BIOPHYSICAL FEATURES OF THE EXTRACELLULAR MATRIX
DIRECT BREAST CANCER METASTASIS

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BIOPHYSICAL FEATURES OF THE EXTRACELLULAR MATRIX DIRECT BREAST CANCER METASTASIS

A Dissertation Presented
By
ALYSSA DANIELLE SCHWARTZ

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2018
Department of Chemical Engineering
Biophysical Features of the Extracellular Matrix Direct Breast Cancer Metastasis

A Dissertation Presented

By

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DEDICATION

To my family for supporting me every step of the way.

To the 14 year-old girl admitted to Black Lion Hospital with aggressive breast cancer.
ACKNOWLEDGEMENTS

I want to acknowledge the incredible commitment that my advisor, Dr. Shelly Peyton, has made to my success in graduate school. The energy that Shelly puts into her work is always apparent, and she has motivated and supported me to do high quality science, travel to conferences to share my work, and helped me be a better mentor to students in STEM. Before I even joined the lab, she invested her most precious resource (time – hers and Lauren Jansen’s!) into my GRFP application. It’s not that hard to understand how Shelly’s passion inspired me to follow her halfway around the world (literally) to learn from and teach graduate students in Ethiopia, which started a project that still has a lot of potential and I have high hopes for its impact.

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Last, but not least, I want to thank my family for their unwavering support. My parents have encouraged me to pursue my interests every step of the way; from cultivating my early love for science, to helping me pick out the right college, and providing me with love and support through graduate school. I also want to acknowledge Brianna and Jason for pushing me to be the best version of myself. It’s been fun to watch their successes come from hard work and it motivates me to work hard and play hard too.
Breast cancer is plagued by two key clinical challenges; drug resistance and metastasis. Most work to date probes these events on an extremely rigid plastic surface, which recapitulates few aspects of these processes in humans. A malignant cell first resides in breast tissue, then likely travels to the bone, brain, liver, or lung, each of which has a distinct mechanical and biochemical profile. Cells transmit mechanical forces into intracellular tension and biochemical signaling events, and here we hypothesize that this mechanotransduction influences drug response, growth, and migration.

To probe the impact of extracellular matrix on drug resistance, we defined a set of biomaterials that allowed us to independently tune stiffness, dimensionality, and cell-cell contact. We screened response to therapeutics across these material systems, and found that these environments increased resistance to targeted, but not cytotoxic, therapies compared to traditional screening on plastic. A systems biology analysis was applied to signaling data across platforms to identify MEK phosphorylation as a key driver of resistance. Comparing robust biomaterial screening to traditional plastic, we identified the role individual features of the tumor microenvironment in adaptive resistance.
The drug response study systematically varied several biomaterial parameters, but the remainder of this work isolated tissue stiffness as a driving force behind metastasis. Here we identified two mechanosensitive proteins that are at least partially responsible for adhesion and motility in breast cancer cells. On soft matrices, integrin $\alpha_6$ and laminin production mimic the aggressive cell behavior induced by epidermal growth factor (EGF). Integrin $\alpha_6$ and the EGF receptor then coordinate to activate calpain 2, which is necessary for migration to distant sites. However, cells likely reside at a metastatic site for long times, so we probed the effect of extended mechanical cues on cell behavior. On biomaterials, cells are selected for phenotypes that mirror primary tumor cells that have never been grown on plastic. This work shows that cells retain memory of their previous mechanical environment, even after being re-challenged with culture in very rigid environments. Using biomaterials, we captured physiological aspects of drug resistance and metastasis, to better understand and ultimately treat breast cancer.
# TABLE OF CONTENTS

| ACKNOWLEDGEMENTS | ......................... | v |
| ABSTRACT | ................................ | viii |
| LIST OF TABLES | ................................ | xiv |
| LIST OF FIGURES | ................................ | xv |

## CHAPTER

1: THE PROCESS OF METASTASIS AND CURRENT MODELS

1.1 The Clinical Problem of Advanced Breast Cancer

1.1.1 An Overview of Metastasis

1.1.2 Clinical Information Derived from Biopsies and Genetic Panels

1.1.3 The Healthy and Tumor Microenvironments

1.2 Malignant transformation at the Primary Tumor Site

1.2.1 Genetic Drivers of Breast Tumorigenesis

1.2.2 The Primary Tumor Stiffens During Cancer Progression

1.3 Dissemination to the Secondary Site

1.3.1 Genetic Heterogeneity and Cell lineages

1.3.2 The Biochemical and Mechanical Microenvironment Facilitates Metastasis

1.4 Selection of Appropriate Models to Study Breast Cancer Metastasis

1.4.1 Identification of Genetic Models that Capture Global Heterogeneity

1.4.2 Optimization of Mechanical Models to Answer Biological Questions

1.5 Hypothesis

1.6 Objectives

1.7 Significance

2: A BIOMATERIAL SCREENING APPROACH REVEALS MICROENVIRONMENTAL MECHANISMS OF DRUG RESISTANCE

2.1 Abstract

2.2 Introduction

2.3 Materials and Methods

2.3.1 Cell culture

2.3.2 Polymerization of 2D and 3D PEG hydrogels

2.3.3 Formation of uniform tumor spheroids

2.3.4 Characterization of Gene Expression by RT-PCR

2.3.5 Hydrogel mechanical characterization

2.3.6 Quantification of drug response

2.3.7 Cell seeding on biomaterials

2.3.8 RNA Sequencing

2.3.9 Gene Set Enrichment Analysis

2.3.10 Multiplex phospho-protein quantification
4: SOFT MATERIAL CONDITIONING OF BREAST CANCER CELLS ALTERS METASTATIC POTENTIAL ................................................................. 92

4.1 Abstract ................................................................................................................................. 92
4.2 Introduction ............................................................................................................................ 93
4.3 Materials and Methods .......................................................................................................... 95
  4.3.1 Synthesis of poly(ethylene glycol)-phosphorylcholine (PEG-PC) Hydrogels ........... 95
  4.3.2 Cell Culture ..................................................................................................................... 96
  4.3.3 Subclonal Cell Selection ............................................................................................... 97
  4.3.4 Proliferation Assays ..................................................................................................... 97
  4.3.5 Random Cell Migration ............................................................................................... 97
  4.3.6 Collective Motility ......................................................................................................... 98
  4.3.7 Traction Force .............................................................................................................. 98
  4.3.8 ECM Remodeling ......................................................................................................... 98
  4.3.9 Cell Invasion ................................................................................................................. 99
  4.3.10 qRT-PCR .................................................................................................................. 99
  4.3.11 Animal Xenografts ....................................................................................................... 99
  4.3.12 Primary Mouse Tumor Cell Culture .......................................................................... 100
  4.3.13 Statistical Analysis ..................................................................................................... 100
4.4 Results ................................................................................................................................. 101
  4.4.1 Design of an extended culture system to model breast cancer metastasis .......... 101
  4.4.2 Extended mechanical conditioning alters cancer cell growth and ECM remodeling ................................................................................................................. 102
  4.4.3 Culture on soft biomaterials promotes motility at conditions similar to growth conditions ..................................................................................................................... 105
  4.4.4 Primary tumor cells are smaller and motile on Soft Biomaterials ......................... 107
  4.4.5 Cells adopt a material-dependent phenotype over time ............................................ 107
  4.4.6 Clonally isolated cells exhibit a range of phenotypic behaviors mimicking mechanical selection .............................................................................................................. 112
4.5 Discussion ............................................................................................................................ 114
4.6 Conclusions .......................................................................................................................... 119

5: CONCLUSIONS AND ONGOING WORK ........................................................................ 121

5.1 Abstract ............................................................................................................................... 121
5.2 Overall Conclusions ............................................................................................................. 121
  5.2.1 Microenvironment mediated drug resistance via MEK .......................................... 121
  5.2.2 Integrating tumor genetics and ECM properties .................................................... 122
  5.2.3 Mechanical Forces Direct Metastasis ....................................................................... 123
5.3 Future Work and Overcoming limitations ........................................................................ 124
  5.3.1 Breast cancer as a global disease ............................................................................. 124
  5.3.2 Development of mechanical drug targets ................................................................. 125
  5.3.3 Further Exploration of Clonality and Heterogeneity in Breast Cancer ............... 126

6: FUTURE DIRECITONS EXPLORING MECHANICAL FORCES ........................................ 131
6.1 Inclusion of Additional Mechanical Forces: Shear Flow Enhances Cell Motility through Calpain 2 ................................................................. 131
  6.1.1 Introduction.............................................................................. 131
  6.1.2 Methods ................................................................................. 132
  6.1.3 Results and Discussion ............................................................ 134
  6.1.4 Conclusions and Future Work .................................................. 138

6.2 Ethiopian Breast Cancer and its Relation to Changes in the Extracellular Matrix ...... 139
  6.2.1 Introduction.............................................................................. 139
  6.2.2 Methods ................................................................................. 141
  6.2.3 Results and Discussion ............................................................ 142
  6.2.4 Conclusions and Future Directions ............................................ 146

BIBLIOGRAPHY .................................................................................. 148
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1. Characterization of gene expression by RT-PCR.</td>
<td>29</td>
</tr>
<tr>
<td>2-2. Two-way ANOVA of drug resistance across biomaterial microenvironments.</td>
<td>36</td>
</tr>
<tr>
<td>3-1. RT-PCR Primers used for gene expression quantification.</td>
<td>66</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1. Schematic of the Metastatic Cascade.</td>
<td>4</td>
</tr>
<tr>
<td>1-2. Mechanisms of Cancer Initiation and Progression span genetic mutations and mechanical pathways.</td>
<td>8</td>
</tr>
<tr>
<td>1-3. Substrate Stiffness Drives Mechanical and Genetic Changes.</td>
<td>13</td>
</tr>
<tr>
<td>1-4. Variables that Determine Model Selection for Genetic and Extracellular Matrix Studies.</td>
<td>17</td>
</tr>
<tr>
<td>2-1. Exploring the role of the Extracellular Matrix in Resistance to Targeted Therapeutics.</td>
<td>22</td>
</tr>
<tr>
<td>2-2. Characterization of Multiple Biomaterial Platforms.</td>
<td>35</td>
</tr>
<tr>
<td>2-3. Drug Response Varies with Biomaterial Platforms.</td>
<td>37</td>
</tr>
<tr>
<td>2-4. Expression of growth and signal transduction genes distinguishes screening platforms but does not explain drug response..</td>
<td>41</td>
</tr>
<tr>
<td>2-5. RNA-Seq on Breast Cancer Cells Reveals Enrichment of Surface Receptor Linked Signal Transduction.</td>
<td>43</td>
</tr>
<tr>
<td>2-6. Spheroid formation method determines expression of cell–ECM and cell–cell adhesion genes.</td>
<td>44</td>
</tr>
<tr>
<td>2-7. Optimization of lysing protocols.</td>
<td>46</td>
</tr>
<tr>
<td>2-8. 3D biomaterials generally suppress signaling.</td>
<td>47</td>
</tr>
<tr>
<td>2-9. Doxorubicin treated cells show limited change in phosphorylation of key signaling analytes.</td>
<td>48</td>
</tr>
<tr>
<td>2-10. Systems analysis reveals that MEK regulates ECM-mediated sorafenib efficacy.</td>
<td>50</td>
</tr>
<tr>
<td>2-11. Multiple biomaterials are required for success of the multiple linear regression.</td>
<td>51</td>
</tr>
<tr>
<td>2-12. PD0325901 and sorafenib are an effective combination therapy to reduce in vivo tumor burden.</td>
<td>53</td>
</tr>
<tr>
<td>3-1. Exploring the role of the Extracellular Matrix in Mechanosensitive Adhesion and Motility.</td>
<td>61</td>
</tr>
<tr>
<td>3-2. RNA-Seq implicates several integrin-signaling related mechano-sensitive genes.</td>
<td>72</td>
</tr>
<tr>
<td>3-3. Cells on soft substrates have increased integrin α6 expression.</td>
<td>73</td>
</tr>
<tr>
<td>3-4. Cells express integrin α6, but not α3 in a stiffness-sensitive manner.</td>
<td>74</td>
</tr>
<tr>
<td>3-5. Cells on soft substrates produce extracellular laminin.</td>
<td>75</td>
</tr>
<tr>
<td>3-6. Media changes do not result in deposited matrix.</td>
<td>76</td>
</tr>
<tr>
<td>3-7. Addition of EGF mimics mechanosensing on soft, laminin containing surfaces.</td>
<td>77</td>
</tr>
<tr>
<td>3-8. EGFR mechanosensitive motility requires integrin α6.</td>
<td>78</td>
</tr>
<tr>
<td>3-9. Extension of integrin α6 cell assays.</td>
<td>79</td>
</tr>
<tr>
<td>3-10. Calpain 2 is downstream of both mechanosensitive integrin α6 and EGFR.</td>
<td>81</td>
</tr>
<tr>
<td>3-11. Quantification of signaling during cell adhesion in the presence of mechanical and biochemical stimuli.</td>
<td>83</td>
</tr>
<tr>
<td>3-12. Knockdown of calpain 2 limits response to EGF on hydrogels.</td>
<td>85</td>
</tr>
<tr>
<td>3-13. Mechanosensitive signaling is dependent on laminin and substrate stiffness.</td>
<td>86</td>
</tr>
<tr>
<td>3-14. Lapatinib dose and timing impacts cell motility.</td>
<td>90</td>
</tr>
<tr>
<td>4-1. Exploring the role of the Extracellular Matrix in Tumor Evolution.</td>
<td>93</td>
</tr>
<tr>
<td>4-2. Biomaterial culture to mimic physiological mechanical cues in breast cancer.</td>
<td>103</td>
</tr>
<tr>
<td>4-3. Extended Mechanical Pressure Alters Cancer Cell Growth and Migration.</td>
<td>104</td>
</tr>
</tbody>
</table>
4-4. ECM remodeling is dependent on culture stiffness. ......................................................... 105
4-5. Proliferation and Integrin Expression show little mechanical memory. ....................... 107
4-6. Motility is altered by culture on biomaterials. ............................................................ 108
4-7. Primary breast tumor cells respond differentially to soft materials and TCPS. .......... 109
4-8. Cells adopt a material-dependent phenotype over time. ............................................. 111
4-9. Clonally isolated cells exhibit a range of phenotypic behaviors mimicking
    mechanical selection. ....................................................................................................... 114
5-1. Mimicking mechanical selection of cells with extreme limiting dilution techniques… 128
5-2. Subclones respond differentially to the tumor microenvironment. ............................... 129
6-1. Matching flow cell properties to blood flow in vivo. .................................................. 135
6-2. Calpain 2 mediates flow-dependent increases in motility......................................... 137
6-3. Ethiopian patients are diagnosed with ER+ breast cancer at a young age. ............... 143
6-4. Mutated genes in Ethiopian breast cancer minimally overlap with commercial SNP
    panels............................................................................................................................... 144
CHAPTER 1

THE PROCESS OF METASTASIS AND CURRENT MODELS

1.1 The Clinical Problem of Advanced Breast Cancer

Patients diagnosed with localized breast cancer have a 5-year survival rate of 99% compared to the general population, but that drops to less than 30% for patients diagnosed with metastatic disease. This highlights the need for improved treatment options, and currently very few therapeutic interventions target either the mechanical or biochemical features of the extracellular matrix (ECM). This work seeks to understand the intersection of cancer genetics and tissue mechanics in metastatic breast cancer to identify potential drug targets to improve patient outcomes.

Cancer is the result of accumulating a set of genetic mutations that induce several specific hallmarks in order to drive uncontrolled proliferation into a tumor mass [4]. There are several driver mutations which can be acquired randomly, through exposure to toxins, or conferred through increased familial or personal risk [6, 7]. The most significant clinical risk factor for developing breast cancer is having dense breast tissue, which is thought to assist the process of malignant transformation [8]. Further, dense breast tissue can promote invasion and metastasis, and tumor stiffness can be further increased through a feedback loop of cancer cells and local stroma depositing and crosslinking additional matrix proteins [9]. This intersection of genetic drivers and mechanical feedback warrants exploration to better understand and combat the process of breast cancer metastasis.

Breast cancer presents many clinical challenges because it is highly heterogeneous in genetic make-up, tissue mechanics, and receptor status both within a single tumor and across patient populations. Each of these factors can impact the propensity of any given patient to develop metastases in critical organs, such as the bone, brain, liver, or lung. However, there
is no clear pattern to predict where any given patient’s primary tumor will metastasize to. In addition to the heterogeneity at the primary tumor site, each of the most common secondary sites also has a distinct mechanical and biochemical profile [1-3, 10-12]. Different cells or cell subpopulations preferentially proliferate or migrate on different stiffness substrates, which can be correlated with their metastasis to specific tissues [13].

Most researchers in the field acknowledge the “seed and soil hypothesis” that there are features of both the tumor cells (seed) and properties of the secondary site (soil) that are necessary for the formation of metastases [14]. However, most previous research has focused on the properties of the cancer cells themselves, not the role of the extracellular matrix in driving tumor progression and metastasis. For example, when a patient is initially diagnosed with breast cancer, the first action is to determine the clinical subtype; luminal (A/B), HER2 overexpressing, or triple negative. More recently, in depth analysis of the cells via genetic sequencing or more stringent molecular subtyping have been employed. While these classifications have been used to predict the optimal therapy options for a given patient (with varied success), they revolve exclusively around the properties of the cancer cell, without any examination of the extracellular space. Given appropriate work on the subject, drugs that are already clinically approved could be used to target tissue stiffness. For example, Tamoxifen treatment can reduce overall breast density, which might improve outcomes for some patients [15]. More work is needed to relate properties of the extracellular matrix to certain features of the cancer cell to better understand and treat metastasis.

1.1.1 An Overview of Metastasis

Metastasis contributes to 90% of cancer deaths, as dissemination can be unpredictable and difficult to treat. Cells undergo malignant transformation at the primary site, where breast epithelial cells acquire a set of driver mutations that lead them to experience uncontrolled growth either innately or in response to factors in the microenvironment (Figure 1-1) [16].
The tumor also recruits stroma [17] to assist in the deposition and crosslinking of matrix proteins, which results in tumor stiffening [9]. Cell clusters and individual tumor cells likely invade the surrounding tissue during all stages of tumor development and stiffening, where they eventually intravasate into blood or lymphatic vessels [18]. These circulating tumor cells then adhere to the vessel wall and extravasate into the surrounding tissue (Figure 1-1) [19]. Tumor cells likely reach a diverse set of secondary sites, but successful colonization of breast cancer cells is largely limited to the bone, brain, liver, and lungs. While this process has been well characterized, we still have limited knowledge of how the interaction of tissue
mechanics with chemokinetic factors, ECM proteins, and the timing of dissemination drive invasion and successful metastasis.

1.1.2 Clinical Information Derived from Biopsies and Genetic Panels

An MRI or mammographic imaging plus the initial biopsy provide most of the information prior to the oncologist deciding on a course of treatment. This provides key information like the clinical diagnosis, subtype, stage, and proliferation status of the sample [20]. Some pathology reports also include amplification or mutation status of other common driver genes in breast cancer including BRCA, ATM, p53, CHK2, PTEN, and CDH1 [21].

![Figure 0-1. Schematic of the Metastatic Cascade.](image)

First, breast epithelial cells undergo malignant transformation, which is catalyzed by oncogenic driver genes that can vary within and across populations. After diagnosis, patients receive a course of treatment that initially shrinks the tumor, but over time the tumor may recur and/or spread to secondary site via the development of resistance by altering kinase signaling. The process of metastasis is mechanosensitive, and the stiffening tumor microenvironment can alter cell adhesion to the ECM and migration via focal adhesion- associated proteins, integrin α6 and calpain 2. Cells are exposed to increased shear forces interstitially as the tumor mass increases, and in the vascular system during metastasis, which enhances cell motility. Finally, cancer cells reside in tissues for extended lengths of time before and during metastatic outgrowth, and this length of exposure to varied mechanical cues selects for a unique subpopulation of cells. Adapted from Barney, Jansen, et al. [5]
Many treatment options target receptors identified by the subtype, so this limited clinical information has been deemed sufficient to make a first pass at patient treatment, although anecdotal evidence from oncologists indicates there is still plenty of room for improvement. Treatments targeting processes associated with the tumor microenvironment, such as angiogenesis and chemotaxis [22, 23], have seen little clinical success thus far.

1.1.3 The Healthy and Tumor Microenvironments

Healthy breast tissue is comprised of soft fatty tissue, epithelial glandular tissue and ducts, and fibrous tissue and ligaments [24]. The amount and dispersal of these tissues can vary both within a single person, as well as between individuals [25]. Additionally, healthy breast tissue contains many different cell types, including epithelial cells, stem cells [26], fat cells [27], fibroblasts [28], immune cells [29], and others, each with distinct roles in maintaining homeostasis. Specifically, healthy glandular tissue maintains organized duct-like morphology, with distinct layers of epithelial cells, myoepithelial cells, basement membrane, and then stroma [30]. However, during malignant transformation, the healthy cells can be coopted by the cancer to promote tumorigenesis and suppress anti-tumor activity [31, 32]. For example, fibroblasts in healthy tissue secrete sufficient collagen to supply structure and density to the tissue, and play key roles in normal processes like wound repair [33]. However, fibroblasts often facilitate the process of epithelial cells undergoing malignant transformation and growth into a tumor mass. Either local or recruited fibroblasts become cancer associated fibroblasts (CAFs), which supply the tumor microenvironment with increased collagen deposition and crosslinking, and thus increased stiffness [34].

Cells sense the stiffness of their local microenvironment, which results in genetic and phenotypic changes [35-38]. For example, fibroblasts can adapt to the stiffness of their substrate by altering intracellular tension [39]. Cells respond to a change in mechanics via focal adhesion proteins, which undergo conformational changes to enhance cell structure by
linking to the actin cytoskeleton and revealing cryptic kinase domains to initiate downstream signaling [40, 41]. Additionally, forces originating from the ECM can directly pull on the nucleus to initiate changes in gene expression [42]. The ability of cells to translate mechanical information from the microenvironment into biochemical signaling events is called mechanotransduction. Changes in ECM stiffness alter mechanical forces and substrate adhesivity experienced by the cancer cells [43], which ultimately impacts cell motility [44, 45]. These processes, which are partially controlled by the stiffness of the surrounding matrix, are important for dissemination of cancer cells, as they respond to signals from the microenvironment in order to invade the surrounding tissue and migrate to a secondary site.

1.2 Malignant transformation at the Primary Tumor Site

1.2.1 Genetic Drivers of Breast Tumorigenesis

New data-intensive methods have been developed to characterize gene expression profiles (RNAseq transcriptome profiling), mutations in protein coding regions of the genome (whole exome sequencing) and mutations across the entire genome (whole genome DNA sequencing). These methods provide large data sets that allow for a top-down approach to understanding both global and local changes that drive tumor formation. For example, sequencing, at high enough coverage, can provide information on all of the mutations in a tumor biopsy, although not all might contribute to the oncogenic nature of that cell. Significant work has been done to identify driver mutations, which actually push a cell towards being cancerous, as opposed to passenger mutations, which are incidental mutations that happen to be present in a cancer cell, but do not contribute to the characteristic uncontrolled growth. Such oncogenes can have one of two main functions; either an inactivating mutation of a tumor suppressor gene, or an increase of a tumor promoting gene. During any single cell division, on average one out of every ten billion bases copied will be
mutated and not repaired by standard machinery. In the 3.1 billion base pairs of the human genome, a cell will acquire a new mutation with approximately every 3 divisions.

Many risk factors for breast cancer are heritable, with the most well-known mutations being those of the BRCA DNA-repair family [6]. Those mutations are well characterized because of their high penetrance in the population, which is defined as the proportion of individuals carrying a mutation that also show the associated phenotype, in this case, breast cancer. Interestingly, not all heritable risk factors are in specific oncogenes. For example, both obesity and breast density are significant contributors to breast cancer risk [46-48]. Tumors have one or more driver mutations that cause malignant transformation, and tumors with subclonal populations that contain distinct driver mutations can act cooperatively to worsen patient prognosis [49]. While there are hundreds of well-evidenced driver genes, some of the most common in breast cancer are: p53 (cell cycle regulator), PTEN (tumor suppressor), KRAS (growth factor response), AKT1 (proliferation signaling), and BRCA1/2 (DNA repair) (Figure 1-2 a).

1.2.2 The Primary Tumor Stiffens During Cancer Progression

The primary tumor is a rigid mass of cells and matrix that is so closely packed that even oxygen and small molecule drugs face extreme diffusion limitations [50]. Researchers
Figure 0-2. Mechanisms of Cancer Initiation and Progression span genetic mutations and mechanical pathways. a. Several oncogenes have been implicated in various stages during the cell cycle, in processes such as DNA repair, cell cycle checkpoints, and quiescence. b. Fibroblasts and other stroma in the tumor microenvironment can deposit and crosslink extracellular matrix proteins, like collagens. Cancer cells respond to the resulting increase in stiffness through mechanosensitive pathways. Integrins transduce extracellular signals intracellularly, where they bind both chemical signaling moieties and proteins that serve as linkers to the actin cytoskeleton. c. Tumors also contain a high amount of heterogeneity that evolves with the tumor, over time. As the tumor progresses, subclones emerge and metastasize, further altering the genomic and mechanical landscapes.

have engineered a plethora of systems to capture select features of this environment, such as stiffness, protein composition, dimensionality, cell-cell contacts, and nutrient availability (Figure 1-2 b). 2D systems have been designed to capture a range of stiffnesses that spans physiological relevance [51], to study aspects of mechanobiology that impact drug response [52], cell migration [45, 53], proliferation [13], and gene expression [47, 54]. Both ECM mechanics [13] and protein composition [55] impact breast cancer cell tropism, defined as cell propensity to metastasize preferentially to specific tissues. Recent work has examined the role of dimensionality in cell behavior, particularly in the area of drug resistance. For
example, cells cultured in 3D spheroid-like environments are more resistant to doxorubicin [56] and paclitaxel [57] than cells assayed as a 2D monolayer. Additionally, in a spheroid model, the matrix deprived cells on the interior were more sensitive to a PI3K inhibitor [58]. Other areas of study concerning 3D systems examine confined cell motility [59], and clonal cell expansion [60]. In addition to understanding the cell-ECM interactions, cells also communicate with one another physically (via cell junctions) and chemically (via soluble factors and receptors). These signals from the microenvironment contribute to drug resistance and collective cell motility and warrant further examination.

The systems described above are all user defined, as the parameters are tuned in the lab. Another promising method is to allow tissue-specific stem cells to define the architecture through the creation of organoids [61]. Although such systems provide less capacity for engineering, they better capture the native architecture and spatial organization of healthy and diseased tissues [62], which can then be used to study disease progression and off-target drug effects on healthy tissues. Combining both user-defined and cell-defined systems, these tissue mimics can be connected in sequence or series to create a model human-on-a-chip [63]. Providing this interaction between organs can simulate often overlooked processes in tumor development, such as the recruitment of bone-derived stem cells [64] and fibroblasts [65] to the primary tumor site. Finally, several systems have been designed to mimic the hypoxic environment of a tumor mass [66-68]. Independently, each system described and referenced above provides a unique contribution to understanding the role of tissue mechanics in cancer progression, but more systematic analyses that compare these features to optimize the balance of simplicity and relevance are needed.

1.3 Dissemination to the Secondary Site

1.3.1 Genetic Heterogeneity and Cell lineages
Distinct gene expression programs contribute to metastatic success of an initial heterogeneous cell population at different secondary sites (Figure 1-2 c) [1-3]. Work in the field has contributed several genes that assist in cell invasion and motility at the secondary site, as well as growth and survival genes. For example, integrins are a class of surface receptors that bind to extracellular matrix proteins to transduce physical and biochemical information into intracellular signaling and tension generation. Changes in the microenvironment are mirrored by changes in the expression of different integrin subunits [69, 70]. In addition to changes in gene expression, cancer cells can have point mutations or copy number enhancements to increase signaling from soluble factors to promote tumor growth. One example of this is the epidermal growth factor receptor (EGFR), where more copies of the gene increases expression, and thus the ability of cancer cells to grow rapidly in the epidermal growth factor-rich tumor environment.

Lineage tracing of cells at the primary tumor and at metastases has revealed distinct mechanisms of metastatic spread. Conventionally, researchers believed that a primary tumor developed and stiffened over time, nurturing aggressive cells until they spread to a secondary site. This has been challenged by genomic analysis, demonstrating that single cells or small clumps of cells are disseminated early, and that metastases likely progress in parallel with the primary tumor [71, 72]. Drug treatment further complicates this analysis and promotes the rise of distinct populations over time [73]. This contributes to a growing body of work that suggests that cancer cells can metastasize early in tumor development, so disseminated tumor cells likely already exist at the time of diagnosis of the primary tumor [74, 75]. This highlights the critical need to 1) understand variation of the primary tumor site and cell populations that drive persister cells after treatment with drugs and 2) understand the features of the secondary tissue sites that enable the survival and outgrowth of disseminated cells.

