6 ± 0.3 and 0.5 ± 0.3, respectively. The weak adhesion between HCT-8 R cells and the substrate, as well as the independence of R cell morphology from substrate stiffness, strongly suggest that R cells lose anchorage-dependence and communication with their mechanical microenvironment. This anchorage-independence can potentially promote R cells survival in suspension, which is an essential hallmark of in vivo metastasis of cancer cells [1, 2, 5, 89, 123].

On stiffness-gradient substrates, both HCT-8 E cells and MKF cells show cell colony formation, especially on stiffer regions (indicated by yellow arrows in Fig. 4.4b and 4.4c). The colony size is positively correlated with the substrate stiffness. On substrate stiffness 1 kPa, 5 kPa, 10 kPa, 15 kPa and 20 kPa gels the cell colony sizes of HCT-8 E cells are 2962.2 ± 1000.5 µm², 3662.1 ± 1105.3 µm², 4249.5 ± 919.5 µm², 9736.5 ± 4032.7 µm² and 11748.7 ± 2144.9 µm², respectively (Fig. 4.4f). For HCT-8 R cells on the same stiffness substrates, the colony
sizes are markedly smaller than their E counterparts even when R cells are in contact with neighboring cells for 3 days (Fig. 4.4a). On substrate stiffnesses of 1 kPa, 5 kPa, 10 kPa, 15 kPa and 20 kPa, the R cell colony sizes are, 1087.4 ± 338.3 µm², 1449.8 ± 343.4 µm², 3062.2 ± 1326.9 µm², 3849.6 ± 919.1 µm² and 3912.1 ± 1183.8 µm², respectively (Fig. 4.4f). We also observed that inside R cell colonies, the cell-cell contact area is not as extensive as in E cell colonies. R cells appear to be just touching each other at point-contacts (Fig. 4.4a). These results suggest R cell-cell adhesion is not sufficient for them to form cohesive colonies or cell islands as do E and MKF cells.

Furthermore, it is interesting to note that as HCT-8 E cells or MKF cells undergo homotypic cell-cell adhesion, their individual cell areas and cell shape factor become remarkably less substrate stiffness-dependent (Fig. 4.4b and 4.4c, indicated by yellow arrows). Individual cell areas and shape factors of single HCT-8 E cells inside cell islands on 1 kPa gels are 785.6 ± 299.4 µm² and 0.7 ± 0.1, respectively, which is similar to those on 20 kPa gels, 892.8 ± 322.1 µm² and 0.6 ± 0.1 (Fig. 4.4e and 4.4h). Same characteristics are observed on intermediate stiffness, the cell area and shape factor of individual HCT-8 E inside islands are 526.7 ± 187.0 µm² and 0.8 ± 0.1 on 5 kPa gels, 633.9 ± 421.4 µm² and 0.6 ± 0.2 on 10 kPa gels, and 723.1 ± 515.2 µm² and 0.6 ± 0.2 on 15 kPa gels. For individual MKF cells inside islands, their cell area and shape factor are 928.5 ± 374.0 µm² and 0.5 ± 0.3 on 1 kPa gels, 892.8 ± 415.7 µm² and 0.5 ± 0.3 on 5 kPa gels, 1098.1 ± 564.6 µm² and 0.5 ± 0.2 on 10 kPa gels, 1008.8 ± 223.7 µm² and 0.3 ± 0.2 on 15 kPa gels, and 1160.6 ± 429.7 µm² and 0.4 ± 0.1 on 20 kPa gels (Fig. 4.4e and 4.4h). Once these cells establish cell-cell contacts, the E and MKF cells show cell spreading on very soft 1 kPa gels, suggesting the cell-cell signals overwhelm the cell-substrate signals (the left region in Fig. 4.4b and 4.4c, indicated by yellow arrows). The majority of HCT-8 R cells;
however, remain rounded, with same apparent cell area and shape factor as those of isolated R cells, even when in contact with neighboring cells (Fig. 4.4a, indicated by yellow arrows). This R cell phenotype results in generally smaller R cell colony area compared to E cell islands consisting of similar cell numbers (Fig. 4.4f). The individual cell areas and shape factors of single R cells inside R cell colonies on 1 kPa gels are 151.8 ± 33.4 µm² and 1.0 ± 0.1, respectively, and is similar to those on 20 kPa gels (169.6 ± 30.5 µm² and 0.9 ± 0.2), respectively, as well as those of single R cells displaying no cell-cell contacts (Fig. 4.4e and 4.4h). On 5 kPa, 10 kPa and 15 kPa gels, the cell area and shape factor of individual HCT-8 R cells inside islands are 156.2 ± 52.3 µm² and 0.8 ± 0.1, 142.8 ± 47.2 µm² and 0.9 ± 0.0, and 160.7 ± 33.4 µm² and 0.8 ± 0.2, respectively. This unique phenotype persists even after R cells are cultured on the very stiff polystyrene substrates (3 GPa) for prolonged culture times (months); again suggesting weak cell-cell adhesion among R cells. Taken together, these results suggest that during or after E-to-R transition, R cells acquire cell autonomy that is characterized by markedly reduced cell-cell and cell-substrate adhesive contacts.
Figure 4.4: Comparison of single cell shape and area of HCT-8 E and R cells and MKF cells on substrates of various elastic moduli. HCT-8 E and R cells and MKF cells cultured on stiffness-gradient PA substrates with stiffness varying continuously from 1 to 20 kPa (left to right). (a-c) Phase contrast images of the harvested HCT-8 R cells, HCT-8 E cells, and normal MKF cells on the gradient-stiffness PA gel substrates. The respective 3 square panels (enclosed
by yellow dash boxes) show the representative magnified views on 1-5 kPa, 8-12 kPa, and 15-20 kPa stiffness domains. The white arrows in magnified views indicate the single, non-contact cells, while the yellow arrows indicate the contacting cells in colonies. Scale bars in magnified view panels are 100 µm. (d) The single cells’ projected area of 3 cell types across the stiffness range are shown. Here they do not have any contact with their neighboring cells on different stiffness substrates. (e) The spread area of single cells in contact with neighboring cells on different stiffness substrates. (f) The apparent cell colony area of 3 cell types on different stiffness substrates. (g) The cell shape factor of 3 cell types, which are not in contact with their neighboring cells on different stiffness substrates. (h) The cell shape factor of single cells, which are in contact with neighboring cells on different stiffness substrates.

4.6 Anoikis resistance

A critical step for successful metastasis is the survival of cancer cells in absence of substrate attachment [128]. Normal cells undergo programmed cell death, i.e., anoikis, when kept unattached to a substrate matrix or neighboring cells. Anoikis, a physiologically essential function, prevents detached cells from forming dysplastic colonies at places other than their correct anatomical locations, and ensures tissue homeostasis [128-130]. Metastasizing cancer cells, however, acquire the ability of anoikis resistance and survive even after detaching from the primary tumor; an ability that enhances their dissemination through the circulatory and lymphatic systems [129, 131]. To test whether HCT-8 R cells have acquired anoikis resistance, we performed cell suspension growth assays for both R and E cells. PA gels without any ECM coating were used to culture both cell types for 8 days followed by cell viability measurements. Since ECM-free gel surfaces are inert and non-adhesive, cells do not form any specific or non-
specific anchorage and are thus kept in suspension. The anchorage-free growth and viability of cells on gels were confirmed by daily imaging.

We found, after 7 days culture in suspension, 95.4 ± 5.07 % of single HCT-8 R cells were alive (Fig. 4.5), and maintained cell proliferation (Fig. 4.5b lower panel) throughout this suspension culture period. In contrast, only 11.8 ± 10.9 % of single HCT-8 E cells were alive after the same period of suspension culture (P value = 0.0001, Fig. 4.5c). As E cells form aggregates in suspension (Fig. 4.5a), their viability increased 7-8 fold, and 85.6 ± 18.6 % of the E-cell aggregates were alive, indicating that cell-cell contact might trigger certain survival pathways to partially evade anoikis in vitro [132]. For R cells in loosely packed aggregates (Fig. 4.5b); however, their viability was not significantly different from those in single cell form, and their cell division was not impaired due to suspension culture. Furthermore, the R cell numbers in suspension increased significantly due to sustained proliferation as the suspension culture continued (Fig 4.5b). These results are consistent with the RNA-Seq data (Chapter 5), indicating R cells up-regulate apoptosis inhibition gene expression compared to E cells. The results suggest R cells are potentially capable of surviving and growing in vivo as would be expected of a metastasizing cancer cell.
Figure 4.5: Suspension test for HCT-8 E and R cells. HCT-8 E (a) and R cells (b) were separately cultured on non-functionalized PA gels for 8 days to investigate anchorage independent growth and viability. (c) The percentage of viable E and R cells were determined by microscopy following harvesting from gels and Trypan-blue staining.

4.7 Discussion and Conclusion

To our knowledge, the present study is the first to describe and evaluate the change in mechanosensitivity and some essential metastasis-associated biophysical properties in human colon cancer cells during a metastasis-like transition produced by solely changing the mechanical microenvironment during in vitro culture. The present study employs a combinatorial assay
system approach using stiffness-gradient substrates, atomic force microscopy (AFM), cell traction force microscopy and suspension assay to explore the quantitative biophysical properties change of human colon carcinoma HCT-8 epithelial E cells as they transit to rounded-shape R cells. We found, triggered by the appropriate substrate rigidity cues, that HCT-8 R cells lose their sensitivity to both the substrate microenvironment as well as their interaction with neighboring R and E cells. As a result, HCT-8 R cells acquire autonomy for survival as anchorage-independent, mobile cells, which is an essential feature of the early events of cancer cell metastasis [1, 2, 4, 9, 59, 87-89, 133].
CHAPTER 5. GENE ANALYSIS

5.1 Motivations

In this Chapter, we first present a comprehensive, comparative investigation revealing that HCT-8 R cells are remarkably more invasive and tumorigenic compared to E cells. The HCT-8 R cells express many of the molecular signatures associated with resistance to hypoxia, apoptosis, as well as genes linked to metastasis and poor clinical outcome in colon cancer patients. Second, we confirm that similar E-to-R transitions can be induced in other cancer cell lines. They are human colon cancer cells, HCT116 and SW480, and human prostate cancer cell, DU145.

Our results suggest that the E-R transition, accelerated by in vitro mechanical cues, may mimic the early stages of metastasis. This model advances our understanding of the physical mechanisms that initiate colon cancer metastasis, and may enable us to identify molecular markers for early diagnosis, and ultimately design anti-metastatic therapeutics.

5.2 Design, Materials and Methods

Human colon adenocarcinoma SW480 cells (Cat. No. CCL-228; ATCC, Manassas, VA) were cultured in L-15 Medium (Cat. No. 30-2008; ATCC) consisting of 10% fetal bovine serum and Penicillin Streptomycin. Human colon carcinoma HCT-116 cells (Cat. No. CCL-247; ATCC, Manassas, VA) were cultured in McCoy’s 5a Medium (Cat. No. 30-2007; ATCC) consisting of 10% fetal bovine serum and 1x antibiotic-antimycotic (Cat. No. 15240-062; Gibco). All cells were cultured at 37 °C, with optimal humidity and 5% CO2.

Whole Transcriptome Sequencing (RNA-Seq) analysis was carried out for RNA derived from cells cultured on soft PA gel and hard polystyrene (PS) substrates. Harvested cells were put in RNA protect reagents (Qiagen Inc., Valencia, CA) to preserve the integrity of RNA. Total
RNA samples were isolated from E and R HCT-8 cells using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer’s recommendation. Briefly, confluent E cells at serial passage 18 (approximately 3-4x10^6 cells per P60 polystyrene dish) following 3 or 17 days of culture were rinsed with PBS followed by scraping off the cell layer in 750 µl of RNA Protect. Cells were pelleted by centrifugation. Cell pellets were suspended in 350ul of RLT buffer from the RNeasy kit and were homogenized using the Qiashredder spin column. The homogenized cell lysate effluent from the Qiashredder spin column was then processed as per RNeasy mini kit manufacturer’s directions, which included a gDNA eliminator column and total RNA was obtained in water. Likewise, total RNA from E cells grown on 20kPA polyacrylamide gels for 3 days was obtained. Total RNA from R cells was harvested following growth on 20kPA polyacrylamide gels for 15 days followed by filtering through 40 µm mesh (BD Falcon #352340) and expanded by culture on PS for 4 days. Concentrations and initial quality assessment of the RNA were determined by measuring absorbance at 260, 230 and 280 nm. RNA quality of the samples was checked using Nano6000/Pico bioanalyzer chips (Agilent 2100 Bioanalyzer, Santa Clara, CA) at the Keck Center for Comparative and Functional Genomics, University of Illinois. These RNA samples are denoted as PS3 (culture on polystyrene for 3 days), PS17 (culture on polystyrene for 17 days), Gel3 (culture on polyacrylamide for 3 days), and R (cells harvested after 15 days of culture on 20 kPA gels and expanded on PS for 4 days). RNA sequencing is performed at the King Abdullah University of Science and Technology (KAUST).

We sued ALDEFLUORTM reagent kit to identify human cancer cells that express high levels of the enzyme aldehyde dehydrogenase (ALDH). 5 µL of ALDEFLUORTM reagent per milliliter of cells were added to cultures at appropriate time points and incubated for 30 to 60 minutes at 37 °C followed by storage on ice or at 4 °C. An epi-fluorescent microscope and the
Metamorph imaging software were used to quantify fluorescent intensity of stained E and R cells.

We used the carboxy-H$_2$DCFDA (C$_{25}$H$_{16}$Cl$_2$O$_9$; No.: C400; Invitrogen, Molecular Probes), carboxy derivative of fluorescein as cell-permeating indicator for reactive oxygen species (ROS). The non-fluorescent 5-(and-6)-carboxy-2’, 7’-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) permeates live cells to enter the cytoplasm and become deacetylated by nonspecific intracellular esterases. The reduced fluorescein compound is oxidized once it detects nonspecific ROS in targeted cells and emits green fluorescence (504/530) [134, 135]. We determined an optimal dye-loading concentration as 5.0 µM (504/530, green) for HCT-8 cell monolayers. The working solution was freshly prepared using high quality anhydrous dimethylsulfoxide (DMSO) prior to experiments, and the excess diluted probe was discarded at the end of the experiment. We first replaced the phenol-red-free HCT-8 growth medium (Catalog number R8755, Sigma-Aldrich, St. Louis, MO) by the working solution, and allow incubation at 37°C for 15 min (empirically determined as optimal). Then, the working solution was removed and cells were incubated with pre-warmed growth medium for 5-10 min recovery time followed by confocal microscopy imaging.

