CELLULAR MECHANOSENSITIVITY IN VITRO: CELL-ECM, CELL-CELL, AND CELL-MATERIAL INTERACTIONS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Theoretical and Applied Mechanics
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

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ABSTRACT

In recent years it has become increasingly evident that physical cues like mechanical micro-environment and geometry, in addition to bio-chemical factors, plays an important role in regulating cell functionalities. Cancer cells also respond to 2D and 3D matrix stiffness in a complex manner using a coordinated, hierarchical mechano-chemical system composed of adhesion receptors and associated signal transduction membrane proteins, the cytoskeletal architecture, and molecular motors. Mechanosensitivity of different cancer cells *in vitro* are investigated primarily with immortalized human cancer cell lines or murine derived primary cells, not with primary human cancer cells. Hence, little is known about the mechanosensitivity of primary human colon cancer cells *in vitro*.

In the first part of this dissertation, an optimized protocol is described that demonstrates the isolation of primary human colon cells from healthy and cancerous surgical human tissue samples. Isolated colon cells are then successfully cultured on soft (2 kPa stiffness) and stiff (10 kPa stiffness) polyacrylamide (PA) hydrogels and rigid polystyrene (~3.6 GPa stiffness) substrates functionalized by an extracellular matrix (fibronectin in this case). Fluorescent microbeads are embedded in soft gels near the cell culture surface, and traction assay is performed to assess cellular contractile stresses. Our findings suggest that both the healthy and tumor cells are mechanosensitive. Their average spread area increased with increase in substrate stiffness, and they displayed actin stress fibers and elongated focal adhesions on rigid polystyrene substrates only. Traction cytometry results on soft gels are the first experimental evidence that primary colon tumor cells can generate augmented traction compared to their healthy counterparts. In addition, the contrast between traction patterns and metastatic staging
raises the possibility of introducing a potent biophysical marker of cancer metastasis with other molecular biomarkers.

In the second part, we study the role of cell-cell adhesions on the substrate elasticity mediated metastasis-like phenotype (MLP) of human colon carcinoma (HCT-8) cells. HCT-8 cells on PA gels is an attractive in vitro biomaterial platform as they exhibit a dissociative, metastasis-like phenotype (MLP) when cultured on extra-cellular matrix (ECM) coated gels with appropriate mechanical stiffness (20–47 kPa), but not on very stiff (3.6 GPa) polystyrene substrates. We ask the question whether similar morphological transition occurs on cell-cell adhesion molecule, i.e., E-cadherin coated PA gels and if so, how the actin cytoskeleton and focal adhesions compare with ECM mediated response on gels. Experimental results suggest that MLP of HCT-8 cells on PA gels is independent of cell to gel adhesion in 2D in vitro culture.

Finally, we challenge the classical readouts of cellular mechanosensing by examining cell response on soft biological gel, namely, collagen. Our results show that different types of fibroblasts can exhibit spread morphology, well defined actin stress fibers, and larger focal adhesions even on very soft collagen gels (modulus in hundreds of Pascals) as if they are on hard glass substrate (modulus in GPa, several orders of magnitude higher). Strikingly, we show, for the first time, that augmented cell spreading and other hard substrate cytoskeleton architecture on soft collagen gels are not correlated with cell proliferation pattern unlike PA gels and do not require nuclear transcriptional regulator YAP (Yes associated protein) localization in cell nucleus. HCT-8 cell clusters also show augmented spreading/wetting on soft collagen gels unlike PA gels and eventually form confluent monolayer as on rigid glass substrates and MLP is completely inhibited on soft collagen gels. Overall, these results in the third part suggest that cell-material interaction (soft collagen gels in this case) can induce cellular phenotype and
cytoskeleton organization in a remarkably distinct manner (do not require higher traction for spreading, actin fiber formation and larger adhesions) compared to a classical synthetic polyacrylamide (PA) hydrogel cell culture model and may contribute in designing new functional biomaterials.
ACKNOWLEDGMENTS

“All praise and thanks be to God”. First and foremost, I like to thank God for giving me the strength and courage to pursue PhD at UIUC.

This dissertation would not have been possible without the help and contributions of several people. I would like to express deep gratitude and thanks to Prof. M. Taher A. Saif, Gutgsell Professor at University of Illinois at Urbana-Champaign for his supervision, guidance, invaluable suggestions, constructive criticisms and sharing his wisdom throughout this work. Special thanks are due to Dr. Brendan Harley, Assistant Professor, Department of Chemical & Biomolecular Engineering at UIUC for providing access to his lab facilities. The author is also deeply grateful to Drs. Iwona Jasiuk and Alison Dunn of Mechanical Science & Engineering at UIUC for taking the time to serve in the doctoral committee.

The author is greatly indebted to Prof. Jamil Khan of University of South Carolina, current and previous Saif lab members, Dr. Sara Pedron, Dr. Elise Corbin, Dr. Scott MacLaren, and Dr. Myandi Sivaguru of UIUC for their valuable help in experimentation, computation, analysis, training, and discussion. I would also like to thank Kathryn Smith of Mechanical Science & Engineering at UIUC for her assistance in my paperwork towards graduation.

This work is financially supported by National Science Foundation ECCS grant 10-02165, and the Interdisciplinary Innovation Initiative Program, University of Illinois grant 12035 and NIH National Cancer Institute Alliance for Nanotechnology in Cancer ‘Midwest Cancer Nanotechnology Training Center’ Grant R25 CA154015A. Finally, I would like to express my special thanks and dedicate this dissertation to my family for their support, constant encouragement and unconditional love.
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CHAPTER 1

INTRODUCTION

In recent years it has become increasingly evident that physical cues like mechanical micro-environment and geometry, in addition to bio-chemical factors, plays an important role in regulating cell functionalities. Cells can sense and respond to the substrate stiffness on which they are adhered to (as in 2D culture) or surrounded by (as in 3D culture) [1-18]. By doing so, cells can modulate their differentiation [3], morphology [4, 5, 9], migration/motility [10, 15], bio-physical properties [16], growth [17], and other processes [12]. The mechanosensing response is often unique to cell type [4], dimensionality (2D or 3D) [18, 19] and even type of ECM (fibronectin or collagen-I) used for substrate functionalization i.e. adhesion receptors [20]. Geometric cues can also influence important cell functionalities including cell adhesion and spreading [21, 22], cell proliferation and differentiation [23], cell fate switching between apoptosis and growth [24], cell polarity [25, 26], migration [27], cellular internal compartment organization [28] and the orientation of the axis of division [29, 30].

1.1 Mechanics of Cancer Cells

Cancer cells also respond to 2D and 3D matrix stiffness in a complex manner using a coordinated, hierarchical mechano-chemical system composed of adhesion receptors and associated signal transduction membrane proteins, the cytoskeletal architecture, and molecular motors [13, 17, 31-34]. For example, mammary epithelial cells (MECs) form normal acinar parenchyma when cultured on 150 Pa substrates that is similar to the stiffness of healthy mammary tissue. Interestingly, they exhibit the hallmarks of a developing tumor, both structural and transcriptional, when cultured on stiffer substrates (> 5000 Pa) that mimic the stiffness of a

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1 Bracketed numbers refer to the list of references at the end of each Chapter.
tumor stroma [35]. Another study shows that in vivo proliferative and dormant breast cancer cells readily proliferate when cultured on 2D plastic dishes, uncorrelated to their in vivo behavior. Strikingly, when these cells are grown in a 3D matrix, they display distinct growth properties strongly correlated to their proliferative or dormant behavior at metastatic sites in vivo [36]. In addition, recent experiments show that breast tumorigenesis is accompanied by collagen crosslinking and ECM stiffening [37]. These results raise the possibility of linking the translocation phase of cancer metastasis [38] with the mechanical micro-environment of parent tumor, in addition to the intrinsic genomic alterations.

A recent experiment indeed shows that human colon carcinoma (HCT-8) cells show metastasis like phenotype (MLP) when they are cultured on 2D substrates having stiffness (20–47 kPa), but not on very stiff (3.6 GPa) substrates [1]. These cells first form tumor-like cell clusters and then dissociate from one another, starting from the periphery. As this epithelial to rounded morphological (E to R transition) change occurs, they reduce cell-cell and cell-ECM adhesion, and proliferate. HCT-8 cells cultured on ECM functionalized very hard substrates do not exhibit these malignant traits. Thus it has been hypothesized that HCT-8 cell become metastatic due to their exposure to appropriate micro-environment. It is worth noting that these experiments are carried out with immortalized cancer cell lines or murine derived primary cancer cells, not with primary human cancer cells.

Recently, Kraning-Rush et al. proposed that augmented cellular traction stress may be used as a potential biophysical signature for metastatic cancer cells [39]. The study measures traction force for different human cancer cell lines on polyacrylamide gels and shows that metastatic cancer cells can exert significantly higher traction stress compared to non-metastatic cells in all cases [39]. However, these results directly contradict to earlier published findings on
murine derived breast cancer cell lines [40]. Also, another paper highlights remarkable differences in cell cytoskeletal remodeling, and cell survival protein expression between immortalized and primary human cells [41].

In this work, an optimized methodology is developed to isolate primary human colon normal and cancer cells from surgical tissues and assess their mechanosensitivity consequently. Next, cellular contractility of human colon cancer cells is quantified and is proposed as a potential biophysical marker of cancer prognosis. In addition, efforts are made to understand the role of cell-cell adhesions on the substrate elasticity mediated metastasis like phenotype of human colon cancer cell line (HCT-8) on polyacrylamide (PA) gels. Further, we examine the response of HCT-8 cells on soft biological material, namely, collagen gels by substituting the underlying PA gel with an aim to learn how cells sense and respond to such fibrillar non-linear matrices.

1.2 Organization of the Dissertation

Chapter 2 describes a protocol to extract primary human cells from surgical colon tumor and normal tissues. Isolated colon cells are then successfully cultured on soft (2 kPa stiffness) and stiff (10 kPa stiffness) polyacrylamide hydrogels and rigid polystyrene (~3.6 GPa stiffness) substrates functionalized by an extracellular matrix (fibronectin in this case). Fluorescent microbeads are embedded in soft gels near the cell culture surface, and traction assay methodology is discussed to assess cellular contractile stresses. In addition, immunofluorescence microscopy assays’ materials and methods on different stiffness substrates are provided. These assays yield useful information about primary cell morphology, cytoskeleton organization and vinculin containing focal adhesions as a function of substrate rigidity. The contents of this chapter are accepted to appear as Ali et al., Journal of Visualized Experiments 2015 [42].
Materials presented in Chapter 3 are currently in review for publication [43]. In this chapter, we seek the answer of following two key questions using the methodology described in Chapter 1: a. Are primary colon cells (tumor and healthy) mechanosensitive? Does substrate rigidity modulate cell spreading and cell cytoskeleton architecture? If so, how do colon tumor cells compare with healthy colon cells from the same patient? And b. Are primary tumor cells contractile, and if so, is contractility a signature of their metastatic potential? Our findings suggest that both the healthy and tumor cells are mechanosensitive. Their average spread area increased with increase in substrate stiffness, and they displayed actin stress fibers and elongated focal adhesions on rigid polystyrene substrates only. Traction cytometry results on soft gels are the first experimental evidence that primary colon tumor cells can generate augmented traction compared to their healthy counterparts. In addition, the contrast between traction patterns and metastatic staging raises the possibility of introducing a potent biophysical marker of cancer metastasis with other molecular biomarkers.

In Chapter 4, we switch our efforts to understand the mechanosensing of human colon carcinoma cell lines. We ask the question whether cell-cell adhesion, i.e., E-cadherin mediated mechanosensing can result epithelial to rounded morphological transition of human colon carcinoma (HCT-8) cells on PA gels. ECM (fibronectin) coated substrates were used as control. Our results show that HCT-8 cells are able to produce the dissociative MLP on specific stiffness (20 kPa) E-cadherin coated PA gels on 6th day of culture, but not on E-cadherin coated stiff glass (~70 GPa) substrates. The stable and irreversible transition in cellular phenotype occurred on ECM coated 20 kPa gels on 7th day of culture. Confocal laser scanning microscopy (CLSM) results suggest distinct cytoskeletal organization near the E-cadherin coated gel surface compared to that on identical stiffness ECM coated PA gels. However, cell spreading area on 20
kPa gels for both cases remains more or less same before MLP. We then studied spatial organization of vinculin on 20 kPa PA gels before MLP. Results suggest differential vinculin distribution with distinct punctate structures on ECM (fibronectin) coated gels only, whereas vinculin signal is primarily localized around cell-cell contact regions on E-cadherin coated gels. Overall, these results in Chapter 4 implicate that MLP of HCT-8 cells on PA gels is independent of cell to gel adhesion system. The materials in this chapter are adapted from Ali et al., Cellular and Molecular Bioengineering 2014 [44].

Chapter 5 discusses cellular response on soft fibrous biological gel, namely, collagen. We show for the first time that fibroblasts can display hard substrate like cell morphology and cytoskeleton organization on very soft fibrous collagen gels, without YAP localization in cell nucleus. YAP is localized in cytoplasmic region and cell proliferation rate is low, as expected on soft substrates. Further, we show that HCT-8 cells on soft collagen gels also display hard substrate like phenotype, i.e., augmented spreading, and confluent monolayer formation and no E-R transition. Overall, the results in this chapter suggest that cell-material interaction (soft collagen gel in this case) can induce cellular phenotype and cytoskeleton organization in a remarkably distinct manner compared to a classical synthetic polyacrylamide hydrogel cell culture model and may contribute in designing new functional biomaterials. The contents of this chapter are published as Ali et al., Soft Matter 2014 [45].

We report a simple two-step method to pattern cell adherent ECM proteins on PA gels in Chapter 6. The method utilizes a hydrophilic glass substrate that is patterned via μCP of ECM proteins using a PDMS stamp. The patterned glass substrate is filled with a droplet of pre-polymer PA gel solution, which is then sandwiched by another functionalized glass slide that adheres to the gel. The composition of the PA gel solution dictates the stiffness of the gel. After
curing the gel at 37 °C, the glass slides are separated from each other, when the gel remains attached to the functionalized glass and peels off the protein patterns from the hydrophilic glass. Thus, the protein pattern is transferred to the gel. The process avoids any functionalization of the inert gel surface for attachment of the protein pattern using the toxic chemicals. This tool enables the defined cellular traction stress distribution on hydrogels as predicted by finite element modeling. This work is originally published in Soft Matter 2012 [46].

*Chapter 7* summarizes the key findings and results presented in this dissertation. Also, the outstanding issues are discussed. Potential future research topics are also considered.

### 1.3 References


CHAPTER 2

METHODS TO STUDY PRIMARY CELL MECHANOSENSTIVITY\(^1\)

**Summary.** Cancer cells respond to matrix mechanical stiffness in a complex manner using a coordinated, hierarchical mechano-chemical system composed of adhesion receptors and associated signal transduction membrane proteins, the cytoskeletal architecture, and molecular motors [1, 2]. Mechanosensitivity of different cancer cells *in vitro* is investigated primarily with immortalized cell lines or murine derived primary cells, not with primary human cancer cells. Hence, little is known about the mechanosensitivity of primary human colon cancer cells in vitro. Here, an optimized protocol is developed that describes the isolation of primary human colon cells from healthy and cancerous surgical human tissue samples. Isolated colon cells are then successfully cultured on soft (2 kPa stiffness) and stiff (10 kPa stiffness) polyacrylamide hydrogels and rigid polystyrene (~3.6 GPa stiffness) substrates functionalized by an extracellular matrix (fibronectin in this case). Fluorescent microbeads are embedded in soft gels near the cell culture surface, and traction assay is performed to assess cellular contractile stresses using free open access software. In addition, immunofluorescence microscopy on different stiffness substrates provides useful information about primary cell morphology, cytoskeleton organization and vinculin containing focal adhesions as a function of substrate rigidity.