1.3.2 The Biochemical and Mechanical Microenvironment Facilitates Metastasis
1.3.2.1 Tissue Biochemical Properties Contribute to Tropism

The extracellular matrix of each tissue is composed of a unique set of proteins at different concentrations, which has been well characterized by the human protein atlas project [76]. For example the brain generally has few extracellular matrix proteins, but a large amount of that protein is laminin, especially near the blood-brain-barrier. On the other hand, bone marrow is more collagen rich. These distinctions are important in considering interactions between features of the cell, like integrins, and features of the extracellular space, like the matrix proteins, that drive metastasis [77, 78]. Prostate cancer metastasis to the bone depends on the surface expression of integrin $\alpha_2\beta_1$, a collagen binding integrin dimer [79]. Additionally, a specific spliced isoform of a laminin binding integrin subunit, $\alpha_{6B}$, alters motility and disseminated tumor cell survival [80, 81]. Despite compiling information on components of both the cancer cell “seed” and the secondary site microenvironmental “soil”, why cancer cells exhibit tropism is still poorly understood.

Several groups have demonstrated that recruitment of immune cells [82] and stem cells [83, 84], as well as the secretion and deposition of proteins, such as fibronectin [85] and myeloperoxidase [86] assist in the formation of a pre-metastatic niche before tumor cells reach the secondary tissue. Engineers have modeled this niche using biomaterials by capturing early tumor cells from circulation on implanted scaffolds and tuning the chemical and physical parameters to enhance capture [87, 88]. Recent work has also shown that small extracellular vesicles allow communication from tumor cells to distant healthy tissue that contributes to this pre-metastatic niche [85]. This niche is further modified by other factors, such as obesity, which causes chronic lung inflammation and the resident immune cells secrete cytokines locally, which enhances breast-to-lung metastasis [89]. Distinct from the pre-metastatic niche, tissues can also be primed with a dormant niche, which facilitates cellular or tumor dormancy when cancer cells arrive at the secondary site [90]. While cellular
dormancy is characterized by non-proliferative cell or cells, tumor-level dormancy occurs when the rate of cell proliferation and cell death are roughly equal. Both of these processes can delay the onset and detection of metastases, but survival in this state requires a unique extracellular niche that doesn’t promote net cell growth or death [90, 91]. Diverse biochemical profiles contribute to tissue specific metastasis, but must also be contextualized within the physical properties of those tissues, as many of these processes are mechanosensitive.

1.3.2.2 Secondary Site Tissue Mechanics Vary Widely

The primary tumor site and each preferred secondary site for metastasis spans a physiological range of moduli. While health breast tissue can range from 1 kPa to over 15 kPa [47, 92-95] based on factors like breast density and obesity, primary breast tumors tend to be stiffer, ranging from 10 to 50 kPa (Figure 1-3) [92]. These measurements can be dramatically different for tissue at a largely healthy secondary site, where brain tissue is usually less than 1 kPa [96, 97], bone marrow and liver tissue is less than 4 kPa [11, 98, 99], while lung tissue has been reported from 2 kPa to almost 100 kPa [12, 100, 101]. Of note here is that the mechanical properties of the lung can be particularly heterogeneous due to its need to undergo cyclic breathing motion and have a high contact area between alveoli and capillaries [102]. While the above discussion is on a whole-organ level, other groups have also noted that the stiffness of cells, such as fibroblasts, changes to mimic the substrate stiffness, due to changes in cytoskeletal assembly and other stress fibers [39]. Because tissue mechanics vary widely during progression and a pre-metastatic niche at each secondary site will be mechanically unique, evaluating the timing of metastasis is crucial to an understanding of the physical background and current mechanical environment of a cell.
As mentioned briefly above, breast cancer likely metastasizes early, although work in breast cancer to date has focused on HER2 overexpressing breast cancer [74, 75, 103]. These new mouse models out of the Aguirre-Ghiso group are the first robust systems used to demonstrate early metastasis, where the cancer cell derives important mechanical and biochemical cues from the secondary site. Other groups have separately examined the role of timing on ECM mechanics and cell phenotype. For example, cells can stiffen biomaterials by producing and crosslinking collagenous matrices and can increase the young's modulus from 200 to 800 kPa over 12 weeks [104]. While in vitro systems have been used to examine the impact of matrix stiffness and culture time on stem cell differentiation [105] and mechanical

![Figure 0-3. Substrate Stiffness Drives Mechanical and Genetic Changes.](image)

Cell Mechanics: Cells respond to the mechanical environment via changes in focal adhesion formation and turnover, changes in downstream signaling, and tension generation. On soft surfaces, cells form transient adhesions and have fewer actin stress fibers. At intermediate stiffnesses, cells exhibit the fastest migration through optimal catch-bond formation of key mechanotransducers. On stiff surfaces, cells receive proliferation signals via ERK and Akt pathways, and cells have a high degree of actin polymerization via RhoA-ROCK phosphorylation, resulting in more cell spreading and traction. Genetics: On soft materials, cells retain a stem-like morphology, as identified by markers for pluripotency. As the stiffness begins to increase, cells begin expressing more integrins that bind to structural extracellular matrix proteins, such as fibronectin and collagen. Additionally, stem cells preferentially differentiate down an osteogenic lineage on these stiffer surfaces. Of critical importance to cancer research, is that cells undergo a transition from an epithelial state (with markers like E-cadherin and laminins) to a mesenchymal-like state (with markers like N-Cadherin and fibronectin). This epithelial to mesenchymal transition has been implicated in processes like metastasis and drug resistance.
memory [106], one study determined how cancer cells modify their gene expression over 9 days of culture to optimize expression of genes responsible for growth and response to the tumor microenvironment [107]. Recent work has scaled culture time to 3 passages of priming on polyacrylamide biomaterials, and found that many phenotypes, including proliferation and morphology were still changing after 9 days [108]. This suggests that analyses over longer time periods would be necessary to capture the full range of behavior alterations. While these and other biomaterial models have provided a valuable framework to relate stiffness, ECM composition, and metastasis, existing studies do not consider the length of biomaterial exposure on cell behavior beyond a time scale of days. This dissertation aims to fill a knowledge gap, examining the scale from the early minutes while cells are initially adhering to the biomaterials, to several months where cells have an opportunity to fully adapt to a different mechanical environment.

1.3.2.3 Shear Forces are Present at Many Stages of Metastasis

While the stiffness of the microenvironment is important to examine, other physical forces, such as shear stress, can influence the metastatic cascade. Tumors are often regions of high fluid pressure, as the increased mass grows to fill in a small volume and develops leaky vasculature, generating a net flow away from the primary tumor. This shear force imparts physical pressure that can alter cell migration (known as rheokinesis) [109], and can also create an autocrine signaling gradient, where cell-secreted soluble factors flow downstream, creating a chemical attractant that can influence migration (known as chemokinesis) [110]. This flow also impacts the local stroma, including fibroblasts [111], where they are increasingly activated in response to the shear forces and can provide protection against therapeutics for cancer cells [112]. Together, these physical forces encourage cell migration
away from the primary tumor by directly facilitating directional cancer cell motility, or by activating fibroblasts to deposit more matrix to indirectly enhance cell motility.

Cancer cells reach distant tissues via the vascular or lymphatic systems [113], where they experience additional shear flow. Cancer cells or clusters of cells must adhere to the vascular wall and extravasate across the endothelial layer to successfully colonize the underlying tissue. Researchers have engineered vascularized tissue models to mimic and study that process [19, 114], but there is limited information on the response of cancer cells to shear forces and the impact those forces have on cells ability to successfully colonize secondary sites.

1.4 Selection of Appropriate Models to Study Breast Cancer Metastasis

The choice of parameters that are defined in a biomaterial model can impact the results of studies. At least partially due to inconsistencies in model selection, the existing literature on breast cancer metastasis contains many conflicting reports with contradictory conclusions. Much of current cancer knowledge derives from biological assays that seek to understand the relationship between genetic mutations and uncontrolled proliferation. Two primary model systems are cancer cells cultured in vitro, which are simple and link the genotype of a single cell or population to its phenotype, and in vivo mouse models that add complexity and are more similar to the human body, although it is more difficult to isolate single parameters. While researchers have become experts in curing mouse cancer, there are significant limitations on the applicability of these systems to human cancer.

1.4.1 Identification of Genetic Models that Capture Global Heterogeneity

Genetic mutations introduced to a cell population or animal can either be forced, by altering a targeted gene, or spontaneous by providing a general pressure for cells to undergo malignant transformation (Figure 1-4 a). Forced mutations employ techniques such as lentivirus transduction to increase or block gene copies, small interfering RNA molecules to
block translation, or targeted CRISPR loci to knock out or knockin/constitutively activate the gene(s) of interest [115-118]. Spontaneous mutations can be induced by culture or feeding with low doses of a toxin, like a cancer drug, or providing other stimuli from the microenvironment to select for cells with a growth advantage [119]. For example, the Massagué lab generated stable cells lines that each metastasize repeatedly to the brain [1], bone [2], or lung [3], through multiple rounds of selection. This spontaneous methodology is a top-down approach, where researchers identify a phenotype, and back out the relevant genetic information, while targeted mutation methods work from the bottom up by first identifying an oncogene of interest. While this bottom up method allows for an in depth analysis of a specific target, it has limited utility in identifying new tumor suppressor or promoters that may be impacted by environmental factors. On the other hand, working with a spontaneous tumor model can provide large amounts of information with new sequencing technologies, making it challenging to identify the true cause or causes. Genetic models of tumor initiation and metastasis provide a platform to examine cancer cell behavior, and in combination with models of the tumor microenvironment can further elucidate what microenvironmental factors contribute to cancer progression.

### 1.4.2 Optimization of Mechanical Models to Answer Biological Questions

Several important parameters dictate the ease of use and relevance of data derived from any experimental model, such as: stiffness [120], ECM proteins and density [55], dimensionality [121], pore size [122], time [106], material stability [123], water content, homo- or heterotypic cell-cell interactions [60], medium, and others (Figure 1-4 b). Biomaterial platforms provide an excellent base system, with water content greater than 90%, on which these other parameters can be layered. These systems can be comprised of either naturally derived materials (like collagen or fibrin) or from synthetic polymers (like poly(ethylene glycol (PEG) or polyacrylamide (PAA)). Natural polymers easily recapitulate
several aspects of native tissue mechanics, structure, and biochemical moieties and are inherently non-toxic and optimized for use at physiological pH. Cells can remodel these biomaterials in the same manner as the \textit{in vivo} microenvironment, leading to changes in mechanical properties, and the adhesion and mobility of cells [124]. Despite the inherent functionality of natural biomaterials, synthetic hydrogels present a different set of advantages, and thus are often the model of choice for engineers. Unlike naturally derived materials, synthetic materials have selectivity and tunability, as the user can determine polymer chain length, crosslink density, and stability with a high degree of batch-to-batch consistency that is not attainable in poorly-defined materials derived from living organisms.

\textbf{Figure 0-4. Variables that Determine Model Selection for Genetic and Extracellular Matrix Studies.} a. \textit{Genetics:} In order to select an optimal genetic profiling technique and subsequent cell model, several parameters need to be considered. Adapted from the ‘Hallmarks of Cancer’ [4], breast cancer cells can use several genetic techniques to engage in the uncontrolled growth that is characteristic of tumors. Determining appropriate driver genes and the corresponding mutations can allow researchers to select genetic features that are most appropriate to answer the question at hand. These parameters include, but are not limited to; Surface-Receptor Overexpression, Population Heterogeneity, Genomic Instability, Tumor Promoter Genes, Reduced Tumor Suppressor Genes, and Replicative Immortality. b. \textit{Extracellular Matrix:} Several features of the tumor microenvironment can be modified by natural or synthetic extracellular systems. These parameters greatly impact cell phenotype and must be carefully considered; Material Stiffness, Chemokinetic Factors, Protein Type, Protein Density, Model Dimensionality, and Time of Exposure to the Material.
In natural systems the density of biofunctional moieties, such as binding sites, is dependent on the amount of protein, so the ligand density is inherently linked to stiffness, making it impossible to tune each parameter independently.

Further, models of the tumor microenvironment need to address contradictions in the literature on the role of certain features, like stiffness, on cancer cell aggression. Some work reports that compliant primary tumors are more likely to be latent and metastatic [9, 125, 126]. One potential population is comprised of stem-like cancer cells, which flourish in a softer microenvironment [127] and are of particular concern due to their contribution to drug resistance and tumor recurrence. On the other hand, stiffer environments are known to confer drug resistance and a growth advantage to tumor cells [128-130]. The primary tumor is highly heterogeneous, suggesting that several populations may arise within a single tumor [131]. This intratumor heterogeneity, where both genetic subclones and mechanics vary between different parts of the tumor [132], can influence drug response [133], metastasis, and ultimately patient prognosis [134]. This work will investigate these competing hypotheses across several times scales and mechanical microenvironments.

1.5 Hypothesis

Many features of breast tumor cells (gene expression changes) and the surrounding ECM (protein composition and mechanical changes) have been identified. However, previous studies have been largely limited in both looking at the primary tumor over common metastatic sites and only examining short term or transient cell behaviors. I believe that we can expand the current working knowledge of breast cancer progression and metastasis by challenging these current model limitations. Here, I hypothesize that by using biomaterial models I can uncover new genetic and mechanical contributors to breast cancer metastasis and drug resistance. Specifically, I postulate that matrix stiffness and dimensionality will
alter cell growth and signaling to drives changes in drug response. I further hypothesize that creating and employing mechanical models of metastatic sites will alter and select for cancer cell behavior that cannot be identified using hard plastic and other non-physiological models.  

1.6 Objectives

The following were objectives of this dissertation:

1. Design a robust set of biomaterial platforms to screen for and overcome mechanisms of drug resistance mediated by the tumor microenvironment.

2. Identify mechanically responsive proteins that facilitate the cell adhesion and motility necessary for breast cancer spread.

3. Drive the selection of cancer cell subpopulations that preferential migrate on softer materials by creating mimics of the extracellular tumor environment.

4. Characterize tumor cell mechanical memory by challenging primary cells and cell lines selected on biomaterials with culture on rigid plastic.

1.7 Significance

The work in this dissertation expands the knowledge base surrounding mechanical regulation of drug resistance and metastasis. First, kinase targeting therapies are an emerging class of drugs, but they are plagued by the frequent development of resistance. One proposed mechanism to overcome this resistance is the use of combination therapies to prevent cell and tumor adaptation. Most drug screening to date has occurred on rigid plastic, but adaptive kinase signaling is dependent on the extracellular context, so the development and testing of biomaterial environments that are extremely tunable and reproducible is essential to move this field forward. However, such materials add a lot of cost to drug screening, so discriminating between conditions where these platforms add value is essential. Second, oncogenic signaling is complex and this work seeks to understand new mechanisms of cell mechanosensing used to facilitate growth and motility. Using techniques like RNA
Sequencing and functional assays, genes and proteins that respond to extracellular mechanics were identified, which could be potential new therapeutic targets that are more specific to cells in stiff tumor environments. Cells also experience tissue stiffness over time, and this work seeks to understand behaviors that respond to these mechanics at later time points and also exhibit mechanical memory. Finally, these additions to the field should be contextualized within the framework of intratumor heterogeneity, where both genetic clones and tissue mechanics can vary across a single lesion and varied cell culture methods or experimental set ups may incidentally assay only a fraction of the original tumor cell population. This work challenges the notion that cells selected for growth advantage on hard plastic over several years present perfect physiological information when exposed to biomaterials for a day, or even a week. This work explores the timeframe for which a population begins to acquire new behaviors in soft systems and the contributions that individual clones might make to those phenotypes. In sum, this work examines genetic and mechanical variations across heterogeneous populations and within a single clone to produce new knowledge on aggressive breast cancer and introduce new paradigms for optimizing treatment.
CHAPTER 2

A BIOMATERIAL SCREENING APPROACH REVEALS

MICROENVIRONMENTAL MECHANISMS OF DRUG RESISTANCE

2.1 Abstract

Traditional drug screening methods lack features of the tumor microenvironment that contribute to resistance. Most studies examine cell response in a single biomaterial platform in depth, leaving a gap in understanding how extracellular signals such as stiffness, dimensionality, and cell-cell contacts act independently or are integrated within a cell to affect either drug sensitivity or resistance. This is critically important, as adaptive resistance is mediated, at least in part, by the extracellular matrix (ECM) of the tumor microenvironment. We developed an approach to screen drug responses in cells cultured on 2D and in 3D biomaterial environments to explore how key features of the ECM mediate drug response. This approach uncovered that cells on 2D hydrogels and spheroids encapsulated in 3D hydrogels were less responsive to receptor tyrosine kinase (RTK)-targeting drugs sorafenib and lapatinib, but not cytotoxic drugs, compared to single cells in hydrogels and cells on plastic. We found that transcriptomic differences between these in vitro models and tumor xenografts did not reveal mechanisms of ECM-mediated resistance to sorafenib. However, a systems biology analysis of phospho-kinome data uncovered that variation in MEK phosphorylation was associated with RTK-targeted drug resistance. Tissue culture plastic, the 2D hydrogels, and at least one 3D condition were necessary for this systems biology analysis to achieve significance. We identified 2 unique mechanisms of spheroid formation that may contribute to drug resistance; cells either upregulate genes associated with cell-cell contacts, or genes associated with increased ECM production and binding, which is the likely mechanism here. Using sorafenib as a model drug, we found that
co-administration with a MEK inhibitor decreased ECM-mediated resistance *in vitro* and reduced *in vivo* tumor burden compared to sorafenib alone.

Many factors of the ECM are known to influence cell signaling and drug response. However, the goal of this work was to systematically vary specific parameters in order to compare drug response across many ECM features, which has been lacking in many previous studies. In order to achieve that goal, we selected three specific parameters with robust evidence of altering kinome signaling, biomaterial stiffness, dimensionality, and the presence or absence of mitogenic factors (Figure 2-1). While those features are the focus of this work, it is important to note that other parameters play a role in how cells respond to biomaterials. For example, in the 3D hydrogel we included protein fragments (peptides) to stimulate cell binding and signaling, which can be altered to tune response, similarly to changing the full length proteins available to cells on 2D hydrogels. Additionally, while we didn’t explicitly change the length of the assays, we identified two phenotypes where the timing likely plays a

**Figure 0-1. Exploring the role of the Extracellular Matrix in Resistance to Targeted Therapeutics.** Several components of the extracellular matrix drive signaling changes and alter drug response. We selected 3 components of the microenvironment to probe the range of stiffness, dimensionality, and growth factors on drug response. Previous work from the Peyton lab has demonstrated that higher substrate stiffness increases resistance to sorafenib. Additionally, the group has identified heterogeneous responses to drugs when cells are grown into spheroids and encapsulated in a 3D hydrogel. Finally, signaling that drives changes in drug response can also be triggered by exogenous mitogenic factors, so we tracked the role of chemokine-rich serum on drug response.
role. First, is that the spheroids were grown over 14 days, which provides opportunities for cells to secrete and bind to ECM proteins, so we evaluated drug response in spheroids that were dissociated back onto plastic for the assay, and found that drug response in those cells looked most similar to cells grown entirely on plastic. Second, in clustering of cell gene expression across our set of biomaterial platforms, in the SkBr3 cells, a mouse xenograft tumor (which required 21 days to grow) is most distantly related to the other samples, while in the MDA-MB-231 cells, the spheroid samples were most different from the others (spheroids grew over 14 days, MDA-MB-231 xenograft only required 10 days to grow). In sum, we provide a novel strategy for identifying and overcoming ECM-mediated resistance mechanisms by performing drug screening, phospho-kinome analysis, and systems biology across specifically selected biomaterial environments.

2.2 Introduction

Adaptive resistance poses a significant challenge for targeted drugs as tumor cells can alter their signaling pathways to bypass the effect of therapeutics. This adaptive signaling occurs quickly and independently of acquired mutations, making the timing of therapeutics critical to sensitize tumor cells to drugs via rewiring from an oncogene-addicted state [135]. For instance, triple-negative breast cancer cells treated with a MEK inhibitor undergo a rapid kinome reprogramming of many receptor tyrosine kinases (RTKs), cytokines, and downstream signaling pathways [136]. Inhibition of this adaptive response by lapatinib prevents downstream Src, FAK, and Akt signaling [137]. Mechanistically, kinase inhibitor treatment can decrease shedding of RTKs, thus enhancing bypass signaling [138]. Drug treatment can also cause changes in the cell cycle, promoting resistance in the quiescent cells adapting to the treatment [139]. These examples demonstrate that quick and widespread changes in signaling can occur in response to anti-cancer drugs.
The tumor microenvironment is a complex, heterocellular system with known roles in modulating tumor progression and drug response [16]. The extracellular matrix (ECM) is one feature of the microenvironment that can alter cell signaling and facilitate adaptive resistance. For example, ECM stiffness can activate RTKs and engage integrin binding to promote cell growth and survival through the Raf/MEK/ERK and PI3K/Akt/mTOR pathways [9, 140]. Our group and others have employed engineered biomaterials to elucidate drug resistance in response to biophysical and biochemical stimuli from the ECM. Two-dimensional (2D) hydrogels allow for the presentation of controlled integrin binding, a physiological stiffness, and are amenable to drug screening. Three-dimensional (3D) hydrogels also present physiological dimensionality and biochemistry through matrix degradability, matrix adhesion, and homotypic or heterotypic cell-cell interactions. No single system can capture all features of the tumor microenvironment, and complex systems do not allow for isolation of the effect of individual cues. Here, we propose a biomaterial-based method to systematically vary stiffness, dimensionality, and cell-cell contacts to analyze matrix-mediated adaptive resistance by measuring genetic and phospho-signaling changes.

All of these extracellular cues can alter the signaling networks targeted by cancer therapeutics. For example, \( \beta_1 \) integrin-mediated tolerance to a BRAF inhibitor in vivo can be recapitulated by stiff synthetic matrices in vitro [141]. We recently developed a high-throughput drug screening platform based on a poly(ethylene glycol)-phosphorylcholine (PEG-PC) hydrogel and identified that increased substrate stiffness imparts cellular resistance to sorafenib [51, 52]. Similarly, others have demonstrated decreased drug sensitivity in 3D hydrogels compared to 2D hydrogels and tissue culture polystyrene (TCPS) [130]. These studies demonstrate the vast opportunities that synthetic biomaterials provide for exploring drug resistance and kinome rewiring mediated by features of the ECM but have yet to
capitalize on the potential for a systematic analysis of stiffness, geometry, and cell-cell contacts afforded by such systems to understand mechanisms of ECM-mediated resistance.

Many systems have been implemented to create multicellular tumor spheroids (MCTS) to understand tumor biology and increase complexity in drug screening, but most scientific studies select a single method without regard for how the formation impacts experimental results [142-144]. Some research focuses on the inherent cell-cell contacts present in MTCS [145], which is more representative of an epithelial cell phenotype, while others examine the role of increased ECM production in facilitating the structure [146], which is more similar to a tumor-like microenvironment. Tight junctions and cell-cell contacts lend themselves to limited diffusion of nutrients and drugs [146], while changes to the ECM are known to regulate drug resistance via intracellular signaling [147]. Therefore, a robust examination of multiple methods of spheroid formation may better explain observed phenomena in drug resistance of MCTS.

It is imperative to consider multiple components of the ECM while studying drug response, as variations in matrix complexity can produce considerably different predictions for in vivo drug response to anti-cancer drugs. Since no single system can capture all features of the tumor ECM, we investigated drug resistance and adaptive reprogramming of breast cancer cells across multiple biomaterial microenvironments to analyze the genetic and phospho-signaling contributions to adaptive resistance. Multiple linear regression modeling revealed that MEK signaling explained the difference in RTK-targeted drug response across the ECMs, and co-administering a MEK inhibitor with sorafenib improved efficacy of the single agent in vitro and in vivo. This is the first report to combine systems biology analysis with screening across biomaterial platforms to identify MEK as a mediator of ECM-driven resistance to RTK inhibitors.

2.3 Materials and Methods
2.3.1 Cell culture

All supplies were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise noted. Human breast cancer cell lines MDA-MB-231 and SkBr3 cells were generous gifts from Dr. Sallie Smith Schneider at the Pioneer Valley Life Sciences Institute and Dr. Shannon Hughes at the Massachusetts Institute of Technology, respectively, and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) at 37°C and 5% CO₂.

2.3.2 Polymerization of 2D and 3D PEG hydrogels

2D PEG-phosphorylcholine (PEG-PC) hydrogels were prepared and protein was coupled as described previously [52]. Glass-bottom 96-well plates (no. 1.5 coverslip glass; In Vitro Scientific, Sunnyvale, CA) were plasma treated (Bioforce Nanosciences, Salt Lake City, UT) and subsequently methacrylate-silanized with 2 vol % 3-(trimethoxysilyl) propyl methacrylate (Sigma-Aldrich, St. Louis, MO) in 95% ethanol (pH 5.0) for 5 min, washed 3 times with 100% ethanol, and dried at 40°C for 30 minutes. PEGDMA (Mn 750, Sigma-Aldrich), is added at 1.1 wt % (10 kPa, 0.015 M) or 3.2 wt % (33 kPa, 0.043 M) to a solution of 17 wt % 2-methacryloyloxyethyl phosphorylcholine (PC) (Sigma-Aldrich) in phosphate-buffered saline (PBS). Solutions were sterilized with a 0.2 μm syringe filter (Thermo) and degassed by nitrogen sparging for 30 seconds. Free-radical polymerization was induced by addition of 0.05 wt % ammonium persulfate (APS, Bio-Rad Laboratories, Hercules, CA) and 0.125 vol % tetramethylethylenediamine (TEMED, Sigma-Aldrich). Hydrogels of 40 μL per well in the 96-well plates were polymerized under nitrogen for 30 minutes. Post-polymerization, hydrogels were allowed to swell for 24 hours in PBS, then treated with 100 μL of sulfo-SANPAH (ProteoChem, Denver, CO; 0.6 mg/mL in pH 8.5 HEPES buffer) under
UV light for 20 minutes, rinsed twice with HEPES buffer, and followed immediately by incubation with type I collagen at 3.3 μg/cm² (Thermo) overnight.

3D PEG-maleimide (PEG-MAL) hydrogels were prepared at 10 wt % (3 kPa) and 20 wt % (5 kPa) solution with a 20K 4-arm PEG-MAL (Jenkem Technology, Plano, TX) and 2mM of the cell binding peptide CRGD (Genscript, Piscataway, NJ), and was crosslinked at a 1:1 ratio with 1K linear PEG-dithiol (Sigma-Aldrich) in 2mM triethanolamine (pH ~ 7.4) [148]. Hydrogels were polymerized in 10 μL volumes for 5 minutes before swelling in cell culture medium.

Bulk diffusion of Rhodamine 6G (R6G) (Stokes’ radius 0.76 nm, Sigma-Aldrich) in 3D PEG-MAL hydrogels was measured by encapsulating 0.1 g/L R6G in the hydrogel and sampling the supernatant at 5-minute intervals. The samples were analyzed on a fluorescent plate reader at excitation: 526 nm/emission: 555nm. The diffusion coefficient of R6G was calculated using the Stokes’-Einstein equation.

2.3.3 Formation of uniform tumor spheroids

To make polyNIPAAM spheroids, lyophilized poly(N-isopropylacrylamide)-poly(ethylene glycol) (polyNIPAAM, Cosmo Bio USA, Carlsbad, CA) was reconstituted in cell culture medium according to the manufacturer’s instructions and kept at 4°C until use. Cells were suspended in polyNIPAAM solution placed on ice at a density of 100,000 cells/mL for SkBr3 cells and 167,000 cells/mL for MDA-MB-231 cells, and each gel was made at a volume of 150 μL. Gelation occurred after 5 minutes at 37°C, and gels were swollen in cell culture medium. Single cells were grown into spheroids for 14 days in the gels with regular medium changes. Spheroids were either dissociated back onto TCPS (for RNA-Seq and drug dosing) or encapsulated in 3D PEG-MAL gels (for RNA-Seq, drug dosing, and signaling) for 24 hours.

To make microwell spheroids, square pyramidal microwells (400 μm side-wall
dimension) were fabricated as described previously [149, 150] or purchased (AggreWell, Stem Cell Technologies, Canada). For fabrication, master molds containing square-pyramidal pits were generated by anisotropic etching of 100 crystalline silicon in potassium hydroxide (KOH). Microwell surfaces for tissue culture were then generated from poly(dimethylsiloxane) (PDMS) using a two-stage replica molding process of the master mold as described previously. Microwells were arranged in a square array with no space between adjacent wells and placed in 6 or 12-well plates. To prepare microwells for cell seeding, microwell surfaces were UV sterilized and pretreated with 5% Pluronic F-127 (Sigma-Aldrich) for 30 min at room temperature and then washed twice with sterile water. Cells were distributed over microwell surfaces at concentrations of 1.03 × 105 cells/cm2 or 1.00 × 104 cells/cm2. After 24 h, spheroids were collected by shaking the plate gently to dislodge most of them, and gently aspirating medium and spheroids. Spheroid solution was spun down at 400 rpm for 5 min. Medium was removed, and the spheroid pellet was lysed for RT-PCR, or encapsulated in 3D hydrogels. Spheroids were handled using cut pipet tips to minimize shear stress.