5.3 Overexpression of metastasis-associated genes in R cells

To further characterize HCT-8 R cells, comparative differential gene expression analyses following E-R transition was carried out using whole transcriptome shotgun sequencing (RNA-Seq). Four cell populations, PS3, PS17, Gel3, and R; under selected temporal-spatial culture conditions were compared. The PS3 and PS17 cell populations are HCT-8 cells cultured on polystyrene (PS) substrate for 3 and 17 days, respectively, without any exposure to soft polyacrylamide (PA) gels. Cells under both conditions display the E cell phenotype and do not
show an E-R transition. The Gel3 cell population is HCT-8 cells cultured on 20 kPa PA gels for 3 days, but still remained in the E cell phenotype. The R cell population is derived from HCT-8 R cells harvested from 20 kPa PA gels on 17th day, followed by expansion on PS substrate. In an effort to discover differential gene expression patterns in R cells due specifically to the E-R transition, we compared gene expression profiles of R cells with those of Gel 3, PS3, and PS17 cell populations (cell populations not exhibiting an E-R transition). To be scored as a differentially expressed gene, the gene had to be 1), observed as differentially expressed in R cells in all three comparisons (R vs Gel3, R vs PS3, and R vs PS17), and 2) could not show differential expression when PS17 and PS3 cell populations were compared. In this manner, we focused on the genes that were more likely differentially expressed due to the E-R transition rather than extended culture time on PS or initial exposure to PA.

We found that 11 genes were up regulated by 3 fold or greater in R cells with respect to those in Gel 3, PS3, and PS17 cells (Fig. 5.1; positive Y axis; q-value < 0.05). These genes are associated with tumor promotion and invasion, repression of apoptosis, cell migration and proliferation, and generation of radical species (Fig. 5.1). Noticeably, one colon cancer stem cell marker, ALDH3A1 [136-138], was markedly up regulated in R cells. Two other genes, TNS4 and CLDN2, which are known as clinical markers for colon cancer staging [139-142], are also shown up-regulated in R cells. Ten other genes also were down regulated by 3 fold or greater in R cells (negative Y axis; q-value < 0.05; Fig. 5.1). Their functionalities are associated with promotion of apoptosis, maintenance of tissue homeostasis, anti-proliferation, and tumor suppression (Fig. 5.1). Of all those genes, down-regulation of CKB gene was reported to promote epithelial-to-mesenchymal transition (EMT) in colon cancer [143]. These data suggest, following culture on the appropriately soft mechanical microenvironment, a metastasis-
enhancing gene pattern is activated in R cells and this activation is likely associated with the characteristics of in vivo EMT [25, 89, 144]. Thus, the E-R transition is exemplified not only by a metastasis-like phenotype, but also a metastasis-like gene expression profile.

To confirm the RNA-Seq results, we used RT-qPCR to verify the differential expression of some selected up-regulated genes in R cells. The representative genes used were ALDH3A1, TNS4, CLDN2, and ALDKETO, and are known to play important roles in cancer cell de-differentiation, migration, invasion, proliferation and apoptosis suppression [137-142, 145-147]. Our qPCR results are consistent with the over-expression of these genes as observed in the RNA-Seq experiments.

![Figure 5.1 Summary of differentially expressed genes in HCT-8R cells as compared to E cells.](image)

RNA extraction and RNA-Seq analyses were carried out as described in Section 5.2. The data are expressed as fold expression. The bar graph represents the average fold expression.
changes of R cells compared to E cells cultured under different conditions (PS 3, Gel3, and PS17) that do not allow an E to R transition. The graphical and tabulated data summarize the up-regulated (green) as compared to down-regulated (red) genes. The composite results suggest that, most of the differentially up-regulated genes in R cells are associated with the functionalities of cell proliferation, motility, metabolism, invasive phenotype, colorectal adenocarcinoma and tumor metastasis. The differentially down-regulated genes in R cells are associated with tumor suppression and inhibition of apoptosis.

5.4 Cancer Stem Cell Marker Enzyme Activity

ALDH3A1 was identified by RNA-Seq and RT-qPCR as over expressed in R cells. ALDH3A1 has been identified as an important enzyme serving as a marker of various types of cancer stem cells [138, 148-150]. In addition to confirming its over-expression in R cells, we also used direct enzyme activity staining to test whether R cells or E cells on soft substrates possess increased ALDH3A1 enzyme activity. We found greater than 90% of R cells, as well as a small portion of E cells in the cell islands prior to E-R transition, show high levels of ALDH3A1 (Fig. 5.2a-b). The integrated whole-cell fluorescent intensities of ALDH3A1 for R cells are 4-5 fold higher than E cells (Fig. 5.2c). These results indicate, in addition to its high mRNA expression, ALDH3A1 enzyme activity is enhanced in R cells. These results also suggest that in vitro culture on soft substrates may promote the selection or production of cancer stem cells.
Figure 5.2: Fluorometric ALDH enzyme assays were used to directly measure the relative expression of ALDH in R and E cells in cell monolayers undergoing E-R transition on soft substrates. (a) 90% of R cells, as well as some E cells in the cell islands undergoing E-R transition express relatively high amounts of ALDH activity. Scale bar: 100 µm. (b) The integrated cellular fluorescence intensity of ALDH for R cells was 4-5 fold higher as compared to E cells.

5.5 Reactive oxygen species (ROS) production is up regulated in R cells

The RNA-Seq gene expression profile showed, following E-R transition, R cells express genes associated with cell survival and inhibition of apoptosis. It is known that the activation of
cell survival signaling pathways is often accompanied with the release of ROS from cell mitochondria [151-153]. To determine relative ROS expression, we used a stable fluorogenic marker, 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA) to stain both HCT-8 E and R cells followed by imaging using high-resolution multi-photon confocal microscopy. The marker distinguishes the oxidatively stressed and non-stressed cell populations through fluorescent-intensity ratio-metrics (Fig. 5.3 a-b). Our data show $57.8 \pm 5.84\%$ of R cells exhibit high ROS expression (fluorescent intensity = 40000 ± 5000 (A. U.)). Only $2.32 \pm 1.07\%$ of E cells express weak ROS (maximum fluorescent intensity = 5000 ± 300 (A. U.)). The integrated whole-cell fluorescent intensity in R cells is 6-8 fold higher than in E cells (p-value = 0.0001; Fig. 5.3c). It is known that expression of ROS can facilitate cell proliferation, mutagenesis, evasion of apoptosis and malignant transformation [154, 155].

**Figure 5.3:** (a-b) Reactive oxygen species of HCT-8 E and R cells were stained using fluorogenic marker: 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (carboxy-
H$_2$DCFDA) agent. Left: Fluorescent pictures. Right: DIC pictures. Scale bar: 20µm. (c) Comparison of ROS production in E and R cells.

5.6 Comparison of Invasive and Metastatic Activity of E and R Cells

We also compared the invasiveness of E and R cells using both *in vitro* and *in vivo* cell invasion assay. The results of *in vitro* invasion experiments show that R cells are significantly more invasive, an essential metastatic phenotype [89, 156, 157], compared to E cells: the number of R cell foci penetrating through basement membranes is more than 10 fold greater than observed with E cells (P value = 0.0075).

In order to evaluate *in vivo* hepatic invasiveness and metastatic potential, HCT-8 E and R cells were surgically injected into the spleen of athymic nude mice. After 9-10 weeks, all animals were euthanized and sacrificed. Spleen, liver, and other tissues with tumor development were fixed in formalin and embedded in paraffin to prepare histological slides. Incidences of tumor development and metastasis between HCT-8 E and HCT-8 R groups were evaluated with Fisher’s exact test. Necropsy and histological evaluations showed 69% of the mice injected with HCT-8 R cells and 71% injected with HCT-8 E cells developed tumor(s), indicating a similar rate of tumor implantations/development (Table 5.1). On the other hand, 73% of mice injected with HCT-8 R cells had more than one implantation sites and/or metastases, compared to 40% for mice injected with HCT-8 E cells (Table 5.1). Regarding liver metastases, there was a mean of 5.1 metastases in the liver of HCT8-R-injected mice and 3.4 metastases in HCT8-E-injected mice. The mitotic rate (number of mitoses per 400x magnification field), reflecting the growth of neoplastic cells in tumors, was identical in both HCT-8 E and HCT-8 R groups (Table 5.1). Interestingly, the rate of implantation of tumors in mice injected with E or R cells were similar.
Also, there were no morphological differences (growth pattern, stroma collagen production, mitotic index) between R and E cell tumors in our *vivo* model. Thus, the higher rate of tumor implantation with R cells was not due to cell survival in the spleen after injection or to different growth capacity of neoplastic cells, but likely due to higher migration and invasiveness. These results suggested that HCT-8R cells were more inclined to spread and migrate to different organs compared to E cells.

**Table 5.1: Comparison of tumor development in nude mice.** HCT8-E and HCT8-R cells were injected into the spleen of nude mice as described in Methods. Spleen tumor implantation and liver metastases numbers were based on the evaluation of H&E stained slides following 9-10 weeks after injection.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Number of animals</th>
<th>Mice (%) developing tumors</th>
<th>Mice (%) with more than one implantation site</th>
<th>Mean number of metastases in the liver</th>
<th>Mean mitotic rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT8-E</td>
<td>7</td>
<td>71 (5/7)</td>
<td>40</td>
<td>3.4</td>
<td>7.6 ± 2.0</td>
</tr>
<tr>
<td>HCT8-R</td>
<td>16</td>
<td>69 (11/16)</td>
<td>73</td>
<td>5.1</td>
<td>7.3 ± 2.7</td>
</tr>
</tbody>
</table>

5.7 Other Cancer Cells Also Exhibit MLP
To explore whether E-R transition is peculiar only to HCT-8 cells, we tested an E-R transition in three other cancer cell lines (HCT116, SW480 colon and DU145 prostate cancer cells) cultured on substrates with various softness. We found colon cancer cell lines, SW480 and HCT116, show E-R transition on 1.0 and 10 kPa gels, respectively, after 10 days of culture, whereas the prostate cancer cell line, DU145, exhibits E-R transition on 10 kPa gel after 19 days (Fig. 5.4).

Figure 5.4: Multiple cancer cells lines show E-R transition on appropriate soft substrates.
(a) HCT-116 cells cultured on 10 kPa PA gel substrates (coated with fibronectin) form cell colonies in 2-5 culture days. (b) HCT-116 cells begin to dissociate from colonies on the 10th day. On PS substrate or other stiffness PA gels under same culture condition, they do not show...
dissociation. (c) DU-145 cells cultured on 10 kPa PA gel substrates (coated with fibronectin) form cell colonies in 3-7 culture days. (d) DU-145 cells begin to dissociate from colonies on the 19th day. On PS substrate or other stiffness PA gels under same culture condition, they do not show dissociation. Scale bar: 100 µm.

5.8 Discussion and Conclusion

In this Chapter, we have demonstrated that soft substrate culture for 7-17 days can elicit remarkable functional and gene expression changes in HCT-8 colon cancer cells. Remarkably, in our in vivo metastatic animal model, HCT-8 R cells acquired strikingly efficient tumorigenic capacity (i.e. capacity to produce tumors in multiple organs) after injection. This mechanically-induced transition is not only limited to HCT-8 cells, as we observed the transition in other cancer cells lines as well, although the time to transition and the optimum substrate softness were dissimilar.

Our RNA-seq and protein expression analysis showed that a soft mechanical microenvironment stimulated HCT-8 cells to express a number of metastasis-enhancing genes associated with functions such as apoptosis inhibition, motility, metastatic activity and cancer stem cell traits. Among those, ALDH3A1, CLDN2 and TNS4 (CTEN), consistently found up regulated in R cells, are of particular interest.

Aldehyde dehydrogenases (ALDHs) are cytosolic enzymes responsible for oxidizing aliphatic and aromatic aldehydes to carboxylic acids. The expression of the ALDH isoform 3A1 (ALDH3A1) has been used as a cancer stem/progenitor marker from multiple organs, including colon and liver [146, 158-162]. High ALDH3A1 expression and activity have also been found to be closely correlated with cell proliferation [163], increased cell metabolism [164], and
prevention of apoptosis [138, 148]. Interestingly, in our model, R cells showed prolonged survival in suspension, and resistance to apoptosis with high elevation of ROS that likely correspond to acquired important traits for successful metastasis.

CLDN2 is a transmembrane protein that has been shown to result in epithelial permeability, neoplastic transformation, significant increases in cell proliferation and anchorage-independent growth when overexpressed in colorectal cancer cells [140].

These results, combined with our molecular data, suggest the R cells can be reasonably considered as a metastatic cell variant. Similar data were obtained by others with a rounded melanoma cell line, previously cultured on 3D soft fibrin gels, that were highly tumorigenic (tumor development with very low numbers of cells injected subcutaneously or intravenously in mice) [24]. However, the tumorigenicity of this model was partially due to survival of neoplastic cells in tissue; indeed melanoma cells, not cultured in 3D fibrin gels, never induced tumors after injection in mice. In contrast, our model clearly demonstrates that colon cancer R cells, produced from E cells through soft substrate culture, displayed a metastatic phenotype that was not linked to cell survival or mitotic rate of neoplastic cells in vivo. Our results raise the possibility that cells from different origins may undergo an accelerated metastatic transition dependent on their respective optimum mechanical microenvironmental niche.
CHAPTER 6. DISCUSSION and FUTURE WORK

6.1 Discussion and Conclusions

The discussions and conclusions drawn from the current work have been presented in the previous chapters. The current chapter is to reflect on the overall work and highlight the major contribution, followed by recommendations for future investigation.

Cancer deaths are primarily caused by metastases, not by the parent tumor[1, 2, 5]. The physical-chemical mechanisms, signals and parameters within the cellular microenvironment that initiate the onset of metastasis, however, remain largely unknown[11, 89]. The works reported in this dissertation describe a rather remarkable influence of the mechanical microenvironment on the tumorigenic promotion of HCT-8 human colon carcinoma cells in vitro. Exposure of human colon carcinoma HCT-8 cells to PA gel substrates of appropriate mechanical stiffness triggers a profound, stable cell state transition or selection, from an epithelial phenotype (E cells) to a metastasis-like phenotype (MLP; R cells). This morphology change is partially similar to epithelial-to-mesenchymal transition (EMT) during the early stage of in vivo metastasis, where epithelial cells undergo loss of cell polarity [128, 157, 165]. Although the rounded shaped R cells discovered in our study, mixed with a portion of spindle-shaped dissociative cells, is not 100% morphologically identical to mesenchymal cells [128, 157, 165], we suspect they might define an intermediate phenotype of EMT and represent cells that have passed only partly through an EMT as we have confirmed R cells possess some of the key EMT functionalities, such as enhanced invasiveness, elevated resistance to anokisis, and reduced E-Cadherin expression, etc. Furthermore, we reported that this mechanically-induced E-to-R cell state transition or selection is not only limited to HCT-8 colon cancer cells, but has also been observed in other cancer cells.
lines originated from different organs, though the time to transition and the optimum substrate softness were dissimilar.