2.1 Motivation

In recent years it has become increasingly evident that mechanical micro-environment, in addition to bio-chemical factors, plays an important role in regulating cell functionalities. Cells can sense and respond to the substrate stiffness on which they are adhered to (as in 2D culture) or

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\(^1\)This chapter is adapted from the following publication: M. Y. Ali, S. V. Anand, K. Tangella, D. Ramkumar, and T. Saif. Isolation of primary human colon tumor cells from surgical tissues and culturing them directly on soft elastic substrates for traction cytometry. *J. Vis. Exp.* 2015 (in press).
surrounded by (as in 3D culture) [3-7]. By doing so, cells can modulate their differentiation [3], morphology [4], migration/motility [5], bio-physical properties [6], growth [7], and other processes.

Cancer cells also respond to the 2D and 3D matrix stiffness using a coordinated, hierarchical mechano-chemical combination of adhesion receptors and associated signal transduction membrane proteins, the cytoskeletal architecture, and molecular motors [1, 2]. For example, mammary epithelial cells (MECs) form normal acinar parenchyma when cultured on 150 Pa substrates that is similar to the stiffness of healthy mammary tissue. Interestingly, they exhibit the hallmarks of a developing tumor, both structural and transcriptional, when cultured on stiffer substrates (> 5000 Pa) that mimic the stiffness of a tumor stroma [8]. In addition, another experiment shows that breast tumorigenesis is accompanied by collagen crosslinking and ECM stiffening [9]. Recent experiments show that human colon carcinoma (HCT-8) cells display metastasis like phenotype (MLP) when they are cultured on 2D substrates having physiologically relevant stiffness (20–47 kPa), but not on very stiff (3.6 GPa) substrates [10-12]. These cells first form tumor-like cell clusters and then dissociate from one another, starting from the periphery. As this epithelial to rounded morphological (E to R transition) change occurs, they proliferate, reduce cell-cell and cell-ECM adhesion, and become migratory. HCT-8 cells cultured on very hard polystyrene substrates do not exhibit these malignant traits. Thus it has been hypothesized that HCT-8 cells become metastatic due to their exposure to appropriate micro-environment. It is worth noting that these experiments are carried out with immortalized cancer cell lines or murine derived primary cells, not with primary human cancer cells.

A recent study proposes that augmented cellular traction stress may be used as a potential biophysical signature for metastatic cells [13]. The study involves measuring traction force for
different human cancer cell lines on polyacrylamide gels. It is found that metastatic cancer cells can exert significantly higher traction stress compared to non-metastatic cells in all cases [13]. However, these results directly contradict the earlier published findings on murine derived breast cancer cell lines [14]. Also, a recent study highlights remarkable differences between immortalized and primary human cells in their cytoskeletal remodeling protein profiling and cell survival protein expression [15]. Hence, it is important to revisit many of the biophysical assays including traction for primary human cancer cells. This will address the question whether the primary cells recapitulate immortalized cancer cell lines traction trend.

The protocol described here is optimized for isolation of primary human colon cells (both healthy and cancerous), and for culturing them on soft substrates (polyacrylamide hydrogels) as well as on petri dishes. The protocol is based on digestion and consequent enzymatic dissociation of surgical tissue sample into single cell suspension [16]. To our knowledge, this is the first demonstration of culturing isolated primary colon tumor and normal cells directly on soft hydrogel substrates with embedded fluorescent microbeads for traction cytometry. Transparent gel substrates also allow immunostaining. This assay revealed differences in F-actin organization and focal adhesions in primary human colon cells as substrate stiffness changes. This cell culture platform opens up the possibility of exploring various biophysical properties of primary human cells such as cell stiffness and traction as parameters for cancer prognostics.

2.2 Materials and methods

The protocol described below follows the guidelines of UIUC human research ethics committee.

2.2.1 Collection and digestion of surgical tissue sample

1. Collect the tumor tissue sample right after colon resection (Figures 2.1A and 2.1B). Collect
tissue from an adjacent healthy site as well.

2. Transfer the tissue immediately to a 15 ml vial containing 12 ml HBSS solution. Keep the vial on ice inside an insulated foam box.

3. Transport the tissue containing vial to a tissue culture hood for further processing within 45 minutes. Keep the vial on an ice block inside the hood.

4. Pour the supplied tissue into a 6 well plate containing 6-7 ml of HBSS solution using a pipette.

   Note: Amount of tissue per well is not critical in this rinsing step.

5. Keep the 6 well plate on an ice block. Rinse the tissue in HBSS solution twice.

6. Cut tissue cleanly into separated sections with sterile scissor. Mince tissue into smaller sections no greater than 1-2 mm$^3$ in size with a sterile scalpel blade.

7. Weigh a labelled 0.1% trypsin vial (containing 1 ml of 0.1% trypsin solution) before transferring the tissue. Transfer ~20 mg tissue into each of the 0.1% trypsin vials with a pipette.

8. Try to minimize the transfer of HBSS into the trypsin vial to avoid further dilution of trypsin.

9. Weigh the trypsin vials after tissue transfer; document the difference as the tissue mass.

10. Pour ~20 mg tissue and 1 ml trypsin into each well of a 24 well plate.

11. Put the 24 well plate on shaker in fridge at 4 °C for 16-20 hours. During this wait period or at an earlier time, prepare polyacrylamide gel substrates and functionalize them with ECM proteins as described in section 2.2.3.
2.2.2 Enzymatic dissociation of tissue sample into single cell suspension and culturing isolated cells on ECM functionalized elastic substrates and rigid polystyrene dishes

1. After 16-20 hours of incubation in trypsin well at 4 °C on shaker, transfer tissue from 4 °C trypsin to 4 °C complete growth media vial (containing 2-3 ml of complete growth media) using a pipette. Ensure minimal transfer of trypsin into growth media.

Note: Complete growth media formulation is as follows: RPMI 1640 base medium supplemented with horse serum to a final concentration of 10% and penicillin–streptomycin to 1% of total solution.

2. Place the tissue containing media vial in a warm water bath at 37 °C for 12-15 minutes.

3. Transfer the tissue to a 15 ml vial containing HBSS solution using a pipette, shake gently.

4. Repeat step 3.

5. Remove tissue from HBSS solution and transfer to 0.1% collagenase solution (in HBSS) vial containing 1 ml solution. Incubate for 45 minutes at 37 °C and 5% CO₂ environment in a humidified cell culture incubator. During the wait period, warm growth media in a 15 ml vial at 37 °C in water bath.

6. Pull out all tissue fragments into pipette minimizing collagenase carryover. Eject just the tissue fragments into pre-warmed culture media vials.

7. Place a 40 μm well-insert filter into each well of a 6-well plate. Moisten the filter with 1 ml cell culture media.

8. Triturate tissue in media with a 10 ml glass pipette several times until tissue is completely dissociated.

9. Maintain the pipette tip as close as possible to the base of the vial.

10. Deposit the solution onto the filter to remove debris and undissociated parts.
11. Centrifuge filtered cell suspension in media at 150 x g for 5 minutes at room temperature.

12. Remove the supernatant afterwards and resuspend cell pellet with 2 ml fresh culture media.

13. Count the cells using a hemocytometer (if required). Seed isolated primary cells on extracellular matrix (ECM) functionalized gels and polystyrene dishes at desired concentration.

2.2.3 Preparation and functionalization of different stiffness polyacrylamide (PA) gels and polystyrene substrates

For visual demonstration of Section 2.2.3, the readers are referred to a recent Journal of Visualized Experiments (JoVE) article [17].

1. Adopting published protocols [18, 19], activate 12 mm² glass cover slips chemically to ensure covalent binding of the hydrogel.

2. First, treat glass cover slips with 3- Aminopropyltrimethoxysilane (ATS) for 7 min at room temperature.

3. Remove the ATS completely with DI water rinse and treat cover slips with 0.5% Glutaraldehyde (diluted in PBS from 70% Glutaraldehyde stock solution) solution for 30 min.

4. Obtain 2 kPa gel solutions by mixing 5% w/v acrylamide solution and 0.05% N, N’-methylenebisacrylamide (bis) solution in 10 mM HEPES-buffered saline [20]. Use 8% w/v acrylamide and 0.13% N, N’-methylenebisacrylamide (bis) solution in 10 mM HEPES-buffered saline for 10 kPa gel [20].

5. In both cases, use 1:200 ammonium persulfate (10% w/v) and 1:2000 N, N’, N’-tetramethylethlenediamine (TEMED) as the initiator and catalyst for the polymerization process, respectively. Also, add 100 µl fluorescent beads to 2 kPa gel solution as fiduciary
markers [21, 22].

6. Deposit a drop of 20 µL pre-polymer PA gel solution on the 12 mm² activated glass cover slip. Place another 12 mm² regular glass cover slip on the drop. Ensure that the drop spreads between the cover slips due to capillarity. Invert the sandwich for 2 kPa gels to ensure that most of the beads come near the cell culture surface.

7. Cure the PA gel for 45 mins at room temperature.

8. Peel off the top cover slip using a single edge razor.

   Note: During peeling, detachment proceeds from one edge of the sandwich. The gel remains adherent to the activated glass slide. Store the gels in PBS solution before use.

9. Functionalize PA gels and glass with ECM molecules, human fibronectin at a concentration of 50 µg/ml following published methods [23]. Briefly, incubate substrates with 2 ml pure hydrazine hydrate overnight. Remove hydrazine hydrate and rinse the substrates thoroughly with DI water.

10. Wash the substrates with 5% acetic acid for 30 mins. Remove acetic acid and rinse the substrates thoroughly with DI water. Keep the substrates immersed in DI water for 30 mins.

11. Incubate the substrates with oxidized fibronectin for 35 minutes at a concentration of 50 µg/ml. Rinse the substrates with PBS on shaker at low r.p.m. for 10 minutes. Incubate all substrates at 37 °C in 2-3 ml culture media for 30 minutes before plating the cells.

   Note: Plate cells in a sparsely populated manner (1000-3000 cells/cm²). Each gel-covered glass slip needs to be contained in a 35 mm petrie dish. Allow cells to adhere completely before any microscopy (at least overnight).
2.2.4 Traction force microscopy and immunofluorescence microscopy assays

2.2.4.1 Traction force microscopy assay

1. Warm 0.25% trypsin-EDTA / 10% SDS solution at 37°C in water bath for 10 minutes before traction experiments start.

2. Remove one gel from the cell culture incubator at a time and place on the fluorescent inverted microscope stage (32X magnification).

3. Find a single cell in field of view. Remove the petri dish lid.

4. Take a phase contrast image of cell (e.g., Figure 2.3).

5. Switch the imaging mode to fluorescence and select appropriate filter. Don’t move the microscope stage or sample during this time.

6. Take an image of the fluorescent beads displaced by cellular traction (Figure 2.4C).

7. Add 1 ml trypsin/SDS solution to petri dish to detach the cell from gel. Don’t move the microscope stage or sample during this time. Also, take a control image of the cell to ensure complete removal from gel.

8. Take a reference (null force) image of the beads after the cell is removed.

9. Repeat steps 2-8 for all other gels.

10. Make an image stack using ImageJ from the two images acquired in step 6 and 8 for each case. To generate the image stack, use following sequence of commands in ImageJ after opening the images: Image → Stacks → Images to stack.

11. To obtain the displacement field and traction, use ImageJ plugins following published methods [21, 22]. Obtain codes and detailed tutorials for these plugins using the following link: https://sites.google.com/site/qingzongtseng/imagejplugins.

12. Align the images in the stack using the ‘Template Matching’ plugin. Use following
sequence of commands in ImageJ after opening the image stack generated in step 10: Plugins → Template Matching → Align Slices in Stack → OK. Save the image stack consequently. Use this new image stack in the following steps.

13. Obtain the displacement field using the PIV (particle image velocimetry) plugin (Figure 2.4D). Use following sequence of commands after opening the image stack saved in step 12: Plugins → PIV → Iterative PIV (Basic) → OK → Accept this PIV and output → Ok. Save the PIV output in the same directory as in the original image stack. Use this as input in next step.

14. Finally use the FTTC (Fourier transform traction cytometry) plugin to obtain the traction map (Figure 2.4E). Use following sequence of commands: Plugins → FTTC → Insert material properties, i.e., Young’s modulus and Poisson’s ratio of PA gel → OK → Select the PIV output file saved in step 13 → OK. Save FTTC results in the same directory as in the image stack and PIV output.

2.2.4.2 Immunofluorescence microscopy assays

1. Take the substrates to be immunostained to the laminar hood and remove the culture media.

2. Rinse the substrates with PBS and fix the cells with 4% paraformaldehyde in PBS for 20 min at room temperature.

3. Wash the substrates with PBS for 3 times (5 minutes each time). Consequently, incubate the substrates with 500 μl signal enhancer for 30 min and rinse with PBS.

4. Incubate cells with monoclonal anti-vinculin antibody at a 1:250 dilution in PBS for 45 min at room temperature. Wash the substrates with PBS for 3 times (5 minutes each time).

5. Incubate the samples with secondary antibody alexa fluor 488 goat anti-mouse IgG at a 1:200 dilution in PBS at room temperature for 30 min. Wash the substrates with PBS for 3
times (5 minutes each time).

6. To visualize the F-actin structure, incubate cells with TRITC phalloidin conjugates at a concentration 50 µg/ml for 45 min at room temperature. Wash the substrates with PBS for 3 times (5 minutes each time).

7. Image the samples using confocal scanning laser microscope (Figures 2.5A-2.5D).

2.3 Representative results

The protocol described above is successfully employed for multiple tissue samples (n=12) from four different patients under guidelines of the institutional review board. Figure 2.1A illustrates a representative colorectal tumor right after surgery from which the tissue sections for cell cultures are obtained. A typical tissue section in HBSS solution after transfer to the laminar hood for further processing is shown in Figure 2.1B. A schematic of digestion and enzymatic dissociation of surgical tissue sample into single cell suspension is shown in Figure 2.2.

After successful tissue dissociation, isolated primary human cells are seeded on PA gels and polystyrene dishes. Phase contrast micrographs (Figures 2.3A and 2.3B) illustrate the representative morphology of primary human colon cancer and normal cells as a function of substrate rigidity. It is evident that primary colon cells (both healthy and cancerous) spread more on polystyrene dishes compared to the PA gels (Figures 2.3A and 2.3B).