To make spheroids using suspension culture, single cells were seeded at 1.05 × 104 cells/cm2, 1.05 × 103 cells/cm2, or 1.05 × 102 cells/cm2 in a 6-well flat ultralow attachment plate (Corning, Tewksbury, MA). After 3 days, spheroids were collected by aspiration of medium and spheroids. Spheroid solution was spun down at 400 rpm for 5 min. Medium was removed, and the spheroid pellet was lysed for RT-PCR or encapsulated in 3D hydrogels. Spheroids were handled using cut pipet tips to minimize shear stress.

2.3.4 Characterization of Gene Expression by RT-PCR

The expression of cell–cell adhesion molecules and ECM mRNA transcripts was measured by quantitative RT-PCR. Total RNA was isolated using the GenElute mammalian total RNA kit (Sigma-Aldrich) and 0.5 μg total RNA was reverse transcribed using the RevertAid
reverse transcription system (Thermo Fisher). Ten ng cDNA was then amplified using 10 pmol of integrin-specific primers (Table 2-1) and the Maxima SYBR green master mix (Thermo Fisher) on a Rotor-Gene Q thermocycler (Qiagen, Valencia, CA) as follows: 50 °C for 2 min, 95 °C for 10 min followed by 45 cycles at 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s. Both β-actin and ribosomal protein S13 were included as reference genes to permit gene expression analysis using the 2−ΔΔCt method [151].

2.3.5 Hydrogel mechanical characterization

Mechanical compression testing for 2D and 3D hydrogels was performed with a TA Instruments AR-2000 rheometer (New Castle, DE) at a 2 μm/s strain rate and analysis as previously described [51]. Indentation testing for polyNIPAAM hydrogels was performed using a custom-built instrument previously described [11]. For this application, a flat punch probe with a diameter of 1.5 mm was applied to samples at a fixed displacement rate of 5 μm/s, for maximum displacement of 100 μm. PolyNIPAAM samples were placed on a heated surface to maintain a sample temperature between 37-40°C throughout testing.

2.3.6 Quantification of drug response
At 24 hours post-seeding, cells on biomaterials were treated with lapatinib (0-64 μM, LC Laboratories, Woburn, MA), sorafenib (0-64 μM, LC Laboratories), temsirolimus (0-80 μM, Selleckchem, Houston, TX), doxorubicin (0-32 μM, LC Laboratories), or dimethyl sulfoxide (DMSO, Sigma-Aldrich) as a vehicle control. Where noted, inhibitors were included in the medium: PD0325901 (3 μM, Sigma-Aldrich), SP600125 (20 μM, LC Laboratories). After 48 hours of drug treatment, cell viability was quantified by CellTiter Glo Luminescent Cell Viability Assay (Promega, Madison, WI), and luminescence was read on a Biotek Synergy H1 plate reader after incubation for 10 minutes. Separately, at 24 hours, viability of untreated cells was determined via CellTiter Glo for a measurement of initial seeding density. IC₅₀ and GR₅₀ [152] values were calculated using the initial seeding density and drug treated measurements using GraphPad Prism v6.0h.

2.3.7 Cell seeding on biomaterials

Cells were seeded on and in biomaterial microenvironments in serum-free DMEM (1% P/S, 20 ng/mL of epidermal growth factor (EGF), R&D Systems, and 20 ng/mL of platelet-derived growth factor BB (PDGF-BB), R&D Systems, Minneapolis, MN) or serum containing DMEM (1% P/S and 10% FBS) where noted. Cells were seeded at 30,000 cells/cm² on PEG-PC gels and on tissue culture plastic (TCPS), or as single cells at 500 cells/μL in 3D PEG gels. Spheroids were recovered via addition of cold serum free medium (1% P/S) on ice for 5 minutes for gel dissolution, and then gravity sedimentation for 30 minutes on ice. The supernatant was removed and the spheroids collected from each polyNIPAAM gel were re-suspended in PEG-MAL solution to make 9 3D hydrogels.

2.3.8 RNA Sequencing

Total RNA was isolated using Gen Elute mammalian total RNA miniprep kit (Sigma-Aldrich). The Illumina TRUseq RNA kit was used to purify and fragment the mRNA, convert it to cDNA, and barcode and amplify the strands. Quality and length of the inserts
was confirmed with an Agilent Genomics 2100 bioanalyzer, followed by single-end reads on a NextSeq 500 to generate a complete transcriptome from each sample. Transcripts were aligned to the hg19 human reference genome using the Tuxedo Suite pathway [153].

2.3.9 Gene Set Enrichment Analysis

Aligned samples were converted to GCT and CLS file formats using useGalaxy.org [154]. Samples were separated by material condition across both cell lines to determine biomaterial microenvironment-specific gene categories. GSEA software was used to analyze enriched KEGG pathways as previously described [155], and pathways with significant enrichment of expressed genes were selected [156].

2.3.10 Multiplex phospho-protein quantification

Cells were seeded onto 2D hydrogels at 56,600 cells/cm², or into 3D gels at 200,000 single cells in 20 μL volume gel. Spheroids were grown for 14 days in polyNIPAAM, and then transferred to 3D PEG-MAL gels, where spheroids from three polyNIPAAM gels were transferred to four 20 μL 3D PEG-MAL gels. Samples were seeded in serum free DMEM. After 24 hours, cells were dosed with either 8 μm sorafenib, 8 μm lapatinib, 25 μm temsirolimus, or DMSO control in serum free medium (or serum containing medium where noted) for 4 hours, then washed once with ice cold PBS and lysed in RIPA buffer supplemented with protease (EDTA-free protease inhibitor cocktail tablets, 1 tablet in 10 ml, Roche, Indianapolis, IN) and phosphatase (1x phosphatase inhibitors cocktail-II, Boston Bioproducts, Boston, MA) inhibitors, 1 mM phenylmethylsulfonyl fluoride (Thermo Fisher Scientific), 5 μg/ml pepstatin A (Thermo Fisher Scientific), 10 μg/ml of leupeptin (Thermo Fisher Scientific), 1 mM sodium pyrophosphate (Thermo Fisher Scientific), 25 mM β-glycerophosphate (Santa Cruz, Dallas, TX). Protein concentration was determined with a BCA assay (Sigma-Aldrich). PEG-MAL gels were manually dissociated with a pipet tip for 30 seconds after addition of lysis buffer.
Lysate concentrations were adjusted to 520 μg/ml and then analyzed with the MILLIPLEX MAP Multi-Pathway Magnetic Bead 9-Plex - Cell Signaling Multiplex Assay (Millipore, Billerica, MA; analytes: CREB (pS133), ERK (pT185/pY187), NFkB (pS536), JNK (pT183/pY185), p38 (pT180/pY182), p70 S6K (pT412), STAT3 (pS727), STAT5A/B (pY694/699), Akt (pS473) supplemented with additional EGFR (pan Tyr) and MEK1 (pS222) beads according to the manufacturer’s instructions, with the exception that beads and detection antibodies were diluted four-fold. Controls were performed to ensure that samples were within the linear dynamic range. Data is reported as the Mean Fluorescent Intensity (MFI) or fold change in MFI relative to the vehicle control.

SkBr3 cells on TCPS, 33 kPa 2D gels, and spheroids in 3 kPa PEG-MAL were serum starved for 24 h and then stimulated with 100 ng/mL of EGF (R&D Systems, Minneapolis, MN), and cell lysates were collected at 0, 5, 15, 60 minutes, and 24 hour time points. Lysate concentrations were adjusted to 160 μg/mL, and samples were quantified the MILLIPLEX MAP Phospho Mitogenesis RTK Magnetic Bead 7-Plex Kit (Millipore; analytes: c-Met/HGFR (panTyr), EGFR (panTyr), ErbB2/HER2 (panTyr), ErbB3/HER3 (panTyr), ErbB4/HER4 (panTyr), IR (panTyr), and IGF1R (panTyr) according to the manufacturer’s instructions, with the exception that beads and detection antibodies were diluted four-fold.

### 2.3.1.1 Experimental Model And Subject Details

Multiple linear regression modeling was performed using ‘stats::lm’ within R, regressing each compendium of signaling measurements against the corresponding viability measured in the same conditions. All relationships were assumed to be additive, and no interaction or intercept terms were included in the models. Each measurement was z-score normalized before regression. The two-sided p-values presented were calculated based upon the significance of each parameter being non-zero. The overall performance of each model (as calculated by the F-statistic) corresponded well to the significance of individual terms. All
code and raw measurements for model development are available on Github (https://github.com/meyer-lab/Barney-Peyton).

2.3.12 Immunofluorescence and immunohistochemistry

Cells were grown in serum for 24 h in the indicated material condition, fixed, and stained for Ki67. Cells were serum-starved and stimulated with EGF as described above, and stained according to standard protocols for total EGFR or HER2. The following antibodies were used for immunofluorescence: Ki67 (ab16667, 1:200, Abcam), EGFR (D38B1, 1:100, Cell Signaling Technology), and HER2 (29D8, 1:200, Cell Signaling Technology). All tumor samples were fixed and paraffin embedded. Ki67 staining (ab16667, 1:100, Abcam) was done on 6 μm tissue slices. All AlexaFluor secondary antibodies (Thermo Fisher Scientific) were used at 1:500. Samples were imaged on a Zeiss Cell Observer SD (Carl Zeiss AG, Oberkochen, Germany).

2.3.13 Animal Xenografts

All animal experiments were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst. UMass has an approved Animal Welfare Assurance (#A3551-01) on file with the NIH Office of Laboratory Animal Welfare. MDA-MB-231 cells were suspended in Matrigel (Corning, Corning, NY), and 10^6 cells were subcutaneously injected into the mammary fat pad of 8-10 week old female NOD scid gamma mice from a breeding colony. Each mouse was injected with two tumors. All mice were housed in ventilated cages with sterile bedding, food, and water. Injected cells were grown in vivo for 7 days then drugs were suspended in DMSO and administered with an intraperitoneal (IP) injection daily for 14 days using a 27-gauge needle. Mice received 100 μL of one of five different treatments: vehicle (100% DMSO), sorafenib at 10 mg/kg, PD0325901 at 10 mg/kg, or a combination of the drugs at 5
or 10 mg/kg each. A minimum of 4 mice were analyzed for each drug dosing condition, and each tumor was considered one replicate.

2.3.14 Quantification And Statistical Analysis

Prism v5.04 (GraphPad Software) was used to perform unpaired Student’s t-test, a one-way analysis of variance (ANOVA) with a Tukey post-test. A two-way ANOVA determined how the $IC_{50}$ changed with respect to material modulus, geometry, and medium condition, with a Bonferroni post-test (GraphPad Prism v5.04). A two-way ANOVA, performed in R, was used to determine drug contribution to tumor burden. Data are reported as mean ± standard error, where $p \leq 0.05$ is denoted with *, $\leq 0.01$ with **, and $\leq 0.001$ with ***.

2.4 Results

2.4.1 Geometry impacts innate breast cancer cell response to RTK-targeting drugs

Distinct differences across in vitro screening methods, including stiffness, dimensionality, and cell-cell interactions prevent comparisons across existing studies. Therefore, we developed a system to independently evaluate the effects of both the geometry and stiffness of the microenvironment on breast cancer cell response to targeted and non-targeted drugs. We measured the GR$_{50}$ (concentration of drug which dampens growth by 50%) [152] for four drugs (sorafenib, lapatinib, temsirolimus, and doxorubicin) across multiple biomaterials: TCPS, a 2D PEG-PC hydrogel, and a 3D PEG-MAL hydrogel with encapsulated single cells or tumor spheroids (Figure 2-2 a). To create tumor spheroids, single cells were encapsulated sparsely in polyNIPAAM hydrogels and grown into uniform clonal spheroids over 14 days in culture (Figure 2-2 b-d). This 14 day endpoint achieves a relatively homogeneous population of viable multicellular tumor spheroids with an average diameter less than 100 μm (Figure 2-2 b-c) [60]. This diameter was chosen to ensure drug [50] and oxygen diffusion into the spheroids. Spheroids were transferred to PEG-MAL hydrogels for
dosing, where bulk diffusion measurements of rhodamine 6G suggest small molecules diffuse through the 3D hydrogel at \(2.5 \times 10^{-6} \text{ cm}^2/\text{s}\) (data not shown), which means that the drug will reach the cells within 10 seconds. Further, immunofluorescent staining of Ki67 expression revealed that there were proliferating cells throughout the entire spheroid, suggesting there were no significant nutrient or oxygen diffusion limitations within the 3D PEG hydrogel (data not shown).

We previously found that breast cancer cells on stiffer substrates were more resistant to sorafenib [52], so we included two different moduli for the 2D and 3D hydrogels.

![Diagram of biomaterial drug screening platforms](image)

**Figure 0-2. Characterization of Multiple Biomaterial Platforms.** a. Schematic of biomaterial drug screening platforms, including TCPS, 2D hydrogels linked with collagen I, and 3D hydrogels with RGD for cell adhesion, either with encapsulated single cells or tumor spheroids. Each hydrogel condition was screened at two different moduli (2D: 10 or 33 kPa, 3D: 3 or 5 kPa). b. Quantification of mean spheroid diameter for MDA-MB-231 spheroid growth in polyNIPAAM gels over 14 days. c. Viability of MDA-MB-231 spheroid after 14 days of growth in polyNIPAAM, and encapsulation in 3 kPa 3D PEG-MAL hydrogel for 3 days. Green: live cells, red: dead cells. Scale: 100 μm. d. Experimental time investment required for the different screening platforms. Timeline displays preparation time required for each system prior to treating with varying concentrations of drug. Figure produced in collaboration with Dr. Lauren Barney.

Although the effect of altered proliferation across the biomaterials (Figure 2-3) would confound comparisons of traditional IC\(_{50}\) calculations, it is accounted for in the GR\(_{50}\)
calculation [152]. Therefore, the differences we report in drug response are a direct result of ECM-driven resistance, independent of a growth advantage. In Figure 2-3, drug response is reported as a fold change in $GR_{50}$ from the indicated condition compared to TCPS, where red indicates resistance and blue indicates sensitivity compared to TCPS. Response to doxorubicin did not vary significantly across geometry, modulus, or medium (with or without serum, varied for the spheroid model only) in either cell line (Figure 2-3). MDA-MB-231 response to temsirolimus was not dependent on the biomaterial platform we tested, while SkBr3 $GR_{50}$ had small but significant changes. However, sorafenib and lapatinib response varied considerably across the biomaterial platforms, particularly in the MDA-MB-231 cells (Figure 2-3). It is notable that the SkBr3 response to sorafenib was only dependent on geometry, and not the addition of serum, suggesting a unique role for the ECM in mediating drug response, independent of exogenous mitogenic factors. A two-way analysis of variance (ANOVA) showed that the changes in geometry and medium had a larger effect on total variance than the change in modulus as reported by the percent of variation across biomaterial platforms (Table 2-2). Within each geometry, we observed no significant difference in drug response at either stiffness, but our analysis indicated that cancer cell

<table>
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<th>Culture Format</th>
<th>SkBr3 (%)</th>
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<tr>
<td>All Conditions</td>
<td>Geometry</td>
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<tr>
<td>Proliferation</td>
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<td>Sorafenib</td>
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<td>Lapatinib</td>
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Table 0-2. Two-way ANOVA of drug resistance across biomaterial microenvironments. Percent of total variation (%). * denotes significance by two-way ANOVA. Data gathered in collaboration with Dr. Lauren Barney and Dr. Thuy Nguyen.
resistance to sorafenib and lapatinib was most sensitive to changes in geometry within this modest stiffness range.

**2.4.2 Gene expression is not responsible for ECM-driven differences in drug response**

We performed RNA-Seq on MDA-MB-231 and SkBr3 cells cultured on TCPS, on 2D hydrogels, as tumor spheroids, and as a tumor xenograft (Figure 2-4 a). Hierarchical clustering of differentially expressed genes first separated samples by the cell line, then by material platform, which we used as a guide to order the ECMs in Figure 2-4 a. The biomaterial platform dictated clustering within each cell line and dimensionality had greater influence on clustering than stiffness. Gene expression on the 2D hydrogels (10 and 33 kPa) was similar for both cell lines, and these samples were closely related to cells on TCPS. Toward understanding whether this was an immediate effect of signals from the ECM or

![Figure 0-3. Drug Response Varies with Biomaterial Platforms.](image)

*a-b. SkBr3 (a) and MDA-MB-231 (b) proliferation after 3 days of culture (top row), and GR$_{50}$ in response to doxorubicin, temsirolimus, sorafenib, and lapatinib across the different biomaterial platforms. Red: increase from TCPS control (resistance compared to TCPS), blue: decrease from TCPS control (sensitivity compared to TCPS). TC: TCPS, 10: 10 kPa 2D hydrogel, 33: 33 kPa 2D hydrogel, 3: 3 kPa 3D hydrogel, 5: 5 kPa 3D hydrogel, +FBS: cells were treated in 10% serum-containing medium. Unless noted by +FBS, all cells were treated with drugs in serum-free medium containing EGF and PDGF-BB. Data gathered in collaboration with Dr. Lauren Barney and Dr. Thuy Nguyen.*
from selective growth of a subset of cells in different environments, we also analyzed cells initially grown into spheroids, but then dissociated and cultured back on TCPS for 24 hours. The gene expression profile from these dissociated spheroids was also closely related to the other 2D conditions, indicating this expression was likely derived from the assay platform, rather than the culture platform (Figure 2-4 a).

Gene set enrichment analysis (GSEA) identified several significantly enriched pathways in one or more of the biomaterials. For example, genes associated with the regulation of growth and receptor-linked signal transduction were upregulated in MDA-MB-231 cells on plastic compared to MDA-MB-231 cells in 3D spheroids (Figure 2-4 b). While this is not an exhaustive list, cells on TCPS expressed more cytokine and associated receptor genes and cells in the xenograft upregulated expression of ECM components, particularly collagens and keratins. In Figure 2-4 c and d, the normalized enrichment scores are plotted as pairwise comparisons, enriched in the row condition over the column platform in red for MDA-MB-231 cells and in blue for SkBr3 cells. As expected, both cell lines upregulated genes associated with growth regulation on TCPS compared to all the other conditions we examined (Figure 2-3, Figure 2-4 b-c). Furthermore, the MDA-MB-231 xenograft tumors upregulated growth-related genes compared to cells grown in vitro as spheroids, suggesting that the mouse host environment provided additional growth cues (Figure 2-4 c). However, both geometry and stiffness influenced surface receptor linked signal transduction. For example, cells on TCPS universally showed enrichment for these genes compared to the other biomaterials, and our spheroids showed enrichment over the 2D hydrogels in the SkBr3 cells (Figure 2-4 d).
We then used this expression data to determine if ECM-mediated drug response was a
result of changes in the gene expression of drug targets for lapatinib and sorafenib across the
Figure 0-4. Expression of growth and signal transduction genes distinguishes screening platforms but does not explain drug response. a. Clustering and normalized expression values of significantly differentially expressed genes across the biomaterial platforms for MDA-MB-231 and SkBr3 cell lines. Platforms were ordered according to the above hierarchical clustering, and genes were ordered by expression on TCPS, and each cell line was independently filtered for the top 250 genes by maximum expression across all platforms. All significantly differentially expressed genes (p<0.01) are shown in Figure S4. MDA-MB-231 order of platforms: spheroids, xenograft, 2D hydrogels at 10 kPa, and 33 kPa, spheroids reseeded to TCPS, TCPS. SkBr3 order of platforms: xenograft, 2D hydrogels at 10 kPa, and 33 kPa, spheroids, spheroids reseeded to TCPS, TCPS. Blue: low expression, red: high expression b. Representative gene set enrichment curves for regulation of growth (MDA-MB-231, TCPS over 3D spheroids) and cell surface receptor linked signal transduction (MDA-MB-231, TCPS over 3D spheroids). c-d. Normalized enrichment scores in pairwise platform comparisons for (c) regulation of growth (d) and cell surface receptor linked signal transduction. MDA-MB-231 comparisons are enriched in red and SkBr3 comparisons are in blue. Comparisons are shown as ROW condition over COLUMN (i.e., Top row shows MDA-MB-231 enrichment on TCPS over other platforms). – indicates no data. e-h. Expression of known targets for (e) lapatinib and (g) sorafenib and previously published off-target genes for (f) lapatinib and (h) sorafenib across the biomaterial platforms with linear correlations. Data gathered in collaboration with Dr. Thuy Nguyen.

biomaterials (Figure 2-3). We found no correlation between expression of these target genes
and the GR₅₀ (Figure 2-4 e,g). However, both of these drugs are known to alter signaling of many other pathways, and a literature search identified several known unintentional targets or resistance mechanisms for each drug [157-161] (Figure 2-4 f,h). Although many of these genes showed little to no change in expression across the biomaterials, we did note that AXL expression showed a slight positive trend, but not a significant correlation, with lapatinib response in the MDA-MB-231 cells (Figure 2-4 f). AXL has previously been shown to predict drug resistance to ErbB targeted inhibitors in many cancer types [161], but does not appear to significantly contribute to the drug responses examined here.

2.4.3 Fibronectin and claudin 4 expression depend on spheroid formation method

To better understand the processes involved in forming cancer cell spheroids, we compared three distinct methods of formation; 1) encapsulating single cells into polyNIPAAM, as described above, 2) seeding cells into microwells and allowing for cell aggregation over 24 hours, or 3) suspending cells in a non-adherent plate and allowing them to aggregate and grow over 3 days. Gene expression was quantified in breast, prostate, and ovarian cancer cell lines in the three spheroid formation methods and compared to basal gene levels in cells on TCPS. To select the relevant genes for this study, the RNA-Seq data from breast cancer cell lines MDA-MB-231 and SkBr3 was analyzed, after cells were grown on TCPS, in polyNIPAAM for 14 days, or grown in polyNIPAAM and then dissociated and plated back onto TCPS, as described above (Figure 2-5). Using Gene Set Enrichment Analysis, we observed that cell surface receptor-linked signal transduction genes, including several integrins, were enriched on TCPS when compared to spheroids formed in polyNIPAAM for 14 days, whereas cell–cell adhesion genes, such as claudin 4, were enriched in cells grown into spheroids in polyNIPAAM compared to TCPS (Figure 2-5 b-c). From this data set, we selected a subset of cell adhesion genes including integrin subunits (α₂ (ITGA2), β₁ (ITGB1) and β₄ (ITGB4)), cell–cell junction proteins (cadherin 3 (CDH3))
and 5 (CDH5) and claudin 4 (CLDN4)), and the ECM protein, fibronectin (FN1), to examine in each spheroid formation method.

We found that either claudin 4 or fibronectin were upregulated in all spheroid formation methods compared to TCPS (Figure 2-6 a) with the few exceptions of OVCAR-3 in polyNIPAAM, and BT549 and PC-3 in microwells. Gene expression patterns varied with the time required for robust spheroid development across the three methods. Fibronectin was downregulated for all the cell lines in microwells, while claudin 4 was upregulated in the

**Figure 0-5. RNA-Seq on Breast Cancer Cells Reveals Enrichment of Surface Receptor Linked Signal Transduction.** a. Principal Component Analysis shows separation by cell type and cell format of MDA-MB-231 and SkBr3 cells grown on TCPS or in polyNIPAAM. b. Gene Set Enrichment Analysis shows cell surface receptor linked signal transduction is enriched on TCPS over spheroids and cell-cell adhesion is enriched in spheroids as compared to TCPS, in MDA-MB-231 and SkBr3 cells. c. Several gene set categories are significantly enriched on TCPS (positive values) or in spheroids in 3D (negative values). Data gathered in collaboration with Dr. Thuy Nguyen.
polyNIPAAM method for all the cells lines, except for AU565 and OVCAR-3. These gene expression changes were dependent on the cell line as well as the spheroid establishment method. Variations in gene expression with each method was confirmed by dendrogram clustering for all combinations of examined cell lines and methods, which revealed that cells that have likely reached some homeostasis on TCPS or in polyNIPAAM primarily clustered together, and the shorter methods of microwells and suspension also clustered together (Figure 2-6 b). This was further confirmed by principal component analysis (PCA), which revealed that samples did not cluster by cell line or cancer type (data not shown), but rather that PC1 separated samples by method, with those that had the shortest and longest times of culture being the most distinct from one another (Figure 2-6 c). Although all of these systems formed spheroids of similar size, the expression of cell-cell contact genes varied across methods, which may have affected the compactness of these spheroids.

Figure 0-6. Spheroid formation method determines expression of cell–ECM and cell–cell adhesion genes. a. RT-PCR of genes collected from 2 breast (AU565 and BT549), 2 prostate (PC3 and LNCaPcol), and 2 ovarian (SKOV-3 and OVCAR-3) cell lines reported as log2(fold change from TCPS). Genes are ordered based on expression clustering. Shades of red indicate gene upregulation compared to TCPS and shades of blue indicate gene downregulation compared to TCPS. b. Dendrogram of RT-PCR data by platform in TCPS (black), in polyNIPAAM (green), in microwells (red), and in suspension (blue). c. Principal component analysis (PCA) of gene expression by MCTS formation method. Ovals represent 0.5 probability for each group of polyNIPAAM (green triangle), microwells (red circle), and suspension (blue square), N ≥ 3. Data Gathered in collaboration with Dr. Maria Gencoglu and Dr. Christopher Hall.
These contrasting phenomena of ECM production or cell-cell interactions have been previously demonstrated in other cell types, although to the best of our knowledge, this is the first work to identify these independent behaviors across breast, prostate, and ovarian cancer cells. Fibronectin 1 is a cell secreted protein that is important in the invasive phenotype [162] and metastasis via collective invasion [163]. Fibronectin also lends structure to cell clusters to facilitate cell adhesion and signal transduction, as previously demonstrated in fibroblasts, where integrin binding to fibronectin was necessary for fibroblast activation [164]. Claudin 4 is essential for tight junctions is expressed in the majority of ovarian cancers [165], particularly in multicellular ovarian cancer patient ascites and knockdown slowed the rate of *in vitro* spheroid formation [166]. Meanwhile, breast cancer cells grown into spheroids using the overlay method, which is most similar to our microwell and suspension methods, for 6 days downregulated integrin α₂, while they upregulated several cell-cell adhesion genes, including claudin 4 [167]. We thus hypothesized that the spheroids that upregulated cell–cell contact genes and took longer times to form, may be more resistant to drugs than cells on TCPS because of their compact morphology.

2.4.5 Multiple linear regression reveals that using a MEK inhibitor with sorafenib is an effective combination therapy

Since there were large differences in GR₅₀ across the screening microenvironments, and known drug targets were not responsible for ECM-mediated resistance, we hypothesized that plasticity in intracellular signaling networks was responsible for the differential responses. We measured a panel of phospho-proteins associated with RTK signaling at basal levels and in response to drug treatment across the biomaterial ECMs. Here, we chose to closely examine sorafenib and lapatinib across material platforms, and added temsirolimus to compare to a drug whose response was largely independent of ECM (Figure 2-3).
Initial signaling data provided a complex and inconsistent picture of cell response to biomaterial, and linear regression modeling yielded no analytes whose phosphorylation varied significantly with platform. Several challenges are present in this system, including dose timing and difficulty of harvesting sufficient amounts of protein from 3D biomaterials to generate a robust signaling. To that end, I will outline here some of the troubleshooting techniques employed to ultimately optimize the process. First, the final optimized system demonstrated that the phosphorylation of EGFR in response to dosing cells with EGF is dependent on the environment. While cells on TCPS, a 2D hydrogel, and in a 3D hydrogel all exhibited pEGFR after 5 minutes, the magnitude was diminished on 2D biomaterials compared to TCPS, and in 3D compared to both other systems (Figure 2-7 a). Several pieces of this process were optimized; the first being the lysing process as demonstrated here. For the original samples, gels were collected from the well plates at the appropriate time, and immediately frozen to -80°C, and then partially thawed before adding lysis buffer containing protease inhibitors and phosphatase inhibitors (process labeled freeze). For comparison, a separate lysing protocol was employed, where gels were washed, and then transferred from

**Figure 0-7. Optimization of lysing protocols.** a. Phosphorylation of EGFR was measured in the MDA-MB-231 cells using the Luminex MAGPix system from cells on TCPS, cell on 33 kPa PEG-PC hydrogels, and from cells encapsulated in a 3 kPa hydrogel. Lysing cells off the 2D surfaces (TCPS and 33 kPa), was performed as described in the text. Lysing of cells encapsulated in 3D is further explained by parts b and c of this figure. b-c. Phosphorylation of EGFR (b) and HER2 (c) was measured after preparing samples using multiple protocols as described, where gels containing cells were either immediately frozen or treated with lysis buffer. Data gathered in collaboration with Dr. Lauren Barney.
the plate immediately into lysis buffer before freezing and mechanical dissociation of the gel (process labeled lyse). Both processes yielded some signal in pEGFR, but the maximal signal from first freezing the 3D samples (~100 MFI, achieved at 15 minutes), was about 6-fold lower than using the lysing protocol (~600 MFI, achieved at 5 minutes) (Figure 2-7 b). Further, the freezing protocol resulted in almost no pHER2 signal, compared to a clear and robust signal from the lysing process (Figure 2-7 c). Other components of the process were also optimized, such as the amount of total protein loaded was increased from 3 µg to 4 µg, after confirming that this still fell within the linear range of read out on the MAGPix. After optimization of 3D phospho-protein extraction, we compared signaling across our biomaterials to characterize the varied cell response to drug.