We found the dissociated HCT-8 R cells display a number of in vivo biophysical and biochemical metastatic hallmarks: cell dissociation from parent colonies, sustained proliferation (both on substrates and in suspension), increased motility, attenuation of mechanosensitivity, down-regulation of E-Cadherin expression, reduction of cell adhesion (both specific adhesion rate and non-specific adhesion strength), gain of anchorage-independence capability, decrease of cell elasticity accompanied by loss of actin filaments, and promoted in vivo tumorigenic capacity (i.e. capacity to produce tumors in multiple organs). Similar E-to-R transitions can also be induced in other cancer cell lines. They are human colon cancer cells, HCT116 and SW480, and human prostate cancer cell, DU145. The physical properties of culture substrates are found to widely affect the phenotypes and gene expression of a number of normal and cancerous cells. However, to the best of our knowledge, our results on HCT-8 E-R transition on substrates with appropriate mechanical rigidity is the first study which suggests that in vitro 2D culture on soft substrates may promote the selection or production of human colon cancer cells with high tumorigenic capacity. Our works may aid in the understanding of the initiation of the early events in cancer metastasis progression.

6.2 Future Work

It is rather surprising to discover that, the HCT-8 R cells harvested following 7 days of culture on soft substrates show stable irreversibility of their rounded morphology and altered expression of proteins such as E-Cadherin and actin cytoskeleton filaments. This interesting phenomenon
raises another question: Is it a transition or selection process promoted by soft substrates? Instead of initiating E-to-R transition *de novo*, soft substrate might also enhance the selection of a pre-existing R cell population, which possesses the rounded morphology and invasive characteristics. Our RNAseq analyses, qPCR and enzyme activity studies consistently show that R cells express cancer stem cell markers ALDH3A1. Very recently, it was reported that an appropriately soft fibrin gel microenvironment selects a metastatic variant of murine B16-F1 melanoma cells that possess the traits of cancer stem cells and are highly tumorigenic in animal models [123]. In addition, during our culture of HCT-8 E cells in standard tissue culture flasks, we and other independent groups [61, 166, 167] also showed that a few rounded isolated cells (R-like cells) appear only on top of confluent epithelial monolayer (E cells). Although future work has to be done to investigate the similarity between R cells appeared on confluent epithelial monolayer (E cells) and on soft PA substrates, we think the above evidences raise an intriguing possibility that the soft PA substrates might enhance the selection and proliferation of a pre-existing stem-like R cell population too. Stem-like R cells, instead of epithelial-like E cells, might have preferred proliferation on the mechanically soft microenvironment. However, this hypothesis appears to contradict against our experimental evidences that solely R cells are presenting on soft PA gels after 2-3 weeks of culture, while there are nearly no E cells remaining. Therefore, we propose it is likely that two processes might co-exist. It is recommended for future investigators to perform a comprehensive cell live-dead assay, combined with time-lapse imaging on a daily basis, to differentiate the two mechanism of E-to-R process as well as their respective contribution. The cellular viability, proliferation rate and migration trajectories of both E and R cells on the same substrate are the key parameters to be examined.
Our finding raises a fundamentally important question: How can a change in culture substrate rigidity induce such a stable metastasis-like phenotype (MLP)? Although the exact downstream signal pathways of MLP are yet to be discovered, we propose that integrins as well as their associated proteins are the first set of mechanosensors involved in triggering E-to-R transition, as they are at the frontier of cell-environment interaction, and transduce extracellular microenvironmental cues to intracellular cytoskeleton via focal adhesion complexes[43, 168-170]. Depending on the stiffness of microenvironment, cells exert corresponding extent of contractile forces[171-173]. Stiffer the substrates are, larger forces that they can sustain and in turn higher force cells could apply to reach homeostasis[174, 175]. For very soft substrates, cells can barely apply any force, and thus results in the spherical cell shape followed by apoptosis[176, 177]. The varied cytoskeleton forces in cells tuned by substrate stiffness may cause conformational changes in both the extracellular and intracellular components, which may activate cryptic reaction sites, and initiate biochemical and genetic signaling cascades.

We hypothesize that, on soft substrates, the integrins of HCT-8 E cells undergo conformational stretch once being mechanically pulled by the desired cellular force. The process of integins conformational change starts after cells spread and settle down on soft substrate. Given the fact that integrins are spatially co-localized with epidermal growth factor (EGFR) inside the focal adhesion domains[5, 88, 141], the conformational change of integrin in turn results in the structure alteration of neighboring EGFR and mechanically opens their hidden sites for extra EGF binding. The resulting EGF stimulation triggers the onset of TEN3-to-CTEN switch[139, 142]. Note CTEN lacks the N-terminal actin binding domain of TEN3, and thus can lead to the disconnection between integrins and actin filaments, which are initially bridged by TEN3[139,
CTEN induction also associates with a reduction in E-cadherin protein expression (but not the amounts of E-cadherin mRNA) and results in the dissociation of cells[139, 141, 178]. Simultaneously, the stimuli of EGF or its phospholipases can also activate the cofilin pathway in HCT-8 cells and leads to the local cofilin release from its complex with PIP2 in the plasma membrane[179]. The released cofilin de-polymerizes the actin filaments and generates free G-actin[180]. Both processes largely enhance a morphology change of HCT-8 cells from epithelial-like to rounded-shape. Once the cells round up and begin to reduce cell-substrate contact area with substrates, they are on the risk of anokisis [176, 181]. To avoid it, some cancer cells start to activate multiple anti-anokisis activities by (1) releasing reactive oxygen species (ROS) from cell mitochondria[135, 151, 153, 155]; (2) over-expressing anchorage-independent growth gene CLDN2[140]; and (3) increasing cell-cell contact through activating proliferation gene Aldo-keto [145, 147] and ALDH3A1[138, 148, 158, 159]. The activation of such oncogenes triggers the invasiveness progression pathways and hence cells express a metastasis-like phenotype. The whole process does not complete immediately, but takes consequential steps to accomplish in the timeframe of 5-7 days.

Along the line of this model, it will be important for future mechanistic studies to examine the synergistic mRNA and protein expression kinetics of CTEN, CLDN2, ALDH, ROS, Cofilin and Aldo-keto, following initial exposure and culture of HCT8 cells on soft substrates. Such synergistic kinetics of those indentified markers is likely to reveal the development of mechsano-erosening of HCT-8 cells. In addition, specifically engineered microfluidic co-culture systems using colon cancer cells and relevant endothelial cells and stromal cells to attempt to at least partially simulate the in vivo tumor microenvironment, will also be valuable to explore the
combinatorial mechano-chemical mechanisms in angiogenesis development and \textit{in vitro} metastasis. Furthermore, our results clearly show this mechanically-induced E-R transition is not limited to HCT-8 colon cancer cells, but also occurs in cancer cell lines from other distinct origins when cultured on soft substrates. It would be essential to investigate whether and how these transitions of different cancer cells from distinct origins are dependent on the respective optimum mechanical microenvironmental niche. The bioengineering techniques and platforms developed in this work form the solid basis for such investigations.

In summary, our current results suggest that the E-to-R transition of HCT8 colon cancer cells, promoted by \textit{in vitro} mechanical microenvironmental cues, may mimic some aspects of the early stages of \textit{in vivo} colon cancer metastasis. This simple but powerful \textit{in vitro} model advances our understanding of the physical-molecular mechanisms of the tumor microenvironment that may initiate or promote the early phase of colon cancer metastasis. This model may enable us to identify molecular markers for early metastasis diagnosis, and ultimately design novel anti-metastatic therapeutics.
CHAPTER 7. CARDIAC MYOCYTE MECHANOTRANSDUCTION

7.1 Introduction

Growing evidences suggest that mechanical microenvironment and mechanical forces, both intracellular and extra-cellular, influence a wide range of cell functionality, including cell locomotion [57, 182], growth [42, 183], proliferation [184, 185], apoptosis [176, 177], and differentiation [177, 186, 187]. Cells show dramatic sensitivity to the substrate stiffness in 2D cultures [188]. For example, naive mesenchymal stem cells (MSCs) differentiate to neurons, muscle cells and osteoblasts when cultured on 1, 20 and 40 kPa polyacrylamide gel substrates, respectively [186]. Cells “feel” the stiffness of the environment by generating force on the substrate and by deforming it [189]. In turn, intracellular forces develop which cause conformational changes in stretchable force sensing molecules, resulting in gene expression and signaling cascades [190-192]. Thus stiffness information is transduced to cell functionality, such as, increased cell stiffness, higher intracellular forces, and stress fiber organization with higher substrate stiffness [41, 185, 188, 193].

The effects of substrate stiffness on cardiac cells bear important physiological significance. Myocardiac infarction results in scar tissues and stiffening of the microenvironment [194-196]. The effects of such stiffening on cardiac cells have been explored in [197] using 2D cell culture in vitro. It was found that cardiac cells beat on soft substrate for much longer times compared to the cells on hard substrate. However, cardiac cells on hard substrates bridged by fibroblasts can beat for longer times than those without fibroblasts connection [198], possibly

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due to softer microenvironment rendered by the fibroblasts. Fibroblasts of cardiac origin are also known to be capable of synchronizing electrical activity of multicellular cardiac tissue over extended distances (~ 600 \( \mu \text{m} \)) [199]. The time scale of such communication is within minutes [200, 201]. Such communication might be due to mechanical force coupling between the cardiac cells through the fibroblasts, besides the coupled bio-chemical pathways.

Mechanical stimulations, e.g. the stretch or shear loading, result in the excitatory electrophysiological response in cardiac cells, known as mechano-electric feedback (MEF) [202-205]. For example, mechanical stretch is known to cause trans-membrane cationic currents, altered action potential [206, 207], and activation of Cl\(^-\) channels [208]. Shear fluid pulses on a monolayer of neonatal rat ventricular cells, induced by localized fluid jets, evoke action potentials that propagate across the monolayer, and reentrant arrhythmia [209]. Although to date the mechanism of MEF remain elusive, mounting evidence indicates that the mechanosensitive ion channels (MSCs), mostly Cl\(^-\) or K\(^+\) selective are mediators of MEF [203, 204], [210-212]. MSCs are found in a wide variety of cardiac cells, including chick [213-215], rat [211, 216], frog [217], guinea pig [218, 219] and human [220-222].

Stretch activation of cardiac cells raises the possibility that a beating cell on a soft substrate may induce stretch on its distant neighbor by deforming the flexible substrate. The neighbors thus can “see” each other mechanically through the substrate. In this Chapter, we addressed questions. First, can a quiescent cardiac cell (cell not beating) on a soft substrate be actuated (set to beating) by a local mechanical stretch of the substrate by an inert probe? The probe was physically distant from the cell so that it represented the stretch induced by another cardiac cell. The inert probe also served the purpose of delivering solely the mechanical signal, without any bio-chemical cues, to the quiescent cardiac cells. We found, the probe caused the
quiescent cell to beat in contrast to the control cell that remained quiescent. Second, how far two neighboring cardiac cells can mechanically influence each other on a soft substrate? We addressed this question by culturing cardiac cells on a soft substrate without any fibroblast, and by noting the beating patterns of pairs of cells. We found, closer the cells were, longer they beat as a pair, i.e., the cells kept each other awoke (beating) for a longer time. With increasing stiffness of the substrate, this mechanical interaction decreases. This distance dependence or the range of “seeing each other” could be well predicted by the theory of linear elasticity and finite element analysis (FEM). Our study suggested that in vivo, cardiac cells could be mechanically coupled to each other in addition to their electrical and chemical coupling, and that the cells interacted with each other through the deformation field of the in vivo soft tissue. The softness of the tissue not only determined their functionality but also their range of interaction, both of which might be essential for the normal functioning of the heart.

7.2 Design, Materials, and Methods

Cardiac cells were extracted from chicken embryo between the 32-35 Hamburger Hamilton stage, roughly when the chicken embryo was 8 days old [223]. These primary cells were plated on the laminin coated substrates with culture media consisting of minimum essential medium (MEM) with alpha modification (Sigma), 10% fetal bovine serum (FBS) (Sigma) and antibiotics (penicillin, 1000 IU/mL and streptomycin, 1000 µg/mL) (Gibco). The cells were left undisturbed in the incubator overnight and measurements were started only on the next day. During data acquisition, the gels and the culture dishes were kept on heated microscope stage to maintain a temperature of 37°C and a tube releasing 5% CO₂ was kept over the dishes to maintain a physiologically relevant pH. The cell culture media was changed every two days. The
videos of the beating cells were taken using a digital Cannon camera and a high speed SPOT camera. The image analysis and processing are done using ImageJ, Matlab 7, and Photoshop CS2.

A rigid Tungsten probe was mounted on the X-Y-Z micro-positioning stage. The X-Y-Z micro-positioning stage was mounted beside the inverted phase-contrast microscope (Olympus IX81). The probe tip was immersed into culture medium in culture dish and lightly indented the soft substrate at the region 40 ~ 70 µm away from the quiescent cardiac cells (Figure 1a). The target quiescent cardiac cells were selected after more than 3-minute observation to ascertain they were not beating with very low frequency. Driving by the X-Y-Z micro-positioning stage, the Tungsten probe cyclically pulled the soft substrate beside the cells in a pulsatile or sinusoidal fashion at the frequency 20 ~ 45 cycles per minute. As the soft and flexible substrate was cyclically pulled, the cells which adhered on substrate and near the probe pulling region (40 ~ 70 µm away) were in turn cyclically stretched. The Tungsten probe was selected because of its high stiffness and effective force application. The Tungsten probe was not chemically functionalized and sterilized by Ultra-violet ray (UV) before experiment. Therefore, no biochemical cues but only mechanical stimulus were introduced during study.