Traction assays are performed on ECM (fibronectin) coated soft 2 kPa gels after confirmation of invasive adenocarcinoma from pathological H & E staining (Figure 2.4A). PA gels are uniformly coated with fibronectin as shown in Figure 2.4B. For ease of comparison, all traction experiments for tumor and healthy colon cells should be performed at the same time. Figure 2.4C shows a snapshot of the nanoscale fluorescent beads embedded inside the gel. From
the displaced and reference bead images, the displacement fields are obtained using the ImageJ PIV plugin [21, 22]. A representative bead displacement field generated by invasive colon tumor cells on 2 kPa gel is displayed in Figure 2.4D. Figure 2.4E shows traction stress obtained using ImageJ FTTC plugin corresponding to displacement field in Figure 2.4D [21, 22].

Figure 2.5 shows the F-actin and vinculin containing focal adhesions of primary human colon cells on soft 2 kPa gels and rigid polystyrene substrates. No actin fiber was present in the less spread cells on soft gels (Figures 2.5A1-2.5A2). Punctate vinculin containing focal adhesions are present on soft gels (Figures 2.5B1-2.5B2). Conversely, primary cells show well spread morphology, well defined actin stress fibers and discrete elongated focal adhesions on rigid polystyrene substrates (Figures 2.5C-2.5D).

2.4 Discussion

Cellular traction stress has recently emerged as a potential biophysical indicator of metastatic state [13]. However, no experimental traction data with primary tumor cells exists in literature to date. Also, directly culturing isolated primary colon cells on different stiffness polyacrylamide gels is not reported yet. Hence, we establish an optimized primary colon cell culture conditions on gels and polystyrene (Figure 2.2). Fluorescent microbeads encapsulation near cell culture surface during soft gel preparation enables the measurement of displacement field and traction generated by primary human cells (Figure 2.4C, 2.4D and 2.4E). In addition, immunofluorescence assays can provide important information regarding cytoskeleton organization and focal adhesions as substrate stiffness changes (Figures 2.5A-2.5D). Note that different cancer and normal immortalized cell lines also show augmented spreading, actin stress bundle formation and mature elongated focal adhesions with increased substrate stiffness [4, 24-25].
The protocol can be easily adopted/modified for isolation of primary cells from surgical tissues of different human organs or other animals. The incubation time of tissue in the dissociation agents -trypsin and collagenase- needs to be optimized empirically for each application. In addition, the filter size for removing debris and undissociated tissue parts is also an important parameter to choose based on expected maximum cell size in suspended state (40 μm in this protocol).

One limitation of the method is that a few soft gels may lack consistent localization of the fluorescent beads near cell culture surface which may adversely affect the following traction cytometry measurements. This can be easily circumvented by examining the gel surface for bead density and distribution using fluorescent microscopy before cell culture. Consequently, gels with relatively non-homogenous bead distribution can be discarded. Another approach is to use a recently proposed method that allows precise localization of the beads near the cell culture surface [17].

Caution should be taken to start processing the surgical samples as soon as they are received, preferably within 45 minutes. The most critical step in the protocol is the optimum exposure time of the tissue in the trypsin and collagenase solution to achieve isolation yield, yet retaining sufficient cell viability. Before culturing the cells on soft hydrogels, the bead density and distribution needs to be confirmed using fluorescent microscopy. Also, a null force image needs to be acquired after confirming that the cell is completely detached from the gel surface.

2.5 Conclusion

In summary, a protocol is described that results dissociated single cells suspension from surgical cancerous/normal tissue sample. This isolation of single primary tumor cells from surgical specimens followed by their culture on soft and hard substrates allows various
downstream biophysical measurements, e.g., cell stiffness using AFM, intracellular rheology using microinjected particles, cell migration/motility, and vesicle dynamics analysis. These measurements may be used for cancer prognosis. The protocol has been successfully used for the extraction and culture of murine cardiomyocytes as well [26]. The method presented here may be used to isolate primary human cells from surgical tissues of various organs and to culture them directly on different stiffness substrate for mechanobiology studies.

2.6 References


2.7 Figures

Figure 2.1. (A) Tumor after colon resection. (B) Tissue sections from the tumor for cell isolation.
Figure 2.2. Schematic of digestion and enzymatic dissociation of surgical tissue sample into single cell suspension.
**Figure 2.3.** Successful primary human colon cell culture (both cancer and normal) on different stiffness gels and on polystyrene dish. Phase contrast images show typical morphology of tumor cells (upper row) and normal cells (bottom row) on different stiffness substrates.
Figure 2.4. (A) H & E staining confirming invasive adenocarcinoma. (B) Fibronectin staining reveals that gels are uniformly coated with ECM molecules. (C) Nanoscale beads embedded inside gel as fiduciary markers. (D) Representative displacement field generated by tumor cell on soft PA gel using PIV plugin in ImageJ. (E) Traction stress exerted by tumor cell on soft gel corresponding to displacement field in (D) obtained via FTTC plugin in ImageJ.
**Figure 2.5.** Immunofluorescent staining of F-actin and vinculin on soft gel and on hard polystyrene substrates. No actin fiber was present in the less spread (A1) tumor cell or (A2) normal cell on soft 2 kPa gels. Punctate vinculin containing focal adhesions are present on soft gels (B1-B2). Conversely, primary cells show well spread morphology, well defined actin stress fibers (C1-C2) and discrete elongated focal adhesions (D1-D2) on rigid polystyrene substrates.
CHAPTER 3

CELL TRACTION AS A BIOMARKER FOR CANCER PROGNOSIS

Summary. Here, we have extracted primary cells from healthy and cancerous colon tissues from two patients, P1 and P2, soon after colon resection using the protocol described in Chapter 2. The cells were cultured directly on rigid polystyrene (~ 3.6 GPa stiffness) and polyacrylamide (PA) hydrogel substrates (2 and 10 kPa) functionalized by an extracellular matrix (fibronectin). We found that both the healthy and cancer cells are mechanosensitive. Their average spread area increased with increase in substrate stiffness, and they displayed actin stress fibers and elongated focal adhesions on rigid polystyrene substrates only. We also measured traction stresses generated by the primary cells (both healthy and cancer) on soft 2 kPa PA gels embedded with nanoscale fluorescent beads using Fourier transform traction cytometry. We found, for P1, the average traction of tumor cells was ~ 11 times higher than that of the normal cells (775.37 Pa vs 67.29 Pa) from the same colon. For P2 the ratio was ~ 2:1 (135.26 Pa vs 73.34 Pa). Histopathology and H & E staining of the tumors revealed that P1 and P2 had invasive adenocarcinoma with pathological staging pT3 and pT3 pN1a pMX, respectively. However, no cancer cells were found in any of the 34 lymph nodes inspected in P1, but one node was found with colon cancer cells out of 15 inspected for P2, i.e., cancer had metastasized in P2, but not in P1. This is the first experimental data of a possible correlation between the primary tumor cell traction and colon cancer metastasis. These finding suggest that the current clinical approach of detecting metastasis through lymph node search may be significantly improved by including bio-

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1 This chapter is adapted from the following publication:
physical signatures of the cells from the parent tumor.

3.1 Key questions

In this study, we isolate primary cells from surgically resected colons of two patients (P1 and P2). Each patient was identified with colon tumors. Tissues from these tumors and nearby healthy colon regions were extracted. Isolated cells from these tissues were plated on polyacrylamide gels with two different stiffness, 2 kPa and 10 kPa, and rigid polystyrene dishes. All the substrates were functionalized by an ECM. We ask the following two questions:

1. Are primary colon cells (cancer and healthy) mechanosensitive? Does substrate rigidity modulate cell spreading and cell cytoskeleton architecture? If so, how do colon cancer cells compare with healthy colon cells from the same patient?

2. Are primary tumor cells contractile, and if so, is contractility a signature of their metastatic potential?

3.2 Results

3.2.1 Substrate rigidity modulates primary human colon cells morphology, cytoskeleton organization and focal adhesions

Single cell suspension from surgical tissues is obtained by adopting the protocol described in Chapter 2. Briefly, tissue from tumor and healthy colon are minced into smaller sections and digested with 0.1% trypsin on shaker at 4°C for 16-20 hrs. Afterwards, tissue sections are incubated in enzymatic dissociation agent (0.1% collagenase solution) for 45 min at 37°C and 5% CO₂. Trituration and consequent filtering with 40 μm filter finally yields single cell suspension. Cells are immediately plated on ECM functionalized elastic substrates (PA gels) and polystyrene. Live/dead assay confirms that both healthy and cancer cells are alive irrespective of substrate stiffness/morphology (Figure 3.1). To our knowledge, this is the first demonstration of
successful primary human colon cancer cell culture on fibronectin coated PA gels. Primary colon cells isolated in this manner can be maintained in culture for three weeks.

Next, we ask the question whether primary human colon cells (both normal and cancer) are mechanosensitive, and if so, how are their spreading area, cytoskeleton architecture and vinculin adhesion sites modulated by substrate rigidity. Also, we screen for any possible signature that can distinguish colon cancer cells from their healthy counterpart.

ImageJ (a public domain Java based image processing program developed in NIH, http://imagej.nih.gov/ij/) was used for determining cell spreading area [1]. Cell spreading area analysis (Figures 3.2A-3.2B) clearly demonstrate that both healthy and cancer cells from colon show a monotonous increase in mean spread area with increase in matrix stiffness. There is only a moderate increase when matrix stiffness changes from 2 kPa to 10 kPa. However, significant increase in projected area is observed on polystyrene compared to both 2 kPa and 10 kPa gels. This trend exists for both the patients that we tested. Not surprisingly, less spread cells (healthy and cancer cells) on soft 2 kPa gel do not show any visible actin fibers (Figure 2.5A in Chapter 2). Diffuse vinculin staining on soft gels correlate well with their rounded morphology (Figure 2.5B in Chapter 2). Conversely, spread cells (both healthy and cancer) on polystyrene show clearly visible actin stress fibers and discrete, elongated vinculin containing focal adhesion complexes (Figures 2.5C and 2.5D in Chapter 2). These results imply that both healthy and cancer cells can sense and respond to substrate stiffness. However, no distinct morphological, cytoskeletal or adhesive traits can be identified that distinguish primary colon cancer cells from the normal ones in the cases we examined. Note that different cancer and normal immortalized cell lines also show augmented spreading, actin stress bundle formation and mature elongated focal adhesions with increased substrate stiffness [2-4].
3.2.2 Primary human colon cancer cells can exert augmented traction on fibronectin coated gels

Finally, we ask whether contractility of tumor cells differs from that of the healthy cells of the same colon, and whether such difference correlates with their metastatic potential.

Figures 3.3A and 3.3B show representative pathological H & E staining images of the tumors of the two patients confirming invasive adenocarcinoma. The summary of their pathological diagnosis is presented in Table 3.1. In order to diagnose metastasis, 34 lymph nodes were examined for P1, and none was found to have been invaded by the tumor cells. It was thus concluded that cancer has not metastasized in P1. Conversely, one lymph node was found invaded out of 15 examined for P2. Thus P2 was diagnosed with metastasis, and was recommended for chemotherapy.

For both patients, primary colon cancer cells generate larger traction on same stiffness gels compared to the control group (normal cells from the same patient) (Figure 3.4A-3.4C). However, non-invasive (lymph node not invaded) colon cancer cells from P1 exhibit significantly larger traction stress (775.37 Pa) compared to that by the healthy ones (67.29 Pa) on 2 kPa gel (Figure 3.4C). Conversely, invasive (lymph node invaded) colon cancer cells from P2 show a moderate increase in traction (135.26 Pa) compared to that of the control normal cells (73.34 Pa) (Figure 3.4C).

We finally quantify contractility or the net force, $|F|$ of the cells using Eqn (1).

$$|F| = 0.5 \times \iint (\sqrt{F_x^2 + F_y^2}) \, dx \, dy$$

(1)

Here, $|F|$ is half of an integral of the traction field over the entire projected cell area. $(F_x, F_y)$ are the local traction vectors defined at a spatial point $(x, y)$ in the projected cell area. The coordinate system is shown in Figure 3.4.
The net force for the tumor cells from P1 is ~ 3 times higher compared to that from the normal cells (166.73 nN vs 47.032 nN) (Figure 3.4D). Whereas, the net force of tumor cells from P2 is just slightly higher (54.78 nN vs 47.08 nN) compared to the normal counterparts (Figure 3.4D).

3.3 Discussion

Colon tumors are detected by colonoscopy. Once detected the primary tumor is generally resected (cut out) from the colon surgically. Lymph nodes are searched for invading cancer cells. If a single node is detected with cancer cells, the patient is typically referred to chemotherapy, otherwise to further observation. It is well known that lymph nodes with cancer cells are often missed. This is where a significant gap exists in prognosis as it relies on a hit and miss technique. Our preliminary studies show that patients with very similar histopathology of colonic tumors may have different lymph node findings, i.e., one lymph node (among many) may be detected with cancer cells in one patient, whereas none is found in another (Table 3.1). However, contractility of the tumor cells with invaded lymph nodes is dramatically different from that without lymph node invasion (Figures 3.4C-3.4D). This implies that contractility of tumor cells may carry metastatic signatures of the cells that have left the tumor, and are en route to invasion. Our experiments reveal that contractility is higher for the tumor cells that have not yet invaded the lymph nodes (Figures 3.4C-3.4D). Contractility reduces when the cells have metastasized. It is thus plausible that the tumor cells undergo a high contractile phase during tumor development prior to becoming invasive. This development phase may accompany a stiffening of the tumor micro environment as well. The increasingly contractile cells may remodel the tumor local stiffness by generating local strains resulting in collagen cross-linking [5]. The increasing local stiffness of the micro environment may in turn serve as a biophysical cue to the cancer cells. The
cells respond by generating more force, thus setting up a dynamic reciprocity [6]. Increasing intracellular force may eventually result in altered gene expression, metastatic transformation and acquisition of invasive traits. The transformed cells may reduce their contractility or even loose stiffness sensitivity. A few of the cells may start the invasion process by joining the circulatory network while others remain in the tumor.

It is clear that the current study with human primary tumor and healthy cells with two patients is limited. More patient data is necessary to draw conclusions. Nevertheless, the study raises the possibility of using resected and to-be-discarded tumor tissues of colon cancer patients for the biophysical studies of the cells. Contractility of the tumor cells in contrast to the healthy cells of the same tumor might serve as an indicator of invasion. A measure of cell contractility is by no means a replacement of lymph node inspection, but it may serve as an additional parameter for detecting cancer metastasis or the potential for this.

3.4 Conclusion

We show, for the first time, that primary human colon cancer and normal cells display mechanosensitivity when cultured on 2D substrates. Their spread area and cytoskeleton architecture change in a substrate stiffness dependent manner. Strikingly, invasive primary colon cancer cells can generate larger traction compared to their healthy counterpart on same stiffness substrate. These interesting results raise the question whether traction magnitude can be a potential biophysical marker of cancer metastatic state/ stage along with classical pathological gold standards.

3.5 References


3.6 Table

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**Table 3.1.** Summary of the pathological diagnosis for the patients
3.7 Figures

**Figure 3.1.** Live/dead assay demonstrate that primary cells are healthy/alive on all stiffness substrates irrespective of their morphology (less/more spread).
**Figure 3.2.** Primary colon cells sense and respond to substrate rigidity. Primary colon cells are plated on PA gels (2 and 10 kPa stiffness) and rigid polystyrene substrates (~3.6 GPa) functionalized with ECM molecules (fibronectin). (A) Mean cell spreading area increases with increase in substrate stiffness for both cancer and healthy primary colon cells. This trend exists for both cases we examined (B).
Figure 3.3. (A-B) Representative pathological H & E staining for the patients confirming invasive adenocarcinoma.
Figure 3.4. Primary colon cancer cells can generate augmented traction compared to their healthy counterparts. (A-B) Representative traction stress map generated by primary invasive cancer and normal cells on 2 kPa gel. Cancer cells can generate larger traction stress on the same stiffness gel compared to their healthy counterparts. (C-D) Cancer cells can generate augmented contractility in terms of traction stress (C) or net force (D). However, traction magnitude shows interesting pattern with lymph node invasion. Invasive cancer cells from the patient with no lymph node invaded can generate significantly larger traction compared to the patient with one lymph node involved (C).
CHAPTER 4

ROLE OF CELL-CELL ADHESIONS ON SUBSTRATE ELASTICITY MEDIATED METASTASIS LIKE PHENOTYPE

Summary. Growing experimental evidences suggest that cells can feel and respond to the mechanical stiffness of the substrate on which they adhere. Human colon carcinoma (HCT-8) cells can exhibit a dissociative, metastasis-like phenotype (MLP) in vitro when cultured on extracellular matrix (ECM) coated polyacrylamide (PA) hydrogels with appropriate mechanical stiffness (20–47 kPa), but not on very stiff (3.6 GPa) polystyrene substrates. In this study, we ask the question whether similar morphological transition occurs on cell-cell adhesion molecule, i.e., E-cadherin coated PA gels and if so, how the actin cytoskeleton and focal adhesions compare with ECM mediated response on gels. First, we culture the HCT-8 cells on E-cadherin coated PA gels of specific mechanical stiffness (20 kPa) and very stiff glass (~70 GPa) substrates. Interestingly, HCT-8 cells show the distinct dissociative in vitro MLP on 20 kPa gel only (not on stiff glass) on sixth day of culture; slightly earlier than the control (ECM / fibronectin coated 20 kPa gels). The complete inhibition of MLP on E-cadherin coated gels by pharmacological agent, blebbistatin, implicates the involvement of non-muscle myosin II activity in MLP. Confocal laser scanning microscopy and quantitative image analysis results suggest that the actin cytoskeletal architecture was characteristically different near the gel surface of E-cadherin and fibronectin coated gels of similar stiffness before dissociation. Conversely, identical cortical actin only structure was observed in the dissociated cells in both cases. Overall, these results suggest that MLP of HCT-8 cells on PA gels is independent of cell to gel adhesion in 2D in vitro culture.

1 This chapter is adapted from the following publication: M. Y. Ali and T. Saif. Substrate stiffness mediated metastasis like phenotype of colon cancer cells is independent of cell to gel adhesion. Cell Mol. Bioeng. 7:532-543, 2014.
4.1 Background

In recent years it has become increasingly evident that mechanical micro-environment, in addition to bio-chemical factors, plays an important role in regulating cell functionalities. Cells can sense and respond to the substrate stiffness on which they are adhered to (as in 2D culture) or surrounded by (as in 3D culture) [1-18]. By doing so, cells can modulate their differentiation [3], morphology [4, 5, 9], migration/motility [10, 15], bio-physical properties [16], growth [17], and other processes [12]. The mechanosensing response is often unique to cell type [4], dimensionality (2D or 3D) [18, 19], and even type of ECM (fibronectin or collagen-I) used for substrate functionalization i.e. adhesion receptors [20].

Cancer cells also respond to 2D and 3D matrix stiffness in a complex manner using a coordinated, hierarchical mechano-chemical system composed of adhesion receptors and associated signal transduction membrane proteins, the cytoskeletal architecture, and molecular motors [13, 17, 21-24]. For example, mammary epithelial cells (MECs) form normal acinar parenchyma when cultured on 150 Pa substrates that is similar to the stiffness of healthy mammary tissue. Interestingly, they exhibit the hallmarks of a developing tumor, both structural and transcriptional, when cultured on stiffer substrates (> 5000 Pa) that mimic the stiffness of a tumor stroma [25]. Another study shows that in vivo proliferative and dormant breast cancer cells readily proliferate when cultured on 2D plastic dishes, uncorrelated to their in vivo behavior. Strikingly, when these cells are grown in a 3D matrix, they display distinct growth properties strongly correlated to their proliferative or dormant behavior at metastatic sites in vivo [26]. In addition, recent experiments show that breast tumorigenesis is accompanied by collagen crosslinking and ECM stiffening [27]. These results raise the possibility of linking the
translocation phase of cancer metastasis [28] with the mechanical micro-environment of parent tumor, in addition to the intrinsic genomic alterations.

A recent experiment indeed shows that human colon carcinoma (HCT-8) cells show metastasis like phenotype (MLP) when they are cultured on 2D substrates having stiffness (20–47 kPa), but not on very stiff (3.6 GPa) substrates [1]. These cells first form tumor-like cell clusters and then dissociate from one another, starting from the periphery. As this epithelial to rounded morphological (E to R transition) change occurs, they reduce cell-cell and cell-ECM adhesion, and proliferate. HCT-8 cells cultured on ECM functionalized very hard substrates do not exhibit these malignant traits. Thus it has been hypothesized that HCT-8 cell become metastatic due to their exposure to appropriate micro-environment.

Here, we screen for similar epithelial to rounded morphological transition and also examine cytoskeletal organization of HCT-8 cells on E-cadherin coated substrates. ECM (fibronectin) coated substrates were used as control. Our results show that HCT-8 cells are able to produce the dissociative MLP on specific stiffness (20 kPa) E-cadherin coated PA gels on 6th day of culture, but not on E-cadherin coated stiff glass (~70 GPa) substrates. The stable and irreversible transition in cellular phenotype occurred on ECM coated 20 kPa gels on 7th day of culture. Confocal laser scanning microscopy (CLSM) results suggest distinct cytoskeletal organization near the E-cadherin coated gel surface compared to that on identical stiffness ECM coated PA gels. However, cell spreading area on 20 kPa gels for both cases remains more or less same before MLP. We then studied spatial organization of vinculin on 20 kPa PA gels before MLP. Results suggest differential vinculin distribution with distinct punctate structures on ECM (fibronectin) coated gels only, whereas vinculin signal is primarily localized around cell-cell
contact regions on E-cadherin coated gels. Overall, these results implicate that MLP of HCT-8 cells on PA gels is independent of cell to gel adhesion system.

4.2 Materials and methods

4.2.1 PA gel and functionalized glass preparation

PA gel preparation and glass cover slip activation for covalent attachment of gels were performed following protocols described elsewhere [29-31]. In brief, gel solutions were obtained by mixing 8% w/v acrylamide solution (Sigma, St. Louis, MO) and 0.27% N, N’-methylenebisacrylamide (bis) solution (Midwest Scientific, MO) in 10 mM HEPES-buffered saline (Lonza, Walkersville, MD). 1:200 ammonium persulfate (10% w/v) and 1:2000 N, N, N’, N’-tetramethylethylenediamine (TEMED) (both from Bio-Rad Laboratories, Hercules, CA) were used as the initiator and catalyst for polymerization process, respectively. Glass cover slips were chemically activated to ensure covalent binding of the hydrogel as described earlier [29]. Briefly, glass slips were treated with 3- Aminopropyltrimethoxysilane (ATS) from Sigma, MO for 7 min at room temperature. Followed by the removal of the ATS completely with DI water rinse, cover slips were treated with 0.5% Glutaraldehyde (diluted in PBS from 70% Glutaraldehyde stock solution from Polysciences, Inc.) solution for 30 min. A drop of 20 µL pre-polymer PA gel solution was deposited on the 12 mm² activated glass cover slip. Another 12 mm² regular glass cover slip was placed (floated) on the drop. The drop spread between the cover slips due to capillarity and was sandwiched with uniform thickness [32]. Curing of the PA gel was performed for 45 mins at room temperature. The top cover slip was manually peeled off using a single edge razor. During peeling, detachment proceeded from one edge of the sandwich. The gel remained adherent to the activated glass slide and was stored in phosphate-buffered saline (PBS; Lonza, Walkersville, MD) before use. AFM (Asylum Research, Santa Barbara, CA) with a pre-
calibrated silicon nitride tip was used to characterize the stiffness of PA gels in PBS solution [33, 34]. The nominal stiffness of the PA gels was ~20 kPa [31].

PA gels and glass were functionalized either with cell-cell adhesion molecules, recombinant human E-cadherin Fc chimera (Cat. No. 648-EC, R&D Systems, Inc., Minneapolis, MN) or ECM molecules, human fibronectin (BD biosciences) at concentrations of 1.5 µg/ml and 25 µg/ml, respectively. The surface functionalization protocols for binding fibronectin and E-cadherin were described elsewhere [30, 35]. Briefly, substrates were incubated with pure hydrazine hydrate (Sigma-Aldrich, MO) overnight. Substrates were then washed with 5% acetic acid (Sigma-Aldrich, MO) and DI water for 1 hour. Oxidized fibronectin was deposited on top of substrates for 35 minutes at a concentration of 25 µg/ml and rinsed with PBS on shaker for 10 minutes. For E-cadherin binding, substrates were incubated with anti-Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) overnight at 4°C at a concentration of 2.5 µg/cm². Consequently, Recombinant human E-cadherin Fc chimera was added at a concentration of 1.5 µg/ml on substrates and incubated for at least 2 hours. All substrates were incubated at 37°C in culture media for 30 minutes before plating the cells.

4.2.2 Cell culture

Human colon carcinoma HCT-8 cells (Cat. No. CCL-244, ATCC, Manassas, VA) were cultured in RPMI 1640 (Cat. No. 30-2001, ATCC, Manassas, VA) base medium supplemented with horse serum (Cat. No. 30-2040, ATCC, Manassas, VA) to a final concentration of 10% and Penicillin-Streptomycin (Cat. No. 30-2300, ATCC, Manassas, VA) to 1% of total solution [36].

Cells in suspension were preincubated with β₁, β₃ and α₆ integrin function blocking antibodies at a concentration of 10 µg/ml for 15 min at 37°C before plating on E-cadherin coated substrates [37-39]. Consequently, cells were plated on E-cadherin coated substrates and were
maintained with continued presence of $\beta_1$, $\beta_3$ and $\alpha_6$ integrin blocking antibodies at a concentration of 10 µg/ml to prevent interactions with cell secreted ECM proteins in long term culture. $\beta_1$ integrin function-blocking rat monoclonal antibody AIIB2 developed by Caroline Damsky (University of California, San Francisco, CA) was obtained from the Developmental Studies Hybridoma Bank, IA [37]. $\beta_3$ integrin function-blocking monoclonal antibody and rat monoclonal antibody against human integrin $\alpha_6$ (GOH3) were purchased from Chemicon International, Temecula, CA (Cat. No. MAB2023Z) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively [38, 39].

4.2.3 Live/dead assay

Cell viability was assessed by a classical live/dead assay [40] and was performed using a kit from Invitrogen, CA (Cat. No. L-3224). A working solution was prepared with 4 µM calcein AM and 2 µM ethidium homodimer-1 in PBS and was applied to adherent cells on PA gels and glass substrates for 30 mins.

4.2.4 Immunofluorescence microscopy

HCT-8 cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cells were then incubated with Image-iT FX signal enhancer (Invitrogen, CA) for 30 min. Next, cells were incubated in appropriate primary antibody at required dilution for 45 min at room temperature. To label fibronectin and E-cadherin, monoclonal anti-human fibronectin (Cat. No. F0791, Sigma-Aldrich, MO) and anti-Ecadherin (Catalog number: 18-0223, Invitrogen, CA) antibodies were used at 1:100 dilution in PBS. Following three times rinse in PBS, the samples were incubated for 30 min with secondary antibody alexa fluor™ 488 goat anti-mouse IgG (Cat. No. 11001, Invitrogen, CA) at a 1:200 dilution in PBS at room temperature in each case. For labeling
vinculin, monoclonal anti vinculin (Cat. No. 700062, Invitrogen, CA) antibody was used at a 1:250 dilution in PBS. Following three times rinse in PBS, the samples were incubated for 30 min with secondary antibody alexa fluor™ 568 goat anti-rabbit IgG (Cat. No. 11011, Invitrogen, CA) at a 1:200 dilution in PBS at room temperature. To visualize the F-actin structure, cells were incubated with TRITC phalloidin conjugates (Cat. No. P1951, Sigma-Aldrich, MO) at a concentration 50 µg/ml for 45 min at room temperature. In all cases, cells were finally incubated with DAPI (1:1000) for 15 min at room temperature before mounting in ProLong gold antifade reagent (Invitrogen, CA) to prevent photobleaching. All the samples were imaged either using the Zeiss LSM 700 confocal scanning laser microscope (Carl Zeiss, Inc.) or Olympus IX81 microscope.

4.2.5 Inhibition of acto-myosin contractility

To investigate whether intracellular force serves as mechanical cue for cell state transition, we used blebbistatin (EMD Biosciences), a potent inhibitor of non-muscle myosin II ATPase [41, 42] to remove the intracellular force generated by myosin II in HCT-8 cells on 20 kPa gels. We adjusted the intracellular force levels by varying the dosages (0 µM, 2.5 µM, 5 µM and 10 µM) of blebbistatin (diluted from 100mM stock solution in dimethylsulfoxide, DMSO). Blebbistatin was applied at different concentrations after HCT-8 cells were plated for 12 h on 20 kPa gels. It is known that blebbistatin can disrupt directed cell migration, but it does not block cell movement [7, 43]. Blebbistatin was added during every media change at 48 hours interval. Earlier study reports the inhibitor stability in the culture media up to 2 days [3].

4.2.6 Image analysis and statistical analysis

ImageJ was used for determining cell spreading area (n > 25 cells per condition) [44]. F-actin alignment for cell cluster was quantified using a plugin in ImageJ, namely, OrientationJ
The degree of F-actin alignment was assessed in terms of coherency in OrientationJ. The value of coherency varies between 0, implying isotropic distribution, and 1, implying highly aligned structures [45, 46]. At least n=3 independent experiments were performed for each case. Student’s t test was performed to evaluate statistical significance. Error was reported as standard deviation unless otherwise mentioned.

4.3 Results

4.3.1 HCT-8 cells show metastasis like phenotype (MLP) on E-cadherin coated 20 kPa PA gels, but not on stiff glass substrates

In this study, we used two experimental model systems for HCT-8 cell culture- E-cadherin coated substrates and fibronectin coated substrates (control). Human colon carcinoma HCT-8 cells were plated on 20 kPa PA gels and stiff glass substrates. We chose the stiffness of PA gels as 20 kPa based on a recent paper [1]. The paper reports that HCT-8 cells can display epithelial (E) to rounded (R) morphological transition, namely, metastasis like phenotype (MLP) on 20-47 kPa gels functionalized with ECM after a week of culture. Here, we want to screen for cellular phenotype transition on E-cadherin coated gels of same stiffness without active participation of ECM. Cells were plated and maintained with continued presence of $\beta_1$, $\beta_3$ and $\alpha_6$ integrin blocking antibodies at a concentration of 10 µg/ml to prevent interactions with cell secreted ECM proteins in long term culture on E-cadherin coated substrates. In all cases, ECM (fibronectin) coated gels of identical stiffness and glass substrates were used as control.

Initially, HCT-8 cells adhere to E-cadherin coated gels and adhere to each other. As time progresses, they start to proliferate generally within twenty hours and eventually form cell island/colonies in 2–4 days (Figure 4.1A). Cell islands on gels consist of numerous cells ranging from hundreds to thousands in number, correlated with initial seeding density. In contrast, cells
form confluent monolayer on E-cadherin coated stiff glass substrates (Figure 4.2A). Time required to reach confluence varies from 48-72 hours depending on initial plating density. Similar epithelial phenotype was observed in cells on control substrates (ECM coated 20 kPa PA gels and glass) (Figure 4.1E and Figure 4.2C).

Strikingly, on the sixth day of culture, mitotically competent and motile single cells start to disassociate from the edge of the cell colonies on E-cadherin coated gels (Figure 4.1B). The dissociated cells lose their original epithelial phenotype and become more rounded. As time progresses, more cell islands display the dissociative phenotype (Figure 4.1C). Within two weeks of culture, 30-50% of cell islands show the cellular phenotype transition with some islands completely dissociated into numerous single cells (Figure 4.1C). R cells retain full viability on gels as assessed by live/dead assay (Figure 4.1D). Unlike gels, cells on E-cadherin coated glass substrate maintain the confluent layer (Figure 4.2B). This in vitro transition in cellular phenotype is analogous to the first phase of in vivo metastasis cascade, namely, translocation [28]. On the other hand, cells on ECM coated control 20 kPa PA gels exhibit similar phenotype transition after a week of culture (Figure 4.1F, 4.1G and 4.1H), but remain confluent on stiff glass substrates consistent with earlier report (Figure 4.2D) [1]. 3T3 fibroblasts are also cultured on E-cadherin and fibronectin functionalized 20 kPa polyacrylamide gels and stiff glass substrates. Fibroblasts attach to the substrates, proliferate and form cell clusters on 20 kPa PA gels and confluent layer on glass by 2-3 days depending on initial seeding density in both cases (Figure 4.3A1-4.3D1). However, normal fibroblasts do not show any phenotype transition on 20 kPa gels/stiff glass substrates throughout the extended culture period (10 days) (Figure 4.3A2-4.3D2).
The dissociated HCT-8 rounded cells show significantly reduced E-cadherin expression compared to their epithelial counterpart (Figure 4.4). We harvested the rounded (R) cells from E-cadherin coated PA gel surface by trypsinaization. The cells were replated onto fresh 1 kPa, 20 kPa, 40kPa PA gels and hard polystyrene (~ 3.8 GPa) substrates (functionalized with ECM). The rounded HCT-8 cells retain their phenotype and viability regardless of the stiffness of the new substrates (Figure 4.5A-4.5D). Hence, the phenotype transition is stable and MLP can be propagated in culture irrespective of substrate stiffness (Figure 4.5A-4.5D).

These results are consistent with the emergent notion that cell-cell adhesions and associated proteins, in addition to cell-ECM adhesions, can play a significant role in cellular mechanotransduction [35, 47-49].

4.3.2 Involvement of Nonmuscle myosin II in the MLP of HCT-8 cells on E-cadherin coated gels

Myosin II is the key motor protein responsible for cell traction force generation [50-52]. To test whether cell contractility is involved in MLP process on E-cadherin coated PA gels, we used blebbistatin, a known pharmacological inhibitor of non-muscle myosin II ATPase. The inhibitor was applied at three different dosages (2.5, 5, and 10 µM concentrations) after 12 hrs of cell plating on 20 kPa gels. No transition in cellular phenotype was observed on E-cadherin coated gels (Figure 4.6A, 4.6B and 4.6C) or fibronectin coated gels (results not shown here) throughout the extended culture period (9 days) regardless of the strength of the drug used in this investigation.

We then ask the question whether the changes induced by drug application were reversible. To address this, the inhibitor was removed on day 6 and day 7 of culture from different dishes. Interestingly, HCT-8 cells display the cellular phenotype transition within 5-6
days of drug withdrawal in either case (Figure 4.7A and 4.7B). From the above observations, it is apparent that cellular contractility, a function of substrate stiffness, is essential in the exhibition of MLP on PA gels.

4.3.3 Actin cytoskeleton on E-cadherin and fibronectin coated gels

We examined the actin cytoskeleton organization of HCT-8 cells before and after MLP on both E-cadherin and fibronectin functionalized 20 kPa PA gels using laser scanning confocal microscopy (LSCM).

Figure 4.8 illustrates the actin structure of HCT-8 cell colonies (before MLP) at different altitudes on E-cadherin and fibronectin coated 20 kPa gels. Cell colonies are generally 12-16 µm thick in both cases. Cell colonies (before MLP) on 20 kPa PA gels display intense actin networks near the gel surface on both E-cadherin and fibronectin coated gels (Figure 4.8A2 and 4.8B2). In both cases, the network spans the entire cell colony. However, the network is distinctly more diffuse, disarrayed, and does not show any specific directional bias in case of E-cadherin functionalized gel (Figure 4.8A2), whereas the F-actin network on fibronectin functionalized gels clearly displays local polarization in spatial organization (indicated by arrow in Figure 4.8B2). HCT-8 cells in the uppermost layer of the colonies display cortical actin around the cell membrane in both cases, but no stress bundles (Figures 4.8A1 and 4.8B1, shown by arrow).

To further analyze and quantify the F-actin spatial organization near PA gel surface, we use a plugin in ImageJ, namely, OrientationJ [45, 46]. The extent of F-actin alignment was reported in terms of coherency in OrientationJ. The value of coherency varies between 0, implying isotropic distribution, and 1, implying highly aligned structures. Figure 4.8E shows the F-actin alignment comparison near gel surfaces on E-cadherin and fibronectin coated gels. Clearly, the measured coherency implies random F-actin distribution on E-cadherin coated gels,
whereas specific directional preference for fiber alignment does exist on fibronectin coated gels. Average coherency increased significantly from 0.07 to 0.39 (Student’s t test, p < 0.05) for E-cadherin to fibronectin functionalized gels, implying the polarization of F-actin structures near gel surface on fibronectin coated gels only. Taken together, the imaging and quantitative analysis results indicate that E-cadherin mediated mechanosensing can give rise to distinct actin cytoskeleton organization of HCT-8 cells near substrate surface compared to ECM mediated mechanosensing on 20 kPa gels before cell dissociation. A recent study with cardiac myocytes plated on different stiffness N-cadherin and ECM (fibronectin/collagen) coated PA gels also highlight that cadherin mediated mechanotransduction is capable of displaying a characteristically distinct local and global cytoskeletal structure, but the magnitude of cadherin mediated traction force is still comparable to ECM mediated forces [35].

In contrast to the distinct actin spatial organization before MLP, dissociated HCT-8 cells on PA gels show similar cortical actin only structures, and no actin networks on both E-cadherin and fibronectin coated substrates (Figure 4.9A and 4.9B, shown by arrows), implying significant remodeling in cytoskeleton structure after MLP [1].

4.3.4 Average cell area on PA gels

Cell spreading area is a known readout of cellular mechano-sensitivity to substrate rigidity [5, 35]. We measure average cell area on both E-cadherin and fibronectin functionalized 20 kPa gels before MLP. Interestingly, average cell area measured before MLP on E-cadherin and fibronectin coated 20 kPa gels is of similar magnitude, 101 ± 9.66 µm² and 94.53 ± 19.97 µm², respectively (Figure 4.10, n > 25 cells per condition).

4.3.5 Vinculin expression of HCT-8 cells on 20 kPa PA gels

Vinculin is a protein molecule found in cell-cell and cell–ECM contacts [47, 49]. Hence,
HCT-8 cells were stained for vinculin on 20 kPa PA gels functionalized with ECM and E-cadherin on day 3 of culture (i.e., before MLP). Imaging results reveal completely different vinculin distribution on gels. On E-cadherin coated gels (with integrin blocking antibodies), vinculin is primarily localized around cell-cell contact regions (Figure 4.11A, shown by arrows). In contrast, more diffuse vinculin with distinct punctate structures appear only on fibronectin coated 20 kPa PA gels (Figure 4.11B and 4.11C, shown by arrows). Hence, cellular phenotype transition is apparently less dependent on spatial distribution of vinculin on gels.

4.4 Discussion and conclusion

A recent experiment shows that human colon carcinoma HCT-8 cells can show metastasis like phenotype (MLP) driven solely by appropriate substrate stiffness (20-50 kPa) [1]. We hypothesize that classical cell-cell adhesion molecule, E-cadherin, and associated functional proteins e.g. α catenin, β catenin and p120 mediated mechanotransduction may play a key role in exhibiting MLP [53-55]. Hence, we plated HCT-8 cells on E-cadherin functionalized substrates and looked for the morphological E to R transition without any active participation of ECM. Cells were plated and maintained with continued presence of β₁, β₃ and α₆ integrin blocking antibodies at a concentration of 10 µg/ml to prevent interactions with cell secreted ECM proteins in long term culture on E-cadherin coated substrates. Our results indeed show that HCT-8 cells can display MLP on E-cadherin coated 20 kPa gels (Figure 4.1C and 4.1D), but not on very stiff glass substrates (Figure 4.2A and 4.2B). To ensure that gels are uniformly coated with fibronectin and E-cadherin, we use monoclonal antibody to label fibronectin and E-cadherin. Imaging results suggest that the surfaces are indeed uniformly coated with fibronectin and E-cadherin, respectively (Supplementary Figure 4.12A – 4.12D).
In this study, the actin cytoskeletal structure and spatial distribution of vinculin of HCT-8 cells on gels are examined in both cell-cell and cell-ECM adhesion models via immunostaining and laser scanning confocal microscopy. F-actin spatial organization near the gel surface suggests that although E-cadherin mediated mechanosensing is capable of eliciting a distinct actin cytoskeletal structure of HCT-8 cells on similar stiffness gels before MLP (Figure 4.8A2 and 4.8B2), yet transition in cellular phenotype does occur in both cases in response to substrate stiffness alone (Figure 4.1C and 4.1G). However, R cells on gels in both adhesion systems show similar actin structures after MLP (Figure 4.9A and 4.9B). This significant remodeling of the cytoskeletal structure in epithelial to rounded phenotype transition is probably attributed to loss of substrate stiffness sensitivity of R cells [1]. An earlier study with myocytes also report that cell-cell contact mediated mechanosensing can result in distinct cytoskeletal architecture response compared to cell-ECM contact mediated one [35].

While role of cell-ECM adhesions in cellular mechanotransduction process is already well appreciated and widely investigated, cell-cell adhesions and associated protein complexes have only recently emerged as a new class of mechanosensor [35, 47-49]. For example, cardiac cells are shown to remodel their cytoskeletal architecture on N-cadherin coated gels in response to change in stiffness as in ECM coated substrates. In addition, it is also shown that the magnitude of traction forces generated by cardiac cells on N-cadherin and ECM coated substrates are of comparable magnitude [35]. Cardiac myocytes display the maximum striation in physiologically relevant stiffness substrates (5-10 kPa) independent of cell-cell/cell-ECM adhesions. Their study highlights that biological activity of cardiac cells (in terms of myocyte striation) responds to appropriate mechanical forces and remains independent of adhesion subsystem [35]. Our analysis shows that cell spreading area (Figure 4.10) before dissociation is
of similar magnitude on both E-cadherin and fibronectin coated gels. Also, the complete inhibition of the MLP by blebbistatin treatment indicates the involvement of acto-myosin contractility of HCT-8 cells in the dissociation process (Figure 4.6). Dissimilarity in actin architecture, but similarity in cell spreading and the effect of blebbistatin on E-cadherin and fibronectin coated gels suggest that MLP is more likely determined by the overall intra and inter cellular forces, but is insensitive to the details of actin architecture.

Earlier, two research groups independently showed that during culture of HCT-8 cells in standard tissue culture flasks (stiffness ~ 3.8 GPa), a few rounded isolated cells (R cells) appear only on top of confluent epithelial monolayer (E cells) [56-59], which is consistent with our tissue culture flask observations as well (Figure 4.13). We suspect that the E to R transition might have been triggered by soft environment of the apical surface of HCT-8 cell monolayer (stiffness ~4 kPa, measured by AFM [1]) and the transition is primarily cell-cell interaction mediated. Interestingly, the R cells showed high metastatic potential in both animal models and in vitro embryonic heart invasion assays [56-59]. Moreover, R cells lack α-catenin which links cell-cell adhesion molecule, E-cadherin, to actin cytoskeletal via β catenin, p120 and other molecular complexes [57]. E-cadherin expression is also reduced by a factor of five in the R cells [1]. Functional cadherin-catenin complexes are clinically known inhibitor of invasion and any perturbation in these complexes lead colon and other cancer cells to acquire a more invasive trait [57, 59]. Hence, appropriate force transmission via cadherin-catenin complexes may cause outside-in signaling on 20 kPa gels and may initiate biochemical signaling cascades. Another recent study also proposes that alpha-catenin may behave as a strain sensor to external force stimuli and consequently able to remodel the adhesion junctions [60]. Thus, it is conceivable that the outside-in signal of appropriate mechanical microenvironment is transduced into HCT-8 cells
via cadherin/catenin complexes and cells may modulate their cadherin-catenin complexes with reduction of both E-cadherin and α-catenin in response[57], eventually enabling them to display E to R transition on gels only. The appropriate molecular mechanisms are yet to be discovered, but the molecules that may be involved are α-catenin, β-catenin, and p120.

In summary, we have shown that in vitro phenotype change exhibited by HCT-8 cells on PA gels is independent of cell to gel adhesion. Also, the exhibition of the MLP can be completely suppressed by inhibition of non-muscle myosin II activity of cells. The appropriate mechanical microenvironment rather than adhesion sub-system appears to determine in vitro cellular phenotype transition decision for HCT-8 cells on gels. The model system presented in this study may be used as a potential in vitro biomaterial platform to dissect colon cancer phenotype transition in cellular and molecular details which may provide valuable insights. This platform may be used for studying early phase of tumor progression for other types of cancers as well.

4.5 References


4.6 Figures

![Images of micrographs showing cell colony formation and live/dead assay results for HCT-8 cells on different substrates.](image)

**Figure 4.1.** MLP of HCT-8 cells is independent of cell to gel adhesion. HCT-8 cells are plated on soft PA gels (20 kPa stiffness) functionalized either with cell-cell adhesion molecule, i.e. E-cadherin or ECM molecules (fibronectin). (A) Phase contrast micrographs showing the cell colony formation on E-cadherin coated gels by 3-4 days of culture (inset shows live/dead assay image of cell island. Green fluorescence indicates viable cells, while red fluorescence indicates dead cells, Scale bar: 10 µm). (B-C) Cells show the metastasis like phenotype (MLP) on E-cadherin coated 20 kPa PA gels on day 6 of culture (B, indicated by arrows). (D) R cells retain their full viability on E-cadherin coated gels (Scale bar: 50 µm). (E-G) Similar HCT-8 cell colony formation on fibronectin coated 20 kPa gels (E, inset shows live/dead assay image of cell island, Scale bar: 10 µm) and exhibition of MLP on day 7 of culture on 20 kPa gels only (F, G), (H) R cells retain their viability on ECM coated gels as well. Scale bar: 100 µm.
Figure 4.2. (A and B) Phase-contrast images of HCT-8 cells cultured on E-cadherin coated stiff glass after 4 and 10 days, respectively. (C and D) Phase-contrast images of HCT-8 cells cultured on fibronectin coated stiff glass after 4 and 18 days, respectively. Cells do not show the MLP on stiff glass substrates irrespective of the adhesion sub-system. Scale bar: 100 μm. At least three independent experiments were performed in each case.
Figure 4.3. Fibroblasts do not show MLP on E-cadherin or fibronectin coated gels/glass. (A1-D1) 3T3 fibroblasts are cultured on E-cadherin and fibronectin functionalized 20 kPa polyacrylamide gels and stiff glass substrates. Fibroblasts attach to the substrates, proliferate and form cell clusters on 20 kPa PA gels and confluent layer on glass by 2-3 days depending on initial seeding density in both cases. However, normal fibroblasts do not show any phenotype transition on 20 kPa gels/stiff glass substrates throughout the extended culture period (10 days) (A2-D2). Scale bar: 50 μm. At least three independent experiments were performed in each case.
Figure 4.4. E-cadherin expression in colony associated and dissociated cells. (A-D) E-Cadherin expression in R cells was significantly reduced compared to the colony-associated E cells. The red lines in A and B represent an arbitrary path along which the intensity profiles were measured (C and D). Comparison of the peaks clearly demonstrates the loss of E-cadherin expression in rounded cells. Scale bar: 5 μm. At least three independent experiments were performed in each case.
**Figure 4.5.** (A-D) R cells can be propagated in culture irrespective of substrate stiffness. R cells were harvested from E-cadherin coated PA gel surface by tripsinaization. The cells were replated onto fresh 1 kPa, 20 kPa, 40kPa PA gels and hard polystyrene substrates (functionalized by ECM). The rounded HCT-8 cells retain their phenotype and viability regardless of the stiffness of the new substrates (inset shows live/dead assay images of R cells. Green fluorescence indicates viable cells, while red fluorescence indicates dead cells). Scale bar: 50 μm. At least three independent experiments were performed in each case.
**Figure 4.6.** Involvement of nonmuscle myosin II in metastasis like phenotype (MLP) of HCT-8 cells on E-cadherin and fibronectin coated 20 kPa gels. Different dosages of blebbistatin (2.5 μM, 5 μM, and 10 μM) are applied to cells on 20 kPa gels to inhibit the myosin II activities and reduce intracellular force. Application of blebbistatin on cells plated on E-cadherin coated 20 kPa gels inhibits the dissociative phenotype completely regardless of inhibitor concentration throughout the extended culture period (A-C). Whereas, cells without blebbistatin show MLP after 6-7 days (as shown in Figure 4.1B and 4.1C). Similarly, the drug inhibits the dissociative phenotype on control (ECM coated gels) as well (results not shown here). Scale bar: 100 μm. At least three independent experiments were performed in each case.
Figure 4.7. Changes induced by myosin II inhibitor drug application on 20 kPa PA gels were reversible. (A and B) Blebbistatin was removed from E-cadherin coated 20 kPa PA gel on Day 6 and Day 7, respectively. In both cases, HCT-8 cells were able to display the morphological phenotype transition within 5-6 days of drug removal. The arrows point to the dissociated cells. Scale bar: 100 μm. At least three independent experiments were performed in each case.
Figure 4.8. (A-B) HCT-8 cell colonies show dense actin network near the surface on both E-cadherin and fibronectin coated 20 kPa gels on fifth day of culture (before MLP) (A2 and B2). However, the network is more diffuse, disarrayed, and don’t show any specific directional bias in case of E-cadherin functionalized gel (A2), whereas the F-actin network on fibronectin functionalized gels clearly displays local polarization in spatial organization (indicated by arrow in B2). HCT-8 cells in the uppermost layer of the colonies display cortical actin around the cell membrane in both cases, but no stress bundles (Figures A1 and B1). (C-D) Merged images of DAPI stained nuclei with F-actin. Scale bar: 10 µm. (E) Quantitative analysis of actin fiber alignment before MLP near gel surfaces as shown in A2 and B2. The average coherency increased significantly from 0.07 to 0.39 (Student’s t test, p < 0.05) for E-cadherin to fibronectin functionalized gels.
Figure 4.9. Laser scanning confocal microscopy images of actin cytoskeleton structure after MLP. (A-B) Unlike cell colonies (as shown in Figure 4.8), dissociated HCT-8 cells on 20 kPa gels only show cortex actin (arrows) in both cases (A and B). No actin network is present in the dissociated cells, implying the disruption of force sensing machinery after morphological E to R transition. (C-D) Merged images of DAPI stained nuclei with F-actin. Scale bar: 10 µm. At least three independent experiments were performed in each case.
Figure 4.10. Average cell area analysis. Average cell area on E-cadherin and fibronectin coated 20 kPa PA gels before MLP. Interestingly, the average area before MLP on E-cadherin and fibronectin coated 20 kPa gels is of the similar magnitude, $101 \pm 9.66 \, \mu m^2$ and $94.53 \pm 19.97 \, \mu m^2$, respectively (n > 25 cells per condition).
**Figure 4.11.** Spatial distribution of vinculin on gels. (A-C) HCT-8 cells on 20 kPa gels were stained for vinculin before MLP. On E-cadherin coated gels (with integrin blocking antibodies), vinculin is primarily localized around cell-cell contact regions (Figure A, shown by arrows). In contrast, more diffuse vinculin with distinct punctate structures appear only on fibronectin coated 20 kPa PA gels (Figure B and C, shown by arrows). At least three independent experiments were performed in each case.
Figure 4.12. Immuno-staining for fibronectin and E-cadherin on E-cadherin and fibronectin coated 20 kPa gels. Gels are stained for fibronectin and E-cadherin. (A) No detectable fibronectin on E-cadherin coated 20 kPa gels. (B) Uniform fibronectin coating on fibronectin coated gel surfaces. (C) Uniform E-cadherin coating on E-cadherin coated gel surfaces. (D) No trace of E-cadherin was detected on fibronectin coated 20 kPa gels. Scale bar: 100 μm. At least three independent experiments were performed in each case.
**Figure 4.13.** R cells on the top of monolayer of HCT-8 cells in regular tissue culture flask. R cells on the top of monolayer of HCT-8 cells in regular tissue culture flask as reported earlier by two research groups independently [56-59]. Scale bar: 50 μm. At least three independent experiments were performed in each case.
CHAPTER 5

REPROGRAMMING CELLULAR PHENOTYPE BY SOFT COLLAGEN GELS

Summary. A variety of cell types exhibit phenotype changes in response to the mechanical stiffness of the substrate. Many cells excluding neurons display increase in spread area, actin stress fiber formation and larger focal adhesion complexes as substrate stiffness increases in sparsely populated culture. Cell proliferation is also known to directly correlate with these phenotype changes/change in substrate stiffness. Augmented spreading and proliferation on stiffer substrates require nuclear transcriptional regulator YAP (Yes associated protein) localization in cell nucleus and is tightly coupled with larger traction force generation. In this study, we show that different types of fibroblasts can exhibit spread morphology, well defined actin stress fibers, and larger focal adhesions even on very soft collagen gels (modulus in hundreds of Pascals) as if they are on hard glass substrate (modulus in GPa, several orders of magnitude higher). Strikingly, we show, for the first time, that augmented spreading and other hard substrate cytoskeleton architecture on soft collagen gels are not correlated with cell proliferation pattern and do not require YAP localization in cell nucleus, i.e. not coupled to higher cell traction. Finally, we examine the response of human colon carcinoma (HCT-8) cells on soft collagen gels. Recent studies show that human colon carcinoma (HCT-8) cells form multicellular clusters by 2-3 days when cultured on soft polyacrylamide (PA) gels with a wide range of stiffness (0.5-50 kPa) and coated with extracellular matrix, ECM (collagen monomer/fibronectin). These clusters show limited spreading/wetting on PA gels, form 3D structures at the edges, and eventually display a remarkable, dissociative metastasis like phenotype (MLP), i.e.,

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1 This chapter is adapted from the following publication:
epithelial to rounded morphological transition after a week of culture on PA gels only, but not on collagen monomer coated stiff polystyrene/glass where they exhibit enhanced wetting and form confluent monolayer. Here, we show that HCT-8 cell clusters also show augmented spreading/wetting on soft collagen gels and eventually form confluent monolayer as on rigid glass substrates and MLP is completely inhibited on soft collagen gels. Overall, these results suggest that cell-material interaction (soft collagen gels in this case) can induce cellular phenotype and cytoskeleton organization in a remarkably distinct manner compared to a classical synthetic polyacrylamide (PA) hydrogel cell culture model and may contribute in designing new functional biomaterials

In this dissertation, we aimed to study strongly nonlinear mechanisms for intense energy exchanges between weakly interacting uncompressed granular chains. Our theoretical approach was based on direct analysis of the discrete and non-smooth equations of motion (i.e., without resorting to continuum limit approximations), and our experimental studies recovered some of the theoretical findings.

5.1 Motivation

In recent years it has become increasingly evident that mechanical micro-environment, i.e., substrate rigidity plays an important role in regulating cell functionalities. Cells can sense and respond to the substrate stiffness on which they are adhered to (as in two dimensional or 2D culture) or surrounded by (as in three dimensional or 3D culture) [1-16]. By doing so, cells can modulate their differentiation [3], morphology [4-6], migration/motility [9, 13], bio-physical properties [16], growth [15], and other processes [10].

Many cell types such as fibroblasts [4], cardiac myocytes [17], and glioma cells [18] show an increase in spread area as substrate elasticity increases in sparsely populated culture. In addition to morphology, cellular cytoskeleton organization at single cell scale is also mediated by
substrate rigidity. Cells show well defined actin stress fibers on stiffer substrates only [4, 18]. Conversely, cortical actin is primarily observed on softer substrates [4, 18]. Further, cells show discrete, elongated and larger focal adhesion complexes on hard substrates [9, 18]. Whereas, small, punctate, and dot like focal adhesions are generally visualized on softer substrates [9, 18].

Cell proliferation rate also increases with increase of substrate modulus in general and is shown to be tightly coupled with enhanced traction force generation on stiffer substrates [15]. Recent discovery demonstrates important role of nuclear transcriptional regulator YAP in cellular mechanotransduction process [19]. YAP is primarily localized in cytoplasmic region in less spread cells on soft polyacrylamide gels [19, 20]. Conversely, it becomes localized primarily in nucleus in well spread cells on stiffer substrates [19, 20]. Hence, one can infer that force dependent augmented cellular spreading, well defined actin stress fibers, and focal adhesions formation are directly correlated with higher cell proliferation rate and YAP localization in cell nucleus.

Recent experiment shows that fibroblasts can spread on soft fibrin gels of low modulus as if they are on substrates with very high modulus (glass) [21]. It has been hypothesized that cell mediated local stiffening of non-linear elastic fibrin gels result augmented spreading and hence the phenomenon is force dependent. This hypothesis is refuted by another recent paper which uses non-linear elastic material modeling to claim that non-linear strain stiffening alone cannot explain such spreading on fibrous soft gels [22]. However, none of these studies explored the experimental correlation of cell proliferation and nuclear transcriptional regulator YAP activity with cell morphology and detailed cytoskeleton organization. It is conceivable that if augmented spreading and hard substrate cytoarchitecture on fibrous biological gels (e.g., fibrin/collagen) are force mediated, cell proliferation on these soft gels is expected to be higher. Also, YAP must be
localized in cell nucleus. Otherwise, it can be implied that cells interact with these fibrous biological gels in a unique manner that induce hard substrate like cell morphology and cytoskeleton organization without the need for high force.

In addition to single cell spreading, multicellular aggregate spreading/wetting on ECM coated substrates is mediated by substrate stiffness as well [23]. On softer substrates, cell clusters show less wetting, i.e. don’t spread well. Conversely, cell clusters show enhanced wetting/spreading on stiffer substrates presumably due to increased cell-substrate adhesions compared to cell-cell adhesions [23]. The hypothesized mechanism is explained with the value of a single parameter, \( S = W_{cs} - W_{cc} \), where \( W_{cs} \) and \( W_{cc} \) represent cell-substrate and cell-cell adhesions energy respectively. Complete wetting occurs for \( S > 0 \). For \( S<0 \), partial wetting takes place. Recent studies show that human colon carcinoma (HCT-8) cells cultured on soft PA gels (0.5 kPa-50 kPa), functionalized by ECM, form multicellular clusters with well-defined boundaries within 2-3 days due to less wetting [1, 24]. Cells from the cluster dissociate from one another after a week of culture, starting from the periphery. As this metastasis like phenotype (epithelial to rounded, E-R morphological transition) occurs, they reduce cell-cell and cell-ECM adhesion, and proliferate. However, on hard substrates, functionalized by ECM, HCT-8 cells form confluent monolayer and do not exhibit any metastatic phenotype transition. The formation of bounded clusters on soft gels and monolayers on hard substrates might be due to the difference between the cell-substrate wettability for the two types of substrates [1, 24]. E-R transition might be a consequence of this wettability and not due to low force on soft substrates. If so, then HCT-8 cells on adhesive soft collagen gels may not show the transition as well.

Here, we show for the first time that fibroblasts can display hard substrate like cell morphology and cytoskeleton organization on very soft fibrous collagen gels, without YAP
localization in cell nucleus. YAP is localized in cytoplasmic region and cell proliferation rate is low, as expected on soft substrates. Finally, we show that HCT-8 cells on soft collagen gels also display hard substrate like phenotype, i.e., augmented spreading, and confluent monolayer formation and no E-R transition. Overall, these results suggest that cell-material interaction (soft collagen gel in this case) can induce cellular phenotype and cytoskeleton organization in a remarkably distinct manner compared to a classical synthetic polyacrylamide hydrogel cell culture model and may contribute in designing new functional biomaterials.

5.2 Materials and methods

5.2.1 Collagen gel preparation

Formulation and synthesis of collagen gels were performed using a protocol described elsewhere [25].

Briefly, collagen gels were synthesized using high concentration collagen-I from rat tail (BD Biosciences, San Jose, CA). Collagen-I was diluted to two final concentrations of 2 and 4 mg/mL as follows. Equal volume of collagen-I and 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer solution in 2X phosphate buffered saline, PBS (pH 7.3) were mixed to reach the final concentration. Gel solution was then placed on a 35 mm glass bottom petri dish (In vitro scientific, Sunnyvale, CA) and allowed to polymerize completely for 90 mins at 37°C and 5% CO₂. Consequently, cells were seeded on polymerized gels and were incubated at 37°C and 5% CO₂. The reported shear modulus values were ~104 and 391 Pa corresponding to final collagen concentrations of 2 and 4 mg/ml in precursor solution [25].

5.2.2 PA gel and functionalized glass preparation

PA gel preparation and glass cover slip activation for the covalent attachment of gels were performed following the protocols described elsewhere [26-28]. In brief, 2 kPa gel solution was obtained by mixing 5% w/v acrylamide (Sigma, St. Louis, MO) and 0.05% N, N’-
methylenebisacrylamide (bis) solutions (Midwest Scientific, MO) in 10 mM HEPES-buffered saline (Lonza, Walkersville, MD) [29]. 8% w/v acrylamide and 0.27% N, N’-methylenebisacrylamide (bis) solution in 10 mM HEPES-buffered saline were used for 20 kPa gels.[29] Finally, 8% w/v acrylamide and 0.48% N, N’-methylenebisacrylamide (bis) solution in 10 mM HEPES-buffered saline were used for 40 kPa gels [29]. 1:200 ammonium persulfate (10% w/v) and 1:2000 N, N, N’, N’-tetramethylethylenediamine (TEMED) (both from Bio-Rad Laboratories, Hercules, CA) were used as the initiator and catalyst for polymerization process, respectively.

Glass cover slips were chemically activated to ensure covalent binding of the hydrogel as described earlier [27]. Briefly, glass slips were treated with 3- Aminopropyltrimethoxysilane (ATS) from Sigma, MO, for 7 min at room temperature. Followed by the removal of the ATS completely with DI water rinse, cover slips were treated with 0.5% Glutaraldehyde (diluted in PBS from 70% Glutaraldehyde stock solution from Polysciences, Inc,) solution for 30 min. A drop of 20 µL pre-polymer PA gel solution was deposited on the 12 mm² activated glass cover slip. Another 12 mm² regular glass cover slip was placed (floated) on the drop. The drop spread between the cover slips due to capillarity and was sandwiched with uniform thickness. Curing of the PA gel was performed for 45 mins at room temperature. The top cover slip was manually peeled off using a single edge razor. During peeling, detachment proceeded from one edge of the sandwich.

PA gels and glass were functionalized with ECM molecules, rat tail collagen (BD biosciences) at a concentration of 100 µg/ml. The surface functionalization protocol for binding collagen was described elsewhere [27]. Briefly, substrates were incubated with pure hydrazine hydrate (Sigma-Aldrich, MO) overnight. Substrates were then washed with 5% acetic acid
Collagen was deposited on top of substrates overnight at 4°C at a concentration of 100 µg/ml and rinsed with PBS on shaker for 10 minutes. All substrates were incubated at 37°C in culture media for 30 minutes before plating the cells.

5.2.3 Cell culture

3T3 fibroblasts (3T3Fs) and monkey kidney fibroblasts (MKFs) (ATCC, Manassas, VA) were cultured in DMEM medium (Cat. No. 30-2002, ATCC, Manassas, VA) supplemented with 10% serum (Cat. No. 30-2040, ATCC, Manassas, VA) and 1% Penicillin-Streptomycin (Cat. No. 30-2300, ATCC, Manassas, VA) of total solution. Human colon carcinoma HCT-8 cells (Cat. No. CCL-244, ATCC, Manassas, VA) were cultured in RPMI 1640 (Cat. No. 30-2001, ATCC, Manassas, VA) base medium supplemented with horse serum (Cat. No. 30-2040, ATCC, Manassas, VA) to a final concentration of 10% and Penicillin-Streptomycin (Cat. No. 30-2300, ATCC, Manassas, VA) to 1% of total solution.

5.2.4 Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cells were then incubated with Image-iT FX signal enhancer (Invitrogen, CA) for 30 min. Next, cells were incubated in primary antibodies against α-tubulin (Cat. No. A11126, Invitrogen, CA), vinculin (Cat. No. V9131, Sigma-Aldrich, MO), or YAP (H-9, Santa Cruz) for 45 min at room temperature, respectively. Following three times rinse in PBS, the samples were incubated for 30 min with secondary antibody (alexa fluor™ 488 goat anti-mouse IgG/alexa fluor™ 647 goat anti-mouse IgG (Invitrogen, CA)) at a 1:200 dilution in PBS at room temperature in each case. To visualize the F-actin structure, cells were incubated with tetramethylrhodamine (TRITC) phalloidin conjugates (Cat. No. P1951, Sigma-Aldrich, MO) at a concentration 50 µg/ml for 45 min at room
temperature. To visualize cell nucleus, cells were finally incubated with 4',6-diamidino-2-phenylindole, DAPI (1:1000) for 15 min at room temperature. All the samples were imaged either using the Zeiss LSM 700 confocal scanning laser microscope (Carl Zeiss, Inc.) or Olympus IX81 microscope.

5.2.5 Proliferation assay

Proliferation assay was performed using the Click-iT EdU (5-ethyl-2'-deoxyuridine) cell proliferation assay kit (Invitrogen, CA) according to manufacturer’s instructions (http://tools.lifetechnologies.com/content/sfs/manuals/click_it_edu_imaging_kit_man.pdf) [30].

Briefly, MKFs were plated on soft collagen gels and glass from a confluent T25 flask culture., After 24 hrs of culture, cells were incubated with 10 μM EdU in complete media for 60 minutes [31]. Then, cells were fixed, permeabilized, and incubated with alexa fluor 488 azides to detect EdU. Finally, cells were counterstained with DAPI. Cells which show double fluorescence (both EdU and nuclei) were considered to be synthesizing new DNA (Deoxyribonucleic acid).

5.2.6 Image analysis and statistical analysis

ImageJ was used for determining cell spreading area [32]. Average number of focal adhesions (FAs) per cell and average size of FAs are measured using ImageJ [33]. Student’s t test was performed to evaluate statistical significance. Error was reported as standard deviation unless otherwise mentioned.

5.3 Results and discussions

5.3.1 Fibroblasts show augmented spreading, well defined actin stress fibers and larger focal adhesions on very soft collagen gels as if they are on stiff glass substrates

We assess cell spreading, F-actin organization, and focal adhesions on collagen gels. Collagen monomer coated glass substrates are used as control.
5.3.1.1 Cell spreading

Cell spreading is a known readout of cellular mechano-sensitivity to substrate rigidity [34]. Cells remain less spread/rounded on ECM functionalized polyacrylamide hydrogel substrates with low modulus (hundreds of Pa) [4, 18]. Cells show a monotonic increase in area with increase in substrate rigidity unless it reaches a plateau. This maximum value and the corresponding substrate stiffness depend on cell type. In this study, we culture monkey kidney fibroblasts (MKFs) and 3T3 fibroblasts (3T3Fs) on collagen gels with modulus of 104 Pa and 391 Pa. The gels were ~600 µm thick ensuring that the cells do not feel the underlying rigid glass substrate. Collagen monomer coated glass substrate (~ 70 GPa) is used as control. Interestingly, MKFs and 3T3Fs show well spread morphology on both soft collagen gels (Figure 5.1A-5.1B) as if they are on rigid glass substrates in sparsely populated culture (Figure 5.1A-5.1B).

Further, we quantify cell spreading area using ImageJ. Both MKFs and 3T3Fs on soft collagen gels with two different moduli (104 Pa and 391 Pa) show similar quantitative spreading with no statistically significant difference (Figure 5.1C, Student’s t test, p > 0.1). No statistically significant difference with glass is observed compared to either formulation of collagen gels (Figure 5.1C, Student’s t test, p > 0.1), consistent with qualitative well spread morphology. This altered spreading behavior on soft collagen gels implies that the interaction between cells and soft collagen gels is distinct compared to ligand coated polyacrylamide hydrogels and glass substrates.

5.3.1.2 Visualization of Cytoskeleton organization

F-actin structure and microtubule organizations of fibroblasts adherent to thick collagen gels and collagen monomer coated glass substrates are visualized by staining with phalloidin and anti α-tubulin antibody respectively after fixation.
Confocal microscopy imaging results suggest that fibroblasts (MKFs) exhibit large and well-organized actin stress fibers on both soft collagen gels and glass (Figure 5.2A1-5.2A3). Further, microtubule organization on soft collagen gels also resembles hard substrate architecture (Figure 5.2B1-5.2B3). Previous studies suggest that fibroblasts on ECM functionalized compliant PA gels (in a range 0.5-5 kPa stiffness) show mostly cortical actin, but no actin stress fiber [16]. Actin stress fibers become visible on stiff PA gels (~ 10 kPa) and larger, well organized stress fiber bundles are apparent on rigid glass substrate in a stiffness dependent manner [16]. Here, we show that when we replace the underlying matrix material with soft biological material, namely, collagen gels of different compliance (yet of very low modulus, 104 Pa and 391 Pa), fibroblasts are able to display hard substrate cytoskeleton architecture in addition to augmented spreading as shown in Figure 5.1.

5.3.1.3 Focal adhesions

Focal adhesions (FAs) are sites of adhesion expressed by different types of cells in culture. They provide the linkage between the ECM components to intracellular cytoskeleton (F-actin) via integrin receptors. FAs are composed of a variety of proteins, such as vinculin, talin, and paxillin [35]. However, vinculin depletion leads to dramatic changes in FA sizes and also in cell functionality[36-38]. In addition, vinculin is the most abundant focal adhesion protein [39]. Hence, adherent cells on soft collagen gels and collagen monomer coated glass are labeled with anti-vinculin antibody and are imaged via confocal microscopy.

Interestingly, fibroblasts (MKFs) on collagen gels show discrete, elongated focal adhesions as on rigid glass substrates (Figure 5.3A-5.3C). Focal adhesion (FA) size and number for each condition are assessed using ImageJ [33]. Quantitative results suggest that there is no
statistically significant difference in FA size and number (Figure 5.3D-5.3E, Student’s t test, p > 0.1), consistent with qualitative imaging results as shown in Figure 5.3A.

Cells are known to exhibit small, dot like punctate vinculin structures on compliant PA gels. Conversely, discrete and elongated focal adhesions are characteristics of sparsely populated cell culture on very stiff gel or rigid glass substrates [4, 9, 18]. These results imply that unusual fibroblast spreading and actin stress fiber formation on soft collagen gels (Figures 5.1 and 5.2) is orchestrated with discrete, elongated and mature focal adhesion formation (Figure 5.3).

5.3.2 Cell proliferation on soft collagen gels is not correlated with hard substrate morphological and cytoskeleton organization phenotype

Substrate rigidity modulates cell proliferation rate, i.e., new DNA synthesis [15]. It is inferred that cell proliferation rate is directly coupled to cell spreading and traction force generation [15]. Hence, we ask the question whether augmented cell spreading and hard substrate cytoskeleton architecture on soft collagen gels can induce increased cell proliferation rate disregarding macroscale/global material softness. We examine MKFs cell proliferation rate on collagen gels of 104 Pa and 391 Pa, soft (2 kPa) and stiff PA gels (40 kPa), and glass after 24 hrs of cell plating. Our results show that fibroblast cell proliferation rate on collagen gels is very low unlike stiff polyacrylamide gels and glass substrates (Figure 5.4A-5.4C). On PA gels, cells show a higher proliferation rate as substrate rigidity increases (Figure 5.4C), consistent with previously published results [15].

5.3.3 Augmented cellular spreading on soft collagen gels doesn’t require YAP localization in cell nucleus

Recent discovery demonstrate that nuclear transcriptional regulator YAP plays an important role in cellular mechanotransduction [19]. Traction force mediated augmented
spreading either on stiffer substrates or on larger ECM micropatterns require YAP localization at cell nucleus [19]. Conversely, for less spread cells that exert smaller traction at cell-substrate interface (either on soft PA gels or on smaller ECM micropatterns on hard substrate), YAP is primarily localized in cytoplasmic region [19]. Thus intracellular YAP localization is also tightly coupled with traction force generation. Hence, to further explore the relationship between the spread cell morphology and traction force generation on collagen gels, we label the adherent cells on collagen gels and glass with anti YAP antibody. Imaging results suggest that YAP is primarily localized in the cytoplasmic region in well spread 3T3Fs on soft collagen gels (Figures 5.5A1-5.5A2). However, YAP is primarily localized in cell nucleus in well spread cells on glass, consistent with earlier observations. We further quantify the percentage of cells with YAP localized in nucleus. Consistent with qualitative observations, spread cells on collagen gels clearly don’t require YAP localization in cell nucleus unlike cells on glass (Figure 5.5C).

5.3.4 HCT-8 cell clusters show increased wetting as on hard substrate and don’t show MLP on soft collagen gels

In addition to single cell spreading, cellular aggregate/cluster spreading or wetting is also shown to be regulated by substrate rigidity [23]. Cell clusters exhibit poor wetting on ECM functionalized softer substrates [23]. Increased wetting of cellular clusters is observed on stiffer substrates only [23]. The hypothesized mechanism is explained with the value of a single parameter, $S = W_{cs} - W_{cc}$, where $W_{cs}$ and $W_{cc}$ represent cell-substrate and cell-cell adhesions energy respectively. Complete wetting occurs for $S > 0$. For $S<0$, partial wetting takes place. Recent experiments with human colon carcinoma cells (HCT-8) show that these cells form tumor like cell clusters with peripheral 3D structures on ECM functionalized PA gels of stiffness ranging from 0.5-5 kPa in 2-3 days (Figure 5.6C1). These cell clusters show relatively poor
wetting (Figure 5.6C2) and after a week of culture show a dissociative metastasis like phenotype (epithelial to rounded, E-R, morphological transition, Figures 5.6C3-5.6C4) [1-24]. Conversely, HCT-8 cells cultured on glass spread (Figures 5.6D1-5.6D2), and eventually form a confluent monolayer by 2-3 days depending on initial seeding density (Figure 5.6D2). Cells on glass do not show any MLP over extended period of cell culture (Figures 5.6D3-5.6D4) [1]. Inspired from unusual single cell scale fibroblast spreading on soft collagen gels, we ask the question whether HCT-8 cell clusters show similar increased wetting on collagen gels at multicellular level and if so, is the MLP completely inhibited by soft collagen gels. Interestingly, cell clusters on both collagen gels (104 Pa and 391 Pa) show augmented wetting (Figures 5.6A1-5.6A2 and 5.6B1-5.6B2) forming confluent monolayer (Figures 5.6A2 and 5.6B2) as on hard substrate, and cells do not display MLP upon extended culture period (Figures 5.6A3-5.6A4 and 5.6B3-5.6B4). These results imply that soft collagen gel can induce increased wetting of multicellular aggregates as well.

5.4 Conclusion

To summarize, we have shown that fibroblasts can display morphological phenotype and cytoskeleton architecture similar to very hard glass substrate on very soft collagen gels. Yet, lower cell proliferation rate and YAP localization in cytoplasmic region on collagen gels are characteristics of being on soft substrates and are tightly coupled with lower cellular traction. Further, HCT-8 cell clusters also show increased spreading on soft collagen gels as on hard substrate and do not show metastasis like phenotype (epithelial to rounded morphological transition) on collagen gels that is otherwise observed on ECM functionalized soft PA gels (0.5-50 kPa). Overall, these results suggest that cell-material interaction (soft collagen gel in this
case) or cell-substrate wettability may determine cell spreading and cytoskeletal architecture, independent of substrate softness.

5.5 References


5.6 Figures

**Figure 5.1.** Cells spread on soft collagen gels as if they are on rigid glass substrate. (A-B) Phase contrast micrograph showing spread morphology of different types of fibroblasts on soft collagen gels and collagen monomer coated glass. (C) Spread area quantification for collagen gels and glass reveal no statistically significant difference for both MKFs and 3T3Fs (Student’s t test, p > 0.1). Total n = 63 and 67 cells were analyzed for MKFs and 3T3Fs, respectively. At least two independent experiments were performed per condition.
**Figure 5.2.** F-actin, and microtubules organization on soft collagen gels. (A) Fibroblasts (MKFs) on soft collagen gels show actin stress fibers (A1-A2) similar to rigid glass substrates (A3). (B) microtubule organization on soft collagen gels (B1-B2) also resemble glass substrates (B3). At least three independent experiments were performed per condition.
Figure 5.3. Elongated focal adhesions formation on collagen gels that are generally seen on very hard glass substrates.
Figure 5.4. Cell proliferation rate of fibroblasts is very low on soft collagen gels. Fibroblasts (MKFs) show lower proliferation rate on soft collagen gels and PA gels. However, the proliferation rate is significantly higher on stiff PA gel and glass (Student’s t test, p < 0.05). Total n = 781 cells were analyzed. At least two independent experiments were performed per condition.
Figure 5.5. YAP is not localized in cell nucleus on soft collagen gels. (A-B) Despite augmented spreading and hard substrate like cytoskeleton architecture, YAP is not localized in nucleus of fibroblasts (3T3Fs) on soft collagen gels (A1-A2 and B1-B2). Conversely, on hard substrates YAP is primarily localized in cell nucleus (A3-B3). (C) Quantification of percentage of cells expressing nuclear YAP. Consistent with qualitative imaging results, YAP is localized in cytoplasmic region of fibroblasts on soft collagen gels unlike glass substrates. Total n = 176 cells were analyzed. At least two independent experiments were performed per condition.
Figure 5.6. Increased wetting of HCT-8 cells on soft collagen gels and inhibition of E-R transition. Cell clusters on both collagen gels (104 Pa and 391 Pa) show augmented wetting (Figures A1-A2 and B1-B2), and confluent monolayer formation (Figures A2 and B2), and they do not display MLP upon extended culture period (Figures A3-A4 and B3-B4) as on very hard substrate like glass (Figures D1-D4). On PA gels, these cells form tumor like cell clusters with peripheral 3D structures in 2-3 days (Figure C1). These cell clusters show relatively poor wetting (Figure C2) and after a week of culture exhibit a dissociative metastasis like phenotype (epithelial to rounded morphological transition, Figures C3-C4). At least three independent experiments were performed per condition.
CHAPTER 6

MICROPATTERNING PROTEINS AND CELLS ON POLYACRYLAMIDE GELS¹

Summary. We present an experimental technique that enables spatial confinement of extracellular matrix proteins/cell adhesion molecules and consequently living cells in defined geometries on tunable stiffness compliant hydrogel substrates, namely, polyacrylamide gels. The protein patterns are first printed on a hydrophilic glass using a polydimethylsiloxane (PDMS) stamp via microcontact printing (µCP). Pre-polymer hydrogel solution of appropriate composition is applied on the protein patterned glass and is then sandwiched by a functionalized glass slide that covalently binds to the gel. The hydrophilic glass slide is then peeled off from the gel. The protein patterns detach from the glass during the process, but remain intact on the gel. The pattern is thus transferred to the gel. As a demonstration, microscale lines of cell adhesion proteins with characteristics dimensions varying from 5-500 µm are patterned on hydrogel surface with stiffness ranging from 1-50 kPa. Living healthy and cancer cells are cultured on the patterned hydrogel surfaces. Cell attachment and proliferation are confined within these patterns for long term (~ 2 weeks). This approach allows defined cellular traction stress distribution on hydrogels as predicted by finite element analysis. This simple yet effective technique of micropatterning extracellular matrix proteins and cells on polyacrylamide hydrogels may serve as a useful tool to study the role of geometry on primary colon cancer cell traction and MLP of HCT-8 cells.

6.1 Background

In recent years it has become increasingly evident that physical cues such as mechanical

micro-environment and geometry, in addition to bio-chemical factors, plays an important role in regulating cell functionalities. Cells can sense and respond to the substrate stiffness on which they are adhered to (as in 2D culture) or surrounded by (as in 3D culture). By doing so, cells can modulate their differentiation, morphology, migration/motility, bio-physical properties, growth, and other processes. The mechanosensing response is often unique to cell type, dimensionality (2D or 3D), and even type of ECM (fibronectin or collagen-I) used for substrate functionalization i.e. adhesion receptors. Geometric cues can also influence important cell functionalities including cell adhesion and spreading [1, 2], cell proliferation and differentiation [3], cell fate switching between apoptosis and growth [4], cell polarity [5, 6], migration [7], cellular internal compartment organization [7, 8], and the orientation of the axis of division [9, 10].

Here, we report a simple two-step method to pattern cell adherent ECM proteins on PA gels. The method utilizes a hydrophilic glass substrate that is patterned via µCP of ECM proteins using a PDMS stamp. The patterned glass substrate is filled with a droplet of pre-polymer PA gel solution, which is then sandwiched by another functionalized glass slide that adheres to the gel. The composition of the PA gel solution dictates the stiffness of the gel. After curing the gel at 37 °C, the glass slides are separated from each other, when the gel remains attached to the functionalized glass and peels off the protein patterns from the hydrophilic glass. Thus, the protein pattern is transferred to the gel. The process avoids any functionalization of the inert gel surface for attachment of the protein pattern using the toxic chemicals. This tool enables the defined cellular traction stress distribution on hydrogels as predicted by finite element modeling.

6.2 Material and methods

PA gel preparation and slide activation for the covalent gel attachment were performed
following the protocols described elsewhere [11]. In brief, gel solutions were obtained by mixing 8% w/v acrylamide solution (Sigma, St. Louis, MO) and 0.27% N, N’- methylenebisacrylamide (bis) solution (Midwest Scientific, MO) in 10 mM HEPES-buffered saline (Lonza, Walkersville, MD). 1:200 ammonium persulfate (10% w/v) and 1:2000 N, N, N’, N’-tetramethylethylenediamine (TEMED) (both from Bio-Rad Laboratories, Hercules, CA) were used as the initiator and catalyst for polymerization process, respectively. AFM (Asylum Research, Santa Barbara, CA) with a pre-calibrated silicon nitride tip was used to characterize the stiffness of PA gels in phosphate-buffered saline (PBS; Lonza, Walkersville, MD) solution [12, 13]. The nominal stiffness of the PA gels was 20 kPa. Glass slides were chemically activated to ensure covalent binding of the hydrogel as described earlier [11]. Briefly, slides were treated with 3- Aminopropyltrimethoxysilane (ATS) from Sigma, MO for 7 min at room temperature. Followed by the removal of the ATS completely with DI water rinse, slides were treated with 0.5% Glutaraldehyde (70% Glutaraldehyde from Polysciences, Inc, diluted in PBS) solution for 30 min.

6.3 Results

6.3.1 Mechanical stress distribution analysis on different micropatterned cells

A 3D finite element model was developed using COMSOL software to predict traction stress generated by patterned cells on hydrogels. The model consists of two layers – top layer/active layer and bottom/passive layer as described somewhere else [14, 15]. The top layer is termed as ‘active’ as this will be used to mimic cellular contractility in the computational model. The bottom surface of the passive layer is fixed. The ratio of height for passive to active layer is used as 1:5. Other dimensions are used from the experimental geometry to be patterned. Cellular contractility is introduced in the model by introducing a temperature drop of 5k. The other
physical properties of the active and passive layers used in modeling are used from earlier literature [14, 15]. Von Mises stress map at the bottom surface is reported in this study. Minimum mesh size is varied from 0.02-28 µm in the simulations to verify the convergence of results.

Figure 6.1A shows the displacement field of a linear rectangular two component model after induction of thermal strain. Figure 6.1B shows the von Mises stress distribution pattern at the bottom surface of the passive layer. Clearly, for the linear/rectangular geometry the stresses are higher at the edges compared to the center. These results are consistent with the reported principal stress distribution at the bottom of similar models [14, 15]. From these results, we can conclude that maximum stress is localized at the edges.

6.3.2 Optical and soft lithography, Micro-contact printing (µCP) and protein patterning

A Si master with desired geometric patterns was fabricated using standard photolithographic technique as shown in Figures 6.2A1-6.2A4. 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 and baked at 110ºC (4 min). Descumming of the Si wafer was performed 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. 6.2A1). After the post exposure bake at 95ºC for 2 min, the wafer was developed using SU-8 developer (MicroChem, Newton, MA) for 10s (Fig. 6.2A2) 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-hydradecyltrichlorosilane) using MVD System (Applied MST, San Jose, CA) to ensure easy removal of the cured PDMS stamp in the subsequent steps (Fig. 6.2A-6.2A4) [16, 17]. 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 [18, 19]. The prepolymer solution was poured over the Si master mold (Fig. 6.2B1) and cured in the oven at 70°C for 12 h. The elastomeric stamp was then peeled off carefully (Fig. 6.2B2) 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 [20]. 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. 6.2B3-6.2B4). The stamp was brought into complete conformal contact with a glass substrate for 45 min at 37°C (Fig. 6.2B5). 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 [21, 22]. 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 $42^\circ \pm 3^\circ$ and $0^\circ$, 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 (Fig. 6.2B6). Curing of the PA gel was performed for 45 mins at elevated temperature, 37ºC (Fig. 6.2B7). 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. 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.

Figures 6.3A and 6.3B 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 is 1.98 ± 0.05 µm. The structures are well defined as is evidenced from their sharp edges and flat surfaces (Fig. 6.3B). Micrographs of the well-defined geometric patterns on silicone elastomers (PDMS) are shown in Figure 6.3C. As a demonstration, typical phase contrast and fluorescent micrographs of the FN patterns transferred onto PA gel are displayed in Figure 6.4. 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. Both the diffusion of ECM protein into aqueous surrounding and the possible deformation on hydro-gel surface due to stamp
indentation are avoided using current two-step method. 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.

6.3.3 Culturing living cells on patterned hydrogels

To demonstrate that the micro-patterned ECM zones (FN, LN and CN) were able to produce high quality spatial organization of cells, normal fibroblast cells (MKF) and HCT-8 cells were plated and cultured on PA gel surface with patterns of varied widths. The cells were imaged on day 3 as illustrated in Figure 6.5. Clearly, the cells adhered to ECM patterns only, and they form well-defined linear clusters.

6.4 Conclusion

We have presented an experimental technique that enables spatial confinement of cells on hydrogels. Our successful cell culture on micro-patterned PA gels indicates that ECM properties are not altered after the transfer and patterning. This straightforward technique of micro-patterning ECM and cells on PA gels will serve as a useful tool to study the effect of geometric cues on primary colon cancer cell traction and MLP of HCT-8 cells.

6.5 References


Figure 6.1. Prediction of mechanical stress distribution on micropatterned cells. (A) Displacement field of a linear rectangular two component model after induction of thermal strain and (B) Von Mises stress distribution pattern at the bottom surface of the passive layer.
Figure 6.2. Optical and soft lithography, Micro-contact printing (µCP) and protein patterning schematic. (A1-A4) Schematic diagram illustrating the basic steps of optical lithography process for Si master fabrication. 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.
Figure 6.3. Characterization of micropatterns. (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.
Figure 6.4. ECM patterns on gels. 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 are (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, prove that the FN patterns are completely transferred without major defects. Scale bars were shown on each image.
Figure 6.5. Spatially patterned cells on gels. 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; (C, D) MKF cultured on 100 and 10 μm laminin (LN) line patterns on 3rd culture day; (E, F) MKF cultured on 1000 and 30 μm collagen I (CN) line patterns on 5th culture day.
CHAPTER 7

CONCLUSIONS AND FUTURE WORKS

7.1 Summary and future directions

In this dissertation, we aim to study different aspects of mechanosensitivity of human colon cancer cells \textit{in vitro} and assess the potential for introducing a biophysical marker for cancer prognosis.

An optimized protocol is developed initially that demonstrates the isolation of primary human colon cells from healthy and cancerous surgical human tissue samples. Isolated cells are consequently cultured on engineered substrates for mechanobiology studies (Chapter 2). This cell culture platform opens up the possibility of exploring other biophysical properties of primary human cancer cells like cell stiffness using AFM, intracellular rheology using microinjected particles, cell migration/motility, and vesicle dynamics analysis as parameters for cancer prognostics in future.

Chapter 3 provides first experimental data of a possible correlation between the primary tumor cell traction and colon cancer metastasis. It is clear that the current study data with human primary tumor and healthy cells from two patients is limited. More patient data is necessary to draw more concrete conclusions in future. Nevertheless, the study raises the possibility of using resected and to-be-discarded tumor tissues of colon cancer patients for the biophysical studies of the cells. Contractility of the tumor cells in contrast to the healthy cells of the same tumor might serve as an indicator of invasion. A measure of cell contractility is by no means a replacement of lymph node inspection, but it may serve as an additional parameter for detecting cancer metastasis.
We examine the mechanosensitivity of human colon cancer cell line (HCT-8) on linear elastic and non-linear elastic substrates at multicellular scale in Chapters 4 and 5. The model system presented (HCT-8 cells on PA gels) may be used as a potential in vitro biomaterial platform to dissect colon cancer phenotype transition in cellular and molecular details which may provide valuable insights. This platform may also be used for studying early phase of tumor progression for other types of cancers as well. From our experimental observations discussed in Chapter 4 and Chapter 5, we hypothesize that appropriate mechanical force or traction stress is a necessary cue for the MLP of HCT-8 cells on PA gels. To test this hypothesis, future experiments may include examination of spatial traction map and net cell contractile force on both fibronectin and E-cadherin coated gels.

We report a straightforward yet novel technique of micropatterning ECM and cells on PA gels in Chapter 6 that may serve as a useful tool to explore the role of geometric cue on MLP of HCT-8 cells on hydrogel substrates with appropriate elasticity. Also, this tool may be used to understand how geometric cue influence spatial traction of micropatterned primary colon tumor and normal cells in future studies.

A recent study suggests that mechanical memory can play a remarkable role in dictating stem cell fate with YAP/TAZ working as ‘mechanical rheostat’ [1]. Stem cells can remember the past mechanical microenvironment history along with the time of culture on that environment (so called ‘dosing’) and thus often can modulate their downstream lineage overriding the current mechanical microenvironment [1]. In this context, it will be a significant advance in ‘reverse engineering’ the metastatic cascade if we can dissect the role of mechanical memory and ‘dosing’ on the fate of metastatic cancer cells in details. To do so, the following experimental metrics are proposed. First, primary tumor tissues at early and late stage of cancer (before and
after metastasis) need to be de-cellularized adopting published protocols [2]. Consequently, the micro architecture and stiffness of the remaining scaffolds needs to be evaluated and the relative difference between the two types of tissues have to be quantified. Next, primary cancer cells with varying metastatic potentials can be potentially seeded in both types of scaffolds to test any bias towards specific micro structure, i.e., highly metastatic cells proliferating and expressing invasive cancer markers in scaffolds from metastatic tumors implying a memory of their past microenvironment. Finally, expressions of cancer markers in cells cultured in both types of scaffolds will be assessed to test whether low grade tumor scaffolds suppress expressions in highly metastatic cells and high grade scaffolds enhance metastatic expressions in cells with low metastatic potentials. The study will reveal the roles of current microstructure in determining the fates of cells, and raises the possibility of reversing metastasis by engineering the tumor micro architecture.

7.2 References