The basal levels of nearly every analyte were significantly lower in 3D than on 2D ECMs (Figure 2-8), which matches growth and signal transduction gene signatures from our expression data (Figure 2-4 c-d). As expected, drug treatment reduced the phosphorylation level of the intended targets (Figure 2-10 a-b). However, we only observed significant reduction in known targets and increases in adaptive pathways in 2D, suggesting that the 3D environment suppresses drug efficacy through dampened signaling and kinome rewiring.

![Figure 0-8. 3D biomaterials generally suppress signaling.](image)

**Figure 0-8. 3D biomaterials generally suppress signaling.** a-b. Basal p-ERK (pT185/pY187), p-Akt (pS473), and p-JNK (pT183/pY185) signaling for MDA-MB-231 (a) and SkBr3 (b) cell lines across the five screening microenvironments. Black: TCPS, dark blue: 2D gel, green: 3D gel with single cells, teal: 3D gel with spheroids, red: 3D gel with spheroids in serum. 2D hydrogel: 33 kPa, 3D hydrogel: 3 kPa. Data gathered in collaboration with Dr. Lauren Barney.
Our lab has previously demonstrated the role of JNK in stiffness-dependent sorafenib resistance [52]. Because we observed increased pJNK in response to the drug treatments in both cell lines, we hypothesized that JNK may mediate drug response across the biomaterials. Co-administration of JNK inhibitor SP600125 sensitized the MDA-MB-231 cells to sorafenib on 2D biomaterials, as spheroids with serum, and on TCPS (Figure 2-10 c). SP600125 only sensitized the SkBr3 cells in the spheroid and 2D hydrogel conditions, but not the stiff TCPS environment, perhaps a result of the very high increase in pJNK in the sorafenib-treated SkBr3 cells in that environment (Figure 2-19 e). As an additional control, we quantified JNK phosphorylation after treatment with doxorubicin, a drug that was associated with low GR_{50} variability across platforms, and found that JNK signaling was still altered by this non-specific drug treatment (Figure 2-9). Combined with our signaling data from the targeted therapeutics, we hypothesize that JNK phosphorylation is indirectly sensitive to drug-induced stress, but does not significantly contribute to the ECM-mediated resistance observed here (Figure 2-9, Figure 2-10 a-b)[168, 169]. Importantly, the efficacy of the JNK inhibitor in the

**Figure 0-9.** Doxorubicin treated cells show limited change in phosphorylation of key signaling analytes. a-b. The log_{2} fold change of cells treated with doxorubicin (Dox) compared to the vehicle control (DMSO) across 5 screening environments; TCPS (black), 2D gel (dark blue), single cells in 3D (green), spheroids in a 3D gel (light blue) and 3D gel spheroids with serum (red) in MDA-MB-231 cells (a) and SkBr3 cells (b).
MDA-MB-231 spheroids in the 3D hydrogel was captured in the TCPS assay, and did not need screening across biomaterials for discovery. Thus, we sought to identify an efficacious combination therapy using this multi-environment screening approach that could not be identified through simple TCPS screening.

Multiple linear regression (MLR) models revealed non-intuitive relationships between the sorafenib-, lapatinib-, and temsirolimus-treated signaling and drug-treated viability data for both cell lines across the biomaterials. This model was constructed from phosphorylation data of 10 analytes after 4 hours of targeted drug treatment across all 15 conditions (3 drugs and 5 biomaterial platforms per drug). Although targeting the MEK/ERK pathway was not an obvious choice from the signaling data (Figure 2-10 a-b), these models revealed that variability in MEK phosphorylation was the key factor associated with viability in response to drug treatment in MDA-MB-231 cells (Figure 2-10 d,f). It is important to note that the MLR output includes the contribution of each signaling analyte to drug response, and while MEK was identified as the key contributor to variance, other analytes have non-zero contributions to the model. To determine the necessity of each component to the model prediction, a subsequent analysis was performed where we independently excluded either all 3D conditions (single cells, spheroids, spheroids with serum) or two of the drugs (lapatinib and temsirolimus). Neither of these modified models were able to achieve significance (Figure 2-11 a). Returning to our model that includes all three drugs in the MDA-MB-231 cells, we determined that each 3D condition could be excluded independently, but that TCPS, 2D biomaterials, and at least one 3D condition were necessary to predict MEK.
50 phosphorylation as a likely mechanism of ECM-mediated resistance (Figure 2-11b).

We next co-administered both sorafenib and the MEK inhibitor PD0325901 to cells in the different biomaterials. In both cell lines, there was no improved efficacy when co-treating sorafenib and PD0325901 on TCPS (Figure 2-10 c,e). However, both cell lines were sensitized to sorafenib when co-administered with PD0325901 in the 2D hydrogel and spheroid conditions (Figure 2-10 c,e). This suggests MEK may be a more physiological

Figure 0-10. Systems analysis reveals that MEK regulates ECM-mediated sorafenib efficacy. a-b. Phospho-signaling in response to drug treatment and GR\textsubscript{So} for MDA-MB-231 (a) and SkBr3 (b) cell lines. TC: tissue culture polystyrene, 2D: 2D PEG-PC hydrogel, 3D: single cells encapsulated in 3D hydrogels, Sp: spheroids encapsulated in 3D hydrogels, +: spheroids in 3D hydrogels in serum containing medium. Phospho-proteins measured include: CREB (pS133), ERK (pT185/pY187), NFκB (pS536), JNK (pT183/pY185), p38 (pT180/pY182), STAT3 (pS727), STAT5A/B (pY694/699), Akt (pS473), EGFR (pan Tyr), and MEK1 (pS222). Red: increase compared to vehicle control, blue: decrease from vehicle control. c.e. Sorafenib GR\textsubscript{So} alone or in combination with SP600125 (JNK inhibitor, 20 \textmu M) or PD0325901 (MEK inhibitor, 3 \textmu M) for MDA-MB-231 (c) and (e) SkBr3 cell lines. Black: control, blue: SP600125, red: PD0325901. d,f. MLR models of signaling data for MDA-MB-231 (d) and SkBr3 (f). Data gathered and analyzed in collaboration with Dr. Lauren Barney and Dr. Aaron Meyer.
resistance mechanism to sorafenib, as SkBr3 cells did not show significant variability of MEK across platforms, but were still responsive to co-treatment in our biomaterials. Interestingly, the MDA-MB-231 cells are KRAS mutant, while the SkBr3 cells are not, meaning this treatment is independent of both this mutation and their clinical subtype.

Altogether, the identification of MEK as an effective target for combination therapy was only realized through screening across many biomaterials and using a systems approach to analyze a phospho-signaling dataset. Likewise, the efficacy of the MEK inhibitor in combination with sorafenib was only achieved through screening in the more complex 2D or 3D biomaterial microenvironments, and would be missed using TCPS or via genetic analysis alone.

2.4.5 MEK inhibition improves sorafenib efficacy in vivo
Given the promising *in vitro* results with the MEK inhibitor, we tested PD0325901 as a combination therapy with sorafenib in an *in vivo* model. MDA-MB-231 tumors were inoculated in the mammary fat pad of NSG mice and allowed to grow for 7 days, at which point all mice had palpable tumors. From days 7 to 21, we treated daily with one of four regimens: 1) vehicle control (DMSO), 2) 10 mg/kg sorafenib, 3) 10 mg/kg PD0325901, or 4) co-treatment of 10 mg/kg each sorafenib and PD0325901 (Figure 2-12 a). As combination therapies are often associated with increased adverse events [170], we also included a fifth treatment group which received a lower dose of the combination therapy at 5 mg/kg each of sorafenib and PD0325901. All of these doses were found to be well tolerated by mice (data not shown), although future work will need to better optimize the doses of this combination therapy to optimally minimize the dose while maintaining tumor burden reduction. PD0325901 was the most effective single agent inhibitor and, as predicted in the 2D hydrogel and spheroid screening models, co-treating mice with PD0325901 and sorafenib had the most significant reduction in tumor burden, cellularity, and proliferation (Figure 2-12 b-d). In the lower concentration combination therapy (5 mg/kg each), reduction in tumor weight was comparable to 10 mg/kg PD0325901 alone (Figure 2-12 b). A two-way ANOVA showed that PD0325901 (F<sub>value</sub>=54.7, p<0.001) contributed more to the decrease in tumor burden than sorafenib (F<sub>value</sub>=8.2, p<0.01). The combination therapy of sorafenib and PD0325901 additively decreased tumor burden (F<sub>value</sub>=0.3, p=0.6).

**2.5 Discussion**
By screening drug response across many ECMs, we identified that breast cancer cell response to sorafenib (Ras/Raf/MEK/ERK targeted) and lapatinib (EGFR-HER2 targeted) was significantly dependent upon the biomaterial environment (Figure 2-3). Gene expression of known drug targets was unchanged across the ECMs (Figure 2-4 e-h), but a systems analysis of phospho-kinome signaling in response to drug treatment across our different biomaterials revealed MEK as a key contributor to the variation in observed ECM-mediated resistance (Figure 2-10 d,f). Although the combination therapy did not improve drug response in cells on TCPS, a MEK inhibitor in combination with sorafenib eliminated the matrix-mediated response in biomaterials in vitro and reduced tumor burden in vivo (Figure 2-12).

**Figure 0-12.** PD0325901 and sorafenib are an effective combination therapy to reduce in vivo tumor burden. a. Schematic for tumor induction of the MDA-MB-231 cells, grown first for 7 days, followed by daily drug dosing for 14 days until a 21-day endpoint. b. Final tumor weight for each drug dosing condition (C: control, S: 10 mg/kg sorafenib, PD: 10 mg/kg PD0325901, and co-dosing at either 5 mg/kg or 10 mg/kg of each therapeutic, p = 0.05; two-tailed t-test on log transformed data). c. Representative images for Ki67 staining in tumors and d. quantification of Ki67 positive cells in drug treatment groups (S/PD is the 10 mg/kg combination therapy). Scale bar is 50 μm. e. Screening for ECM-mediated resistance in drugs like sorafenib can be used in combination with multiple linear regression modeling to find combination therapies with the MEK inhibitor PD0325901. Error bars represent mean and SEM, N≥4. Data gathered by Dr. Christopher Hall and Dr. Lauren Jansen.
While most current literature examines signaling changes within a single platform [57, 58, 171, 172], we applied MLR across five ECM conditions and three targeted drug treatments to determine which phospho-proteins varied significantly with ECM-mediated drug response. This approach captures multiple aspects of the ECM that may contribute to drug response in vivo, including ECM stiffness, dimensionality, and cell-cell contacts using both 2D and 3D biomaterials. Synthetic 2D biomaterials allow for the presentation of full-length proteins, while spheroids encapsulated in 3D better recapitulate tumor geometry. In our experiments, cells seeded onto 2D biomaterials or as single cells in 3D resided in those environments for 24 hours, so they likely relied exclusively on the provided collagen I (2D) or RGD (3D) for integrin-mediated signaling. However, breast cancer cells grown as spheroids upregulate ECM genes, such as fibronectin, suggesting additional ECM proteins, not provided by us initially, contribute to the observed cell response [60]. Recent work has shown that the ECM-mediated signaling can impart resistance to combined inhibition of PI3K and HER2 [173], but our method identified that targeting two nodes in the same pathway, Raf with sorafenib and MEK with PD0325901, is a promising combination therapy for breast cancer. This non-intuitive co-treatment, which would not be identified by screening on traditional TCPS, suggests a similar approach may be beneficial in identifying combination therapies for kinome-targeting drugs, where adaptive resistance is frequently mediated by the ECM [52, 130].

Combination therapies with MEK inhibition are effective in many cancer types [174-177]. In fact, MEK inhibition is currently under evaluation in clinical trials because many cancers activate MEK signaling as a form of acquired resistance [178]. Such combination therapies are often designed to limit adaptive survival signaling, and generally target two distinct pathways. Interestingly, in our work, co-targeting MEK improved sorafenib efficacy in vitro and in a pre-clinical in vivo tumor regression model, even though both drugs target
the same pathway (Figure 2-12). Others have seen similar improved efficacy in HER2+ breast cancer when co-treating the HER2-targeted drugs lapatinib and trastuzumab, suggesting that combination therapies targeting the same pathway can have improved effects by overcoming acquired resistance [179]. Emerging work also demonstrates that co-targeting RAF and MEK is an effective strategy in liver, breast, and other cancers [180-182]. Further targeting strategies, like using nanoparticles to co-deliver sorafenib and a MEK inhibitor are also gaining traction [183]. However, not all cells within a tumor contribute equally to drug response. Tumors are highly heterogeneous and composed of cell subpopulations that can evade both cytotoxic and targeted therapeutics via up-regulation of drug transporters, mutations of drug targets, adaptive signaling, and other mechanisms [136, 184, 185]. Here we identified the additive effect of co-dosing sorafenib with a MEK inhibitor on the bulk tumor, but further work is necessary to understand the contribution of individual populations to resistance.

We measured a large panel of phospho-proteins to see if the biomaterial environment facilitated phospho-signaling changes in response to the drug treatments that were associated with drug sensitivity or resistance. In our soft 3D environments, basal and drug-stimulated signaling were significantly suppressed, in agreement with other data in spheroids and on soft substrates [186-188]. JNK appears to be a broad adaptive response [189, 190]; however, MLR identified MEK as the most significant driver of ECM-mediated resistance to sorafenib. ERK is downstream of both the lapatinib and sorafenib targets, which may explain why MEK/ERK signaling was so critical to drug response in our data. In agreement with our findings, myeloid leukemia cells have a stiffness-dependent response to drugs, including sorafenib and a MEK inhibitor, which target the RAF/MAPK pathway [129, 191]. We have previously shown that sorafenib resistance increased with increasing hydrogel stiffness [52], and many others have also demonstrated that stiffness is a significant driving force for drug
resistance [52, 141, 171, 172, 192-195]. Thus, we were surprised to find that drug resistance was only minimally affected by stiffness within the modest range examined here (Figure 2-3). However, in our data, dimensionality contributed more to drug resistance than stiffness. Previous work has similarly shown that cancer cell spheroids are more resistant to paclitaxel than a monolayer [57], and the matrix-attached cells in spheroids are resistant to PI3K/mTOR inhibition [58].

Because cells are less able to form cell-matrix adhesions on soft versus stiff 2D substrates, and in 3D versus 2D environments [196], genes associated with integrin-mediated adhesion and cell-surface receptor linked signal transduction are varied across our biomaterials (Figure 2-4 b-d). Matrix adhesion via integrins mediates survival and drug resistance through activation of survival signaling, such as FAK, Src, and ERK [197-199]. Disruption of cell adhesion can sensitize tumor cells to drug treatments [52, 173, 200-202]. In 3D, we generally observed greater drug sensitivity for single cells compared to spheroids (Figure 2-3), perhaps because single cells have lower overall adhesion to surrounding cells and matrix [130]. Spheroids and tumors can also exhibit multicellular drug resistance through either cell-cell contact-mediated signaling or drug diffusion limitations [203], and often co-targeting multiple pathways is required to sensitize the outer, matrix-attached cells [58]. While our spheroid size was intentionally kept small to minimize diffusion limitations, the cell-cell contact inherent to the spheroid structure may provide survival signaling, resulting in increased resistance to the RTK-targeted drugs.

In our hands, gene expression of drug targets was relatively unchanged across our materials. Previous work has reported significant changes in gene expression profiles of cell lines cultured in 2D and 3D laminin-rich ECM [204]. This is similar to our own data, where we observed that surface receptor linked signal transduction genes vary with both geometry and stiffness, and are universally upregulated during culture on TCPS compared to the other
biomaterials (Figure 2-4 b). Also, several ligand-receptor pairs were co-expressed and enhanced in vivo, including BMP and IL6 signaling (Figure 2-13). We found that growth context determined the expression of cell surface receptors, while expression of the associated ligands was dependent on the current assay platform and was more sensitive to stiffness.

Interestingly, both gene expression and drug sensitivity were regained when cells grown as spheroids were dissociated and seeded back onto TCPS (Figure 2-4 a, Figure 2-13 c). This demonstrates that drug response in the timeframe we examined is dependent on the current environment and not necessarily a result of stable genetic alterations or subpopulations resulting from a previous growth condition. In our gene expression data, the xenograft was the most distant from other samples for the SkBr3 cells, but not for the MDA-MB-231 cells. This is likely due to the differences in length of time required for the xenografts to reach a similar size. The MDA-MB-231 xenograft tumor reached 50 mm³
within 10 days, and the SkBr3 xenograft took 3 weeks to reach 50 mm$^3$. In an *in vivo* microenvironment, xenograft tumors derived from highly lung metastatic clones of the MDA-MB-231 breast cancer cell line have a distinct ECM signature that up-regulates growth factor signaling including TGF-β and VEGF [78]. We conclude that although gene expression may be useful to inform patient treatment [205], it was not sufficient to predict *in vitro* or pre-clinical drug efficacy here.

Our data suggested that cells employ one of two possible mechanisms for spheroid formation: they either secrete their own local matrix to provide binding sites and structure, or they rely on cell–cell contacts. In spheroids formed in the microwells (briefest culture time), claudin 4 was upregulated, whereas those formed using the polyNIPAAM method (longest culture time), upregulated fibronectin and integrins (Figure 2-6 a). Immunofluorescence staining has shown that in spheroid configurations that are loosely connected and have more void space, fibronectin is distributed within the intercellular space throughout the spheroid. However, in compact spheroids, fibronectin is found on the outer edge [206]. This can be compared with our suspension and polyNIPAAM spheroids, respectively. On the other hand, knocking down claudin 4 reduces *in vitro* spheroid formation [166] because it is essential for tight junctions. Interestingly, claudin 4 is expressed in the majority of ovarian cancers [165], and also in SKOV-3 and OVCAR-3 spheroids. Additionally, breast cancer cells grown into spheroids using the overlay method, similar to our suspension method, upregulated claudin 4 and several other cell–cell adhesion genes [167]. Our results are largely cell-line dependent, without any correlation to cancer type, so this contribution can be applied in other tumor spheroid models. For example, ovarian cancer metastasizes through the abdominal lining and into the abdominal cavity, where fluid contains high numbers of spheroids, called ascites [207]. Understanding the relationship between the spheroid structure and drug
resistance may lead to better therapeutic development and optimization of patient-specific treatment options.

2.6 Conclusions

Though the ECM can impart resistance to some RTK inhibitors (sorafenib and lapatinib), MLR modeling of phospho-signaling identified combinatorial strategies to combat this resistance (Figure 2-12 e). This approach could provide insight into other anti-cancer drugs with varied clinical success, such as neratinib [208, 209] and sunitinib [210, 211]. However, not all the drugs we tested showed ECM-dependent responses, and response to a cytotoxic drug, such as doxorubicin, would have been predicted in a simple, quick, cost-effective TCPS assay. It is therefore critical to weigh the monetary cost and time burden of the screening approach against the potential benefits. The efficacy of the PD0325901 and sorafenib combination therapy would not have been realized using only one screening environment or without a systems biology analysis. We envision this approach can be used to identify drugs with poor in vivo efficacy early in the drug development pipeline and identify novel drug combinations to overcome ECM-induced resistance.
CHAPTER 3
INTEGRIN α6 AND EGFR SIGNALING CONVERGE AT MECHANOSENSITIVE CALPAIN 2

3.1 Abstract

Cells sense and respond to mechanical cues from the extracellular matrix (ECM) via integrins. ECM stiffness is known to enhance integrin clustering and response to epidermal growth factor (EGF), but we lack information on when or if these mechanosensitive growth factor receptors and integrins converge intracellularly. Towards closing this knowledge gap, we combined a biomaterial platform with transcriptomics, molecular biology, and functional assays to link integrin-mediated mechanosensing and epidermal growth factor receptor (EGFR) signaling. We found that high integrin α6 expression controlled breast cancer cell adhesion and motility on soft, laminin-coated substrates, and this mimicked the response of cells to EGF stimulation. The mechanisms that drove both mechanosensitive cell adhesion and motility converged on calpain 2, an intracellular protease important for talin cleavage and focal adhesion turnover. EGF stimulation enhanced adhesion and motility on soft substrates, but required integrin α6 and calpain 2 signaling. In sum, we identified a new role for integrin α6 mechanosensing in breast cancer, wherein cell adhesion to laminin on soft substrates mimicked EGF stimulation. We identified calpain 2, downstream of both integrin α6 engagement and EGFR phosphorylation, as a common intracellular signaling node, and implicate integrin α6 and calpain 2 as potential targets to inhibit the migration of cancer cells in stiff tumor environments.

In the previous section we identified changes in dimensionality and biomaterial platform as the main contributors of ECM-mediated drug resistance, and saw relatively little response to stiffness across the range examined. However, stiffness is known to contribute to
tumor cell aggression, so this work was broadly motivated by the changes in stiffness that occur during cancer progression as the primary tumor stiffens and cells disseminate to distant metastatic cites. However, the impact of stiffness does not occur in a vacuum, so we employed an extracellular matrix model where varied stiffness in a 2D hydrogel system was complimented by varied full-length proteins for cell adhesion and growth factors to stimulate aggressive cell phenotypes (Figure 3-1). Our system allows for independent tuning of each of these parameters, and modifications to the optimal ECM model were generated iteratively through experimental exploration. For example, as we found that cells produce laminin on soft biomaterial systems, we sought to include that signal to determine its functional impact on cell phenotype. Together, this demonstrates that we have compiled an ECM model that is physiologically relevant to understand mechanosensing in the tumor microenvironment and the role of other biochemical signals in altering that cascade.

3.2 Introduction
Carcinoma progression is associated with deposition of ECM that stiffens the local microenvironment [212, 213]. This tissue stiffening results in deposition of additional matrix proteins, initiating a positive feedback loop between cells and the evolving stroma [32]. Cells sense and respond to the stiffness of their environment via RhoA GTPase activation, which feeds back to increase cell contractility via activation of myosin light chain kinase [214]. These ECM-driven changes in cytoskeletal tension regulate motility in a cell-type specific manner [44]. In vitro, synthetic biomaterials have revealed that the type of material, stiffness, and biochemical surface modifications alter the attachment and motility of cells [45, 215-217]. We therefore hypothesized that independently tuning stiffness, while altering integrin-binding sites and the availability of epidermal growth factor could reveal new mechanosensitive proteins that drive adhesion and motility in cancer cells.

Several mechanically responsive genes and proteins have been implicated in breast cancer metastasis [47]. For example, extracellular mechanical forces in the tumor microenvironment alter nuclear stiffness and gene expression [54] and activate adhesion proteins, including focal adhesion kinase (FAK) and talin [8, 40, 218], leading to increased motility. One class of surface receptors, integrins, translate extracellular forces to downstream signaling cascades in a process called mechanotransduction. Increasing substrate stiffness increases integrin binding and clustering, which has implications for several pathways in breast cancer metastasis, including FAK and PI3K signaling [9, 219]. However, most cancer mechanobiology research has focused on collagen- and fibronectin-binding integrins. Integrins that bind to other ECM proteins, including laminin investigated here, have largely been neglected, despite the prevalence of laminins, such as laminin-111 and -511, in the ECM of many tumor types, including breast and prostate cancers [69, 220, 221].
In the breast cancer cell line, MDA-MB-231, laminin-511 enhances cell adhesion and migration [222], while laminin-322 promotes cell survival [223].

EGFR has recently emerged as mechanosensitive [186, 224], and this process is critically relevant to cancer progression, given the known abundance of EGF in breast tumors [225] and frequent acquired resistance to EGFR inhibitors [137]. Residues on EGFR can be phosphorylated by αvβ3 integrin clustering [226], so we postulated that there could be a role for integrins in facilitating EGFR mechanosensing [227].

To uncover whether such a relationship exists, we created a biomaterial system to identify mechanoresponsive proteins working cooperatively downstream of EGFR phosphorylation and laminin-binding integrin engagement in breast cancer. Through a combination of transcriptomics, molecular biology, and quantification of cell adhesion and motility, we found that the intracellular protease calpain 2 is one of these links. Gene expression quantification revealed that both calpain 2 and integrin α6 had an inverse relationship with ECM stiffness. Cell adhesion and motility experiments demonstrated that engagement of integrin α6 resulted in mechanosensitive effects on adhesion and motility in the same manner as EGF stimulation. This suggested coordination of integrin α6 with EGFR and that calpain 2 is downstream of both EGFR phosphorylation and integrin α6 engagement. Further, this indicated that calpain 2 is a common signaling node in the cell that regulates motility and cell adhesion in a mechanosensitive manner. In sum, we highlight the utility of tunable biomaterials systems to uncover previously unrealized relationships between different classes of proteins in breast cancer mechanobiology.

3.3 Materials and Methods

3.3.1 Cell Culture

All cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise specified. MDA-MB-231 cells were a generous gift from Sallie
Schneider at the Pioneer Valley Life Sciences Institute. Highly metastatic and tropic MDA-MB-231 variants were kindly provided by Joan Massagué at the Memorial Sloan Kettering Cancer Center. These cell lines preferentially metastasize to the bone (1833 BoM [2]) brain (831 BrM2a [1]) or lung (4175 LM2 [3]). All cells were cultured in Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) at 37°C and 5% CO₂.

3.3.2 Synthesis of Poly(ethylene glycol)-phosphorylcholine (PEG-PC) Gels.

PEG-PC gels were formed as previously described [51]. For this application, a 17wt% solution of 2-methacryloyloxyethyl phosphorylcholine (PC) (730114-5g, Sigma Aldrich, St. Louis, MO) in pH 7.4 phosphate buffered saline (PBS) was mixed with varying concentrations of poly(ethylene glycol) dimethacrylate (PEGDMA) (Sigma Aldrich). This solution was filtered through a 0.22 µm PVDF syringe filter, and excess oxygen was removed by degassing with nitrogen for 30 seconds. 20wt% Irgacure 2959 (BASF, Florham Park, NJ) was dissolved in 70% ethanol and added to the polymer solution in a ratio of 40 µL Irgacure to 1 mL of polymer solution. 50 µL of this final hydrogel precursor solution was sandwiched between a 3-(trimethoxysilyl)propyl-methacrylate treated 15mm diameter coverslip and an untreated coverslip and UV polymerized for 20 minutes. Gels were swelled in PBS for at least 48 hours before functionalizing the surface using 0.3 mg/mL Sulfo-SANPAH (c1111-100mg, ProteoChem, Hurricane, UT) in 50 mM HEPES buffer at pH 8.5, under UV for 10 minutes. Gels were then flipped onto droplets to achieve a theoretical final concentration of either 10 µg/cm² collagen 1 (A1048301, Thermo Fisher Scientific), 10 µg/cm² collagen 1 + 0.5 µg/cm² β₁-chain-containing laminin isoforms (AG56P, Millipore, Billerica, MA), or 10 µg/cm² collagen 1 + 20 ng/cm² EGF (R&D Systems, Minneapolis, MN), binding via primary amines. These theoretical values are calculated based on the surface area of each hydrogel and amount of protein we supply for functionalization. Gels
were incubated with protein overnight in a hydrated chamber at room temperature, washed in PBS, and sterilized under germicidal UV for 1 hour before cell seeding. For mechanical testing, gels were formed in a cylindrical mold (5µm high, 5µm diameter), allowed to swell for at least 48 hours and compression rheology was performed using an AR2000 (TA Instruments, New Castle, DE), as previously described [51].

3.3.3 RNA Sequencing

Total RNA was isolated using GenElute mammalian total RNA Miniprep kit (RTN70, Sigma Aldrich). The TRUseq stranded RNA LT kit (15032612, Illumina, San Diego, CA) was used to purify and fragment the mRNA, convert it to cDNA, and barcode and amplify the strands. Quality and length of the inserts was confirmed with an Agilent Genomics 2100 bioanalyzer, followed by single-end 75 base pair reads on the MiSeq (Illumina) to generate a complete transcriptome from each sample. Transcripts were aligned to the hg19 human reference genome using the Tuxedo Suite pathway [153, 228-230]. Cufflinks was used to determine statistically significant differential expression of genes (p<0.05) [231-233].

3.3.4 qRT-PCR

Total RNA was isolated from two biological replicates as previously described [55]. 50 ng cDNA was then amplified using 10 pmol specific primers (Table S1) and the Maxima Sybr green master mix (Thermo Fisher Scientific) on a Rotor-Gene Q thermocycler (Qiagen, Valencia, CA) as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 45 cycles at 95°C for 10 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Both β-actin and ribosomal protein S13 were included as reference genes to permit gene expression analysis using the 2^-ddCt method.

3.3.5 Cell Adhesion Quantification
MDA-MB-231 cells were seeded at a final density of 5,700 cells/cm². Cells were either seeded immediately, or pretreated for 30 minutes before seeding with 20 µM ERK inhibitor FR180204 (Sigma Aldrich), 10 µg/mL calpain inhibitor IV (208724, Millipore), 20 µM lapatinib (LC Laboratories, Woburn, MA), or 100 ng/mL EGF. The plate was preincubated on the Zeiss Axio Observer Z1 microscope (Carl Zeiss AG, Oberkochen, Germany) for 1 hour, then cells were seeded onto gels functionalized with 10 µg/cm² collagen 1, 10 µg/cm² collagen 1 + 0.5 µg/cm² laminin, or 10 µg/cm² collagen 1 + 20 ng/cm² bound EGF and imaging was started within 10 minutes of seeding. Images were taken with a 20x objective every 5 minutes for at least 2 hours. Cells were manually traced using Image J (NIH, Bethesda, MD). n ≥ 50 cells per condition.

### 3.3.6 Cell Migration

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Table 0-1. RT-PCR Primers used for gene expression quantification.
Cells were seeded onto gels functionalized with 10 µg/cm² collagen 1 or 10 µg/cm² collagen 1 + 20ng/cm² EGF at a density of 5,700 cells/cm² and allowed to adhere for 24 hours. For inhibitor conditions, cells were treated 2 hours prior to the start of imaging with 10 µg/mL calpain inhibitor IV, 20 µM ERK inhibitor FR180204, 20 µM lapatinib, or 10 ng/mL EGF (R&D Systems). Cells were imaged at 15 minute intervals for 12 hours on the Zeiss Axio Observer Z1 microscope (Carl Zeiss AG). n≥ 48 cells per condition were quantified using the manual tracking plugin for Image J (NIH, Bethesda, MD).

### 3.3.7 Generation of ITGA6 and CAPN2 shRNA transduced cells

The shRNA sets specific to human ITGA6 (Sequence: CCGGCGGATCGAGTTGAT AACGATCTCGAGATGTATCAAACCTCGATCCGTTTTTG) and CAPN2 (sequence: CCGGCAGGAACTACCCGAACACATTCTCGAGAATGTGTTCGGGTAGTTCCTGT TTG) were purchased from Sigma-Aldrich (St. Louis, MO). Plasmids (in pLKO.1-puro) were packaged into virus particles according to manufacturer’s instructions and used to transduce MDA-MB-231-luciferase cells. Stable pools were selected using 1 mg/mL puromycin (Invivogen, San Diego, CA). Cells transduced with a non-targeting shRNA served as a control.

### 3.3.8 Immunofluorescent Staining and Imaging

28,500 cells/cm² were seeded and fixed at 24 hours in 4% formaldehyde. Cells were permeabiliized in Tris-Buffered Saline (TBS) with 0.5% Triton-X and washed 3 times in TBS with 0.1% Triton-X (TBS-T). Blocking was done for 1 hour at room temperature in TBS-T + 2%w/v Bovine serum albumin (BSA, Sigma Aldrich) (AbDil). Cells were then incubated in primary antibody in AbDil for 1 hour at room temperature using 1 or more of the following antibodies; vinculin (V9264-200UL, 1:200, Sigma Aldrich), pEGFR-Y1068 (ab32430, 1:200, Abcam, Cambridge, MA), total EGFR (D38B1, 1:200, Cell Signaling, Danvers, MA),
integrin $\alpha_6$ (ab134565, 1:200, Abcam), integrin $\alpha_3$ (ab131055, 1:200, Abcam), ERK (ab54230, 1:200, Abcam), calpain 2 (MABT505, 1:200, Millipore), or phalloidin 647 (A22287, Thermo Fisher Scientific). For insoluble fractions, cells were permeabilized in cold Tris-Buffered Saline (TBS) with 0.5% Triton-X at 4°C for 1 minute prior to fixing. For immunofluorescent imaging of ECM proteins, cells were fixed at 24 hours (57,000 cells/cm$^2$) or 6 days (11,400 cells/cm$^2$) before staining with a pan-laminin antibody (ab11575, 1:100, Abcam), collagen 1 antibody (ab6308, 1:200, Abcam), or Fibronectin-488 antibody (563100, 1:200, BD Biosciences, San Jose, CA). Molecular Probes secondary antibodies were used at a 1:500 dilution (goat anti-mouse 555 (A21422) and goat anti-rabbit 647 (A21244) or goat anti-rabbit 488 (A11008), Thermo Fisher Scientific). Cells were then treated with DAPI at a 1:5,000 dilution for 5 minutes, and washed in PBS prior to imaging of 2 biological replicates on a Zeiss Cell Observer Spinning Disk (Carl Zeiss AG).

3.3.9 Matrix Decellularization

Cells were cultured on PEG-PC gels of 1, 4, 8, and 41 kPa for 24 hours or 6 days, and the resulting matrix created by the cells was visualized by adapting a previously published protocol [234]. Briefly, cells were washed with warm PBS, then lysed with 500µL warm extraction buffer (PBS with 0.5% Triton-X with 20 mM ammonium hydroxide) for 10 minutes at room temperature. 1 mL of PBS was added and the gels were stored overnight at 4°C before staining as described above.

3.3.10 Protein and Phospho-protein Quantification

Autolyzed calpain 2 was analyzed via western blot. Cells were cultured on PEG-PC gels of 1 or 41 kPa for 24 hours, then lysed in RIPA buffer with protease inhibitors: 1 cOmplete Mini EDTA-free Protease inhibitor cocktail tablet per 10 mL (Roche, Indianapolis, IN) 1 mM phenylmethylsulfonyl fluoride (Thermo Fisher Scientific), 5 µg/mL pepstatin A (Thermo Fisher Scientific), 10 µg/mL of leupeptin (Thermo Fisher Scientific), and
phosphatase inhibitors: Phosphatase inhibitors cocktail-II (Boston Bioproducts, Boston, MA), 1 mM sodium pyrophosphate (Thermo Fisher Scientific), 25 mM β-glycerophosphate (Santa Cruz, Dallas, TX). Lysates were prepared in a 5x reducing sample buffer (39000, Thermo Fisher Scientific), run on Tris-Glycine gels in an Invitrogen mini-Xcell Surelock system, and then transferred to nitrocellulose membranes (88018, Life Technologies, CA). Membranes were blocked with AbDil for 1 hour, then stained overnight at 4°C with antibodies against calpain 2 (MABT505, 1:1000, Millipore), integrin α6 (ab134565, 1:2000, Abcam), EGFR (D38B1, 1:1000, Cell Signaling Technology, Danvers, MA), integrin α3 (343802, 1:1000, Biolegend, San Diego, CA), integrin β4 (ab29042, 1:1000, Abcam), GAPDH (ab9485, 1:2000, Abcam) or β-actin (ab75186, 1:1000, Abcam). Membranes were washed 3 times with TBS-T, stained in secondary antibody (Goat anti-rabbit 680 (926-68021, 1:20,000) and Donkey anti-mouse 800 (926-32212, 1:10,000), LiCor, Lincoln, NE) for 1 hour at room temperature, protected from light, and washed 3 times in TBS-T with a final wash in TBS. Membranes were imaged on the Odyssey CLx (LiCor).

2 biological replicates of lysates were collected during cell adhesion at time 0, at 5 minutes, and at 24 hours. At 5 minutes, the adhered fraction was lysed off the surface, while suspended cells were pelleted, then lysis buffer was added. These 2 fractions were combined for analysis. Phospho-protein levels were quantified with the MAGPIX system (Luminex, Austin, Texas) with MILLIPLEX MAP Multi-Pathway Magnetic Bead 9-Plex - Cell Signaling Multiplex Assay (48-681MAG, Millipore) and added beads against p-EGFR (pan-tyrosine, 46-603MAG, Millipore), p-MEK (Ser222, 46-670MAG, Millipore), and p-ERK1/2 (Thr185/Tyr187, 46-602MAG, Millipore), following the manufacturer’s protocols, with beads and antibodies used at 0.25x.

3.3.11 Co-Immunoprecipitation
MDA-MB-231 cells were grown to confluence in a T-75 cell culture flask. Cells were treated with 10 mL of fresh media containing 10% FBS, with or without an additional 100 ng/mL EGF for 10 minutes. Flasks were lysed in 4.5 mL of lysis buffer, comprised of 50 mM Tris (pH 7.4), 0.15 M NaCl, 0.1% Triton-X, with protease and phosphatase inhibitors as described above. 1 mL of lysate was immunoprecipitated with 10 µL agarose beads (Thermo Fisher Scientific) with either 5 µL rabbit IgG (ab6718, Abcam), 10 µL anti-EGFR (D38B1, Cell Signaling), or with 10 µL anti-EGFR pre-conjugated to sepharose beads (5735S, Cell Signaling). Lysates were precipitated on beads overnight at 4°C on a rotating platform and spun down. Beads (with protein) were washed 3x with TBS, and boiled in 70 µL of DiH₂O and sample buffer. Blotting was done as described above for pEGFR (ab32430, Abcam) and integrin α_6 (ab134565, Abcam).

### 3.3.12 Statistical Analysis

One-way Analysis of Variance (ANOVA) with a Tukey post-test was performed on paired samples using Prism v5.04 (GraphPad software, La Jolla, CA). Data reported is the mean and reported error is standard deviation with significance values of p ≤ 0.05 as *, p ≤ 0.01 as **, and p ≤ 0.001 as ***.

### 3.4 Results

#### 3.4.1 Cells Maximize Expression of Integrin α₆ and Extracellular Laminin on Soft Substrates

To identify ECM and integrin-associated genes involved in breast cancer mechanosensing, we performed whole transcriptome sequencing (RNA-Seq) on MDA-MB-231 cells cultured on collagen 1-functionalized PEG-PC hydrogels ranging in stiffness from 1 to 41 kPa for either 24 hours or 6 days (Figure 3-2). The tumor microenvironment is rich in collagen, which provides structural and biochemical signals to tumor cells to facilitate migration and metastasis [235]. Further, The stiffness range chosen spans that of several
tissues in the body where breast cancer metastasizes (1 kPa - brain [10], 4 kPa - bone marrow [11], 8 kPa - lung [12]), as well as a supra-physiological stiffness (41 kPa) that approximates extremely stiff breast tumors [92] and a tissue culture polystyrene (TCPS) control.
Because the ECM is known to regulate the ability of cancer cells to migrate and metastasize, we focused our in-depth analysis of this RNA-Seq data set on genes that regulate interaction with the ECM. We found that the expression of integrins, other focal adhesion
complex genes, and genes downstream of EGFR phosphorylation became sensitive to stiffness over time (Figure 3-3 a-b). Short-term (24 hour) decreases in expression were clear in integrin α₆, calpain 2, and integrin β₄. However, integrin β₄ expression was 3-fold higher on gels than on TCPS after 6 days of culture, which we confirmed at the protein level (Figure 3-4 a). This implies a switch in dimer pairs, as integrin α₆ preferentially dimerizes with integrin β₄ over integrin β₁ [236]. This is an important finding, as α₆β₄ is known to be important in cancer cell motility and invasion [237]. We compared the day 6 gene expression data from Figure 3-3 b to that at 24 hours from Figure 3-3 a and found that the expression of this subset of genes was generally higher at day 6, suggesting a time-dependent mechanosensing event related to this set of integrins, focal adhesion proteins, and EGFR.

Given that integrin α₆β₄ is laminin-binding, and that we saw mechanosensitive changes in α₆ gene and protein expression (Figure 3-4 b), we hypothesized that we could tune integrin α₆ mechanosensing on these surfaces by supplementing the collagen-functionalized hydrogel surfaces with β₁-chain-containing laminins. On soft gels, integrin α₆ protein
expression was higher at 24 hours with added laminin compared to collagen 1 only, and it was also high after 6 days of culture, even in the absence of supplemented laminin (Figure 3-4 c, Figure 3-5 a). This mechanosensitivity appears to be specific to integrin α6 as other laminin-binding integrins (e.g. integrin α3) did not follow this trend (Figure 3-4 d-e).

As we noted this integrin α6 effect when we supplied only collagen 1, we hypothesized that cells might be producing their own laminin to support integrin α6 binding at the longer culture times. Immunofluorescent staining revealed that laminin, but not collagen 1 (Figure 3-5 b) or fibronectin (Figure 3-6 a), was upregulated on the softer gels at 6

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**Figure 0-4. Cells express integrin α6, but not α3, in a stiffness-sensitive manner.** a. Western blot for protein expression of integrin β4 and integrin β1 on gels of 1, 4, 8, and 41 kPa after 24 hours, compared to TCPS. b. Protein expression of integrin α6 in MDA-MB-231 cells determined via western blot. Cells were cultured on gels or 1, 4, 8, and 41 kPa for 24 hours before lysis and blotting. c. Unmerged images of MDA-MB-231 cells 6 days after plating on gels of 1 or 41 kPa functionalized with collagen 1 only. Vinculin staining is in red and integrin α6 staining in green. Scale bar = 20 µm. d. MDA-MB-231 cells were cultured on gels of 1 or 41 kPa functionalized with 10 µg/cm² for 6 days before fixing. Cells were stained for vinculin (red) and integrin α3 (green) and nuclei were labeled with DAPI (blue). Scale bar = 20 µm. e. Western blot for integrin α3 on gels of 1 kPa, 41 kPa, and on TCPS after 24 hours.
days. We separately confirmed that differential staining was not due to ECM proteins crosslinked to the gels or from serum (Figure 3-6 b). Cultures that were decellularized after 6 days showed the highest amount of extracellular laminin on the softest, 1 kPa gels (Figure 3-5 b), concordant with the increase in integrin α6 expression in Figure 3-5 a.

To understand the impact of laminin and integrin α6 expression on cell phenotype, we quantified cell spreading during initial adhesion to the hydrogel surfaces. We found cells spread to similar extents across the 4 stiffnesses examined here when only collagen 1 was present (Figure 3-5 c). However, when laminin (0.5µg/cm²) was added to the collagen (10µg/cm²) on the gel surfaces, cell spreading was mechanosensitive (Figure 3-5 d). We

![Figure 0-5. Cells on soft substrates produce extracellular laminin.](image)
a. Representative immunofluorescent staining of vinculin (red) and integrin α6 (green) and DAPI (blue) in cells cultured on gels with collagen only for 24 hours, collagen with laminin for 24 hours, or collagen only for 6 days. Yellow denotes overlap of vinculin and integrin α6 staining. Scale bar = 20 µm. b. Representative Immunofluorescent staining for ECM proteins after 1 day of culture (left), decellularized gels after 24 hours (center) or decellularized gels after 6 days (right). Collagen 1 = red, pan-laminin = green, DAPI = blue. Scale bar = 100 µm. c-d. Cell area during adhesion quantified on 1, 4, 8, and 41 kPa gels functionalized with collagen 1 only (c) or collagen 1 + laminin (d). N=3 biological replicates, n=75 cells per condition.
attempted to remove collagen from the experiment completely, but found that cells did not adhere to laminin-only hydrogels (data not shown). This was not surprising given the documented role of laminin being somewhat anti-adhesive [238]. In sum, cells upregulate integrin α₆ expression on soft substrates, which correlates with secretion of extracellular laminin. This coordination appears to take several days, but the mechanosensitivity effect on cell spreading can be accelerated by adding β₁-chain-containing laminin to the culture substrata.

### 3.4.2 Cell Response to EGF Stimulation Mirrors Mechanosensing on Laminin

Given the prevalence of EGF in the tumor microenvironment and its known role in promoting cancer cell motility, we posited that the mechanosensitive expression of EGFR alongside the laminin-binding integrin α₆ implied that they mechanotransduce under a related pathway. Similar to previous reports, we found that MDA-MB-231 cells spread to a smaller area on hydrogels upon addition of EGF prior to and during adhesion [55], achieving a similar size to cells on soft, laminin-coated gels without EGF (Figure 3-7 a). EGFR is known

![Figure 0-6. Media changes do not result in deposited matrix.](image)
to associate with the $\alpha_6\beta_4$ integrin heterodimer to enhance clustering and response to EGF [239], and in agreement with this report, we found that the addition of EGF increased the association of integrin $\alpha_6$ with EGFR in our system (Figure 3-9 a). We separately analyzed the protein expression of integrin $\alpha_6$ and EGFR in variants of the MDA-MB-231 cell line selected for their ability to metastasize specifically to the brain, bone, and lung [1-3]. The brain tropic cells, which metastasize to the softest of these sites, have the highest expression of both integrin $\alpha_6$ and EGFR (Figure 3-9 b).

To test the necessity of integrin $\alpha_6$ in the observed response to EGF, we generated a stable shRNA knockdown cell line (shITGA6, Figure 3-9 c-e). We quantified adhesion of the shITGA6 cells to collagen-functionalized gels, and found that they spread to a larger area...
during adhesion with EGF stimulation, compared to cells transfected with the scramble control (shSCR), suggesting a role for integrin α6 during EGF response on collagen (Figure 3-7 b). Even though there was no laminin present, others have demonstrated a role for integrin α6 in facilitating growth factor response, independent of laminin binding [240]. Combined with our data, this implicates a mechanosensitive role for integrin α6 during EGFR signaling (Figure 3-7 b).

3.4.3 EGF Overwhelms Laminin-Based Mechanosensing and Maximizes Cell Motility on Soft Substrates

To further explore the relationship between integrin α6 and EGFR signaling during

![Cell spreading and migration images](image)

**Figure 0-8. EGFR mechanosensitive motility requires integrin α6.** a. Representative images of cells seeded onto gels of 1 and 41 kPa functionalized with collagen 1 and EGF stained for vinculin (red), EGFR (green), F-actin (purple), and DAPI (blue). b. Area was quantified 40 minutes after adhesion to 10 µg/cm² collagen 1 with no EGF, soluble EGF (sEGF), or bound EGF (bEGF). Statistics are shown relative to the control bar. N=2, n=50 cells. c. Parental MDA-MB-231 cell speeds were quantified on 10 µg/cm² collagen 1 with no EGF, with 10 ng/mL soluble EGF, 20 ng/cm² bound EGF. shITGA6 cell speeds were quantified on 10 µg/cm² collagen 1 + 20 ng/cm² bound EGF. N=2, n=48 cells. Asterisks denote significance (p<0.05) relative to the no EGF control at the same stiffness.
adhesion, we functionalized hydrogels with collagen 1 and laminin, and treated the cells with lapatinib, a dual tyrosine kinase inhibitor against EGFR and HER2. This approach allowed cells to engage integrin α6 during adhesion, while preventing EGFR signaling by obstructing ATP-binding on the intracellular domain of EGFR and HER2. When pretreated with lapatinib, we found that cells adhering on 41 kPa hydrogels spread to a significantly larger

Figure 0-9. Extension of integrin α6 cell assays. a. Co-immunoprecipitation of EGFR and integrin α6 either without EGF (-) or with EGF (+). Lysates were pulled down with Rabbit IgG and agarose protein A beads (IgG), EGFR antibody with agarose protein A beads (EGFR), or with sepharose beads with pre-conjugated EGFR antibody (Seph). b. Western blot of integrin α6 and EGFR from parental MDA-MB-231 cells (231), and cells selected for tissue specific metastasis to the bone (BoM), brain (br), or lung (LM2) by the Massagué group, assayed on TCPS [1-3]. c. mRNA expression of integrin α6 determined via qRT-PCR in integrin α6 knockdown cells (shTGA6) and scramble control (shSCR). Data is normalized to shSCR cells. d. Surface expression (determined by FACS) of scramble control and shITGA6 knockdown cells. e. Permeabilizing the cell membrane before fixing and immunofluorescent staining confirmed that there is less integrin α6 associated with focal adhesions in the knockdown cells compared to a scramble shRNA control. Cells were immunofluorescently stained for insoluble vinculin (red), insoluble integrin α6 (green) and DAPI (blue). f. Cells were cultured on collagen with either no EGF (left), 100 ng mL⁻¹ soluble EGF for 10 minutes (middle), or 20 ng cm⁻² EGF bound to the surface for 24 hours (right). Scale bar = 20 µm. Fixed cells were stained for vinculin (red), EGFR (green), and DAPI (blue). Data gathered in collaboration with Dr. Lauren Barney and Dr. Christopher Hall.
area than cells on 1 kPa hydrogels (Figure 3-7 c). This result mimics the adhesion we observed of cells on laminin without supplemented EGF or lapatinib (Figure 3-5 d). Further, when we supplemented cells with soluble EGF on the surfaces functionalized with collagen 1 and laminin, cells on both 1 and 41 kPa spread to a smaller area (Figure 3-7 d). This EGF-induced reduction in spreading is similar to our observations of cells on collagen 1 only in the presence of soluble EGF (Figure 3-7 a). Together, these data suggest that, even in the presence of laminin, adhesion signaling through EGFR dominates, but in the absence of exogenous EGF, signaling via mechanosensitive integrin α6 controls cell spreading during adhesion.

We observed that soluble EGF (sEGF) caused rapid internalization of EGFR, within 10 minutes of administration (Figure 3-9 f). To promote sustained EGFR signaling to examine its relationship with integrin α6 [239], we covalently linked both collagen 1 and EGF to the surface of the hydrogels. This EGF presentation minimized EGFR internalization and localized both pEGFR(Y1068) and total EGFR to regions of vinculin staining (Figure 3-8 a). EGFR receptor internalization was required for EGF to moderate adhesion, as only cells supplemented with soluble EGF showed measurable differences in cell area after 40 minutes (Figure 3-8 b). However, bound EGF had a measurable effect on cell motility (Figure 3-8 c). First, we observed a biphasic trend in cell speed with respect to stiffness. This phenomenon has previously been described in many cell types, including smooth muscle cells [45] and glioma cells in both 2D [241] and 3D [242]. In this migration study, only collagen 1 was present, suggesting that collagen-binding integrins contributed to this mechanosensitive motility after 24 hours [70]. Our data agree with literature reports demonstrating that cells seeded on collagen 1 exhibit mechanosensitive motility [243], but do not differ in adherent area between 1 and 30 kPa in some melanoma cell lines [244]. The addition of bound EGF increased cell motility across the stiffness range tested here (Figure 3-8 e), and shifted the
biphasic curve to softer substrates, requiring us to expand the mechanical range of the hydrogels to capture the full curve [51]. We also observed a role for integrin α<sub>6</sub> in regulating this EGF-dependent motility, as shITGA6 cells were less responsive to EGF than the control (Figure 3-8 c). The scramble control cells, which peaked at 34 µm/hr on 1 kPa gels, were more responsive to EGF than the shITGA6 cells. Interestingly, shITGA6 cells don’t respond to changes in stiffness from 1 to 18 kPa, either in control media, or in response to soluble EGF (data not shown). Together, this data suggests that the addition of EGF alters mechanosensitive motility and that the MDA-MB-231 breast cancer cells are dependent on

![Figure 0-10. Calpain 2 is downstream of both mechanosensitive integrin α<sub>6</sub> and EGFR.](image)
a. Clustering of PCR data on genes expressed after 24 hours of culture on gels functionalized with 10µg/cm<sup>2</sup> collagen 1 (data in Fig. 1a). b. Calpain 2 activity during adhesion was measured by evaluating the ratio of autolyzed/full length calpain 2 via western blot in both the adhered and suspended cell fractions during the first 60 minutes, or just the adhered fraction after 24 hours. N=2. c-d. Cell area was quantified during adhesion in the presence of an ERK1/2 inhibitor or calpain 2 inhibitor on 1 kPa (c) and 41 kPa (d) gels. N=2, n=50. e. Cell migration speeds quantified the presence of an ERK1/2 or calpain 2 inhibitor. N=2, n=48. f. Representative western blot for full length and autolyzed calpain 2, with loading control GAPDH. In order of lanes from left to right: 1 kPa with collagen 1, 41 kPa with collagen 1, TCPS, 1 kPa with collagen 1 + 100ng/mL soluble EGF for 10 minutes, 41 kPa with collagen 1 + 100ng/mL soluble EGF for 10 minutes, TCPS + 100ng/mL soluble EGF for 10 minutes, 1 kPa with collagen and laminin, 41 kPa with collagen and laminin.
integrin $\alpha_6$ for this response.

### 3.4.4 Calpain 2 is Downstream of both EGFR and Integrin $\alpha_6$ Mechanosensing

Inspired by the trends observed in the RNA-Seq data (Figure 3-2), we clustered our PCR data from the 24 hour time point, which uncovered that ITGA6 and EGFR expression clustered with the intracellular protease CAPN2 (Figure 3-10 a). Calpain 2, which has been implicated in focal adhesion formation and disassembly, is thought to play an important role in breast cancer cell motility and metastasis [245]. Because calpain 2 is activated by ERK [246], we performed phospho-analysis of multiple kinases in integrin- and EGFR-dependent pathways (Figure 3-11 a). Steady-state phosphorylation of EGFR, MEK, and ERK increased on stiffer surfaces, as previously demonstrated [37, 186, 247] (Figure 3-11 b). However, we observed the opposite trend with the activation of calpain 2 (Figure 3-11 c).

We observed higher calpain 2 activity (autolysis) during adhesion to the soft hydrogels compared to tissue culture plastic (Figure 3-10 b). Although repeated multiple times, this trend was not statistically significant. There was a functional role for calpain 2 during mechanosensing on the gels, however, as a pharmacological inhibitor to either calpain 2 or ERK had no effect on cell spreading on 1 kPa gels, but resulted in significantly larger cells on 41 kPa gels (Figure 3-10 c-d). In parallel, to provide evidence of the necessity of calpain 2 in responding to EGF stimulation, we generated a CAPN2 knockdown cell line (Figure 3-12 a-c), and observed that those cells were less responsive to EGF than the scramble control cells during adhesion, independent of stiffness (Figure 3-12 d). Further, calpain 2 inhibition was sufficient to limit cell response to soluble EGF using a pharmacological inhibitor (Figure 3-12 e). However, when we limited calpain 2 activity in integrin $\alpha_6$ knockdown cells, the effect of larger cells during adhesion was exaggerated.

We further hypothesized that altering calpain 2 activity could disturb the typical biphasic relationship between cell migration speed and matrix stiffness given its known role
in mediating focal adhesion turnover [246]. Inhibiting ERK activity entirely eliminated the durokinesis effect, and calpain 2 inhibition shifted the migration curve maximum to lower stiffnesses (Figure 3-10 e). The limiting step during motility on stiff surfaces is retraction of the rear edge [248], and here we demonstrate that inhibiting calpain 2 reduces cell migration on stiffer substrates, likely because of this reduced ability to turn over adhesions. We then
compared migration speeds of the integrin α6 knockdown and scramble control cells from figure 3-8, in response to the calpain 2 inhibitor. We found that the scramble control cells responded to the inhibitor similarly to the parental cells, although the addition of soluble EGF was able to partially overcome that inhibition (data not shown). However, the shITGA6 cells exhibited slower migration at 18 kPa even in the control media, so the calpain 2 inhibitor had no additional effect.

After 24 hours of culture on PEG-PC gels with collagen 1 or collagen 1 and bound EGF, cells adopted a normal morphology with no noticeable protrusions (Figure 3-12 f). However, in the presence of lapatinib, ERK inhibitor, or calpain 2 inhibitor, we observed that cells left behind long, stable protrusions at the rear of the cell (Figure 3-12 f), indicative of their inability to release adhesion sites [249]. There were minimal observable differences in morphology as a function of stiffness, regardless of the pharmacological treatment applied. This suggests that at these concentrations, the inhibitors impact focal adhesion turnover enough to functionally alter motility with marginal impacts on morphology at these stiffnesses.

While we demonstrated earlier that integrin α6 is necessary for both mechanosensitive and EGFR-mediated adhesion, this data suggests that EGFR activates calpain 2 to regulate mechanosensitive motility in breast cancer cells. To some extent on collagen-functionalized hydrogels, and particularly on plastic, calpain 2 activity was low, and EGF stimulation increased calpain 2 activity on the hydrogels (Figure 3-10 f). Finally, when we supplemented the gels with laminin, calpain 2 activity was maximized on both stiffness gels, suggesting an additional role for integrin α6 engagement to activate calpain 2. Interestingly, when integrin α6 is knocked down in a stable manner, the cells still exhibit calpain 2 activity, suggesting that the cells might be compensating for the lack of integrin α6 signaling via another mechanism, not explored here (Figure 3-12 g). Together, these data suggest that the
mechanosensitive receptors integrin α6 and EGFR both activate calpain 2 to facilitate

Figure 0-12. Knockdown of calpain 2 limits response to EGF on hydrogels. a-b. mRNA expression (a; determined via qRT-PCR) and protein expression (b; determined by western blot) of scramble control (shSCR) and calpain 2 knockdown (shCAPN2) cells. c. Permeabilizing the cell membrane before fixing and immunofluorescent staining confirmed that there is less calpain 2 associated with focal adhesions in the knockdown cells compared to a scramble shRNA control. Cells were immunofluorescently stained for insoluble ERK (red), insoluble calpain 2 (green) and DAPI (blue). d. shCAPN2 cells are less responsive to EGF during adhesion than the shSCR control cells. All area measurements are normalized to time 0. e. Adhesion of scramble control cells (shSCR) or integrin α6 knockdown cells (shITGA6) was quantified on gels of 1 and 41 kPa functionalized with 10 µg/cm² collagen 1. Cells were pre-treated with either 10 µg/mL calpain 2 inhibitor or 10 µg/mL calpain 2 inhibitor + 100 ng/mL soluble EGF. f. Cell morphology was analyzed 24 hours after seeding onto PEG-PC gels of 1 or 41 kPa functionalized with either 10µg/cm² collagen 1 or 10µg/cm² collagen 1+ 20 ng/cm² EGF. Cells on collagen only were dosed with lapatinib, ERK inhibitor, or calpain 2 inhibitor 22 hours after seeding onto PEG-PC gels of 1 or 41 kPa. Cell morphology was analyzed 2 hours after treatment with inhibitors. g. Western blot on scramble control cells or integrin α6 knockdown cells on 1 kPa gels functionalized with 10 µg/cm² collagen 1 (with or without 100ng/mL soluble EGF) or 10 µg/cm² collagen 1 + 0.5 µg/cm² laminin, compared to TCPS. Data gathered in collaboration with Dr. Christopher Hall.
3.5 Discussion

This work demonstrates integrin α6 and calpain 2 as mechanosensitive proteins important for stiffness-driven breast cancer cell adhesion and migration (Figure 3-13 a). The mechanosensitivity of calpain 2 is a new finding, as well as the role of integrin α6 in EGFR signaling that facilitates cell adhesion (Figure 3-13 b, Figure 3-1). EGF is known to enhance breast cancer cell adhesion and motility, and here we demonstrate that mechanosensitive integrin α6 and EGFR both signal through calpain 2. We also contribute that in the absence of EGF, breast cancer cells on soft gels, or any stiffness gel supplemented with EGF, were small and motile. Cells on soft gels produced their own laminin to engage α6, or we added it exogenously. Our data suggests this increases activation of ERK and Calpain 2, which feeds back to increase turnover of focal adhesions. During adhesion to substrates, cells were more responsive to laminin on soft substrates but did not adhere as well on stiff substrates with a calpain 2 inhibitor. Cell adhesion was facilitated in the presence of EGF on both soft and stiff gels. After 24 hours on gels, calpain 2 activity decreased with increasing stiffness, while EGFR phosphorylation increased with stiffness without added EGF. Cell motility had a biphasic dependence on substrate stiffness.

Figure 0-13. Mechanosensitive signaling is dependent on laminin and substrate stiffness. a. Breast cancer cells on soft gels, or any stiffness gel supplemented with EGF, were small and motile. Cells on soft gels produced their own laminin to engage α6, or we added it exogenously. Our data suggests this increases activation of ERK and Calpain 2, which feeds back to increase turnover of focal adhesions. b. During adhesion to substrates, cells were more responsive to laminin on soft substrates but did not adhere as well on stiff substrates with a calpain 2 inhibitor. Cell adhesion was facilitated in the presence of EGF on both soft and stiff gels. After 24 hours on gels, calpain 2 activity decreased with increasing stiffness, while EGFR phosphorylation increased with stiffness without added EGF. Cell motility had a biphasic dependence on substrate stiffness.
of EGF stimulation, integrin α\textsubscript{6} and calpain 2 regulate stiffness-dependent adhesion and motility (Figure 3-13 b).

We focused this study on breast cancer cells because of the important roles for ECM binding and stiffness in metastasis. Cancer metastasis begins with individual or collective groups of cells migrating away from the primary tumor site [18, 250, 251]. This motility is driven in part by cell-ECM adhesion via integrins and the creation and turnover of focal adhesions, both of which are known to be sensitive to the stiffness of the surrounding ECM [252]. Integrins have well-documented responses to changes in the microenvironment stiffness. As two examples, the expression of integrins α\textsubscript{2}, α\textsubscript{3}, and β\textsubscript{1} is higher in mouse mammary epithelial cells cultured on TCPS compared to cells on a soft basement membrane-like matrix [70], and integrin α\textsubscript{5} expression was found to increase 5-fold on a 10 kPa stiffness compared to 1 kPa in cancer cells [69] and over a similar range in fibroblasts [253]. Most of this work has focused on collagen- and fibronectin-binding integrins, with the exception of one paper on integrin α\textsubscript{6} in fibroblasts, where expression increased with stiffness, resulting in increased invasion [254]. We demonstrate here the mechanosensitivity of integrin α\textsubscript{6} in breast cancer cells, and the downstream regulation of cell adhesion, migration, and response to EGF (Figure 3-5 a, Figure 3-7 b). Integrin α\textsubscript{6} is a laminin-binding subunit, and so many of the stiffness-sensitive phenotypes we observed either required, or were exaggerated by, addition of laminin to the culture substrate. However, cells exhibited mechanosensitive behavior on collagen alone when cells were on hydrogels for longer times. For example, we observed a biphasic relationship in migration speeds on collagen 1 only (Figure 3-12 e). We present data that on soft surfaces, cells produce extracellular laminin and engage integrin α\textsubscript{6} in these instances, and that knocking down integrin α\textsubscript{6} expression limited response to EGF (Figure 3-8 c).
Previous work has established the role of integrin α₆ in key aspects of the metastatic cascade. Low integrin α₆ expression inhibits the migration and proliferation necessary for establishing metastases at distant secondary sites [255]. Additionally, integrin α₆ expression can protect cells from radiotherapy treatment both through PI3K and MAPK activity [256].

In a cohort of 80 patients, integrin β₄ was co-expressed with integrin α₆ and laminin in primary tumors, and co-expression of α₆β₄ with laminin production was significantly correlated with breast cancer relapse and death [236, 257]. This is further supported by other work in breast cancer, that elucidates the role of laminin-511 in cell adhesion and migration [222], which are key phenomena in the metastatic cascade. Our data demonstrate that this is a mechanoresponsive effect, as the most laminin is secreted by the breast cancer cells on the softest substrate (Figure 3-5 b). Interestingly, this phenomenon is not maintained in other secreted ECM proteins, such as collagen 1 (Figure 3-5 b) or fibronectin (Figure 3-6 a), suggesting a key role for integrin α₆ binding to laminin on soft surfaces. This is possibly mediated through the mechanically responsive transcription factor, TAZ [258], which has been shown to regulate laminin production [259]. Here we found that both integrin β₄ expression and laminin production increased after 6 days of culture on soft gels (Figure 3-3 b, Figure 3-5 b), where EGF enhances migration in an integrin α₆-dependent manner (Figure 3-8 c).

We further observed that during cell adhesion, integrin α₆ engagement with laminin decreased cell size and facilitated adhesion similarly to EGF (Figure 3-7 a-b). Smaller cells can be correlated with a cancer stem cell and invasive phenotype [121], and our work suggests integrin-mediated mechanotransduction contributes to that behavior. The α₆β₄ integrin dimer associates with EGFR [239], and we observed that integrin α₆ engagement increased the sensitivity to EGF (Figure 3-8 c). Further, our data agrees with other work, which has demonstrated that the integrin dimer α₆β₄ plays an important role in cell response
to growth factors, even in the absence of laminin [240]. We also observed that integrin $\alpha_6$ adhesion shows a similar phenotypic response on soft hydrogels as EGF stimulation.

Downstream of integrin $\alpha_6$ engagement and EGFR phosphorylation, cells activate calpain 2, which facilitates focal adhesion turnover, and therefore, motility (Figure 3-10 e-f). EGFR phosphorylation is necessary for mechanosensing [224] and activity in downstream effectors, including MEK, ERK, and calpain 2 at the cell membrane [260, 261], to cleave focal adhesion proteins vinculin, talin, paxillin, and FAK [262, 263]. Additionally, recent work has demonstrated that calpains are recruited to protrusions containing integrin $\beta_4$ [264]. During adhesion, we saw that calpain 2 activity was higher in cells on the hydrogels compared to TCPS, which was consistent with RNA levels (Figure 3-3 a-b, Figure 3-11 c). Calpain 2 inhibition during adhesion resulted in larger, less motile cells on stiff substrates. The opposite trend in adhesion was observed when activating this pathway, as cells spread to a smaller area at both 1 and 41 kPa in the presence of soluble EGF (Figure 3-7 a). Together, these data suggest that EGFR phosphorylation could be a stronger activator of calpain 2 than stiffness. Previous work has shown that calpain 2 has a significant correlation with an epithelial-to-mesenchymal transition [265] and lymphatic or vascular invasion, where patients with basal-like tumors and high calpain 2 expression had a significantly worse prognosis [113]. Others have also found that this is an isoform-specific effect, as calpain 2, but not calpain 1, was upregulated in gastric cancer compared to healthy tissue [266]. Our work demonstrates a mechanosensitive role for calpain 2, downstream of both integrin $\alpha_6$ and EGFR, providing further evidence for its potential as a druggable target for metastatic breast cancer.

3.6 Conclusions

Mechanical forces have long been thought to play a role in tumor progression, and here we shed new light on two mechanoresponsive proteins that influence breast cancer cell
motility: integrin α6 and calpain 2. First, enhanced integrin α6 expression on soft substrates is associated with increased adhesion and laminin secretion. Second, we find that both integrin α6 and EGFR activate calpain 2 in a mechanosensitive manner, which mediates cleavage of focal adhesion proteins necessary for motility. Others have suggested targeting calpains to inhibit cancer cell invasion [267], and here we provide a mechanosensitive role of calpain 2 using tunable hydrogel substrates.

3.7 Future work

Targeting features of the extracellular matrix to limit cancer metastasis has met with little success this far. However, mediating the ability of cells to interact with the microenvironment has resulted in several clinically approved drugs. For example, Lapatinib, the dual-kinase HER2/EGFR inhibitor is widely used, although patients tend to develop resistance, as discussed in depth in chapter 2. Further, lapatinib can have varied effects on the cell phenotypes observed in this chapter, like cell migration (Figure 3-14). There are few good strategies to predict the dose of drug that will reach the tumor, and this data shows that low doses have little effect on cell motility at 24 hours in our system, but can instead

![Migration Speed fold change from control on TCPS with Lapatinib](image)

**Figure 0-14. Lapatinib dose and timing impacts cell motility.** Cells were dosed with 1, 5, 10, or 20 µM laminin at 2, 6, or 24 hours prior to the start of cell migration. Cell migration was tracked over 12 hours on TCPS.
dramatically increase cell motility on a rigid surface at short times. Therefore, more work is needed to better understand how altering tumor cell interaction with the microenvironment, by targeting mechanosensitive kinases or proteases, is necessary for the advancement of the field.
CHAPTER 4
SOFT MATERIAL CONDITIONING OF BREAST CANCER CELLS
ALTERS METASTATIC POTENTIAL

4.1 Abstract

Mechanical features of the tumor microenvironment have been largely neglected in the study of tumor evolution. A breast cancer cell population evolves in parallel at the primary tumor and metastatic sites, so we hypothesized that the timing of dissemination and secondary site tissue stiffness influences whether individual cell clones survive and grow out to form lethal metastases. Changes in stiffness alter many cell behaviors that are essential for success in the metastatic cascade, such as growth, motility, and ECM remodeling. We employed biomaterials to mechanically condition breast cancer cell lines over several passages, ultimately driving short and long term changes in cell behavior. We separately demonstrated that subclones exhibit a range of growth rates and migration behaviors that potentially explain the altered phenotypes we observed over time. We then challenged our mechanically conditioned populations with culture back on rigid plastic, and found they retain motility and chemotactic behaviors, but revert back to their original proliferation and wound healing phenotypes. These phenotypes were compared to primary breast tumor cells from an MMTV-PyMT model, where we found that cells that had never been exposed to rigid plastic looked most similar to cells that we had mechanically conditioned on soft, 1 kPa hydrogels over several months. Together, this data suggests that breast cancer cells adapt to local stiffness over the extended times they reside at a metastatic site, which drives population-level changes that result in altered growth and motility.

In order to explore the role of tumor evolution, the most important parameter to consider is the timing of cell exposure to biomaterials and the length of conditioning (Figure...
4-1). We changed this feature of the model in a few specific ways. First, stable cancer cell lines were conditioned on biomaterials for several months and assayed at early- and late-stage conditioning, before being re-challenged with brief culture on extremely rigid plastic to test cell memory of their previous environments. The second mechanism of changing the experimental timing, is by working with primary tumor cells that have never been conditioned or grown on rigid TCPS. Here, we observed low growth of our primary tumor cells in culture, so they were only exposed to biomaterials for short periods of time before being assayed, which yielded similar, although not identical phenotypes to existing cell lines conditioned over months after coming off plastic. In addition to time, we chose to explore the role of soft biomaterial environments, because similarly to drug treatment stress, soft environments can limit cell growth (Figure 4-1). This challenge to cell adhesion and growth over time likely promotes population-level alterations in phenotype over time, to better understand how tissue stiffness changes tumor evolution over long time scales.

4.2 Introduction
Cancer cells respond to the mechanical properties of the extracellular matrix (ECM) [9], but little is known about how these physical features drive cellular and tumor evolution. Breast tumors are highly heterogeneous and drug treatment is known to enhance mutagenesis and clonal selection [73]. For example, treatment with EGFR inhibitors frequently results in secondary genetic mutations or amplification of parallel pathways, resulting in resistance [268-270]. Further, these drug resistant populations can be obtained and isolated from outgrowth of persisting subclones after drug treatment [119]. The role of these different persisting clones is not fully understood, but they have been implicated in drug resistance and metastasis [72]. Independent of drug treatment, genetic lineage tracing has been used to identify several branch points, within the primary tumor and at distant metastatic sites [71]. However, tumor evolution is not linear, and two landmark studies recently demonstrated that breast cancer cells leave the primary tumor at very early time points [74, 75], and undergo parallel evolution at the secondary site.

The mechanical properties of the ECM inform cell migration, growth, drug resistance, and metastasis [9, 52, 271, 272]. Upon initial malignant transformation, a tumor cell might reside in a soft, healthy-like ECM, which can either stiffen in situ [273], or the tumor cell can migrate to a distant site, with distinct mechanical properties. The most frequent breast cancer metastatic sites have distinct stiffness profiles, ranging from extremely soft brain tissue to stiffer tissues in the lung [10, 12]. Response to the ECM is mediated, at least in part, through focal adhesions, where changes in matrix stiffness induce conformational changes of receptors and associated intracellular proteins that enable binding of structural proteins, like vinculin, actin, and myosin, and activate signaling molecules, like Akt, ERK, and calpains [41, 253, 256, 274, 275]. Engaging structural cell elements enables a force balance, where cell assembly of actin-myosin fibers pull on the ECM to initiate processes like matrix remodeling and cell migration [241]. Increased stiffness also enhances
integrin clustering [276] and survival and growth signaling via downstream cascades like Akt and RAF [129].

Because each secondary site has unique physical properties, the length of time that a cell or cell cluster resides there likely impacts cell behavior. Cancer cells adapt to a soft biomaterial environment over time, and are still changing after 3 passages on compliant polyacrylamide substrates [108]. In addition to extended exposure to the mechanical properties of the secondary site, recent work demonstrates that cells retain a memory of their mechanical environment, termed mechanical memory [106, 277]. It is important to note here that this memory scales with the length of conditioning. Together, these studies suggest that the mechanics of the secondary tissue site and cell residence time can have a dramatic impact on cell phenotype.

While several groups have independently examined the impact of tissue stiffness, timing, or cell clonality on cancer dissemination, to the best of our knowledge, this is the first study to examine the combined effects of these parameters on breast cancer metastasis. Here we mechanically conditioned breast cancer cells on soft or stiff biomaterials over several months to select for a population that is less proliferative in a rigid environment, but more motile when assayed on soft biomaterials. This top-down approach was compared to a limiting dilution selection of single subclones, which exhibited a range of proliferation and migration similar to those cells selected on biomaterials. Together this work suggests that the mechanical properties of the secondary site can select for survival and growth of a set of subclones, which might be altered with time and the diversity of clones that are seeded at a distant metastatic site.

4.3 Materials and Methods

4.3.1 Synthesis of poly(ethylene glycol)-phosphorylcholine (PEG-PC) Hydrogels
PEG-PC gels were formed as previously described [51]. For this application, a 17wt% solution of 2-methacryloyloxyethyl phosphorylcholine (PC) (730114-5g, Sigma Aldrich, St. Louis, MO) in pH 7.4 phosphate buffered saline (PBS) was mixed with varying concentrations of poly(ethylene glycol) dimethacrylate (PEGDMA) (Sigma Aldrich). This solution was filtered through a 0.22 μm PVDF syringe filter, and excess oxygen was removed by sparging with nitrogen for 30 seconds. 20wt% Irgacure 2959 (BASF, Florham Park, NJ) was dissolved in 70% ethanol and added to the polymer solution in a ratio of 40 μL Irgacure to 1 mL of polymer solution. 50 μL of this final hydrogel precursor solution was sandwiched between a 3-(trimethoxysilyl)propyl-methacrylate treated 15 mm diameter coverslip and an untreated coverslip and UV polymerized for 20 minutes. Gels were swelled in PBS for at least 48 hours before functionalizing the surface using 0.3 mg/mL Sulfo-SANPAH (c1111-100mg, ProteoChem, Hurricane, UT) in 50 mM HEPES buffer at pH 8.5, under UV for 10 minutes. Gels were then flipped onto droplets to achieve a final protein concentration of either 5 or 10 μg/cm² collagen 1 (A1048301, Thermo Fisher Scientific). Gels were incubated with protein overnight in a hydrated chamber at room temperature, washed in PBS, and sterilized under germicidal UV for 1 hour before cell seeding.

4.3.2 Cell Culture

All cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise specified. MDA-MB-231 cells were a generous gift from Sallie Schneider at the Pioneer Valley Life Sciences Institute and Hs578T cells were provided by Mario Niepel. All cells were cultured in Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) at 37°C and 5% CO₂. For extended culture conditions, cells were passaged as usual on PEG-PC gels of 1 kPa or 41 kPa functionalized with 10 μg/cm² collagen 1, alongside cells grown on tissue culture polystyrene (TCPS). For cell mechanical memory assays, after growth on biomaterials, cells
were plated back onto TCPS. Cells were maintained and passaged as usual. Cells were assayed for motility and invasion as described below.

4.3.3 Subclonal Cell Selection

mCherry labeled MBA-MD-231 cells were selected in RPMI with 10% FBS and 1% P/S. RFP labeled Hs578T cells were cultured in DMEM with 10% FBS and 1% P/S. Individual cell lines were plated at the density of 1 cell/well in 96-well plates. Plates were imaged to identify wells containing exactly 1 cell. Cell culture media was replenished every 2-3 days. Subclones were expanded from 96-well plates to 12-well plates to 6-well plates to one 10-cm dish. Each plate transfers was performed when the cells were confluent.

4.3.4 Proliferation Assays

2D cell proliferation assays were performed on TCPS in a 96-well plate. Cells were seeded at a density of 3,125 cells/cm² and growth between day 1 and day 4 was quantified using a Cell Titer 96 MTS assay (Promega, Madison, WI). For comparison to proliferation on 2D PEG-PC hydrogels, cells were seeded at a density of 260 cells/cm² in a 24 well plate on gels of 1, 8, 41, or 500 kPa functionalized with 5 µg/cm² collagen 1, and on TCPS. 10 random images per well were quantified on day 1 and day 6 to calculate the fold change in cell number. To quantify 3D growth, cells were suspended at 2x in cold serum free media, and mixed 1:1 with Matrigel (Corning Life Sciences, Corning, NY). 10 µL gels were formed at 37°C for a final cell concentration of 100 cells/µL.

4.3.5 Random Cell Migration

Cells were seeded onto gels functionalized with 10 µg/cm² collagen 1 at a density of 5,700 cells/cm² and allowed to adhere for 24 hours. Cells were imaged at 15 minute intervals for 12 hours on the Zeiss Axio Observer Z1 microscope (Carl Zeiss AG). n≥ 48 cells per condition were quantified using the manual tracking plugin for Image J (NIH, Bethesda, MD).
4.3.6 Collective Motility

Cells were seeded onto TCPS at a density of 30,000 cells/cm² and allowed to adhere for 24 hours. 1 hour prior to the assay start, a scratch was made in the confluent cell layer with a p200 pipette tip. The same frame of cells was imaged at time 0 and after 12 hours with the Zeiss Axio Observer Z1 microscope (Carl Zeiss AG) and the width of the scratch was determined using Image J (NIH). The percent wound closure after 12 hours was determined.

4.3.7 Traction Force

Traction force was analyzed using a polydimethylsiloxane micropost array (PMA) method as described previously [278, 279]. Briefly, PMA substrate was prepared on a coverslip (25 mm, round) and mounted onto a 35-mm petri dish with a 20-mm hole for high magnification imaging. Flat PDMS stamps were coated with fibronectin (50 μg mL⁻¹) for surface functionalization of PMA via microcontact printing. PMA were labeled with DiI solution (5 μg mL⁻¹; Life Technologies) for 1 hr. Non-specific protein absorption were blocked by 0.2% (wt/vol) F127 Pluronic solution (Sigma) for 30 min. Cells were seeded with a density of 0.1M/ml. Phase images were taken using a 40 × objective (Leica DMi8) after 24 h. All images were analyzed using a custom-developed MATLAB program (MathWorks), as described previously.

4.3.8 ECM Remodeling

Cells were grown into spheroids over 14 days in lyophilized poly(N-isopropylacrylamide)-poly(ethylene glycol) (polyNIPAAM, Cosmo Bio USA, Carlsbad, CA), as previously described [60]. Briefly, cells were suspended in polyNIPAAM solution placed on ice at a density of 167,000 cells/mL and each gel was made at a volume of 150 μL. Gelation occurred after 5 minutes at 37°C, and gels were swollen in cell culture medium. Spheroids were harvested by placing plates on ice for 5 minutes in cold serum free media, then diluting the mixture further and allowing spheroids to settle for 30 minutes on ice. The
supernatant was removed and spheroids collected from 1 polyNIPAAM gel were resuspended in 45 µL of fresh media then gently mixed and plated in a 1:1 mixture of Matrigel:spheroids+media. Spheroids were allowed to settle to the plate before it was placed at 37°C to sandwich the spheroids between the TCPS and Matrigel. Cells were allowed to remodel their ECM for 4 days prior to staining for collagen (ab6308, Abcam), pan-laminin (ab11575, Abcam), F-actin (A22287, Thermo Fisher Scientific), and DAPI and imaging on a Zeiss Cell Observer SD (Carl Zeiss AG).

4.3.9 Cell Invasion

MDA-MB-231 cells were seeded into the top of a Boyden chamber in serum free media, at a density of 200,000 cells/mL. The bottom chamber was filled with media containing 10% FBS. Cells were allowed to invade through 8 um pores for 24 hours, then the top chamber was removed, and the bottom chamber washed 1x in PBS and fixed in 4% formaldehyde. Cells and nuclei were permeabilized with Tris-buffered saline (TBS) with 0.5% Triton-X for 20 minutes, then washed briefly in TBS with 0.1% Triton-X (TBS-T). Nuclei were stained with DAPI (1:5000 in PBS) and the entire well was imaged on the Zeiss Axio Observer Z1 microscope for manual quantification of invading cells.

4.3.10 qRT-PCR

Total RNA was isolated and qRT-PCR performed as previously described [55]. 50 ng cDNA was amplified using 10 pmol specific primers (Table S1) and the Maxima Sybr green master mix (Thermo Fisher Scientific) on a Rotor-Gene Q thermocycler (Qiagen, Valencia, CA) as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 45 cycles at 95°C for 10 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Both β-actin and ribosomal protein S13 were included as reference genes to permit gene expression analysis using the 2^\(-\Delta\Delta C_t\) method.

4.3.11 Animal Xenografts
All animal experiments were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst. UMass has an approved Animal Welfare Assurance (#A3551-01) on file with the NIH Office of Laboratory Animal Welfare. MDA-MB-231 cells were suspended in a 1:1 Serum Free Media: Matrigel (Corning, Corning, NY) mix, and $10^3$ cells were subcutaneously injected into the mammary fat pad of 6-12 week old female NOD scid gamma mice from a breeding colony. Each mouse was injected with two tumors. All mice were housed in ventilated cages with sterile bedding, food, and water. Injected cells were grown in vivo until tumors reached 500 mm$^3$. A minimum of 6 tumors were analyzed for each condition, and each tumor was considered one replicate.

4.3.12 Primary Mouse Tumor Cell Culture

MMTV-PyMT mice were sacrificed and tumors extracted within 24 hours. Tumors were finely minced with a scalpel, and incubated at 37°C for 1 hour in digestion media, which is DMEM:F12 containing: 5% FBS, 5 µg/mL Bovine insulin, 50 µg/mL Gentamicin, 2 mg/mL trypsin, and 1 mg/mL collagenase. Samples were spun down at 400G for 10 minutes, and resuspended in fresh DMEM:F12 media containing 10% FBS and 1% P/S. Once larger pieces settled (~5 minutes), supernatant containing spheroids and single cells was plated onto TCPS or biomaterials of 1 kPa or 41 kPa functionalized with 10 µg/cm$^2$ collagen 1. Cells were maintained as usual, and passaged once onto new biomaterial surfaces where indicated.

4.3.13 Statistical Analysis

One-way Analysis of Variance (ANOVA) with a Tukey post-test was performed on paired samples using Prism v5.04 (GraphPad software, La Jolla, CA). Prism was used to perform two-way ANOVA where indicated. Data reported is the mean and reported error is standard deviation with significance values of $p \leq 0.05$ as *, $p \leq 0.01$ as **, and $p \leq 0.001$ as ***.
4.4 Results

4.4.1 Design of an extended culture system to model breast cancer metastasis

To test the combined role of stiffness and timing on cell phenotype, we devised 3 systems that employed interactions between biomaterials and breast cancer cells. First, we plated 2 triple negative breast cancer cell lines, MDA-MB-231 and Hs578T cells, on biomaterials of 1 kPa and 41 kPa, in addition to a tissue culture polystyrene (TCPS) control, and passaged cells at these stiffnesses over several months to condition them to those mechanical environments (Figure 4-2 a'). We were particularly interested in triple negative breast cancer, as basal-like populations have been reported to show extreme stiffening behavior via high levels of collagen deposition [280]. Second, after these populations had been isolated by adhesion and/or growth on these biomaterials, we plated the cells back onto the original, rigid TCPS to determine which phenotypes were maintained in the cell’s mechanical memory (Figure 4-2 a''). Finally, to study cell populations that had never been selected for or grown on TCPS, we isolated primary breast tumor cells from an MMTV-PyMT model. Due to their low growth rates in vitro, we initially isolated these primary cells onto either 1 kPa or 41 kPa hydrogels or onto TCPS, then passaged them onto one of those 3 conditions to determine the role of the stiffness used to isolate the cells and the stiffness used to assay the cells in informing breast cancer cell phenotype (Figure 4-2 a'''). Because the cell lines grow more rapidly, which allows for selection over time (Figure 4-2 b), we assayed these populations for phenotypes like growth, motility, chemotaxis, and ECM remodeling to identify conditions necessary for onset of phenotypic changes and sustained alterations in behavior after we re-challenged those cells with culture on extremely rigid TCPS (Figure 4-2 c).

Heterogeneity makes treatment of breast cancer challenging, because the diversity of genotypes and phenotypes lend themselves to clones that survive and thrive in a wide variety
of conditions. However, this variety of cell behaviors poses an additional challenge in the lab. This problem is particularly present in attempts to examine population-level phenotypic changes in the lab, as the behavior of one individual cell can look extremely different than the population mean. For example, cells migrate at speeds ranging from 5 to 80 µm/hour, even while the population mean only varies by about 50%, patterns that persist even between individually isolated subclones that are genetically distinct. One strategy employed here to overcome this experimental challenge was to employ systems that give either a binary output, or focus on population level behaviors, rather than tracking individual cells. For example, application of a boyden chamber system measures the number of cells that are able to chemotax towards FBS, out of the whole population, which gives a binary read out on a single cell basis (a cell either migrates across the porous membrane towards the chemoattractant or it does not migrate) and this assay also measures population level changes, as the number of cells invading changes between conditions, while the number of cells seeded into the experiment is held constant. A second experiment that is not binary, but asses whole-population rather than single cell behavior is a wound healing assay. Cells migrate to fill in the space left by a scratch, but the speed of any single cell has relatively little bearing on how much of the wound is ultimately closed over the course of the assay. Tracking single cells still provides useful information on the range of behaviors that a heterogeneous cell population is capable of achieving, so here we have employed a combination of single cell tracking and measurement of population-level changes to capture the variety, but also acquire consistent and representative measurements of cell behavior before, during, and after mechanical conditioning.

4.4.2 Extended mechanical conditioning alters cancer cell growth and ECM remodeling
We set out to identify the role of extended exposure to varied ECM mechanics in cell proliferation, as an essential piece of metastatic outgrowth. After culturing cells on biomaterials as outline in figure 4-2 a, we plated cells onto TCPS, and assayed growth. We found that MDA-MB-231 cells grown on soft biomaterials were less proliferative on rigid TCPS than cells maintained entirely on TCPS (Figure 4-3 a), but cells did not retain memory of this phenotype when re-challenged with growth over multiple passages back on TCPS.
However, as cells in the tumor microenvironment grow in 3D, we encapsulated single cells in Matrigel and tracked cluster diameter over 10 days. There was no difference in cell growth in this soft, 3D environment in MDA-MB-231 or Hs578T cells (Figure 4-3 b). We also examined ECM remodeling, as breast cancer patients have highly heterogeneous expression of the protein Rho-Kinase (ROCK), which is necessary for tension generation (Figure 4-4 a). Cell spheroids grown from cells isolated at each of these...
conditions exhibited differential ECM remodeling when plated into a rigid TCPS environment, overlaid with Matrigel (Figure 4-4 b). To capture more physiological aspects of the microenvironment that might promote or inhibit cell growth, we created orthotopic tumors in the 4th mammary fat pad of NSG mice and measured tumor size over time. Here we also found no difference in growth rates between the 3 populations conditioned on either 1 kPa or 41 kPa, or maintained on TCPS (Figure 4-3 c). Together, this data suggests that while a rigid 2D environment discriminates between cell populations based on their mechanical background, growth in a soft, 3D, physiological environment is less dependent on that priming.

4.4.3 Culture on soft biomaterials promotes motility at conditions similar to growth conditions

In addition to growth, traction and motility are essential steps in the metastatic cascade, so we set out to examine the impact of mechanical conditioning on cell migration. First, we assayed traction force with micropillars, and found that at this particular combination of material stiffness and post deformability, cells grown on soft 1 kPa hydrogels
exhibited more traction forces than cells coming from stiffer environments (Figure 4-3 d). Interestingly, other groups have demonstrated that MDA-MB-231 and other metastatic cells exhibit overall higher traction than non-metastatic cell lines [212]. With this traction force data in hand, we also aimed to quantify the expression of integrin receptors in these cell populations to relate our work to extensive computational modeling of the motor-clutch model of cell migration. Cells heterogeneously altered expression of many integrin subunits, with upregulation of integrin genes for receptors that bind to several different ECM proteins, such as collagen (ITGB1), fibronectin (ITGA5), and laminin (ITGB4) (Figure 4-5 b). Each of the subunits mentioned above also maintained slightly increased expression when probed for mechanical memory. Integrins and traction are linked, as different integrin affinity for ECM proteins regulates cell force generation [281]. We then sought to assemble these analyses of the motor parameters (traction) and clutch parameters (integrin expression) into a functional migration assay.

We first observed a biphasic relationship between stiffness and motility for cells that were always maintained on TCPS, which has been previously described in this system and others [45, 275]. The stiffness that produces optimal migration, however, varies widely across cell types, so we hypothesized that the stiffness of optimal migration in our cancer cell lines could be tuned with extended mechanical cues. Indeed, we found that we could tune the stiffness of maximal cell migration of MDA-MB-231 cells by culturing cells on biomaterials of 1 kPa or 41 kPa (Figure 4-3 e). We also observed a similar trend in Hs578T cells, although the change in speeds was not significant (Figure 4-6 a). We were surprised to find that cells grown on 41 kPa exhibited maximal migration speeds on 41 kPa biomaterials, which is stiffer than the 18 kPa hydrogel where cells from TCPS migrated fastest. One possible explanation is that the hydrogels were functionalized with a high amount of additional collagen 1, which was not provided to the cells grown on TCPS. Additionally, we decided to grow cells on
much stiffer gels, of 500 kPa, and then similarly assayed those cells for motility from 1 to 64 kPa (Figure 4-6 b). We found that the cells grown on 500 kPa hydrogels followed a stiffness-dependent migration profile very similar to cells grown on 41 kPa, suggesting we were above a ‘saturation stiffness’ (Figure 4-6 b). This saturation phenomena has been observed in other phenotypes, such as fibroblasts matching their stiffness to the stiffness of their substrate, which was only observed below 20 kPa [39].

**4.4.4 Primary tumor cells are smaller and motile on Soft Biomaterials**
One major limitation of working with stable human cell lines, is that they have been selected for and grown on TCPS for long times before commencement of this study. To better understand if the results presented here represent physiological behaviors, we compared our data to primary tumor cells from an MMTV-PyMT model, as these cells have never been exposed to TCPS. We began by seeding cells directly onto 1 kPa or 41 kPa biomaterials after isolation, and seeded a fraction of the cells on TCPS for comparison, as shown in figure 4-2 a. Initially, isolated cells exhibited an epithelial morphology and maintained strong cell-cell contacts, so we could not assay single cell migration at passage 0. Instead, we observed spreading of cell clusters starting 24 hours after initial seeding on biomaterials of 1 kPa or 41 kPa, as compared to TCPS. Interestingly, we found that cell clusters on soft biomaterials spread much more over the course of this assay than cells on the stiffer surfaces (Figure 4-7 a-b). Once these cells spread from clusters into a monolayer on our biomaterials, we passaged them from each of our 3 substrates (1 kPa, 41 kPa, TCPS) onto a new set of 3 substrates, giving us 9 total conditions with each isolation condition divided into 3 assay conditions. We observed that cells were alive and exhibiting membrane ruffling.
at all 9 conditions, but most were non-motile. However, cells that were isolated at the softest

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**Figure 0-7. Primary breast tumor cells respond differentially to soft materials and TCPS.** a-b. Spheroids isolated from primary MMTV-PyMT cells were seeded directly onto substrates of 1 kPa (red), 41 kPa (blue), or TCPS (black) (a). After adhesion, spheroid spreading was measured by change in area over 12 hours (b). c-f. After 1 passage on a biomaterial surface of 1 kPa (red), 41 kPa (blue), or TCPS (black), mouse primary tumor cells were transferred to a new surface of 1 kPa, 41 kPa, or TCPS, as indicated on the X-axis, and traced to determine cell area (c,e) and circularity (d,f). N=2, g-h. MDA-MB-231 cells selected on biomaterials of 1 kPa, 41 kPa, or TCPS were each seeded onto a new surface and traced to determine cell area (g) and circularity (h). i-j. Two-way ANOVA was performed on data from c and d (i) and g and h (j), with tables reporting the percent contribution to variance of the interaction between current and past platforms, the current platform only, or the past platform only. P<0.05, *, p<0.01, **. Data analysis was done with assistance from Rainy Wortelboer.
1 kPa condition and re-plated onto 1 kPa gels for the assay were significantly more motile, migrating about twice as fast as cells at the other conditions (Figure 4-3 f).

The motility we observed was not correlated with other shape parameters, like cell area and circularity (Figure 4-7 c-f). We probed these shape parameters at passage 1 across the 9 conditions described above. A two-way ANOVA revealed significant dependence of cell circularity on the assay platform, but no significant relation between the stiffness that we used to isolate the primary cells and their morphology at the time of assay (Figure 4-7 i). Upon comparing this data to the human cell lines selected on soft biomaterials, we observed similar trends in the cell area, with the largest contribution to variance coming from the assay platform (Figure 4-7 g,j). However, circularity of these MDA-MB-231 cells was significantly dependent on the substrate the cells came from, but not their current assay condition, in contrast to our primary cell data (Figure 4-7 h,i). This suggests that at the short times we were able to observe primary cells, they relied primarily on their current stiffness for mechanical cues, but when cell lines were conditioned over many passages, they derived important information from the stiffness of their substrate over time.

4.4.5 Cells adopt a material-dependent phenotype over time

Since we had observed changes in 2D proliferation and in motility after cells were cultured on biomaterials, we set out to identify the time-dependence of these phenotypes. To do this, we identified cells early during the cell conditioning process (40-60 doublings), cells at late stages of the conditioning process (130-160 doublings) (Figure 4-2 a'), and also examined cells during the memory stage (25-35 doublings back on TCPS) (Figure 4-2 a''). First, we assayed cells for invasion through 8 µm pores towards media containing 10% FBS, and found that they were equally chemotactic across all 3 populations at the early stage, but culture substrate mechanics began to differentiate between populations at the later time point (Figure 4-8 a). Further, these cells did retain a memory of that culture substrate after being
Cells adopt a material-dependent phenotype over time. a. Cell populations were assayed early (after 40-60 doublings on biomaterials), late (130-160 doublings on biomaterials) or to determine memory (25-35 doublings back on TCPS) for chemotactic invasion towards 10% FBS. b. Cell populations were assayed in the same time frames as (a) with a wound healing assay. c. MDA-MB-231 cells seeded onto biomaterials and assayed for random motility on substrates of 1 kPa or 41 kPa either early (40-60 doublings on biomaterials), or assayed at the same conditions for memory (25-35 doublings back on TCPS). For a(early), b and c, N=2. For a(late conditioning & memory), and c (memory, 41- >1 and 1->41), N=1. p<0.05, *; p<0.01, **; p<0.001, ***. Data was collected with assistance from Sarah Duquette and analysis was done with assistance from Rainy Wortelboer.

plated back onto TCPS and cells from 1 kPa and 41 kPa were less invasive than those that
were cultured on TCPS for the entire length of the study (Figure 4-8 a). While we observed a similar trend during the conditioning phase of these cells in the wound healing phenotype, cell mechanical memory did not play a role after cells were plated back onto TCPS. In fact, cells coming from 1 kPa and 41 kPa closed significantly more of the wound than cells from TCPS during this memory stage (Figure 4-8 b). Wound healing relies on both cell growth and migration over the 12 hour assay, and we had separately noted that the cell proliferation phenotype in 2D reverted back to look like the original cell population, and cell memory did not play a role. This suggests that the reversion in wound healing is at least partly due to the reversion in cell growth rates. We also looked back at the random motility phenotype observed in figure 4-3 e. We observed that this motility phenotype had a much earlier onset than changes in invasion, as cells from soft biomaterials were significantly more motile when assayed at that same stiffness as compared to cells from TCPS, and cells do exhibit memory of this phenotype (Figure 4-8 c). Here we have identified 3 cell phenotypes that are key pieces of the metastatic cascade, with early onset changes in motility and late onset alterations in chemotaxis and wound healing. Interestingly, cells only retained memory of motility and chemotactic behavior, not that of cell proliferation and wound healing.

4.4.6 Clonally isolated cells exhibit a range of phenotypic behaviors mimicking mechanical selection

Most cells that successfully colonize distant tissue sites arrive there as part of a larger cluster containing cells that are not clonally identical. Several recent publications have shown at least 2 distinct populations in micrometastases, each with unique roles that contribute to metastatic efficiency [282, 283]. However, the ratios of those populations change over time at the secondary site, suggesting a selection mechanism dependent on features of the microenvironment. Here we employed techniques commonly used in the field of drug resistance, to isolate individual cell clones by extreme limiting dilution. These subclones
were then grown out into larger populations to better assay their range of phenotypes. We first noted that they have very different growth rates, and we quantified the variation in doubling time (Figure 4-9 a). We found a range of proliferation rates that suggests the possibility that a less heterogeneous population could emerge from our parental line over more than 10 passages, equating to an average of about 50 cell-doublings. Additionally, we assayed these populations for motility and, as expected, there is a large degree of variability in the migrations speeds of individual cells even within a single clone (Figure 4-9 b). Mean cell speed of the clones varied by 30-50%, which indicates that while each clone is able to achieve a large range of migration speeds, there are differences between clones in overall motility (Figure 4-9 b). Finally, we looked at collective cell motility of each single clone, to determine if cell-cell contacts between cells from the same(456,621),(751,650) subclone could facilitate a wound healing phenotype, which relies on a combination of cell growth and migration. We similarly found a range of behaviors, which seemed to mimic the trends observed in figure 4-9 a and b. For all 3 of these phenotypes, cells selected on soft biomaterials fall in the range of individually isolated cell clones, while looking distinct from cells grown entirely on TCPS.

After we identified a high degree of variation in single clones when assayed for growth and motility on rigid TCPS, we hypothesized that these clones might also demonstrate varied mechano-responsiveness when plated onto biomaterials. Proliferation of cells grown on TCPS increased at higher stiffnesses, similar to previous reports (Figure 4-9 d) [128, 186]. However, we found this profile was altered in different subclones. For example, clone 1-10B barely grew at all, while clone 1-8D grew on TCPS, but not on any of the biomaterials. Additionally, clone 1-5C seemed to grow similarly across all 5 substrates examined here. We further assayed the cells selected on biomaterials for comparison, and found that the cells grown on 1 kPa or 41 kPa hydrogels behaved most similar to clone 1-11G and 1-5C, respectively (Figure 4-9 d). Together, these clonal comparisons demonstrate the
possibility of selection of a subset of cells out of a heterogeneous population during culture on soft biomaterials, and the individual subclones mimic the growth and motility phenotypes seen in populations after extended cell growth on soft hydrogels.

4.5 Discussion

Here we set out to identify a set of phenotypes that are altered over time with exposure to soft biomaterials. We used two independent approaches to identify the heterogeneity that contributes to change over time; a top-down approach which identified stiffnesses of interest which were used to apply selection pressures to these cell populations,
and a bottom-up approach that selected individual clones by limiting dilution to identify the existing population-level heterogeneity. We demonstrated that the range of growth and motility phenotypes that were isolated with this bottom-up approach is sufficient to explain the changes in cell behavior observed over time on soft biomaterials. However, the top-down approach most closely mimics in vivo metastatic seeding, as the most successfully formed metastases arise from multi-clonal clusters at both the invasion and seeding steps [282]. Further understanding this clonal diversity and the role of tissue mechanics in selecting these populations can build upon existing efforts to use circulating tumor cells to predict patient outcomes [284].

Heterogeneous populations arise within a primary tumor and increasingly small subsets of these cells leave the primary tumor, survive vascular or lymphatic travel, reside as disseminated cells at distant sites, and ultimately form macrometastases [282, 285]. Transfer of multiclonal populations to a new environment can result in erosion of the original tumor diversity. For example, patient-derived xenograft models, which are thought to most closely recapitulate the original microenvironment, can result in tumors comprised of clones that made up less than 5% of the original tumor after several passages [286]. Here we observed subclones with widely varied proliferation rates on both TCPS and hydrogels, indicating that it is likely that extended culture on biomaterials would shift the population make up towards those that better adhere to, and grow on, soft biomaterials (Figure 4-9 a, Figure 4-9 d). However, reducing studies down to a single clone misses key pieces of information as emerging work demonstrates that two or more distinct subclones can work cooperatively to facilitate growth and drug resistance. As examples, in chronic lymphocytic leukemia, patients with independently evolving subclones had worse clinical outcomes than patients without independent subclonal driver mutations [49], and minor EGFR mutant clones contributed to overall tumor growth via a paracrine mechanism in glioblastoma [287]. While we are likely
narrowing the diversity of subclones in our conditioned populations, it is highly improbable, if not impossible, that we are selecting for a single subclone. This at least partially explains the continued heterogeneity in our results and the memory of some phenotypes, but not others after re-challenging cells with culture on rigid TCPS.

Here our data indicates that cells altered their motility based on culture conditions beginning early in conditioning, and sustained that mechanical memory over several doublings back on TCPS. We observed that the stiffness of maximum migration was shifted to the left for cells grown on 1 kPa hydrogels, and to the right for cells grown on stiffer gels of 41 kPa and 500 kPa (Figure 4-3 e, Figure 4-6 b). We noted that the maximum migration of cells grown on TCPS fell between these conditioned populations, at 18 kPa, and predict this is related to the collagen 1 functionalization that we provided to cells grown on biomaterials, but not to cells grown on TCPS. Cell migration is frequently described by a motor-clutch model, where parameters of cell traction (motor) scale with parameters of cell adhesions (clutch) [288]. Our tension data in a micropillar study indicate that cells from soft materials generate slightly more tension in this particular environment (Figure 4-3 d), although they seem to generate less tension and engage in less ECM remodeling on rigid TCPS surfaces (Figure 4-4 b). Other groups have reported decreases in integrin β1 expression on soft ECMs at short time points [289], but we noted that cells eventually recovered or slightly increased mRNA levels over several passages on our collagen 1 functionalized biomaterials, signifying an increase in the number of available clutch molecules (Figure 4-5 b). In order to maintain homeostasis, changes in clutch parameters must be offset by changes in either other clutch parameters or motor parameters [241]. Because the changes in traction forces that we observed between our 3 populations were small relative to the variation in phenotype, we hypothesize that our cells best fit into a clutch-clutch model, although we do not probe broader clutch-related changes in this work. A related phenotype, chemotaxis towards FBS,
was also retained in cell memory even after cells were re-challenged with culture on TCPS (Figure 4-8 a). Other groups have demonstrated that stiffer substrates enhance chemotaxis in immune cells [290] and limit chemotaxis in mesenchymal stems cells [291], but to the best of our knowledge, this is the first work to show that cells can be primed by a substrate to still invade towards a chemo-attractant several passages later.

While we observed stable phenotypes in cell motility and chemotaxis, growth and wound healing behavior appeared to be more transient. For the purpose of our study, we define memory as sustained presentation of altered phenotypes after cells from soft environments are challenged with extremely rigid culture conditions for at least 5 passages. Mechanical conditioning induced short term changes in cell growth, in 2D but not 3D, and in wound healing, but cells exhibited little or no memory of past stiffnesses after 25-35 doublings back on TCPS (Figure 4-8 b). However, we did observe differences between isolated subclones assayed for proliferation on both rigid TCPS and softer biomaterials (Figure 4-9 d). Higher stiffnesses are known to drive increased proliferation [272], and our data indicates that when assayed on rigid surfaces, mechanically conditioned cells do not retain a memory of the substrate where they were grown. Other groups have shown that serially passaging breast tumor cells in rats resulted in increased growth and invasion over time [292]. One study warrants robust comparison to our work, as they passaged breast cancer cells up to 4 times over 12 weeks in brain, lung, or bone marrow tissue that was implanted in a subcutaneous skin fold chamber [293]. They observed early, but transient, changes in ion transport and homeostasis, and longer term upregulation of gene ontologies associated with cell-matrix adhesions and chemotaxis, which tracks closely to the data presented here. Other recent work found changes in MDA-MB-231 cell proliferation after 3 passages on polyacrylamide gels, but, similarly to our work, cells retained little memory when they were seeded back onto TCPS [108]. Additionally, collective motility is enhanced
in a 3D model at high collagen concentrations [294] and cells respond to substrate stiffness much more efficiently in a collective cell migration model [295], which may explain the propensity of our populations to show short-term dependence on culture stiffness but demonstrate no memory of that phenotype (Figure 4-8 b). Overall, our data demonstrate that cells respond to mechanical conditioning over time, but only retain memory of those phenotypes in a subset of behaviors that are ultimately necessary for breast cancer metastasis.

A recurring problem in the emerging field of mechanical memory is that standard cell isolation and maintenance protocols require exposing cells to an extremely rigid TCPS substrate before conditioning the cells in a biomaterial environment. To that end, we compared data from established breast cancer cell lines to primary breast cancer cells that were isolated and assayed on our hydrogels. Importantly, MMTV-PyMT tumors retain several features of human breast cancer that drive metastasis, including collagen organization and early dissemination [296, 297]. One group has cultured primary breast carcinoma cells in natural ECM gels, and found that collagen, but not basement membrane induced migration [18]. Our system allows the decoupling of stiffness and integrin binding sites, so here we functionalized all of the surfaces with collagen 1 to promote migration, and tested the unique role of stiffness. We found that primary mouse tumor cells were only motile when they were both isolated and assayed at the softest condition (Figure 4-3 e). Additional work has been done in mesenchymal stem cells that were also isolated directly on substrates designed to match physiological stiffnesses [298]. That group identified multiple levels of memory, starting from early onset (transcription factor translocation to the nucleus), to those at longer time frames (changes in gene expression). Here we suggest a mechanism that occurs over longer periods via selection of subclones that preferentially adhere to and proliferate on soft biomaterials. There are likely contributions from all of these factors (transcription factor localization, gene expression, and selection) in the phenotypes observed. For example, as the
wound healing phenotype is reversible, this is likely driven by changes in gene expression across the population. In contrast, memory of chemotaxis behavior suggests more stable selection, likely for cells with an advantage in both growth and response to soluble factors. These stable changes could be a result of selection for cells with higher copy numbers of key growth factor receptors, like EGFR.

Memory of a previous mechanical environment may determine metastatic success or failure based on whether cells are disseminated from a very soft or very stiff tumor, where stiffer tumors [120] and high expression of the cross-linking enzyme lysyl oxidase [299] are associated with worse prognosis. In stem cells, memory of a previous stiffness is maintained by YAP transcription factor nuclear localization, which impacts differentiation in a time-dependent manner [106]. This has further been demonstrated as a mechanism for epithelial reprogramming in a wound-healing environment [300]. While little has been done to translate this work to stiffness changes during tumor progression and metastasis, one group has observed YAP/TAZ dependent memory and noted an impact on motility in non-malignant epithelial cells and invasive tumorigenic cells [277]. YAP/TAZ nuclear translocation has previously been linked to mechanical signaling via control of focal adhesion dynamics [301] and matrix production [259] at short time points. While localization may play a role in our system, the long timeframe required to observe many phenotypic changes indicates that gene expression changes and clonal selection may better explain the functional downstream changes that result from conditioning cells using biomaterials (Figure 4-1).

4.6 Conclusions

Serial passage of breast cancer cells on soft biomaterials promotes a phenotype of low motility and growth on TCPS, enhanced motility at physiological stiffnesses, and mimics behavior of primary mouse tumor cells that have never been exposed to extremely rigid TCPS. Culture in soft environments can yield short term decreases in cell proliferation and
related behaviors, although this phenotype is not retained over long periods of time, suggesting that proliferation is more dependent on the current environment than past mechanical cues. However, motility and chemotaxis change based on mechanical priming, suggesting memory is important for those phenotypes. While more work is needed to fully characterize the impact on overall risk and its’ relation to tissue specific metastasis, this study demonstrates that mechanical conditioning has a broad effect on cell phenotype, with a subset of those behaviors retained even as cells are challenged with new mechanical cues.
CHAPTER 5

CONCLUSIONS AND ONGOING WORK

5.1 Abstract

Breast cancer is really a collection of many diseases and that heterogeneity makes it difficult to treat. While clinical and molecular subtyping provides a host of information to treat a patient, this analysis neglects many druggable aspects of a tumor or the metastases. The field of medicine has become exceptionally proficient at treating primary breast tumors through methods including; early detection, resection, radiation, cytotoxic drugs, and targeted therapeutics, but preventing or treating disseminated tumor cells and ultimately macro-metastases at essential organs has remained elusive. The work in this dissertation aims to broaden the scope of options available by applying engineering methods to this challenging medical problem. I have successfully identified mechanisms of drug resistance in biomaterial systems and targets that respond to mechanical forces in the tumor microenvironment, as important contributions to the large body of research that is characterizing and treating breast cancer.

5.2 Overall Conclusions

5.2.1 Microenvironment mediated drug resistance via MEK

We combined biomaterial platforms, drug screening, and systems biology to identify mechanisms of extracellular matrix-mediated adaptive resistance to RTK-targeted cancer therapies. Drug response was significantly varied across biomaterials with altered stiffness, dimensionality, and cell-cell contacts, and kinome reprogramming was responsible for these differences in drug sensitivity. Screening across many platforms and applying a systems biology analysis were necessary to identify MEK phosphorylation as the key factor associated with variation in drug response. This method uncovered the combination therapy
of sorafenib with a MEK inhibitor, which decreased viability on and within biomaterials *in vitro*, but was not captured by screening on tissue culture plastic alone. This combination therapy also reduced tumor burden *in vivo*, and revealed a promising approach for combating adaptive drug resistance. MEK has independently emerged as a therapeutic target, but is an interesting candidate for combination therapy here because sorafenib and the MEK inhibitor target two nodes within the same pathway, while most combinations aim to target different, but redundant, pathways.

Combining therapies is an established method to combat adaptive resistance, but screening on TCPS is not a perfect tool and does not mimic physiological conditions. Therefore, interdisciplinary approaches that combine drug screening, systems biology and biomaterials engineering are important. However, building these systems adds time and cost to the drug screening process and may not always be necessary. For example, we demonstrated that the biomaterial platform did not impact cell response to doxorubicin, where screening on TCPS alone was sufficient. Additionally, our group has previously identified JNK phosphorylation as a resistance mechanism to sorafenib in very stiff environments, but that work combined with our data here has demonstrated that this could be identified using just 2D systems, and the efficacy of that combined therapy was evident on TCPS.

### 5.2.2 Integrating tumor genetics and ECM properties

Stiffness is one feature of the tumor microenvironment that is known to influence cell phenotype. Relating this information to cancer cell genetics is important because while engineers have characterized the impact of physical forces in and around the tumor, clinicians use sequencing and expression information to inform treatment decisions. For instance, predicting where a patient might develop metastasis would be useful for monitoring and treating that patient, but that information is not immediately obvious from a biopsy of the
primary tumor. Engineers have worked to develop *in vitro* techniques to predict this tropism, but this is not yet applied clinically [55]. This combination of the study of features of the tumor cell and the surrounding matrix is key for integrating current genetic analysis with emerging knowledge in mechanobiology.

Here, I have worked to identify a set of genes and their resulting proteins that respond to the mechanical properties of their environment and have a functional impact on cell behavior. I focused on genes that mediate cell interaction with the ECM, like integrins, and the resulting signaling cascades. While most work thus far has focused on collagen binding integrins, here I identified integrin $\alpha_6$, a laminin binding subunit, as mechanosensitive and confirmed its necessity in EGFR signaling in our system. The ligands for these two surface receptors (laminin and EGF respectively) are abundant in the tumor microenvironment, and so their relation has important implications for cell adhesion and motility in dissemination. Further, these two pathways converge at the intracellular protease calpain 2, which is known to be responsible for focal adhesion turnover. Cells were more sensitive to pharmacological calpain 2 inhibition in stiffer environments, more reminiscent of late-stage primary tumors. Together, these offer new potential drug targets that are unique to the stiff tumor microenvironment that is rich in laminin and EGF.

### 5.2.3 Mechanical Forces Direct Metastasis

Disseminated breast cancer cells reside at the secondary tissue sites for extended periods of time, undergoing evolution and selection at those distant sites as well. Each of the most common sites of breast cancer metastasis (bone marrow, brain, liver, and lung) has a distinct stiffness range, so we designed a set of biomaterials to isolate those tissue properties, and then grew cells in those environments for extended lengths of time. I have demonstrated that the clonal diversity in growth and motility of the parental cell line is sufficient to account
for selection of subpopulations over the course of 32 passages (equating to approximately 160 cell doublings) on biomaterials. I identified that cell growth and collective migration on rigid TCPS is limited in cells selected on biomaterials compared to those maintained on TCPS. However, cells grown on soft biomaterials were more motile when re-plated back onto the same, soft hydrogels, a behavior that was similar to mouse primary cells that had never been exposed to rigid TCPS. As the most successfully metastasizing cells likely arrive at the secondary site as multi-clonal clusters, tumor evolution dynamics at the secondary tumor site are important in understanding how to best target the cells contributing to metastasis and poor patient outcomes.

Clinically-oriented work is beginning to understand how drug treatment drives cell selection and tumor evolution, and here I’ve identified the role of stiffness on these processes, in a time-dependent manner. Cells residing in a stiffer metastatic niche may be more successful at responding to growth signals in their environment, as indicated by cell chemotaxis experiments, and they may also have an advantage in migration and proliferation. This aligns with other recent work that demonstrates the importance of a fibrotic pre-metastatic niche in recruiting and supporting disseminating cancer cells. Together, this demonstrates that significant tumor-level evolution occurs at the disseminated rather than primary tumor site, and that targeting the ECM at common sites of metastasis may be a promising therapeutic strategy.

5.3 Future Work and Overcoming limitations

5.3.1 Breast cancer as a global disease

While the majority of breast cancer research occurs in western nations, globally 1 in 8 women will be diagnosed with breast cancer. Outside the scope of this work, the standard of care varies dramatically between countries, but early systematic studies have begun to identify scientific similarities and differences in breast cancer development in these different
populations. For example the age of diagnosis is significantly lower in black women born in East African nations. Understanding these scientific parameters is important, because in the United States, women typically begin getting mammograms at age 45, which can provide early diagnosis and identify other risk factors, like increased breast density [48].

Beyond the public health challenges facing breast cancer patients in developing nations, there is limited research on genetic and mechanical forces that drive breast cancer in non-western populations. For example, the standard reference genome used for mutational and transcriptomics studies is comprised of 11 white individuals from upstate New York, which is not representative of a global population. Here we were able to identify somatic mutations through comparing cancerous tissue to a matched normal sample, but this limits the number of samples that can be included in such studies. Understanding the change in Ethiopian breast tumors over time and expanding the available population for sampling will benefit future work to build a diverse set of reference genomes to better understand breast cancer on a global level.

One benefit from studying diverse populations is that the types of therapeutics are often different for these groups. For example, African American breast cancer patients tend to have higher expression of ECM related genes, like those associated with angiogenesis. However, inhibitors of these processes have had little clinical success, effectively ending these efforts. Using genetic and phenotypic methods to better understand the relationship between the impacts of the tumor microenvironment on aggressive breast cancer and the differences in those cancers developed by women born in East Africa, black women born in the United States, and white women born in the United States will be of immense benefit.

5.3.2 Development of mechanical drug targets

The field of mechanobiology has identified several mechanisms by which cells translate extracellular forces to biochemical signaling and changes in gene expression
Early clinical work has focused on targeting intuitive mechano-transducers, like ROCK [302], where inhibitors have already been approved for other indications. In this work I have contributed new potential drug targets that respond to the mechanics of their environment, and may show differential expression in tumor ECMs. For example, earlier work proposed calpain 2 as a promising target, but in vitro work had suggested likely off-target effects, as calpain 2 is a key regulator of motility in immune and endothelial cells too [245]. However, knowledge of tumor mechanics can assist in preferential targeting of diseased tissues. Additionally, a common target in breast cancer is EGFR, which is known to have altered expression and activity at different stiffnesses [186], which may have implications for efficacy and resistance. Understanding the mechanical influence on signaling allows a level of specificity for cancer cells over strategies that disrupt the rest of the extracellular matrix and stroma that remodel that matrix [9, 303]. This knowledge of the tumor microenvironment, such as stiffness and abundance of laminin and EGF, can instead be used to limit cancer cell adhesion and motility in a manner that is unique to the tumor. However, integrins are incredibly difficult to target, with all clinical successes thus far targeting integrins on cells in suspension in the blood, rather than in solid tissues, including tumors [304]. Therefore, targeting parallel or downstream nodes, as described in this work, may hold more promise. In the future, the analysis I have done here should be further expanded to include other tissue parameters, like dimensionality and an expanded set of signaling molecules, to identify new therapies and therapeutic combinations.

5.3.3 Further Exploration of Clonality and Heterogeneity in Breast Cancer

Treatment of breast cancer frequently results in resistance and recurrence, either through redundant signaling or via genetic mutations that occur or are selected for over time, known as tumor evolution. This is a key clinical challenge because the diversity of populations within a tumor means there is a chance some cells may already exhibit resistance
to frontline therapies, but also any residual cells may develop further mutations that promote resistance to whole classes of therapies and further support cell survival and growth, even in the presence of other cellular stresses. Even before treatment, cell growth results in somatic mutations through standard proliferative processes, but the addition of stress via drug can accelerate the accumulation of potentially advantageous mutations [72]. This evolution does not only occur at the primary tumor, and recent work has demonstrated that cells likely disseminate from primary breast tumors early [74, 75]. This work, coupled with sequencing of tumors over time demonstrates parallel evolution can occur with a few mutations shared by the primary tumor and metastasis, but a broad range of mutations being unique to each location [71]. Further, in depth genetic analysis of patients over the course of their treatment shows the rise of new mutations within a single patient as they are treated with sequential drugs and drug cocktails [73]. All of this data highlights the necessity of understanding the functional distinctions between subclones to better treat patients.

Here I have investigated the role of cell clonality in key phenotypes associated with metastasis, including migration, proliferation, and response to extracellular features of the tumor microenvironment (Figure 5-1). However, more work will need to be done to understand how these parameters relate to successfully metastasizing populations in vivo and how they promote drug response in a complex tumor environment. For instance, recent work has demonstrated that cells appear as multi-clonal clusters during all stages of metastatic spread; from local invasion, travel through the vasculature, and seeding at distant secondary sites [282]. This suggests that each clone may play a distinct role in providing growth signals, chemotactic signatures for immune or stromal cells, ECM production and deposition, and
tumor growth – all of which are necessary for survival and outgrowth of disseminated tumor cells. Interestingly, each subclone maintains a degree of heterogeneity in cell migration, suggesting any given cell can both produce and receive several cues from the extracellular space, including cell-cell contacts, or soluble factors (Figure 5-2 a). Here, we have done a preliminary experiment with mixing of 4 or 7 subclones, which can achieve increased proliferation, more similarly to the heterogeneous parental line (Figure 5-2 b). Additionally, a lot of work has been done to understand the rise of drug resistant clones in tumors, but this research has focused almost exclusively on cells grown on stiff, 2D, tissue culture polystyrene, which is not representative of the tumor microenvironment [119]. Here I have
done some work to characterize single clone response to features of the tumor microenvironment, like protein type and presence of EGF (Figure 5-2 c-d). This is an important dimension to add because tissue stiffness can alter resistance by changing expression and activity of key kinome signaling nodes for targeted therapeutics, and can change proliferation rates for evasion of cytotoxic drugs [72, 128, 270]. However, this has yet to be applied to understanding clonal selection for drug resistant populations or to induce secondary genetic alterations that allow the survival of persister cells, both of which could be enhanced by the reduced kinome signaling in 3D materials, or by an increased sensitivity to apoptotic signaling in similar systems. The set of material systems that I have described in this work allow for the isolation of specific features of the extracellular matrix, and can help
identify the role of ECM in driving clonal selection, metastasis, and drug response of cancer cells.
CHAPTER 6
FUTURE DIRECTIONS EXPLORING MECHANICAL FORCES

6.1 Inclusion of Additional Mechanical Forces: Shear Flow Enhances Cell Motility through Calpain 2

6.1.1 Introduction

Cells experience many types of mechanical forces during metastasis. One force that is not explored in this work but is of interest to the field is shear flow, which cells experience in both the interstitial space and in the vascular or lymphatic system [305]. Most research on shear flow to date has focused on cells in the vasculature, like endothelial and smooth muscle cells, as these forces are essential to development, homeostasis, and understanding healthy vasculature, which then allows us to better understand disease phenotypes like atherosclerosis [306] and tumor angiogenesis [307].

Sub-cellular structure response to shear flow can be translated from endothelial and immune cell studies to better understand the impact of interstitial and vascular or lymphatic flow on cancer cells. For example, laminar flow promotes a healthy endothelial cell phenotype, while turbulent flow over athero-prone cells induced YAP/TAZ activation and translocation to the nucleus, driving proliferation and pro-inflammatory phenotypes [308]. Further work in cancer cells has demonstrated that flow across the top surface of a cell seeded onto a 2D surface can result in strain on focal adhesions, resulting in increased cell invasion through increased length, but not number, of invadopodia [309]. This increased force across a focal adhesion promotes stability, and shifts mechanosensitive cell migration. Additionally, shear stress can also impact cell proliferation and cell-cycle arrest through altered cyclin and cyclin-dependent kinase (Cdk) expression, downstream of integrin
signaling [310]. MDA-MB-231 breast cancer cells exposed to shear forces showed enhanced adhesion under low-flow conditions, and increased the activity of PI3K and Akt, also through integrin clustering and downstream signaling [311]. Integrin engagement and force transmission is a key part of the shear-sensing machinery, but is difficult to target, and has had little clinical success thus far.

To that end, we set out to identify other components of the shear-sensing machinery in breast cancer, which may make more easily druggable targets. We designed and characterized a flow system that recapitulates some aspects of in vivo blood flow and demonstrated increased cell migration under high-flow conditions. This shear-effect was nullified in a cell line with reduced calpain 2 expression. We attempted to stimulated increased migration at the no-shear condition via two known mechanisms of activating calpain 2 – through the EGFR-ERK axis and via exogenous calcium. EGF-dependent calpain 2 signaling partially rescued the increased motility and smaller cell morphology, suggesting that increased flow either replenished the supply of EGF to a cell or resulted in signaling downstream of EGFR through other mechanisms, such as integrin clustering. Here we propose calpain 2 as a shear-dependent target, although more work is needed to elucidate the full therapeutic potential.

6.1.2 Methods

6.1.2.1 Device Fabrication

An inverse mold of the desired polydimethylsiloxane channel (PDMS, Dow Corning, Midland, MI) was designed in FreeCad and printed on a LulzBot Taz4 (Aleph Objects, Loveland, CO) in acrylonitrile butadiene styrene (ABS, Aleph Objects). The inside surface was smoothed by pipetting 3 mL of acetone into the mold, letting it sit for 15 seconds, and then pouring out the acetone. After 3 acetone washes, the entire mold was sprayed liberally
with 70% ethanol and allowed to set for at least 24 hours. PDMS was mixed in a 10:1 weight ratio of polymer: curing agent and poured into the mold, completely filling the mold. The PDMS solution was degassed in a vacuum chamber at room temperature for 30-60 minutes. Then it was cured at 60°C for at least 16 hours. A 1.5 mm biopsy punch was used to make holes at either end of the chamber, the PDMS was sterilized with 70% ethanol, then both the PDMS and a cleaned 50 mm x 75 mm glass slide were UV ozone treated (Bioforce Nanosciences, Salt Lake City, UT) for 20 minutes. The PDMS and glass were immediately placed together and allowed to set overnight under a weight.

6.1.2.2 Cell Culture

All cells were cultured in DMEM buffered with sodium bicarbonate, with 10% Fetal Bovine Serum (FBS) and 1% Pen/Strep (P/S) at 37°C and 5% CO₂. All experiments were performed in DMEM buffered with HEPES, with 10% FBS and 1% P/S at 37°C. MDA-MB-231 cells were a generous gift from Dr. Sallie Schneider at the Pioneer Valley Life Sciences Institute, and transfected with shRNA to calpain 2 or a scramble control as previously described [275].

6.1.2.3 Cell Migration

MDA-MB-231 cells transfected with shRNA to either calpain 2 (shCAPN2) or a scramble control (shSCR) were seeded into the device at 4,400 cells/cm² in media buffered with HEPES and allowed to adhere for 24 hours. 12 mL of fresh media was prepared to flow over the cells, either with FBS and P/S only (control), with FBS, P/S and 100ng/mL of EGF (+EGF), or with FBS, P/S and 3mM CaCl₂ (+CaCl₂). Two syringe pumps (from syringepump.com) were set to ‘recirculation’, with a direction change with every 10 mL pumped. The syringes were connected to the 1.5 mm holes in the device via tubing (Cole Parmer, Vernon Hills, IL) and flow was started. 1 hour after the initiation of flow, the cells
were imaged every 15 minutes for 12 hours on a Zeiss Axio Observer. Individual cells were tracked and quantified using IMARIS.

6.1.3 Results and Discussion

6.1.3.1 Characterization of Flow Cell and Physiological Relevance

In order to study the impact of shear forces on breast cancer cells, we designed a recirculating flow system to allow for continuous and indefinite perfusion of media across a defined surface (Figure 6-1 a). Cells were seeded onto the glass bottom of the device as single cells on the 2D surface. Media was flowed through the channel at volumetric flow rates ranging from no flow to 1000 µL/min. This flow rate showed a clear correlation with the average linear speed of 20-µm-diameter beads (Figure 6-1 b), selected due to their similarity to the size of cells. The recirculation system used here uses a back-and-forth motion, which requires an occasional change in the direction of the fluid flow. To this end, we characterized the behaviors of the beads in flow to understand the impact of the directional change on the fluid velocity. We identified a 10-15 second stoppage of flow each time the flow changed directions (Figure 6-1 c) although this had minimal impact on the average velocity of particles in flow as characterized in figure 6-1 b. To determine the physiological relevance of this system, we compared the wall shear rate and Reynolds number to reported values for different locations in the vascular system of humans (Figure 6-1 d). Importantly, the Reynolds number fell within the laminar regime, which was confirmed by observation of the 20 µm beads flowing with a clear parabolic profile. Here the Reynolds number was above that in capillaries, although far below the reported values for veins and arteries. However, wall shear fell below most healthy physiological values, but closely matches reported experimental values experienced by circulating tumor cells [312].
The effect of such shear forces has been reported to have contrasting impacts cancer metastasis. Shear decreased the viability of melanoma cells in circulation and increased sensitivity to apoptosis signaling cascades [313, 314]. However, flow can induce autologous chemotaxis to drive motility away from the tumor [315], and shear forces promote adhesion.

![Diagram](image)

**Figure 0-1. Matching flow cell properties to blood flow in vivo.** a. The recirculating flow system designed for this work relies on a base glass slide and a poly(dimethylsiloxane) (PDMS) base with an empty channel that is 4 mm high, 3 mm wide, and 6 cm long. Two syringe pumps communicate to direct volumetric flow with direction changes every 10 mL dispensed. b. 20 µm beads flowed through the device at volumetric flow rates ranging from 100 to 1000 µL per minute, which resulted in linear bead velocities of about 0.5 to 4.5 µm per second through the channel. c. Reversal of the flow at base rate of 750 µL per minute results in a temporary stoppage of bead flow, which is resumed over less than 40 seconds. d. Published data on physiological wall shear and Reynolds number in human blood flow, as compared to calculations for this flow system. Data gathered in collaboration with Elizabeth Swanson.
of cancer cells to the vessel wall, an essential precursor to extravasation [316]. Further, shear stress has been reported to facilitate both upstream and downstream migration relative to the direction of flow. While autologous chemotaxis drives downstream cell migration as described above, cells also undergo a process called *rheotaxis*, where focal adhesions on the upstream side of the cell are under more tension, driving an increase in cell protrusions, local actin polymerization, and mechanosensitive signaling [109]. Together, this suggests that the impact of shear forces on cell movement and survival is a balance of opposing behaviors, and is extremely context-dependent. To that end, we sought to isolate the impact of shear on cell migration specifically through the protease calpain 2, which we have previously identified as mechanosensitive.

### 6.1.3.2 Cancer Cell Response to Shear is Mediated by EGF-Calpain 2 Signaling

Endothelial cells, which line blood vessels, are known to be exposed to high levels of shear in blood flow. Many groups have worked to characterize their response to those shear forces, such as sprouting and migration via the intracellular protease calpain 2 [317]. However, many other cell types also travel through the vasculature, like immune cells, mesenchymal stem cells, and circulating tumor cells. Here we focused on tumor cell migration in response to varied shear flow rates to better understand the biochemical impacts of vascular dissemination of breast cancer cells. First, we identified that increased flow rate resulted in increased migration speed in a control cell (shSCR) population (Figure 6-2 a). The intracellular protease calpain 2 has been proposed as necessary for cell migration in flow in many cell types, including immune and endothelial cells. Here, we used a calpain 2 knockdown cancer cell line (shCAPN2), and tracked migration both with and without shear flow. In the absence of shear flow, calpain 2 expression had no impact on cell migration, but at high flow rates, shCAPN2 cells migrated much slower than the control cell population.
(Figure 6-2 a), suggesting cancer cells use similar motility mechanisms as immune and endothelial cells in the presence of shear force.

Calpain 2 is known to be activated both by calcium signaling and in response to epidermal growth factor (EGF). In highly motile T lymphocytes, which frequently travel through the vasculature, extracellular calcium is essential to activation of calpain 2 [318].

**Figure 0-2. Calpain 2 mediates flow-dependent increases in motility.** a. Either control cells (shSCR) or calpain 2 knockdown cells (shCAPN2) were seeded at 8,000 cells per device, allowed to adhere for 24 hours and then flow was applied for 1 hour prior to imaging for 12 hours. b. Control cells at 0 µL per minute flow were analyzed for migration speeds either with control media, with added EGF, or with added CaCl$_2$ in the media. c-g. Wind-rose plots of random cell migration (top) and cell morphology (bottom) under the following conditions: shSCR cells with no flow (c), shSCR cells at 750 µL/min (d), shCAPN2 cells at 750 µL/min (e), shSCR cells under no flow with EGF (f), and shSCR cells under no flow with CaCl$_2$ (g).
Calpain 2, also known as m-calpain, is activated by millimolar (mM) levels of cytoplasmic calcium, so the exogenous concentration of CaCl$_2$ in this study was increased to 3 mM. This concentration of calcium cannot be sustained within a cell under physiological conditions, but local regions of high calcium can be transiently induced in the presence of shear [319]. In endothelial cells, shear flow of 10 dynes/cm$^2$ induced such localized pockets of high Ca$^{2+}$ and recruitment of calpain 2 to the edge of the cell, so we hypothesized a similar mechanism may have been directing migration in our cancer cell population [320]. We exposed our control cell population to media containing either added EGF (+EGF) or added calcium (+CaCl$_2$) in the device, but with no shear flow. Here we found that the addition of EGF, but not calcium, at least partially mimicked the effect of increased shear on cell migration (Figure 6-2 b).

We also noted differences in cell morphology under shear and these different biochemical treatments. The shSCR cells at high flow were much smaller than no flow or low calpain 2 conditions, which we have previously demonstrated is linked to a more motile and aggressive phenotype in MDA-MB-231 breast cancer cells (Figure 6-2 c-e) [275]. Similarly to our motility results, cells treated with EGF, but not CaCl$_2$, mimicked this morphology (Figure 6-2 f-g). Our data indicates that shear forces increase cell migration via calpain 2 signaling at high flow rates, which may be an important contributor to cell invasion away from the primary tumor and extravasation and seeding at the secondary tumor site.

6.1.4 Conclusions and Future Work

Breast cancer cells experience shear forces at a primary tumor mass and as they travel through the vascular or lymphatic systems to a metastatic site. Little work has been done to understand how cancer cells respond to this class of mechanical forces. Previous work has identified several integrin-signaling mechanisms that drive cancer cell shear-sensing, but as integrins are difficult to target, particularly within a solid tumor environment, here we suggest calpain 2 as a potential target that is required for non-specific integrin turnover, and
responds to changes in stiffness in the tumor microenvironment in addition to shear flow [275].

Future work will need to fully characterize the mechanisms of inhibiting calpain 2 in a tumor-like or flow-based system to confirm EGFR-ERK inhibition, and not calcium chelators, are the appropriate signaling mechanism. Further, in situ systems of extravasation will assist in tissue-level analysis of calpain 2-based motility. For example, previous work out of the Kamm Lab has designed high throughput studies of cell extravasation from a vascular network, which provides a good platform for validation of this work in a 3D system [19]. Further, vascularization of synthetic tissue mimics will contribute knowledge of the tissue-specific nature of metastasis and any relation to extravasation in that process. This work demonstrates a shear-based role for calpain 2 in cell motility, and future studies can further elucidate this process and potentially use it to target metastasizing cancer cells.

6.2 Ethiopian Breast Cancer and its Relation to Changes in the Extracellular Matrix

6.2.1 Introduction

Estrogen receptor positive (ER+) tumors, comprise as much as 80% of breast cancers in the United States [321], but little information exists to determine if this is mirrored globally. Further, only 30% of familial breast cancers can be traced to a known genetic mutation [322], and that number is even lower in non-western populations, as a recent study in Korea found that less than 22% of familial breast cancer could be traced to a BRCA1/2 mutation [323]. Most current work on health disparities in cancer treatment have little ability to distinguish between discrepancies in access to high-quality care and genetic mutations that disproportionately affect an understudied population. For example, in Ethiopia, women wait an average of 1.5 years from noticing a lump to seeking treatment [324]. While this proposed work does not resolve those potential contributions to clinical outcome, here we present a study to isolate somatic mutations that may contribute to poor prognosis.
Recent work has begun to control for several non-biological variables, including income disparities in diagnosing and treating breast cancer in African American women [325]. Here, we probe the biophysical signature of breast cancer in Ethiopian women. About 85% of the Ethiopian population lives in rural areas, which rely on small household farms for food and income [326]. There has been a recent increase in the use of chemical pesticides at such farms in Ethiopia [327], where there are also over 250 sites with at least 1,500 tonnes of obsolete pesticides (defined as old, banned, or not identifiable) in rural areas. Many of these pesticides have also been identified as endocrine disrupting chemicals (EDCs), and the recent uptick has been mirrored by an increase in endocrine related diseases, including, but not limited to, breast cancer [328] and thyroid disorders [329, 330].

One mechanism of EDC action is binding to the estrogen receptor to mimic presence of the hormone, which stimulates cell growth and can induce new mutations. One example of an ER-binding chemical, bisphenol A (BPA), has a well-documented effect on the development of breast cancer through increased cell proliferation and Akt signaling, and this effect is most dramatic when exposure occurs during development [331]. Exposure to BPA during development can also alter the collagen localization. While the overall number of visible collagen fibers was decreased after exposure to BPA, the density of fibers immediately surrounding the epithelial ducts was significantly higher in animals that were exposed to EDCs [332]. This may be due to increased expression of transglutaminase 2 (tgm2) in response to BPA, but only in ER+ epithelial cells, not in the local stroma [333]. Transglutaminase 2 catalyzes crosslinking of the ECM, which could lead to a local stiffening effect [334]. Additionally, exposure to commonly used pesticides, like Fenvalerate, has been shown to promote cell proliferation and collagen production, similar to BPA [335]. Other endocrine disrupting pesticides, like Diazinon, induce lesions with increased macrophage infiltration, a process known to cause regions of stiff and fibrotic tissue [336]. Therefore we
hypothesize that use of pesticides with endocrine disrupting function may serve to stiffen hormone-responsive tissue in young women, leading to an increase in ER+ breast cancer. While the studies described above focused on expression of key genes and proteins, our approach here is to identify mutations that contribute to this aggressive, ER+ breast cancer phenotype in Ethiopian women.

This disparity is further compounded in the laboratory as researchers further explore causes and treatments for breast cancer, as many commonly used panels are limited to the most common genetic markers in European populations. As a result and due to lack of robust research initiatives, these panels exclude markers for other populations. Several published and commercially available panels attempt to characterize the key genes in malignant transformation [337, 338], but often fall short in non-European populations [323, 339]. Additional data bases, such as COSMIC (Catalog of Somatic Mutations in Cancer) are working to further annotate mutations that appear to have a causal, rather than just correlative, relationship with cancer [340]. However, one major limitation of such projects are that they provide and compile a large amount of information on well characterized mutations, rather than addressing the large portion of cancer patients whose tumors are not driven by those specific changes. Gene and protein characterization is the most commonly used clinical tool to inform patient treatment, but this focus on properties of the cancer cell only provides part of the picture, and looking at how the surrounding microenvironment impacts cell behavior can assist in understanding the disease and its variation between populations.

6.2.2 Methods
6.2.2.1 Sample Collection

42 pairs of tumor and matched normal tissue were collected by graduate students at the Black Lion Hospital in Addis Ababa, Ethiopia. Samples were placed in Phosphate buffered saline (PBS) after surgery, and stored down to -80°C. Samples were minimally thawed and sections collected for transport to labs in the United States. Small pieces of the tumor samples were paraffin embedded and sent to the Cooperative Human Tissue Network (CHTN) for hormone receptor characterization.

6.2.2.2 Whole Exome Sequencing

Small pieces of tumor tissue and matched normal tissue for samples 3, 5, 6, and 40, were digested with a DNAeasy blood and tissue kit (69504, Qiagen, Germantown, MD), and assayed for quantity using a Qubit Fluorometer. DNA was sheared to fragments of approximately 150 base pairs using ultrasound pulses of 30 seconds on followed up 30 seconds off, for a total ultrasound exposure of 10 minutes. Samples were then amplified and selected for coding regions with an Illumina Exome TRUseq kit. Samples were run on mid output flow cells on the NextSeq 500 at the University of Massachusetts Genomics Resource Lab. Fastq files were checked for quality, and aligned to the hg38 human reference genome with Bowtie2 and Samtools on the Massachusetts Green High Power Computing Center [230, 341]. Samples were filtered with bcftools and annotated with SNPEff using the online tool ‘Galaxy’ [342-344].

6.2.3 Results and Discussion

Women in Ethiopia develop extremely aggressive breast cancer, and this can occur at a very young age. The most comprehensive study to date followed 1,070 Ethiopian women and determined 2 key contributors to an increased hazard ratio were being diagnosed younger than 50 years old, and being diagnosed with stage 3 breast cancers [345]. Our survey of the
literature revealed that the average age of breast cancer diagnosis in Ethiopian women is 42 years old [346], with almost 2/3 of cases being diagnosed under 50 years old [347] (Figure 6-3 a-b). This matches anecdotal evidence of extremely aggressive breast cancer diagnoses in very young women. In the United States and Europe, hormone receptor negative breast cancer is considered to the most aggressive, and is over represented in African American populations [325]. However, the hormone receptor status of Ethiopian women maps more closely to that of white women in the United States, while breast cancer in other African

![Figure 0-3. Ethiopian patients are diagnosed with ER+ breast cancer at a young age. a-b. Two published clinical data sets show that women born in both East- and West-Africa are diagnosed with breast cancer at a much younger age than their American- and Jamaican born counterparts. Data is plotted from Jiagge et al (a) and Jemal and Fedewa (b). c-d. Ethiopian women, and more broadly, East-African women, are diagnosed with hormone-receptor positive breast cancer at rates more similar to White Americans than other groups examined here. Data is plotted from Jiagge et al (c) and Jemal and Fedewa (d). In a and c, white numbers on the bars represent the number of patients that contribute to that data from each population examined.](image-url)
countries, like Ghana, has a profile closer to that of African American women (Figure 6-3 c). Jemal and Fedewa further make a robust comparison between women of different races and birth locations, and found East African born black women (with the majority of their sampling coming from Ethiopia) had levels of ER expression most similar to that of white women born in the United States (Figure 6-3 d). This indicates that there are both germline and environmental components to the percent of breast cancer cases that are hormone receptor positive.

**Figure 0-4. Mutated genes in Ethiopian breast cancer minimally overlap with commercial SNP panels.** a. 6 ER+ Ethiopian breast cancer cases were selected for analysis via exome sequencing. The work here derives from sequencing of 3 of these patients, cases 3, 5, and 6. Estrogen receptor status (ER) and progesterone receptor status (PR) are given in terms of intensity, % staining, and overall result. b. The somatic mutations from those 3 Ethiopian patients were compared to mutations on commercial SNP panels that are commonly used to study breast cancer in European and North American populations. Colors are matched to samples in a. c. The list of 18 genes that harbor mutations that are shared between all 3 patients and the commercial SNP panels. Data analyzed in collaboration with the Cooperative Human Tissue Network facilities at the University of Virginia, Dr. Courtney Babbitt, and Trisha Zintel.
Because of these notable comparisons between Ethiopian women and American women, we chose to more closely examine gene panels currently used in the United States [337, 338, 348]. First, we selected 3 patients with ER+ staining as our initial sequencing cohort, and sequenced both the cancerous tissue, as well as the matched normal sample (Figure 6-4 a). We then identified genes containing functional mutations of interest, such as non-synonymous mutations, lost start codons, or gained stop codons. When we compared these genes to those identified by commercial panels, we found a total of 18 genes that overlapped, with a very low confidence threshold applied to our samples (Figure 6-4 b-c). This small fraction of overlap from the thousands of genes that we identified as potentially containing driver mutations in our patient samples, indicates that breast cancer in Ethiopian born women is still genetically very different from that of US born women. This analysis relied on comparison of the tumor samples to the matched normal tissue to identify somatic mutations. Future work should also work to identify germline mutations that may also increase a women’s risk for developing breast cancer. However, there are few or no good reference genomes for non-white individuals thus far, and a recent global analysis discovered that most populations differ from the standard reference genome by 4.1 to 5.0 million base pairs, which is consistent with standard human migration models [349]. In that study, individuals from Africa had the largest number of variation sites from the reference genome.

In addition to detecting genetic drivers, high penetrance mutations in understudied populations can identify new therapeutic targets. This has previously been done with an activating mutation in ER+ breast cancer [350], but that work still focused on populations primarily of European descent. There is mounting evidence that the current diagnostics may not capture breast cancer as it presents in African American women [351], highlighting a severe limitation of current genetic panels and treatment options. Treatment targeting the tumor microenvironment, particularly in pathways that are over expressed in African
American women, such as angiogenesis and chemotaxis [339], have seen little clinical success thus far. Here we aim to better capture the spectrum of mutations in non-white, non-BRCA familial breast cancer using whole exome sequencing techniques [7]. One previous example of a small genetic population with non-BRCA familial history of breast and ovarian cancer lays the groundwork for future studies and identified several genes in DNA repair, cell signaling, and apoptosis that were possible predictors for heritable cancer risk [352, 353].

6.2.4 Conclusions and Future Directions

Breast cancer is a global disease that will ultimately affect 1 in 8 women during her lifetime. While most research has focused on western populations, adopting a methodology that examines the global nature of the disease is critical to understanding how breast cancer manifests across populations. Anecdotal and statistical evidence demonstrates that women in East Africa, and specifically in Ethiopia, are diagnosed with aggressive disease at a very young age. Further, in Ethiopia hormone receptor positive tumors are common, which exists in contrast to HR+ tumors in the US being the least aggressive and the statistical evidence that African Americans are more likely to be diagnosed with triple negative breast cancer. Further, pesticide use and improper disposal is common in rural farming communities, which make up a majority of the population in Ethiopia. Many of these pesticides are endocrine disrupting chemicals, which can interrupt or mimic the effect of estrogen, leading cells to remodel their extracellular matrix. We set out to identify the relationship between the breast cancer cells and patient interaction with the environment by performing whole exome sequencing on ER+ tumors from patients in Ethiopia.

Here we demonstrate that in ER+ Ethiopian tumors, there are a set of somatic mutations that minimally overlap with existing genetic panels. Comparing the mutated genes identified here to several commercially available SNP panels, of the 109 genes on at least one panel, 53 genes were not mutated in any of the patients, with only 18 genes mutated in all 3
of the Ethiopian patients. We have further identified mutations in genes associated with gene ontology groupings involved in decreased KRAS signaling (data not shown). This is a promising target, as supplemented by ongoing research in Ethiopia, which identified cytokeratin 18 as a promising biomarker [354]. This methodology is further corroborated by additional studies that examine the frequency of mutations in non-white populations [322, 323]. Together, the exposure to endocrine mimicking agents, which has been shown to facilitate changes in the extracellular matrix, along with identifications of somatic mutations within the cancer cells themselves, provides a promising platform to identify and target breast cancer in an Ethiopian population.


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