7.3 Local Mechanical Excitation of Quiescent Cardiacmyocyte

Primary chicken cardiomyocytes were cultured on inert soft 2D polyacrylamide (PA) substrates (stiffness E = 1.05 ± 0.17 kPa, calibrated by Atomic Force Microscopy). The density of cells plated on 2D substrates was controlled as 50,000 to 100,000 cells per cm², counted by the standard hemocytometer. This range of cell density resulted in the spatial separation of the individual plated cells to be 0 ~ 200 µm from one another. Note that the primary cell samples, extracted from chicken hearts, consisted of a mix of both cardiomyocytes and cells from the
connecting tissues, such as vascular smooth muscle cells, fibroblasts, and endothelial cells, etc. Since most connecting tissue cells were unable to grow on very soft substrate, e.g. at 1 kPa, due to the lack of cell traction necessary for cell spreading, these cells underwent apoptosis [224], resulting in a physical separation between the cardiac myocytes. However, unlike the fibroblasts, the cardiac myocytes survived on mechanical soft substrates with high viability. During culture, some of the cardiac myocytes beat, while others occasionally beat or kept quiescent for most of the culture time. A cell was determined as quiescent if it was found not beating by continuous video observation for 4 minutes prior to any mechanical stimulation. Based on our experience, the cardiac cells which do not beat for these 4 minutes remained quiescent, and did not spontaneously start beating (at least within the duration of video observation, often for an hour).

In order to apply stretch on a quiescent single cardiomyocyte by deforming the substrate, a rigid tungsten probe was mounted on a piezo stage with x-y-z actuators (Fig. 7.1). It contacted the gel surface and exerted a small compressive force on the gel near a cardiac cell. Fig. 7.1a shows such a probe at 45 µm away from a quiescent cardiac cell. Driven by the actuator, the probe cyclically pulled the soft substrate in a pulsatile fashion with the varied frequency, from 20 to 45 cycles per minute. As the soft and flexible substrate was cyclically pulled, the cell adhered to the substrate near the probe was also cyclically stretched. In Fig. 7.1b, the cell stretch was about 6%. This simple but effective method exerted mechanical force onto the cardiac cell while avoiding the direct contact between the rigid probe and the cells. Also, the tungsten probe was not bio-chemically functionalized, and thus only mechanical signal was propagated towards the cell through the substrate. Throughout the experiment, the beating activities of the cardiomyocytes were monitored by an inverted phase-contrast microscope and time-lapse video recording.
Figure 7.1: Awaking a quiescent cardiac cell by a distant probe. The probe stretched the cell by deforming the soft PA substrate. (a) Schematic of a probe at 45 µm away from a quiescent cardiac cell on a soft substrate. The microscope objective was underneath the Petri dish containing the soft substrate and cells to monitor the entire cell excitation process. (b) Phase-contrast images of a chicken cardiomyocyte adhered to a 1kPa gel substrate functionalized by laminin ECM and the probe tip in contact with the substrate. The probe applied a small compressive force on the substrate and was then moved horizontally away and towards the cell. This induced a deformation of the substrate. Consequently, the cell was stretched and compressed in a pulsatile fashion. The probe may slide during motion. The marker, A, on the surface of the substrate moved when the probe was not sliding. Therefore, the motion of A represented the actual deformation of the substrate. Scale bar: 50 µm.

The probe was slightly tilted with the vertical (Fig. 8.1a). Thus, as it was moved away from the cell, the angle of probe increased due to its compliance. Consequently, the contact force between the probe and the substrate decreased, and the probe may slide on the substrate. As the probe was moved towards the cell, the contact force increased which prevented the sliding. In
order to monitor the actual mechanical stimulation on the cell, and for later analysis (next section), we monitored the position of a marker on the soft culture substrate (point A in Fig. 7.1b). The marker moved when the probe deformed the substrate without sliding. During sliding, the probe continued to move leaving the marker stationary. Thus the mechanical stimulation on the cell can be quantified from the motion of the marker. The relation between the motion of the probe without sliding and that of point A was obtained as follows: the probe was initially placed at about 45 µm away from a cardiac cell. It was then moved towards the cell. The corresponding motion of A along the direction of motion of the probe (i.e., a component of its motion) was monitored. The probe was then moved back to the original configuration when A returned to its origin. The motions of the probe and that of A show a linear dependence and the probe displacement was three times that of the marker A (ratio: 1/0.31~ 1/0.34). The linearity is due to the linear elasticity of the gel [225-227]. The continuity of the line is due to no slippage between the probe and the substrate. This relation will be used in the analysis later.

Fig. 7. 2a shows the displacement of marker A and the corresponding activation scenario of the cell as a function of time. Note that the amplitude of A decreased with time due to the increasing sliding between the probe and the substrate. The maximum displacement of A was about 4.5 µm at the seventh stretching cycle when the cell was stretched by 6.5 % (Fig. 7.2b and 7.2c). After seven cycles of such stretches (about 20 seconds), the quiescent cell started to beat, and its self-contraction and stretching were much larger compared to those imposed by the probe (Fig. 7.2c). The cell continued to beat with the beating period of 15-25 seconds for hours after the mechanical stimulation is stopped. This period was much longer than the period of beating during stimulation. Note that the cardiac cell was initially quiescent, at least for 4 minutes prior to the stimulation. The control experiments were also performed: the quiescent cardiac cells,
chosen based on the same rules and cultured under the same condition, were continuously video recorded for 8 minutes in the absence of mechanical probe stretching. We found, they remained quiescent throughout entire 8-minute observation. Thus the beating of the stretched cell was due to mechanical stimulation of the probe, and was not a spontaneous activity of the cell.

**Figure 7.2:** (a, c) Displacement of point A on the substrate in response to the motion of the probe as a function of time. This displacement represented the deformation of the substrate. Its amplitude varied with time due to the sliding between the probe and the substrate. The blue line shows the contraction-expansion strain along the vertical diameter (indicated by the white double-head arrow in (b)) of the cell after it began to beat. Before the stimulation by the probe, the cells were confirmed as quiescent (no beating) based on 3~4 minutes of observation (see Results section). (b) Cell shape at the maximum and minimum substrate stretches. (c) Strains in the cell due to deformation of the substrate before it began to beat. The representative maximum stretching and contraction of soft substrate were labeled.
Out of the fifteen quiescent cells that were stimulated by the probe, 8 were found to beat. The quiescent-to-beating activation time was cell-specific but all were within tens of seconds. The average stretch applied by the probe on the cells was $5.89 \pm 0.62 \%$ of their original size. After activation, the period of beating of the cardiac cells was also cell-specific, varying within $15 \sim 55$ seconds (Fig. 7.3). In all cases, the post-stimulation beating period was much longer than the period during stimulation. These results indicate that chicken cardiomyocytes are sensitive to mechanical stimulation and that they can commit to contraction-relaxation cycles by mechanical cue alone.

![Histograms of the periods of mechanical stretching of cardiac cells by a mechanical probe and the activated cells.](image)

Figure 7.3: Histograms of the periods of mechanical stretching of cardiac cells by a mechanical probe and the activated cells. The cells were confirmed as quiescent, i.e. not beating, by 4 minutes of continuous observation prior to stimulation. The quiescent cardiac cells began to beat after a few cycles of stimulation. The beating frequency of the stimulated cells was high during stimulation. After stimulation was removed, beating frequency dropped. The post-stimulation periods of beating are shown in the histogram.
In the excitation experiments, the cell culture dishes were removed from the 37°C incubator to the microscope stage at room temperature of 25°C. In order to minimize a drop in temperature of the medium, the dish was placed on a hot plate at temperature 37 °C on the microscope stage. Each stimulation experiment took less than 4 ~ 7 minutes. To ascertain whether the change of beating frequency during and after the application of stimulation was not biased by the drop in the culture medium temperature, we continuously monitored the frequency of naturally beating cardiomyocytes with time of exposure to room temperature as the control. It is found that within 7 minutes, the beating frequency dropped by 11.1 ± 9.8 %. This drop due to the temperature change is 5~6 folds less than the drop in frequency due to the removal of mechanical stimulation. This suggests that the frequency drop after the removal of mechanical stimulation was not due to decrease in temperature.

7.4 Neighboring cardiacmyocytes stimulate each other

In the previous experiment, we showed that an inert probe could excite a quiescent cardiac cell to beat by applying mechanical stimulation through the substrate. This suggests the possibility that a beating cell might also stimulate a neighbor by stretching and releasing the soft substrate. Such stimulation might ‘awake’ a quiescent neighbor, which in turn may stimulate the neighbor that awoke it in the first place. Thus the beating of the pair may become coupled, and they might continue to beat for a longer time compared to a cell too far from any neighbor. The coupled beating would depend on the distance between the neighbors - farther the neighbors are, less is the mutual stimulation. Therefore, we also explored the spatial distance between two cells within which they might stimulate each other through the soft mechanical substrate.
To examine whether the mutual stimulation between two close-by cells was due to mechanical interaction alone, in contrast to the possibility that chemical factors released by close-by cardiac cells might also play a signal-transmitting role in communication, we studied the cell-beating patterns on 2 different substrates with low (1kPa) and high (47 kPa) stiffness (confirmed by AFM). If the mutual stimulation is primarily chemical, then the degree of stimulation will depend on the distance between the cells alone, irrespective of the substrate stiffness. If the stimulation is primarily mechanical through deformable substrate, then stiffer the substrate, lesser is the interaction for a given distance between the cells, and the cells in a pair may not beat together for long time.

We performed video imaging of cardiac cells plated on same soft substrate (1 kPa) at 246 different locations on day 1-4 of plating (Fig. 7.4a). For each cell in the view-field, we identified the nearest neighbors, and grouped the cells as pairs with the neighbors at distances 0-5 µm, 5-10 µm, 10-20 µm, 20-30 µm, and 30-40 µm apart. Cells that did not have any cell neighbor within 60 µm were considered as single. We recorded the fraction of pairs within each given group (for example, pairs with 10-20 µm apart partners) with both cells beating (Fig. 7.4b). Similarly, the fractions of single cells that were beating during day 1-4 were determined (Fig. 7.4b inset). Fig 3b shows that the cells with their closest neighbors at, i.e. 0-10 µm, beat (together with the neighbor) for longer times, as hypothesized. For example, on day 4, 100% of the pairs with partners within 0-5 µm beat as couples. The fraction is 50% when partners are 5-10 µm apart. Single cells lacked any mechanical stimulation from each other, and hence lower fraction of them beat together for long time. On day 1, 2, 3, and 4, the percentages of single beating cells were 62.5%, 50%, 34% and 18 % respectively.
Figure 7.4: (a) Determination of cell pairs by measuring the distance between the nearest neighbors. No fibroblasts were present to connect the cardiac cells. (b) On 1 kPa gel, cell pairs were grouped by distances between neighbors, such as a group consists of pairs with partners 0-5 µm, 5-10 µm, 10-20 µm apart, etc. n = 246. Percentages of pairs within each group with both cells beating were plotted for day 1-4. Higher percentages of pairs with close-by cells beat for longer times, possibly by stimulating each other. (b inset) Percentages of single cells beating vs. time. These cells have no partners within 60 µm. (c) On 47 kPa gel, percentage of beating pairs (both cells of the pair beating) within mutual distance 0-10 µm is shown on day 1, 2, 3 and 4, respectively. n = 52.

On the stiffer (47 KPa) substrate, the cells’ beating patterns at 52 different locations were monitored. Since the stiff substrate is 47-fold more rigid than the soft one, the mechanical deformation of the substrate by cellular traction is negligible beyond 10 µm (verified by both
theoretical prediction and Finite Element Analysis; see details in Section 7.5), hence we only studied the beating pattern of cells within 0-10 µm apart. We found, the percentage of beating-cell-couples within 0-10 µm was 17% on day 1 and 14% on day 2, dramatically lower than those on 1 kPa gels. On day 3 and 4, there were no beating-couples within this effective distance range. The data suggest that the chemical factors, if any, released by cardiac cells at small mutual distance, were not sufficient to sustain the long lifetime of beating-couples. These results, together with the finding that an inert probe could stimulate a quiescent cell through the deformable substrate, imply that mechanical stimulation, either from a probe or from a neighboring cell, regulate the beating scenario of cardiac cells.

7.5 Range of Mechanical Interaction

In order to interpret these mutual mechanical interactions between cells in light of the stimulation of quiescent cells by the probe, we carry out the following analysis. Consider a force, $F$, applied at a point on the surface (with Elastic shear modulus $G$ and Poisson ratio $\nu$) of an elastic half space. For the linear-elastic, homogeneous and isotropic material, the elastic modulus, $E = 2G(1 + \nu)$. The displacement, $u$, of a point on the surface of the substrate along the direction of the force at a distance $x$ from the force is given by $u = \frac{F}{2G\pi x}$ [228]. Similarly, a couple with two forces, $F$, acting at a distance $\Delta$ (Fig. 7.5) from one another produces a displacement $u$ at a distance $x$ from the dipole as,

$$u = \frac{F}{2G\pi} \left( \frac{1}{x} - \frac{1}{x + \Delta} \right) = \frac{F}{2G\pi} \frac{\Delta}{x(x + \Delta)} \quad (7.1)$$
Now, consider a segment of length $\Delta$ on the surface at a distance $x$ from the dipole. Then the increase in length of the segment, $d\Delta$, due to the dipole is:

$$
d\Delta = F \left( \frac{1}{x} - \frac{1}{x+\Delta} \right) - F \left( \frac{1}{x+\Delta} - \frac{1}{x+2\Delta} \right) = \frac{F}{G\pi} \frac{\Delta^2}{x(x+\Delta)(x+2\Delta)}
$$

(7.2)

If $\Delta$ represents the size of a cell and $F$ the dipole force it generates, then $d\Delta$ represents a mechanical stimulation by the cell on a substrate at a distance $x$ away. The net stretching ratio, $\varepsilon$, of the substrate due to the cell is:

$$
\varepsilon = \frac{d\Delta}{\Delta} = \frac{F}{G\pi} \frac{\Delta}{x(x+\Delta)(x+2\Delta)}
$$

(7.3)

Thus, the stimulation decays rapidly as $\frac{\Delta}{x(x+\Delta)(x+2\Delta)}$. Here $d\Delta$ is the stretch of the free surface from the original length $\Delta$. If a cell is attached to the surface at the same location, it will resist the substrate deformation. The cell will also get stretched depending on the relative stiffness between the cell and the substrate. In order to obtain the stretch of the cell, we need the stiffness of the cell. We estimated the stiffness of the cardiac cell from the numerical simulation of the experiment with the probe and the single cell as follows.
Figure 7.5: Schematic of force dipole model on an elastic half space. The horizontal displacement of a point on the surface along the direction of the dipole is given by $u$. $d\Delta$ is the stretch of a segment of length $\Delta$ on the substrate due to force dipole. If a cell of length $\Delta$ is attached to the substrate, then it resists the stretch $d\Delta$. However, together with the substrate, it stretches by a lesser amount. Thus $d\Delta$ represents the stimulation on the cell by the substrate.

We modelled (Fig. 7.6) the substrate as an isotropic elastic film with modulus of 1kPa (measured by AFM) attached to a rigid substrate (glass). The film spans an area of $500\mu m \times 500\mu m$ and have thickness of $70\mu m$. The vertical sides of the film and the bottom surface were fixed, i.e., restrained from motion. The cell was considered as a homogeneous, isotropic, linear elastic solid with a diameter of $40 \mu m$ and thickness of $4 \mu m$, similar to the geometry of the cell in Fig. 7.1. The cell was attached to the substrate uniformly along the interface. The probe was modeled as a rigid cylinder with diameter $128 \mu m$ and was attached to the substrate (i.e., no sliding possible). It was $45\mu m$ away from the cell.
Figure 7.6: Finite element modeling of a cardiac cell, the soft substrate, and a mechanical probe. The rigid probe and the cell were attached to the soft substrate. The cell was 4 µm thick, and 45 µm in diameter. The probe was 128 µm in diameter. The probe applied a prescribed in-plane displacement to the substrate. The corresponding displacement and the stress fields of the substrate and the cell, as well as the cell-substrate traction were computed by finite element method. The probe actuation was represented by a prescribed displacement field over a circular region of the substrate. Substrate elastic modulus was 1KPa, and the cell modulus was varied until the computed stretch of the cell due to the displacement of the probe matches the experimentally measured stretch. Point A in the figure represented a reference marker on the substrate found in the experiment.
Figure 7.7: Results of the cell stretch experiment simulated by the Finite Element analysis (see also Fig. 7.8). (a) Cell stretch due to probe actuation was computed for various elastic modulus of the cell material. The cell with modulus of 3-3.5 KPa resulted in a stretch that is comparable to the experimentally measured value of 5.8%. (b) The traction between the cell and the substrate was shown when then cell modulus is 3.5 KPa.

In the experiment with the probe and the cardiac cell, the maximum displacement of the probe without sliding was about 10.82 ± 1.21 µm. The displacement of marker A is 3.61 ± 0.41 µm (Fig. 7.1b). The corresponding stretch of the cell was found to be about 5.82% ± 0.65% of the initial size. In the simulated experiment, we applied 11 µm displacements on the probe. The corresponding stretch of the cell was obtained from Finite Element analysis. For an arbitrary elastic modulus of the cell material, the experimental and the simulated stretches of the cell did not match. Thus, we varied the cell material modulus until the experimental and the simulated stretches matched. This modulus of the simulated cell was found to be $E_{\text{cardiac-cell}} = 3.5 \text{kPa}$ and
3kPa when the stretch was 5.61% and 5.92% respectively, both close to the experimental value of 5.82% ± 0.65% (Fig. 7.7a). This modulus range (3~3.5 kPa) was in excellent agreement with the experimental cardiac myocytes stiffness data reported earlier, e.g. 2.5~ 3.3 kPa [229] or as 2.2~ 5.4 kPa [230]. In the following, we used the cell elastic modulus as 3.5 kPa. Note that it was the stiffness of the cell along the substrate that prevents the free stretch of the substrate during probe displacement. This stiffness resulted from the combination of the cell thickness (4 µm) and the elastic modulus (3.5 kPa). For the simulated cell, finite element analysis provided the traction between the cell and the substrate. The maximum shear stress propagated to the cell due to probe stretch was 77.2 Pa, when the cell was stretched by 5.3% (Fig. 7.7b). The resulting cellular force dipole was 200 nN. With all these information, we can now analyze cell-cell mechanical interaction on the same soft substrate.

Consider a cell C1 attached to the substrate. It beat with an amplitude and generated a force on the substrate. It has a neighboring cell, C2, at a distance x away, also attached to the substrate. We estimated the stretch in C2 due to contraction of C1 for various values of x. C1 induced the stretch in C2 by stretching the substrate. We modeled the cells as discs of size 40µm diameter, 4 µm thick with isotropic elastic modulus of 3.5 kPa. Boundary conditions of the substrate were similar to those used in simulating the probe experiment. The contraction of C1 was simulated by applying a force dipole on the substrate as shown in Fig 6a. The magnitude of the force dipole was 200 nN (derived from the analysis of Fig. 7.7b), acting at 40 µm apart. This magnitude was not critical for the conclusions to be drawn in the following, since the stretch of C2 will be normalized by its stretch at x =5 µm. The stretch of C2 due to the dipole was obtained from finite element analysis for various values of x. The strain in C2, namely, is derived from dividing stretch in C2 by its original size (40 µm) along the dipole as a function of x. Clearly,
the effect of C1 on C2 decayed with x rapidly. The best fit of the cell strain with the basis function \( \frac{\Delta}{x(x + \Delta)(x + 2\Delta)} \) was obtained from Eqn. 7.3, with \( \Delta \) as a fitting parameter. We found \( \Delta = 148 \mu m \) for the best fit curve shown in Fig 7.8b. Note that the large value of \( \Delta = 148 \mu m \) compared to the cell size of 40 \( \mu m \) essentially reflected the large elastic modulus of the cell (3.5 kPa) with respect to the substrate (1 kPa). Fig. 7.8c shows the histograms of Fig 7.4b, which gives the probability of finding a pair of beating cells with members at a given distance away. Superimposed on the histogram is the curve of Fig. 7.8b, but the cell strains were normalized by the strain at 5\( \mu m \) away from the force dipole. The close correspondence between the predicted cell-cell mechanical interaction (Finite Element analysis or the analytical prediction) and the histogram suggests that the close-by cells are more probable to stimulate each other mechanically. The farther apart the cells, the less the probability of their mutual stimulation. Thus the close-by cells were more likely to beat for longer time as a pair. This analysis, however, does not account for the time evolution of mutual stimulation. There must be some time required for a cell to be stimulated by a neighboring cell. Initially (soon after plating), the cells were likely to beat randomly without the effect of a neighbor, and the distance between them did yet not play a role. As time progresses, they became mechanically coupled through the soft substrate.
**Figure 7.8:** How far the cells can see each other? (a) We simulated the cell-cell interaction by applying a force dipole (200 nN at a distance of 45 µm) on the substrate that mimics the contraction of a cardiac cell during beating. The corresponding stretch on a neighboring cell attached to the substrate was analyzed using Finite Element method. (b) The stretch was represented as a percentage of the original size as a function of the distance between the cells. The solid line is the best fit curve with the basis function $1/(x(x+\Delta)(x+2\Delta))$ from Eqn. 7.3. (c) Histogram of Figure 5 is presented, together with the prediction from Fig (b) normalized by the cell stretch ratio at 5 µm. This prediction accounts for mechanical interaction between two cells only (i.e., stretch induced on one cell by the other).
The strong distance-dependence of mechanical interaction between cells suggests that if a cell has two neighbors, one closer than the other (not all three necessarily on the same line), then the nearby one will have significantly higher influence than the farther one. Such is the case with the group of cells in Fig. 7.9 at day 3 of culture on a 1kPa substrate. The nearby pair (cell C1 and C2), about 42 µm apart, beat in synchrony with frequency of 64 cycles /min, although their beating was phase lagged by about half a period, i.e., when one of them contracts, the other relaxes. The cell, C3, which was farther apart, about 65 µm away from C1 and 89 µm away from C2, beat with a frequency of 70 cycles /min. They all contracted by about 5% during their own individual beating. However, when C2 contracted during beating, it caused 2.5 ± 0.8 % strain in C1, while it displaced its edge (closest to cell 1) by about 5 µm (along a direction away from C1). C3 induced no noticeable stretch in C1. We observed this effect of proximity for several 3-cell systems. To test whether this mutual stretch was mechanical, we carried out the FEM analysis of the above 3-cell system to estimate the effect of one on the others. We simulated the contraction of the cell C2 by applying a displacement of 5 µm along the edge of the simulated cell 2. We found that the corresponding induced stretch in cell C1 is 3.1%, close to the experimental value of 2.5 ± 0.8%. The study suggests that the threshold induced strain needed for mechanical coupling might be less than 2.5%, i.e., if one cell can induce 2.5% strain on another, then they might become mechanically coupled. They might keep each other ‘awake’, and they synchronized their beating. Similarly, when the cardiac cells were connected by connecting tissues over long distances, even 150 µm away, they became mechanically coupled, and they could synchronize their beating.
Figure 7.9: Mechanical interaction between beating cardiac cells. (a) A group of beating cardiac cells on soft (1 kPa) substrate. The nearby pair (cell 1 and cell 2), about 42 µm apart, beat at the same frequency (64 cycles/min), although their beating is phase lagged by about half a period. The cell 3, which was farther apart, about 65 µm away from cell 1 and 89 µm away from cell 2, beat at a frequency of 70 cycles/min. Cell 2 was in contractile mode, it stretched cell 1 by about 2%; (b) Both cell 1 and 2 were at relaxed states. (c) Simulation of interaction between the cells using finite element analysis. A displacement of 5 µm was imposed on the boundary of cell 2, mimicking the experimental observation. Corresponding stretch in cell 1 was found to be 3.1%, close to the experimental stretch. The effect of contraction of cell 3 on cell 1 and 2 was negligible.

7.6 Discussions and Conclusions
It is recognized that the cardiomyocytes in various animals are capable of performing mechano-electric feedback (MEF) [203-205, 231]. Namely, the mechanical stimulus applied on cardiomyocytes results in the excitatory response [206-209]. The present study shows that beating cardiac cell could stretch its neighbors by deforming a soft medium in between. Thus they could stimulate each other mechanically through the substrate, possibly by invoking the MEF. The experiment with the inert tungsten probe that awaked a quiescent cell to beat by deforming the substrate clearly shows that such stimulation is mechanical. The underlying mechanism of mechanical stimulation through the substrate is likely to be the activation of stretch sensitive ion channels due to stretch, or equally likely, the activation of cell signaling pathways triggered by cells’ stress sensor due to the mechanical deformation. Thus the coupling between the cells was both mechanical and biochemical. The latter follows the former. The exciting results are, the stimulation can be mediated by mechanical deformation alone over astonishingly large distances.

The answer to the question of how far a cell can see its neighbors mechanically lies in the magnitude of its stretch induced by the neighbors. For a given type of cardiac cell, this distance depends on the stiffness of the medium that bridges the cells. For the case of cells on a 2D substrate, the strain of a cell due to the force dipole of a neighbor is well approximated by a function of the form \( \frac{F}{G\pi x(x+\Delta)(x+2\Delta)} \), where \( F \) is the force of the dipole, \( G \) is the shear modulus of the substrate, and \( \Delta \) is a constant. Thus, as the Elastic modulus of the substrate increases, the effect of a neighbor at a given distance away decreases. If there is a threshold strain, \( \varepsilon_{th} \), needed for the cell to be stimulated, then

\[
\varepsilon_{th} = \frac{F}{G\pi x(x+\Delta)(x+2\Delta)} \tag{7.4}
\]
gives the relation between \( G \) and \( x \) for the cells to be mechanically coupled. Figure 7.10 shows the relation qualitatively for a given \( F \) and \( \varepsilon_{th} \). Thus, for a pair of cardiac cell and a given substrate stiffness, there is a spacing, \( x_{th} \), between the cells that is just enough for the cells to be mechanically coupled. If the distance is higher, \( x>x_{th} \), the cells may become mechanically decoupled, and they may beat incoherently, or stop beating. Similarly, for a given \( x_{th} \), if the substrate stiffness increases, the cells may not “see” each other anymore, and they cannot keep each other awake. This is exactly the case for cell pairs with partners 0-10 \( \mu \)m apart on 1 kPa and 47 kPa gel substrates (Fig. 7.4). On stiff (47 kPa) substrate, the pairs beat with both partners for only 2 days, whereas on soft (1 kPa) substrate most of such pairs beat (both cells of the pairs beating) after 4 days. However, on the same soft substrate, when the cells are far apart from one another (60 \( \mu \)m or higher), only 30\% of the cells beat after 4 days. A recent experimental study shows that cultured quail embryonic cardiomyocytes (without fibroblasts) on soft substrates maintain beating for days, whereas those on a hard substrate that mimic post-infarct fibrotic scar tissue stop beating in two days [197]. Here, cell – cell mechanical interaction might have played a role in long term beating on the soft substrate. Thus, equal-distant cells on soft substrate show much stronger interaction than those on hard substrate, which, together with the finding that quiescent cells can be stimulated to beat by inert mechanical probes, suggest that cell-cell communication is more mechanical than chemical. Hence, beating of cardiac cells can be prolonged by mutual stimulation by neighbors due to mechanical coupling between them through the deformable substrate. As substrate stiffness increases, the mutual coupling decreases, and the duration of mutual beating may decrease. Such might be the case after myocardial infarction when myocardium becomes mechanically stiffer due to the fibrotic rigidification [194-196].
Following infarction, the myocardial tissues gradually stop beating. In human heart failure, ventricular fibrillation often results in the damaged cardiac region with larger-than-micrometer scale. Such long cell-cell separation distance can diminish any mechanical stretching signal produced by beating cardiac cells.

**Figure 7.10:** How far the cells can see each other depends on the stiffness of the substrate and the distance between the cells. The figure shows the qualitative relationship (solid line) between the elastic modulus of a substrate and the distance between two cells to achieve a prescribed stretch (or strain $\varepsilon$) in a cell due to the contraction in the other. Here $\varepsilon_{\text{th}}$ is a threshold strain for which a cell gets stimulated. For $\varepsilon < \varepsilon_{\text{th}}$, the cells become mechanically decoupled. And their beating may become incoherent, or they may cease to beat. If the tissue stiffens, the cells need to be closer for mechanical coupling.
In conclusion, our experiments and computational simulations suggest that cardiomyocytes can interact with one another remotely through the deformation of the soft substrate. The interaction originates from the mechanical stretch induced by one cell on the other. The deformable soft media around the cells transfer the deformation. Mechano electric effect (MEF) in cardiac cells then possibly allows the stretch induced cell to be stimulated, which in turn interacts with the neighbor that stimulated it in the first place. Thus the cells become mechanically coupled. Closer the cells are, higher is the mechanical coupling. These cells keep each other beating for a long time. The range of interaction depends on the stiffness of the medium between the cells. For a given distance between the cells, stiffer the medium, less is the interaction which may result in lack of synchrony and coordination between the cells. Such a mechanically decoupled cell may cease beating. These findings have implications in the understanding of myocardial infarction when cardiac tissues become stiff due to fibrotic scar formation.
CHAPTER 8. SOFT LITHOGRAPHY MICRO-PATTERNING TECHNIQUE

8.1 Motivations

To mimic *in vivo* physical and chemical cues, the precise spatio-temporal control and organization of cells is critical to represent the proper microenvironment (µ-environment) around the cells [104, 168, 169, 232-238]. Soft lithography techniques have been demonstrated to be an inexpensive and efficient technique for patterning a range of substrates starting from metal-coated glass [235, 239-241] or bare glass [37, 234, 242, 243] and polystyrene substrates to flexible PDMS substrates [244, 245] and biomaterials (e.g. chitosan and collagen) [246-248]. However, it is technically challenging to spatially pattern proteins (extracellular matrix, ECM) on polyacrylamide (PA) hydrogels for living cells culture due to the compliant nature of the hydrogels and their aqueous environment. Traditional micro-fabrication process is not applicable.

In this Chapter, we report a novel and simple two-step method to pattern cell adherent ECM proteins on PA gels. The method utilized a hydrophilic glass substrate that was patterned via µCP of ECM (FN, LN or CN in this case) using a PDMS stamp. The patterned glass substrate was covered with a droplet of liquid phase PA gel solution, which was then sandwiched by another functionalized glass slide that adhered to the gel. After curing the gel at 37 °C, the glass plates were separated from each other, when the gel remained attached to the functionalized glass and peeled off the protein patterns from the hydrophilic glass. Thus, the protein pattern was transferred to the gel. The process avoided any functionalization of the inert

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gel surface for attachment of the protein pattern using the hazardous chemicals. The mechanics of the pattern transfer was also studied in light of fracture and interface mechanics. We found that the hydrophilicity of the glass where the proteins were first printed was essential for pattern transfer to PA gel. Hydrophilic glass offered strong enough adhesion with FN such that a pattern could be printed, but weak enough adhesion such that FN could be completely peeled off by the polymerized gel. This balance is essential for successful pattern transfer.

8.2 Soft Lithography

A Si master with designed geometric patterns was fabricated using standard photolithographic technique as shown in Fig. 8.1a1–1a4. The patterns were designed using AutoCAD (Autodesk, Inc., CA). Chrome masks were manufactured by Fine line Imaging (Colorado Springs, CO). The Si wafer was cleaned, degreased, blown dry with N₂ gas, baked at 110ºC (4 min) and re-cleaned using O₂/Ar plasma (flow rate ratio of 2:1) at Reactive Ion Etcher (March Instruments, Concord, CA) for 1 min at 100 W. The Si wafer was then spin-coated (maximum spin speed of 4000 rpm) with a thin layer of SU-8 negative photoresist (SU-8 2002, MicroChem, Newton, MA). After soft baking at 110ºC for 4 min on a flat hot plate, the wafer was brought in hard contact with the chrome mask and was exposed to UV light for 7s using a flood exposure system (ABM, Inc., Fig. 8.1a1). After the post exposure bake at 95ºC for 2 min, the wafer was developed using SU-8 developer (MicroChem, Newton, MA) for 10s (Fig. 8.1a2) and hard baked at 150 ºC for 18 min. Finally the wafer was treated with molecular vapor deposition (MVD) of FDTS (heptadecafluoro-1H, 1H, 2H, 2H-hydroxyltrichlorosilane) using MVD System (Applied MST, San Jose, CA) to ensure easy removal of the cured PDMS stamp in the subsequent steps (Fig. 8.1a3-1a4) [249-252]. PDMS prepolymer was obtained via mixing
Silicone elastomer and curing agent at 10:1 ratio (Dow Corning, Midland, MI) and degassing the mixture in a vacuum jar for 45 min [252-254]. The prepolymer solution was poured over the Si master mold (Fig. 8.1b1) and cured in the oven at 70ºC for 12 h. The elastomeric stamp was then peeled off carefully (Fig. 8.1b2) and cut in 2 × 2 cm² pieces for μCP.

Fibronectin (FN), laminin (LN) and collagen type I (CN) (from BD Biosciences, NJ) were respectively diluted in PBS solution to a concentration of 50 µg/ml. For FN, the diluted solution was oxidized to contain aldehyde groups by using sodium periodate (NaIO₄) to facilitate the FN conjugation to PA gel solution [255, 256]. The PDMS stamp obtained via soft lithography was then inked with the oxidized FN solution for 20 min at room temperature. Then, the excess solution was blown off (Fig. 8.1b3-1b4). The stamp was brought into complete conformal contact with a glass substrate for 45 min at 37ºC (Fig. 8.1b5). Small weights (25 g) were placed over the PDMS stamp to aid complete protein pattern transfer from PDMS to intermediate glass. Two different types of glass cover slips were used: (1) cover slips as received from the manufacturer (No. 1, 2.5 × 2.5 cm², Corning life Sciences, Netherlands) without any surface treatment; (2) cover slips after immersion in piranha bath (96% H₂SO₄+30% H₂O₂ in 3:1 molar ratio) for over 1 day, which makes the glass hydrophilic. Wet treatment of glass in piranha creates both hydroxyl (-OH) and sulfhydryl (-SH) groups on glass surface responsible for the increased surface hydrophilicity [249, 257-259]. The surface hydrophilicity characteristics for both the as-received glass slide and the ones after piranha treatment were quantified by performing the static contact angle measurements. A CAM200 Goniometer from KSV NIMA (Finland) was used and 7 µL droplets of DI water were dispensed on the surfaces to measure the static contact angles. The contact angles of DI water on slides before and after piranha treatment were 42⁰±3⁰ and 0⁰, respectively. A drop of PA gel solution (20 µL) was
placed on the cover slip with the protein patterns. An activated glass cover slip which can covalently bind to gel was placed (floated) on the drop. The drop spread between the cover slips due to capillarity and was sandwiched with uniform thickness [260-262] (Fig. 8.1b6). Curing of the PA gel was performed for 45 mins at two different temperatures (room temperature, 24ºC, and elevated temperature, 37ºC) to explore the effect of curing temperature on the efficacy of protein pattern transfer (Fig. 8.1b7). The cover slips were manually peeled from each other. During peeling, detachment proceeded from one edge of the sandwich. The gel remained adherent to the activated glass slide. The protein patterns were fully peeled off from the piranha-treated glass, and partially from the as-received glass. The patterns were monitored by both phase contrast and immunofluorescence microscopy. The patterning of laminin and collagen type I was done similarly, except the proteins were not oxidized.
Figure 8.1: Schematic diagram illustrating the basic steps of optical lithography process for Si master fabrication. (a1-a4) After development, the SU-8 photoresist surface was treated with FDTS vapor to aid the removal of the cured PDMS stamp. The right side legend shows the color representing the specific components. (b1) and (b2) Soft lithography to make the PDMS stamp.
by casting PDMS pre-polymer against the Si master. (b3) and (b4) Microcontact printing of fibronectin on treated/untreated glass cover slip. (b5), (b6) and (b7) Activated glass cover slip is floated over the precursor gel solution on the protein micro-patterned slide. The polymerization was performed at 37 °C. (b8) The top cover slip is peeled off once the polymerization is complete.

Normal monkey kidney fibroblast cells (ATCC, Manassas, VA) were cultured in a medium with 90% Dulbecco’s Modified Eagle’s Medium (DMEM; ATCC) and 10% fetal bovine serum (FBS; ATCC) [59, 96, 263]. The 0.4% trypan blue solution (Sigma–Aldrich, St. Louis, MO) was applied in the cell medium to test the biocompatibility of the micro-patterned gel substrate with cells.

PDMS, intermediate glass and PA gel with FN patterns were immunolabeled right after the protein transfer in each step. After rinsing with PBS 3 times, the substrates with FN were blocked with Image-iT FX signal enhancer (Invitrogen, CA) for 30 min to eliminate non-specific proteins binding. Samples were incubated in monoclonal anti-human fibronectin (Sigma-Aldrich, MO) at a 1:100 dilution in PBS at room temperature for 45 min. The substrates were then incubated for 30 min alexa fluor™ 488 goat anti-mouse IgG (Invitrogen, CA) at a 1:200 dilution in PBS at room temperature. The immunolabeled samples were mounted in ProLong gold antifade reagent (Invitrogen, CA) to prevent photobleaching. All the samples were imaged either using the Zeiss LSM 710 confocal scanning laser microscope (Carl Zeiss, Inc.,) at 63 ×, NA 1.4 oil immersion objective or Olympus IX81 microscope.

In order to determine the adhesion strength between fibronectin and glass substrates, a particle tip AFM probe was used. The Silicon Nitride (Si₃N₄) AFM cantilevers with a well-
defined spherical SiO$_2$ particle tip (1 µm diameter coated with a 30 nm gold layer) were chosen (Novascan Technologies, Inc., Ames, IA). The gold-coated microsphere was functionalized to ensure its attachment with FN through FN-MUA-EDC+NHS-Au covalent bonding. The functionalization process consists of: (a) self-assembly of a layer of MUA (11-mercaptoundecanoic acid) on the gold coated probe by soaking it in a 1 mM MUA (in PBS) solution for 30 min, (b) immersion of the probe in a mixer of 75 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and 15 mM NHS (N-hydroxysuccinimide) in PBS for 15 min, and (c) exposure of the probe to the 50 µg/ml fibronectin solution in PBS for an hour [264]. MUA, EDC and NHS were obtained from Sigma-Aldrich, MO. All adhesion force measurement experiments were performed using AFM (MFP 3D, Asylum Research, Santa Barbara, CA) and in PBS solution. The particle AFM probes were properly functionalized as described in previous section. Prior to the experiments, functionalized probes were stored at 4ºC. Multiple functionalized probes were prepared at the same time to check the consistency between experiments. Calibration using the thermal tuning method [265, 266] yielded the nominal value of $k = 0.123$ N/m. The probes were inspected with an Olympus IX81 microscope to confirm their integrity before experiments. The force measurements were carried out at randomly selected locations for every sample and a 5 x 2 array of force curves were obtained for each location. In order to measure the adhesion strength between FN and glass (piranha treated hydrophilic glass and as received glass), a drop of PBS solution was deposited on the glass surface. The AFM tip covalently bonded with FN was immersed in the drop and brought in contact with the glass surface. To ensure the complete contact between FN and glass, the AFM tip was bent on the glass surface until reaching a compressive force of 12 nN. The tip was then retracted while the AFM cantilever deflection was monitored to calculate the force of interaction between the tip.
and the glass. The maximum force prior to detachment was considered as a measure of adhesion between the glass and FN. All of the AFM parameters were kept constant during the adhesion experiments.

### 8.3 Interfacial Fracture Mechanics Interpretation of Defects

Figs. 8.2a and 2b show the patterns and surface topography of the Si master measured by profilometer (Alpha-step IQ, KLA Tencor). The nominal thickness of the SU-8 photoresist layer was 1.98 ± 0.05 µm. The structures were well defined as is evidenced from their sharp edges and flat surfaces (Fig. 8.2b). Micrographs of the well-defined geometric patterns on silicone elastomers (PDMS) are shown in Fig. 8.2c. As a demonstration, a typical immune-florescent micrograph of the FN patterns transferred onto PA gel are displayed in Fig. 8.2d3, where one of the glass slides was functionalized for bonding with PA gel, the other was as-received (not piranha treated). Patterning of laminin and collagen type I on gel was carried out similarly. The pattern consists of parallel FN lines, 25 µm wide with 25 µm spacing between them. However, there were discontinuities in FN line patterns along the center region on the gel surface, i.e., not all the proteins were transferred successfully. To investigate at which step the protein patterns lost their integrity, immuno-fluorescence staining of FN patterns were performed on PDMS, glass and PA gels. Images from PDMS and glass substrates revealed uniform and 100% deposition of FN patterns (no defects) (Fig. 8.2d1 and 8.2d2). The discontinuous regions appeared only on the PA gel (Fig. 8.2d3). Based on these step-wise examinations, we believe that the loss of patterns integrity occurs during the step when the as-received glass slide was peeled off from the gel. The effectiveness of the pattern transfer to the gel during the peeling process was determined by the relative adhesion between FN and glass vs. adhesion between FN
and gel. If FN-glass adhesion is weaker than that between FN and gel, FN would stay with the gel. Otherwise, only part of the FN pattern could be transferred to the gel. In the following sections, we explain this partial pattern transfer using the concepts of fracture and interface mechanics.

Figure 8.2: Characterization of Si mold and micro-pattern FN on substrates. (a1-a4) Micrographs of SU-8 line-array micropatterns on Si wafer. Scale bar: 250 µm; (b) Typical 1D surface profile of the Si/SU8 master, measured by the profilometer. The thickness of SU-8 layer is 2 µm; (c) PDMS stamp with well-defined line-array micropatterns of different line widths, 100 µm (c1), 50 µm (c2), 10 µm (c3) and 5 µm (c4), respectively. Scale bar: 100 µm; (d) Fluorescence microscopy images of the immunofluorescent-stained fibronectin line patterns on
PDMS (d1), intermediate glass (d2) and PA hydrogels (d3), respectively. Mid region of the pattern is missing on gel in (d3). Scale bar: 10 µm.

To explore the mechanism of defects generation, we designed and performed a series of gel-glass separation experiments with the peeling force applied at directions as shown in Figs. 8.3a and 8.3b. In all cases, the peeling force was orthogonal to the glass surface. But the location of the force was such that the peeling proceeds either along the pattern lines (Fig. 8.3a) or perpendicular to the lines (Fig. 8.3b). We found, for wide (100 µm) patterns, FN failed to transfer to the gel in the mid region of the patterns. The FN-free region was at the center of the pattern when the peeling proceeded along the pattern lines (Fig. 8.3a and 8.4a1), but was shifted from the center towards the edge (along the direction of peeling) when peeling was orthogonal to the lines (Fig. 8.3b and 8.4b2). Narrow line patterns (5 µm wide) have regions where patterns were either completely transferred to gel or they completely remained on glass (Fig. 8.3c).
Figure 8.3: (a) Phase contrast image of 100 μm fibronectin line patterns transferred to polyacrylamide gels with the intermediate glass peeled from the front. Peeling proceeded along the line patterns (see Fig 8.4). Scale bar: 100 μm. (b) Phase contrast image of 100 μm fibronectin line patterns transferred to polyacrylamide gels with the intermediate glass peeled from the side such that peeling proceeds orthogonal to the line patterns. Scale bar: 100 μm. (c) Phase contrast image of 5 μm fibronectin line patterns transferred to polyacrylamide gels with the intermediate glass peeled from front or side part. Scale bar: 50 μm. (d) Phase contrast micrograph of the intermediate glass slide after peeling off. The pattern was 100 μm wide. The image shows clear evidence of fibronectin residues on glass. Scale bar: 100 μm. In all insets: Immunofluorescent-staining of FN.
The peeling of glass from gel can be considered as an interface fracture problem, where an advancing crack separates the two. Pattern transfer involves detachment or debonding of FN from intermediate glass. Such detachment processes have been studied in the literature as an interface fracture between two solids forming the interface [267-270]. In the case of the gel-glass system, we noted the following: (1) gel was significantly more compliant than glass, i.e., there was a large elastic modulus mismatch between the two materials; (2) adhesion between FN and gel was likely to be stronger compared to that between FN and glass. Recall that FN was oxidized to contain the aldehydes group which allowed covalent conjugation of FN with the gels; (3) the thickness of FN layer was on the order of few nanometers (Fig. 8.5c and 8.5d), orders smaller than the gel layer thickness which was on the order of 100 µm. Thus FN and gel could be considered as one combined layer. Failure of pattern transfer implied that part of the gel remained with the glass together with FN during the peeling process; (4) the spaces between the FN patterns did not adhere to the gel, i.e., these regions detached from the glass surface during peeling without any resistance. In the current pattern, they were the linear spaces between the FN lines and have the same widths as the FN pattern widths (e.g., 10 µm wide linear space when the FN line was 10µm wide). These regions acted as entrapped cracks and generated crack tip stresses on the FN-coated bonded regions during peeling.
Figure 8.4: Normal peeling forces (90° w.r.t gel/glass interface) were applied at different positions for wide line pattern, i.e. 100 um. The cracks propagated along 2 different directions, respectively. (a1) Peeling force was applied as shown. (a2) cracks propagated along the line patterns. The entrapped cracks propagated inward from the sides. (a3) The resulting FN patterns on gel and FN residued on intermediate glass slides. (b1-b2) cracks propagated orthogonally to the line patterns. (b3) The resulting FN patterns on gel and FN residues on intermediate glass slides.
Figure 8.5: (a, b) Alpha-profilemeter shows the FN profile across 100 and 5 µm line patterns on intermediate glass slide respectively.

8.4 Hydrophilic Surface Enhancing Pattern Transfer

During peeling, a force was applied orthogonal to the glass. As a result, the gel stretched orthogonal to the glass and tended to shrink laterally, in-plane to the glass, due to Poisson’s effect. However, the glass substrate, being much harder than gel, restrained the lateral shrinking resulting in shear stresses along the glass-gel interface. The stress got intensified at the tip of the cracks, i.e., the cracks were loaded in mode II (shear mode) in addition to mode I (opening mode) [271, 272]. Thus, the crack tips were loaded in mixed mode [273, 274]. If the compliance of the gel and the glass were similar, then the crack would be loaded in pure mode I only, and thus the debonding or the crack growth would proceed along the interface resulting in complete
pattern transfer. Due to mode mixity, the crack tip tended to kink into the gel to propagate along the maximum resultant mode I (opening) direction. Two competing factors determined this kinking, namely, the interface toughness (depends on the strength of adhesion between FN and glass) and the gel toughness. Interface toughness also depended on the degree of mode mixity. In general, the higher the ratio of mode II to mode I components, the higher was the interface toughness. If the interface strength was weak, then the crack was expected to grow along the interface. If the strength were higher, the crack would kink into the gel and run parallel to the interface. For an intermediate strength, the crack might grow along the interface and then kink due to local interfacial imperfections (Fig. 8.5c). We believe, when the FN was patterned on as-received glass, the adhesion was in the last category resulting in partial pattern transfer. For a given compliance mismatch between glass and gel, one potential parameter to adjust was the adhesion between FN and intermediate glass. The adhesion should to be weak enough for interfacial crack growth, but strong enough for the pattern to be defined. We achieved this goal by using piranha-treated hydrophilic glass.
Figure 8.6: Experimental results from AFM adhesion study. (a) Representative force-distance curves for as received glass cover slip without any modification. Inset: Contact angle is 42°. (b) Representative force-distance curves for the more wettable glass cover slip after piranha treatment. Inset: Contact angle is 0°. (c) Bar chart for the maximum adhesion forces. The results indicate a 7 times decrease in adhesion strength for the more wettable sample.

Micro-patterning of PA gels with FN were repeated using piranha-treated glass to reduce FN-glass adhesion. Polymerization of the PA gel was carried out at 37 °C. Fig. 8.7a-f shows both the phase contrast and immunofluorescent staining images of the FN patterns of different widths transferred onto PA gels. The patterns show no defect irrespective of the pattern widths.
Similarly, the peeled-off intermediate glass did not reveal any notable FN residues. When polymerization was carried out at 24 °C, and hydrophilic glass was used, the FN patterns on PA gels were still not completely transferred. Alternatively, the FN patterns on PA gels were incomplete with as-received glass and at 37 °C curing. Both hydrophilic glass and polymerization at 37 °C are needed to achieve the complete FN pattern transfer. We used same procedure for patterning LN and CN, following the procedure outlined above for FN.

A quantitative AFM measure of adhesion between glass and FN show a seven-fold reduction in adhesion strength when piranha-treated glass was used. Fig. 8.6a and 6b show the typical AFM force-distance curves obtained for the slides without and with piranha treatment, respectively. The maximum pulling-off force of the AFM cantilevers from the substrates was used as a measure of adhesion strength between glass and FN (Fig. 8.6c). The maximum adhesive force for regular glass slide was 48.05 ± 0.94 nN, whereas that for a treated one was 7.57 ± 1.58 nN. These observations of decrease in adhesion force corresponding to the increase in hydrophilicity were consistent with earlier data available for other kinds of protein-substrate interactions [275-278].
Figure 8.7: Phase contrast images of the FN patterns (on PA gels) with different line widths that were transferred with the hydrophilic glass slides. The line pattern widths were (a) 100 µm, (b) 10 µm, and (c) 5 µm, respectively. The results demonstrated significant improvement in the efficacy of the approach irrespective of the patterns width (~ 100%). The immuno-fluorescent staining of FN patterns images (on PA gels) with different line width, (d) 100 µm, (e) 15 µm, and (f) 5 µm, respectively, proved that the FN patterns were completely transferred without major central defects. Scale bars were shown on each image.

8.5 Patterned ECMs and Cells on PA Gels

To demonstrate that the newly micro-patterned ECM zones (FN, LN and CN) were able to produce high quality spatial organization of cells, normal fibroblast cells (MKF) were plated and cultured on PA gel surface with patterns of varied widths. The cells were imaged on day 3 as
illustrated in Fig. 8.8. Clearly, the cells adhered to ECM patterns only, and they form well-defined linear clusters. To test the biocompatibility of the micro-patterned gel substrate with cells, we applied 0.4% trypan blue solution in the cell medium. We found that the cells within the patterns are viable (Fig. 8.8c, f, i).

Figure 8.8: Fibroblasts (MKF) were cultured on micro-patterns with varied line widths and extracellular matrices. The cells are well confined inside the patterns. (a, b) MKF cultured on 400 and 100 μm fibronectin (FN) line patterns on 3rd culture day; (d, e) MKF cultured on 100 and 10 μm laminin (LN) line patterns on 3rd culture day; (g, h) MKF cultured on 1000 and 30 μm
collagen I (CN) line patterns on 5\textsuperscript{th} culture day. (c, f, i) The trypan blue live-dead assay experiments show cells are alive and compatible with the micro-patterned gel substrate.

### 8.6 Discussion and Conclusion

Compared to existing micro-patterning techniques, the advantages of our patterning method are two-fold. Firstly, this method overcomes the difficulties associated with the direct micro-patterning of compliant PA gel using PDMS stamping. Note that micro contact printing generally involves only one step, transfer of pattern from PDMS stamp to certain substrates. Here, printing involves two steps: PDMS stamp to glass, and glass to PA gel. We investigated the latter step using interfacial mechanics. We did not investigate the first step mechanistically. Secondly, it avoids the use of harsh toxic chemicals (e.g. hydrazine hydrate) to functionalize the polymerized gel surface as the current pattern transfer is initiated in the liquid phase of PA gel, thus making the process simpler and safer.

Our study suggests that the pattern transfer process could be explained as a fracture mechanics problem where the transfer was considered as a debonding of the protein layer from the substrate. This debonding occurred through the growth of an interface crack between the substrate and the protein. If the protein-glass adhesion was too strong, then the interface crack kinked into the gel material during peeling, leaving the pattern on the substrate. If the adhesion was too weak, then the protein could not be patterned on the substrate in the first place. Hence, the degree of hydrophilicity or hydrophobicity of the substrate where the protein was temporarily patterned depends on the nature of the protein. If the protein needs a hydrophobic surface for minimal adhesion necessary for patterning on the surface, then the surface must be treated accordingly. If the adhesion is too high with a hydrophobic surface, then the surface may
need to be hydrophilic to achieve the minimal adhesion necessary for patterning on the surface. In all cases, however, it is clear that there needs to be some form of binding between the gel material and the protein. The binding must be strong enough such that the protein can be detached from the substrate, and that the pattern is stable on the gel surface. In the present study, the underlying mechanism of defect minimization in pattern transfer was the reduction of adhesion between glass and FN through enhanced surface hydrophilicity, and increased conjugation between PA gel and FN through curing (gel polymerization) at 37 °C. Our successful cell culture on micro-patterned PA gels indicates that ECM properties are not altered after the transfer and patterning. All the materials and reagents used in the technique are commercially available. Hence, implementation of the technique avoids any chemical synthesis. This straightforward technique of micro-patterning ECM and cells on PA gels may serve as a useful tool in cell mechanics research.

9.1 Introduction

Recent research has demonstrated that cells communicate with each other as well as with their microenvironments through mechanical signaling [279-284], in addition to biochemical ones [59, 285-291]. Many physiological processes, including cell adhesion [170, 292, 293], cytoskeleton polarity [290, 294], cell proliferation [295, 296], cell differentiation [52, 197, 289], embryogenesis [297, 298], cancer metastasis [285, 299], and wound-healing [55, 300], can be significantly influenced by the transmission and sensation of physical forces between the cells and their microenvironments. For example, in this thesis, we demonstrated 2 examples: (1) the exposure of HCT-8 human colon cancer cells to soft substrates resulted in a profound stable E-to-R cell state transition; and (2) cardiac myocytes could sense and respond to mechanical stretch [59, 285, 301-304]. Hence, accurate estimation of the traction forces exerted by the cells on their substrates under various physiological conditions can provide important insight on many fundamental questions regarding the mechanical interactions between various cell types and their microenvironment [181, 305, 306]. Over the past few decades, several seminal techniques to assess the cellular traction forces have been developed (see reviews [65, 291, 307-311]). However, most of them are limited to computation of traction forces exerted by single, isolated cells, whereas most physiological processes are inherently multi-cellular in nature where cell-cell and cell-microenvironment interactions determine the emergent properties of cell clusters. In this Chapter, a newly-developed finite-element-method-based cell traction force microscopy technique was introduced to estimate the traction forces produced by multiple isolated cells as well as cell clusters. We first demonstrated the utility of the technique by computing the traction force fields generated on hydrogels by multiple monkey kidney fibroblasts (MKF) in close
proximity, as well as by large MKF clusters. Second, the accuracy of the technique was shown, through the comparison between the experimentally applied known force on hydrogel surface and the computed traction force obtained from input displacement fields. Our technique is applicable to any flexible cell culture biomaterial, and can be used to better understand the dynamic properties of collective biological processes in vitro, such as metastatic transformation in cancer cells through cell-cell interaction, neuro-muscular junction formation, and cardiac myocyte synchronization.

9.2 Principle, Modeling and Elasticity Theory

Monkey kidney fibroblast (MKF) cells (from ATCC Inc., USA) were cultured following procedure described in Chapter 8. Prior to cell plating, PA gel substrates were incubated in the cell growth medium at 37°C for 15 minutes for equilibration followed by sterilization under a germicidal UV lamp in a tissue culture hood for 20 minutes. Cells were plated in varying densities and incubated for 12-24 hours before imaging. The plating density was governed by the requirement to image single isolated cells (~10,000 cells per coverslip), cell pairs (~120,000 cells per coverslip) and multiple cells and large clusters (>500,000 cells per coverslip). Phase contrast imaging of the cells on PA gel and fluorescence imaging of the microbeads embedded within PA gels were performed using an Olympus IX81 motorized inverted microscope. The cells were detached from the gel surface by the addition of sodium dodecyl sulphate (SDS, Fisher Inc.) and both phase contrast and fluorescent images were taken within 0.5 min following the addition of SDS.

2D displacement field produced by the cells was computed by digital image correlation method (DICM) [312-315] using gray intensity of fluorescent beads embedded beneath the top
surface of soft substrates. The marker subset size, subset shape function, sub-pixel optimization algorithm, and sub-pixel intensity interpolation scheme were optimized. The theoretical resolution of DICM can reach 0.1 pixel size of the acquired images [313]. The fluorescent beads with diameter 1 µm or 0.2 µm were used. Images were acquired by 20× objectives with 1.6× magnifiers (each pixel = 0.2 µm). Under these conditions, 0.2 µm beads spanned more than 4 pixels on the image, with the brightest portion at center, giving the recognizable gray intensity features for correlation tracking. An open source MATLAB program[316] based on the Sum of Squared Differences (SSD) correlation criterion was used to compute the gray intensity correlation of fluorescent beads [317, 318]. Depending on the fluorescent bead density in the gel, fluorescent bead size and in situ gel deformation, both optimal marker subset size and grid size are adjusted. In the present analysis, the markers subset size was selected ranging from 5× 5 pixel$^2$ to 30× 30 pixel$^2$ through trial and error. A uniform grid matrix (with $\Delta x \times \Delta y = 4.84 \times 4.84$ µm$^2$ intervals) encompassing the entire target cells’ contracting area was generated to obtain both transverse and longitudinal displacements of each grid node. During image processing, rigid body motions (translation and rotation (generally 2° to 3°)) of the beads were subtracted to obtain the net cell-induced 2D displacement field ($u_x$ and $u_y$). We verified the displacement result obtained from the MATLAB program by comparing the field with that analyzed by another commercial-available DICM software, Vic-2D [319]. All parameters, such as, marker and grid size, etc., were kept the same during processing the displacement field in both the programs. During FM traction computation, a MATLAB program was used to automatically link the grid points in the DICM to the corresponding FEM nodes using interpolation.

A tungsten flexible micro-needle with known stiffness (10.74 nN/µm) was used to apply increasing deformation on the flexible hydrogel surface under aqueous condition. The tungsten
micro-needle has dimension of 6 mm in length and 22µm in diameter. The position of the deflected micro-needle [59, 301] was measured with respect to a reference needle using an Olympus IX81 motorized inverted microscope.

9.3 Validation of Solution Uniqueness and Force Equilibrium

In this section, we demonstrate computationally that the traction solution from finite element simulation is unique as long as the full 3D boundary conditions are prescribed. We defined two boundaries representing two cells with half-cell distance apart on a soft gel surface. The diameter of each boundary is chosen as 20 µm, close to real cell size. A three-dimensional finite-element (FEM) block model is generated (ANSYS 12.0 Workbench Package) to represent the PA gel substrate. The gel is presumed linear elastic, isotropic, and homogeneous in their mechanical properties for a wide range of deformations [29, 320]. The model height is 70 µm, same as the thickness of PA gel used in experiments. We applied an in-plane force field (Fig. 9.1a) within each boundary, and computed the corresponding displacement field, u_x, u_y, u_z (Fig. 9.1b). We used the computed u_x, u_y and u_z within the cell boundary on the surface (Fig. 9.1c), and zero-traction conditions outside the boundaries to calculate the traction within the cells (Fig. 9.1d). A comparison between the prescribed and the calculated forces from the two steps shows close quantitative agreement (within 1%) (Fig. 9.1e-f).
Figure 9.1: Validity of the accuracy and uniqueness of the finite element solution to extract the 3D traction force fields. (a) A computational model with two regions representing 2 separated cells, each 20 µm in diameter and separated by half-cell distance 10 µm, was established. A self-equilibrated force field was applied within each region. The magnitude and directions of forces were indicated by arrows. (b) The resultant full displacement field was obtained by ANSYS. (c) The displacement fields underneath each cell were chosen and assigned to the same model. The boundary conditions of nodes outside the regions were set traction-free. (d) A new force field was obtained using above mixed-boundary condition. The magnitude and directions of nodal forces were shown by arrows. (e-f) The node-by-node difference between initially applied forces and retrieved forces (in X and Z direction, respectively) are shown. The difference is < 10^{-2} nN (within 1%).
The mixed boundary conditions are applied in the FEM analysis, as described in Section 9.4. Individual cells or cell clusters generate self-equilibrated traction on the substrate. Hence, as a measure of accuracy of the traction solution, we defined the error ratio $\varepsilon$,

$$
\varepsilon = \sqrt{\frac{\left( \sum F_{xi} \right)^2 + \left( \sum F_{yi} \right)^2}{\sum \sqrt{(F_{xi})^2 + (F_{yi})^2}}} \quad (9.1)
$$

where $F_{xi}$ and $F_{yi}$ are the nodal force components within the individual cells. For exact solution, $\varepsilon=0$.

**9.4 Experimental verification of computed traction field:**

To verify the accuracy of traction force computed by FEM, a known force was experimentally applied on hydrogel surface embedded with beads and compared to the numerical force computed from the experimental displacement fields. A Tungsten micro-needle with known stiffness, 10.74 nN/µm, was manipulated by a high-resolution x-y-z piezo-stage to apply incrementally increasing horizontal force (3 progressive deformation) on gel surface (Fig. 9.2a) [59, 65, 301, 304]. At each force increment, both the deflection of micro-needle and the beads displacements near the gel top surface are recorded by phase-contrast and fluorescent microscope, respectively (Fig. 9.2b). The reaction force on the PA gel was calculated, and then compared to the experimental force on micro-needle. The latter force was obtained from micro-needle’s spring constant and the deformation between the needle and a reference. We found good agreement between simulation and experimental results, with relative errors ranging from 4 % to 6.5 % (Fig. 9.2c). This set of experimental verification indicates that the simulated results computed based on displacement maps can reliably provide cell traction field.
Figure 9.2: (a) A Tungsten probe with known stiffness of 10.74 nN/µm (calibrated with weight) was vertically held by a high-resolution x-y-z piezo-stage to apply horizontal force on the flexible hydrogel surface. (b) The deflections of probe tip with respect to reference base, as well as the resultant displacement fields of beads on gel’s top surface, were recorded. The displacement fields were assigned to FEM model to compute the resulting force. The double-headed arrows indicated the gap between micro-needle and reference base. Multiplying this gap with spring constant of the micro-needle provided the force applied on the substrate. (c) The sum of reaction nodal force on PA gel was calculated using present traction force microscopy and compared with needle force. The relative error in force estimation is within 6.5 %.
9.5 Demonstration of 2-cell experiments

In this section, we demonstrate the applicability of the method by evaluating the traction induced by two neighboring cells. Here, two monkey kidney fibroblasts were plated on 1 kPa PA gel (Fig. 9.3a) with Poisson’s ratio of 0.46. Two different regions (two sets of $S_u$ and $S_o$) were selected to prescribe the displacement boundary conditions: (1) displacement field underneath the two cells were prescribed in the model (the white parts in Fig 9.3b), whereas the free-traction condition was applied outside the cells (the black part in Fig. 9.3b); (2) the displacement field within a region enclosing both cells was prescribed (the white part in Fig. 9.3c), whereas the free-traction condition was applied outside this region (the black part in Fig. 9.3c). The out of plane force, $F_z=0$, was prescribed within the cellular regions in (1) and (2). The traction fields were calculated for both cases (Fig. 9.3d, e, g, h), and compared (Fig. 9.3f and i). The root-mean-square (RMS) of node-by-node traction difference inside 2-cell region (superscripts indicate regions 1 and 2) was 21.7 Pa, which is only 5.1 % of maximum traction inside cells (426.8 Pa). They are almost identical.
Figure 9.3: Verification of the uniqueness of solution of the traction field computed from the experimental displacement field. (a) Phase-contrast pictures of 2 spatially isolated MKF cells on 1 kPa PA gels, cultured after 1 day. Scale bar: 15 µm. (b) The displacement fields underneath each cell were chosen for computation. (c) A larger displacement field enclosing both cells and neighboring area was chosen for computation. In both cases, the nodes outside the selected regions were set traction-free. (d-e) The traction field computed by above 2 cases were visualized and compared by 2D contour plots (d-e) and 3D bar representation (g-h). Also, the node-by-node difference of traction fields computed using 2 selection schemes was illustrated by both 2D contour plot (f) and 3D bar representation (i).
9.6 Whole-field displacement boundary conditions method and comparison

In this section we compared our mixed-boundary condition method with traditional whole-field displacement boundary condition method, which requires iterative calculation and has been successfully used by Fredberg, et al. Briefly, the iteration calculation proceeded as follows: (a) we assigned the complete 2D DICM displacement data (u_x and u_y) for all nodes of the top surface of the gel(both intracellular and extracellular regions; Fig 9.4a-b). We prescribe Fz=0 within the cluster for both the mixed and iterative methods, (b) the traction field was solved using FEM. Then all the forces in the extracellular region were replaced by F_x = F_y = F_z = 0 to satisfy the traction-free condition, while the forces in the intracellular traction were retained intact. (c) the new traction field was used to generate a new displacement field using FEM. Thus a new displacement field was computed within the intracellular region. (d) the computed intracellular displacement field was replaced with the DICM displacement field (u_x and u_y), while the computed extracellular u_x, u_y, and u_z from previous step were retained intact. (e) the steps (b), (c), (d) were repeated until solution converged, i.e., the difference between the root mean square (RMS) of surface nodal forces in two consecutive cycles became less than 5% (Fig. 9.4c-e).

Our computational results showed that the solutions from mixed-boundary and iterative methods converge (Fig. 9.4c-e). We found, the difference between the root mean square (RMS) value of traction from the two methods was $1.6 \times 10^{-1}$ kPa (Fig. 9.4f), less than 3.8% of the maximum computed cell traction. The difference between the RMS of the node forces was 0.2 nN, which is 0.25% of the maximum nodal force at cell cluster - substrate interface (Fig. 9.4g). The distribution of traction |t| and forces $F$ at FE nodes (Fig. 9.4h-j) shows good agreement between
the two methods. As a measure of accuracy of the traction solution, we use the same criteria as described for accuracy by defining $\varepsilon$ in Section 9.3.

![Figure 9.4: Comparison of mixed-boundary condition method and full-field displacement boundary condition method.](image)

(a) Phase contrast picture of a single cell cluster to be studied. Scale bar: 50 $\mu$m. (b) The displacement field generated by cell cluster on the top surface of substrate. (c-e) The traction field calculated by mixed-boundary method, and full-field displacement boundary method (with iterative calculation 1 time and 2 times, respectively), were shown respectively. The difference of RMS of the traction between mixed-boundary method and full-field displacement boundary method with 1 time iteration was $1.6 \times 10^{-1}$ kPa, less than 3.8% of the maximum computed traction at cell cluster and substrate interface. The difference of RMS of their nodal force was 0.2 nN, which was 0.25% of the maximum nodal force at cell cluster and substrate interface. (f-g) Histograms of nodal traction and force obtained by the two methods demonstrated good agreement between each other. (h) Summary of net force sum and absolute force sum calculated by the above three conditions. The force equilibrium was best satisfied in
mixed boundary condition method, which is 6.69% of total force. (i) Sum of surface nodal force distribution calculated by above three conditions. The RMS results of nodal force calculated by mixed BC method and 1-time iteration method agreed within 4.96%. (j) Sum of surface nodal traction distribution calculated by the above three conditions. The RMS results of nodal force calculated by mixed BC method and 1-time iteration method agreed within 9.27%.

9.7 Mesh size effect

In FEM, convergence test is required to determine the optimal mesh size needed to obtain the accurate solution. Three mesh sizes, 3.23 μm, 4.84 μm, and 6.45 μm were tested, as shown in Fig. 9a-c, and used to calculate the traction field of the same cell cluster by mixed-boundary condition method. The distribution of nodal traction and forces showed little difference between the three mesh sizes (Fig. 9.5a-c and 9.5e). The values of ε were 4.74%, 6.69%, and 6.12% for mesh size of 3.23 μm, 4.84 μm, and 6.45 μm respectively (Fig. 9.5d). Therefore, in the following computations, mesh size of 4.84 μm was used for analysis. The upper limit of mesh size is dependent on the specific cell size and the gradient of the traction field produced by the cell. A starting point on mesh size can be < 20 % of cells size.
Figure 9.5: (a-c) The convergence test was performed to determine the maximum fine mesh size needed to obtain the accurate solution. The mesh sets with different $\Delta x$ and $\Delta y$ ($\Delta x = \Delta y = 3.23 \, \mu m$, 4.84 $\mu m$, and 6.45 $\mu m$, respectively) were tested respectively. The traction distribution map and traction magnitude histograms from three mesh-size displayed uniform feature patterns. (d) All three cases shown net force sum ratio within 7 %, satisfying the force equilibrium requirement. (e) The root mean square (RMS) difference of traction between 3.23 and 4.84 $\mu m$ meshes was about 64.06 Pa (1.28% of maximum computed traction), and the difference between 4.84 and 6.45 $\mu m$ meshes was about 192.7 Pa (3.86% of maximum traction). The comparison indicates that when mesh size is reduced to 4.84 $\mu m$ or below, the traction output starts to show minimum variation.
9.8 Traction calculation of multiple cell clusters

One essential advantage of the present FEM-based Traction Force Microscopy is its capability to study the traction fields applied by multiple cell colonies. To demonstrate its validity and robustness, we showed the traction field produced by 2 spatially isolated large MKF cell clusters and some small ones around (Fig. 9.6a, b). The mixed-boundary condition method was used to compute the traction fields. The ratios $\varepsilon$ were 5.27% and 9.72% for these two large cell clusters respectively, and thus the traction was close to equilibrium state within each large cell cluster (Fig. 9.6c). The traction applied by the small clusters seems relatively minor with respect to that generated by the large clusters (Fig. 9.6c). This result indicates that the present method can potentially be used to study the cell-cell interaction through mechanical coupling via flexible substrates.
Figure 9.6: (a) Two spatially isolated MKF cell clusters were investigated on 1 kPa PA gels. (b) The displacement field produced by MKF cell clusters on the top surface of substrate. (c) The traction field was obtained using mixed-boundary condition method. The computed forces within each large clusters were close to self-equilibrium state, with $\varepsilon=0.052$ and 0.0972, respectively. The traction force applied by these neighboring small clusters seems relatively minor compared with that generated by the large cell clusters. The results indicate that our TFM method can potentially be used to study the long-distance cell-cell interaction through mechanical coupling on substrates.

9.9 Discussion and Conclusion
The majority of fundamental physiological processes in tissue development, health, and disease are coordinated by the collective activities of multiple cells[321-324], rather than single cells[287, 325]. To understand how mechanical traction applied by neighboring cell cluster groups could specify or mediate the tissue functionalities [59, 285, 288, 326-329], robust cellular traction evaluation method is indispensable. In the present study, we developed a Finite-Element-Based Traction Force Microscopy (TFM) to accurately compute and visualize the traction maps resulting from multiple cell clusters. The uniqueness, convergence, and correctness of traction solutions are substantiated. We showed that as the gel Poisson’s ratio > 0.4, the in-plane traction can be obtained with minimal error from the in-plane displacement field alone. For Poisson’s ratio < 0.4, both in and out of plane traction depend on both in and out of plane displacement boundary conditions, and it is essential to measure these displacements to compute any of the traction components. Our FEM technique is general and is applicable to the Poisson’s ratio < 0.4 case as well. It calculates the full 3D traction field given the 3D displacement boundary condition within cells or cell clusters, Moreover, unlike the classical TFM methods that are based on Boussinesq solutions[307, 308, 330, 331], the FEM takes into account the effect of substrate thickness and nearby environment. It is now known that cells can sense the substrate depth within the cellular length scales by showing distinct morphological variation on the gel substrate with same Elastic modulus but with varying thickness[52, 332].

The proposed finite element based method allows for the study of the traction fields between interacting, but isolated, large cell clusters on soft substrates. There is growing evidence showing that tissue cells can sense the presence of neighboring cells up to hundreds of μm away through mechanical interactions [282, 288, 333-335]. This mechano-sensitivity leads to the establishment of cell-cell communication as well as cell self-patterning and alignment [171, 288,
322, 336]. However, how the cell traction mediates ECM remodeling and cellular morphology remains elusive. Our FEM-based traction force microscopy provides a powerful tool to probe such unsolved questions. The method may be used to study diseases, wound healing, and perturbation of homeostasis, all of which involve assembly/disassembly dynamics of multiple cell ensembles.

In conclusion, we developed a robust FEM-based cell traction force microscopy technique to estimate the traction forces produced by multiple cells and clusters. The utility of the technique is exemplified by computing the traction force fields generated by multiple monkey kidney fibroblasts (MKF) clusters in close proximity. The developed technique is user-friendly and computationally inexpensive. Our technique can be used to better understand a number of dynamic biological processes that involve cell-cell mechanical interactions.
REFERENCES:


