Matrix Rigidity Influences Breast Cancer Cell Behavior at Bone Marrow-Like Microenvironments

By

Logan Alexander Northcutt

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

August 9, 2024

Nashville, Tennessee

Approved:

Fiona Yull, D.Phil. (Chair)

Julie Rhoades, Ph.D. (Co-Advisor)

Marjan Rafat, Ph.D. (Co-Advisor)

Rachelle Johnson, Ph.D.

ACKNOWLEDGEMENTS

The work presented in this dissertation was made possible by several funding sources. This includes the NIH Integrated Biological Systems Training in Oncology Training Grant, NIH Grant #R00CA201304-05, VA MERIT Grant #5I01BX001957-08 and the Susan G. Komen Foundation's A Supplement to Promote Inclusion for Research Excellence Grant.

Whew… it's been a long journey but through the grace of God we completed the PhD. There are several people to thank that would have made this journey possible.

First, I must thank my thesis advisors, Dr. Marjan Rafat and Dr. Julie Rhoades, for their mentorship throughout my time at Vanderbilt. Marjan, thank you for your efforts in pushing me to be the best scientist that I can be and your patience in mentoring. I have enjoyed getting to know you not only as a scientist but also as a kind-hearted individual. Julie, thank you for your unwavering support since you became by co-advisor in 2021. Between all the questions that I had, conversations about science and life, and accepting letters of recommendation requests for opportunities; I am mostly grateful for you viewing me as a whole person and not just a researcher. I am forever grateful and look forward to cultivating our mentor-mentee relationship. I would also like to acknowledge my committee members Dr. Rachelle Johnson and Dr. Fiona Yull. Thanks for your questions and feedback over the years as they have helped shape the scientist I am today.

Along with having two advisors, I had the esteemed pleasure being a member in two labs. To the Rafat Lab – especially the original lab members Dr. Ben Hacker, Steven Alves, Tian Zhu, Kevin Corn, Shannon Martello, Greg Berumen, McKenzie Windham and Candace Grisham – thank you for all the advice, feedback and assistance with projects and the stories along the way. I also want to especially recognize my undergraduate mentee, Ally Questell. Thank you, Ally, for not only allowing me to mentor you but also teaching me things and expanding the possibilities of the projects we developed. I am so proud of you for carving out your own path and providing your intellectual input. I am excited for your next steps as an MD-PhD at Harvard University, and I know that you will be more than exceptional there! To the members of the Rhoades Lab - Dr. Erik Beadle, Dr. Natalie Bennett, Dominique Parker, Jade Miller, Rachel Mangano, Emily Jaremba and Jennifer Baum. If only I had enough pages for all the goofy conversations, playful banter and scientific support that we had given each other over the years. Thank you all for cultivating an environment where I am excited to go to work daily.

I also want to give thanks to my family I developed while at Morehouse College, who continue to support me. Tiara Lacey, Alexis Carey, Cedric Bowe, Roy McReynolds III, Dr. Best Uchehara, Dr. Matthew Young, and Dr. Andy Nwancha – I am grateful for the inspiration and support that we have given each other over the years. It just seemed like yesterday that we were in the Woodruff Library studying for Organic Chemistry exams; now we all have either earned or earning MDs and/or PhDs. I am excited for all of us to see what next for all of us. I also want to thank several of my mentors from Morehouse

– Dr. Juana Mendenhall, Dr. Wallace Sharif, Dr. Lance Shipman-Young, Dr. Triscia Hendrickson, Dr. Brian Lawrence and Dr. Brock Mayers. Thank you all for continuing to pour into me and serving as role models.

To the Vanderbilt family, I am so grateful for the friendships and connections that I have made over my past 5 years here. First, I want to thank my IMSD Program Directors and Coordinators, both former and current. Dr. Roger Chalkley, Dr. Linda Sealy, Dr. Christina Keeton, Dr. Julie Rhoades, Dr. C. Henrique Serezani, Dr. Digna Velez-Edwards and Dr. Kafond Wilder – thank you for taking the time and energy to create an environment for people that look like me know they have a place in this university and the academic world. To the members of the Organization of Black Graduate and Professional Students and the Nashville Chapter of the National Organization for the Professional Advancement of Black Chemists and Chemical Engineers, thank you for entrusting me with leadership positions within these organizations during my time as a student. I hope the communities that we were able to cultivate here will continue to build and become stronger. To my friends that I have made at Vanderbilt – Dr. Isaiah Speight, Dr. Kai Bracey, Dr. Jamal Bryant, Dr. Tolu Omokohinde, Dr. Geena Ildefonso, Dr. Dora Obodo, Hamilton Green, Joshua McDuffie, Joshua Livingston and many more that I have not named – thank you so much for all the laughs, conversations and support other these years. I look forward to fostering these relationships in the future. Lastly, I want to thank Dr. Don Brunson, who gave me an opportunity in 2015 as a rising sophomore to be a part of Vanderbilt's summer program when I did not know anything about research. For that, I am forever grateful.

Outside of Vanderbilt, I am also appreciative for the support of the organizations that I have been a part of to help build my career. The Scientist Mentoring and Diversity Program, the Endocrine Society's FLARE Program, Janssen's Scholars in Oncology Diversity Engagement Program and The Leadership Alliance - thank you for the information and assistance over these years. My world view has been expanded by these programs on what a life in science can look like.

To my family - thank you for the love and support from you all. To my mother, father and sisters - Cynthia Northcutt, Stanley Northcutt, Jasmine Northcutt and Jonei Northcutt – thank you for supporting me though all my ups and downs and always keeping me humble. To my extended family, thank you for supporting "Hammy" throughout his journey in becoming the first in the family to earn their doctorate.

Lastly, I want to thank my amazing girlfriend and partner Danielle Robinson. Words cannot express how much I love you and how much you have supported me throughout my PhD, even in ways that were not intentional. Thank you for being my rock in this process. I love you to the moon and back.

DEDICATION

To my family – thank you for your love and support.

To my friends – thank you for all the encouragements and laughs.

To my mentors – thank you for all insights and direction.

To the cancer patients and survivors – thank you for your bravery in the battles that you fight every day. I hope that this and future work will go towards finding a cure.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii
DEDICATION vii
LIST OF FIGURES x
LIST OF TABLES xi
Introduction 1
1.1 Breast Cancer Metastasis and its Impacts on Human Health 1
1.2 Breast Cancer Metastasis and Colonization to the Bone Microenvironment 3
1.3 Physical Influences of Cancer Progression in the Primary Site 5
1.4 Physical Influences of Breast Cancer Cell Progression in the Bone Microenvironment 9
1.5 Bone Microenvironmental Influences on Breast Cancer Progression 11
1.6 Hormone Status and Microenvironmental Influences 13
1.7 Dissertation Focus 18
Emerging Biomimetic Materials for Studying Tumor and Immune Cell Behavior 19
2.1 Introduction 20
2.2 The Extracellular Matrix (ECM) 22
2.3 Biomimetic Tumor Microenvironments 27
2.4 Organoid and Spheroid Models 27
2.5 Decellularized ECM (dECM) 32
2.6 Naturally-Derived Materials 33
2.7 Synthetic Materials 38
2.8 Immune Cell Behavior 40
2.9 Conclusion 44
Development of an alginate-Matrigel hydrogel system to evaluate cancer cell behavior in the stiffness range of the bone marrow 46
3.1 Introduction 47
3.2 Materials and Methods 49
3.3 Results and Discussion 53
3.4 Conclusion 59
Increased Matrix Rigidities Modulate Interleukin Gene Signatures in ER+ Breast Cancer 60
4.1 Introduction 60

4.2 Materials and Methods **62**
4.3 Results **64**
4.4 Discussion **77**
4.5 Conclusions **79**
Chapter 5 80
Summary and Future Directions 80
5.1 Synopsis of Dissertation Work **80**
5.2 Limitations **83**
5.3 Future Directions **85**
5.4 Concluding Remarks **87**
REFERENCES 90

LIST OF FIGURES

Figure 1.1. Metastasis of breast cancer and common metastatic sites……...2
Figure 1.2. Elongated morphology of breast cancer cells at higher matrix rigidities due to ECM Changes……...7
Figure 1.3. Matrix rigidities of the bone marrow and cortical bone……12
Figure 1.4. Breast Cancer subtyping and common sites of metastasis based on receptor status……15
Figure 2.1. Tumor Microenvironment and its components……..21
Figure 2.2. ECM-cell interactions……23
Figure 2.3. Normal tissue and tumor organoid model systems and uses……30
Figure 2.4. The process of deriving decellularized ECM……34
Figure 2.5. Biomimetic materials to model different physiological stiffness ranges……37
Figure 3.1. Development of crosslinked alginate-Matrigel hydrogels……51
Figure 3.2. Evaluation of alginate-Matrigel hydrogel stiffness with varying alginate and calcium sulfate concentrations……54
Figure 3.3. Determination of the effect of stiffness on breast cancer cell proliferation.. 56
Figure 3.4. Investigating breast cancer cell cluster morphology to determine invasive capacity……58
Figure 4.1. Workflow of evaluating ER+ Cell behavior on bone marrow mimetic stiffnesses……66
Figure 4.2. Bone marrow mimetic stiffnesses does not change *GLI2* and *PTHLH* expression in ER+ breast cancer cells compared to normal tissue culture plates……..67
Figure 4.3. RNA sequencing shows transcriptional changes between marrow mimetic stiffnesses and normal tissue culture plates, but not between 0.5 kPa and 32 kPa in MCF7s……69-70
Figure 4.4 Gene Set Enrichment Analysis shows increased expression of genes associated with Interleukin-1 and Interleukin-6 production pathway in MCF7s at bone marrow related stiffnesses…….....71
Figure 4.5 Increasing rigidities does not modulate cytokine secretion in MCF7s**……**73
Figure 4.6. RNA sequencing from MCF7s show higher estrogen signaling gene set enrichments at lower stiffnesses compared to higher stiffnesses……..74
Figure 4.7. ER+ breast cancer cells show decreased expression of downstream Estrogen outputs at protein level at lower stiffnesses……..75
Figure 4.8. Increasing rigidities does not modulate ERα in MCF7s……...76
Figure 5.1. Proposed model summary figure……88

LIST OF TABLES

Table 1. Components of the ECM……24
Table 2. Common biomimetic materials and their applications……..29

Chapter 1

Introduction

1.1 Breast Cancer Metastasis and its Impacts on Human Health

Breast cancer is a prevalent problem for female patients worldwide. According to the American Cancer Society, breast cancer is the leading cancer among women with an estimated 300,000 new cases being diagnosed in 2024. There are several therapeutic options for breast cancer including hormonal therapies, chemotherapies, immunotherapies and other personalized medicines. However, mortality rates of breast cancer are still less than optimal with deaths of breast cancer in 2024 estimated to be around 42,000. Additionally, breast cancer creates a financial strain on the healthcare system and patients. In 2020, it was estimated that $29.8 billion USD was spent on healthcare related to breast cancer. For patients, the estimated cost of initial care in 2020 was $34,979.50 USD and $76,101.20 USD for care during the last year of life.[1–3] One of the major causes of mortality in breast cancer patients is metastasis to other sites.

Metastasis is the spread of cancer cells from the primary tumor site to a distant site in the body and is a well-studied hallmark of cancer and is associated with higher death rates in patients.[4,5] In breast cancer, distant recurrence is common in sites such as the brain, bone, liver, lymph nodes, and lungs.[6] (**Fig. 1.1**) In order for cancer cells to metastasize, they undergo an epithelial-to-mesenchymal transition and then circulate in the vasculature, exiting an colonizing at a secondary site.[7] This process has been

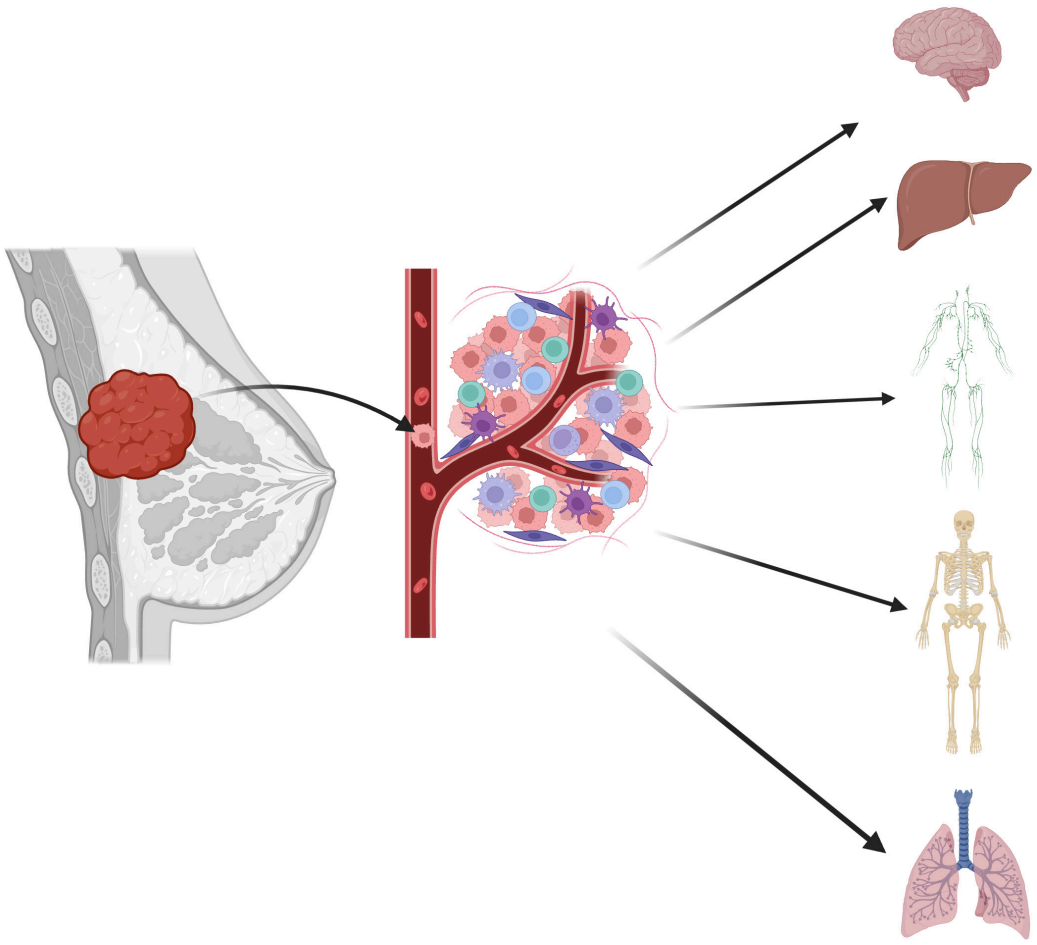

Figure 1.1. Metastasis of breast cancer and common metastatic sites. Breast cancer can undergo the metastasis from the primary breast tissue to other parts of the body. Common secondary sites include the brain, liver, lymph nodes, bones and lungs. Figure created in BioRender.

coined the “metastatic cascade” and is seen in various types of cancers. It is thought that breast cancer cells circulate to visceral organs due to access of blood flow, however, it is also thought that breast cancer cells can establish a premetastatic niche in other sites to prime them for colonization.[8,9] This growth of tumor cells at other organ sites causes disruption in function which leads to complications and mortality for patients. According to the National Cancer Institute’s 2023 Surveillance, Epidemiology and End Results Program, there is a 5-year survival rate of 99.3% when patients present localized tumors. However, the survival rate decreases to 31.0% when patients present distant metastasis.

1.2 Breast Cancer Metastasis and Colonization to the Bone Microenvironment

The bone microenvironment is a common site of metastasis for breast cancer. It is not well understood why breast cancer tends to home to the bone, but it is speculated that the vascularization and growth factors in the bone make it ideal for survival and proliferation.[10] It has been shown that bone metastases are correlated to lower incidences of death in breast cancer patients compared to visceral organs, but 70% of patients who succumb to the disease have bone metastases after autopsy.[11] Patients may exhibit bone metastases 5-15 years after diagnosis.[12,13] Interestingly, this late detection of bone metastases is attributed to tumor cell dormancy, a phenomenon where tumor cells undergo decreased proliferation. This decreased proliferation of the tumor can be contributed to immunological processes mediating the tumor size or intracellular processes inducing quiescence.[14,15] The processes of when and how cells awaken from dormancy and begin proliferation in the bone microenvironment is still widely debated.

Under normal skeletal remodeling, osteoblasts (bone-forming cells) directly interact with osteoclasts (bone-degrading cells) to create structurally sound bone. After breast cancer cell dissemination to the bone marrow, breast cancer cells will interact with the bone microenvironment through the process known as the 'vicious cycle of bone destruction'.[16,17] In this process, cancer cells will disrupt normal bone remodeling by interacting directly with the osteoblasts via expression of parathyroid hormone-related protein (PTHrP), causing osteoblasts to increase their expression of Receptor activator of nuclear factor kappa-B ligand (RANKL). The RANK receptors on the osteoclast precursors bind to RANKL from the osteoblasts. This causes the osteoclast progenitors to begin fusion into mature osteoclasts and begin resorption of the bone.[18] Tumor cells can also bypass the osteoblast-RANK mediated signaling and communicate directly with the osteoclasts. This is typically done by using cytokines such as IL-1 and IL-6 to cause osteoclastogenesis and activity.[10] This pathway can disrupt normal bone remodeling by resorption outcompeting formation. In addition to bone degradation, the byproducts of this process such as extracellular calcium and transforming growth factor beta (TGF-β) can be released from the bone matrix and feed the proliferation and invasion of these metastatic breast cancer cells.[19] Although prevalent in breast cancer, this process is also seen in other cancers that can home to the bone microenvironment, such as prostate cancer.[20] The vicious cycle of bone destruction can cause several ailments for patients. These include pathological factures and hypercalcemia that can lead to renal failure. Typically, patients will have a life expectancy of 18 months after diagnosis with bone metastases. [21,22]

1.3 Physical Influences of Cancer Progression in the Primary Site

Although there have been several studies evaluating the intracellular signaling that generates the vicious cycle, it is not clear how the bone microenvironment itself contributes to the progression of the tumors. In this project, we considered analyzing if the physical factors of the bone microenvironment can influence the tumor cell progression. This started due to current evidence about how the primary site influences tumor progression through a variety of processes associated with its physical characteristics.

Tumorigenic ECM and Biophysical Parameters

The extracellular matrix (ECM) is a network of macromolecules that provide biophysical and biochemical support for cells.[23,24] In mammals, the ECM primarily consists of mainly fibrous collagen, but also laminin (which makes up the basement membrane), and fibronectin (which have integrin-binding sites), that provide a scaffold for cells to form tissues and migrate. In the context of a tumorigenic breast environment, tumor cells can manipulate or dysregulate the ECM to their advantage for proliferation and metastasis. This can be done directly by the tumor cells that are proliferating and even dormant. One study showed that dormant cancer cells created a fibronectin matrix and can release matrix metalloproteases (MMPs) to degrade the matrix.[25] This degradation is a key process in allowing cancer cells to move through the stroma and undergo intravasation and extravasation.

Tumor cells can also utilize fibroblasts which are stromal cells responsible for generating connective tissue though the deposition of collagen. When normal fibroblasts encounter tumor cells, they can transition into cancer-associated fibroblasts (CAFs), fibroblasts that can secrete cytokines and can remodel the ECM to allow growth and motility. This is done by causing upregulation of transcription factors in the fibroblasts. For example, when breast cancer cells were co-cultured with normal fibroblasts (NFs), there was an upregulation of Friend leukemia integration 1 (Fli-1) and Transcription Factor 12 (TCF-12) genes that are associated with cancer-associated fibroblasts (CAFs) and tumor progression.[26]

The surrounding architecture of the ECM is also important to the movement and morphology of the cancer cells. (**Fig. 1.2**) It has been shown that higher confinement of cancer cells can affect their motility. [27] With these factors taken into consideration, it is important to make sure that the physical properties of the environments are well understood to better understand certain premetastatic niches, environments primed for cancer metastasis.

Mechanotransduction and Breast Cancer Progression

The process by which mechanical cues affect biological responses is called mechanotransduction. Mechanotransduction is integral to cell development in all parts of the body, and it is also highly prevalent in looking at tumor progression in the primary site.[28] To promote growth, tumor cells utilize degradation and the formation of collagen, numerous mechanotransduction processes are taking place and can contribute to the phenotype of breast cancer cells.[29] This is mainly due to the signaling pathways that are

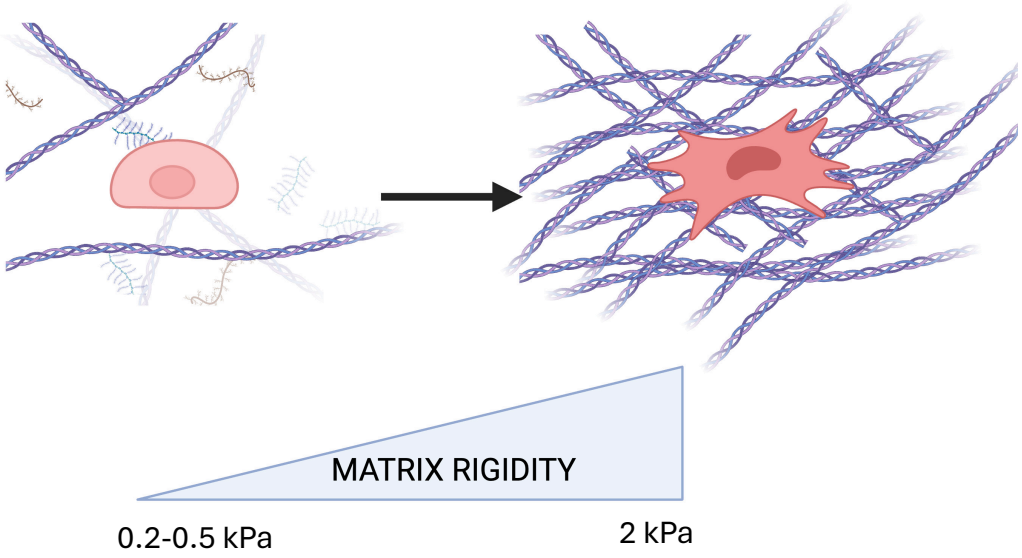

Figure 1.2. Elongated morphology of breast cancer cells at higher matrix rigidities due to ECM changes. Increased matrix deposition and degradation of tumorigenic microenvironments can cause increased rigidities that tumor cells sense. This can cause breast epithelial cells to change their morphologies to become more invasive though the activation of mechanotransduction pathways. Figure created in BioRender.

affected when factors such as fibrotic macromolecules and stress are increased in the tumorigenic environment. These pathways usually include integrin signaling, phosphoinositide 3-kinase (PI3K)/Akt mechanosensitive pathway, and yes-associated protein (YAP) and transcriptional coactivators with PDZ-binding motif (TAZ) that is associated with Hippo and Wnt signaling. The upregulation of these pathways can cause an increase in metastasis, epithelial-to-mesenchymal transition, and tumor cell proliferation by inducing pro-tumorigenic proteins such as STAT3, gp130, MMP7 and Myc. [30] Additionally, mechanosensitive genes can be expressed though mechanotransducers, which are mechanically activated proteins and attach to transcription factors in the nucleus and influence gene expression, such as YAP/TAZ.[31]

Matrix Rigidity and Tumor Progression in Breast Cancer

The stiffness of the environment is commonly associated with tumor progression.[32] Cancerous breast tissue is generally stiffer (2 kPa) than normal tissue (0.1 kPa), and this is highly correlated to the metastatic potential, which is mostly due to the upregulation of fibrillar macromolecules such as collagen and fibronectin.[33] These macromolecules are deposited by cancer-associated fibroblasts and provide a track for cancer cell motility. It has also been shown in breast mimetic microenvironments that breast cancer cells increase their invasive capacity when seeded at higher stiffnesses via the usage of invadopodia and the upregulation of genes that are used for ECM remodeling and cell adhesion such as matrix metalloproteinase-1 (MMP1) and fibronectin 1 (FN1).[34,35] It is also thought that some cancer therapies, including radiotherapy, can affect the stiffnesses of the tumor microenvironment. For example,

irradiated tissues have been shown to contribute to tumor cell recruitment.[36] Stiffness not only affects the cancer cells but also the cells that are responsible for the development of the ECM, including fibroblasts and macrophages.

Researchers have shown that stiffnesses within a microenvironment can cause stromal and immune cells to change their phenotypes. For example, He et al. showed that macrophages seeded in higher stiffnesses (60.54 kPa) are not as proliferative as in softer matrices (1.5 kPa).[37] Macrophages with a more pro-tumorigenic phenotype can deposit matrix metalloproteases for angiogenesis, ultimately increasing metastatic potential of the surrounding tumor cells. Another component of the microenvironment that is highly affected by stiffness are the fibroblasts. At higher stiffnesses, fibroblasts increase in their spreading and production of angiogenic factors.[38] It has also been shown that the combination of stiffness and factors associated with cancer can cause the transition into CAFs. One study showed that microvesicles from MDA-MB-231 breast cancer cells promoted cancer-associated fibroblasts when NFs were seeded on stiffnesses associated with breast cancer (1 kPa – 20 kPa).[39] These CAFs can deposit more fibrous matrix, ultimately increasing the stiffness of the environment. Overall, the stiffnesses in the environment causes cells to behave differently and promotes cancer progression via different mechanisms.

1.4 Physical Influences of Breast Cancer Cell Progression in the Bone Microenvironment

In the body, various tissues have different matrix rigidities ranging from 0.5 kPa in the marrow and breast, 2-8 kPa in the lungs, and cortical bone ranging to 20,000 kPa.[40] These rigidities are associated primarily with the macromolecules and cell types

that give the organ its function. The bone marrow microenvironment is highly dynamic and is developed from many fibrous macromolecules and progenitor cells.[41,42] It is also highly variable in its physical properties such as stiffness, three-dimensional (3D) architecture, and fluid flow. However, few studies have discussed the physical influences of breast cancer progression at their metastatic sites, particularly in the bone and bone marrow niche. In this this section, I will discuss how the physical parameters of the primary and metastatic sites can influence tumor cell progression.

Bone Marrow Composition

The bone marrow is one of the largest organs within the body, accounting for 5% of the weight of humans.[43] It is responsible for the production of hematopeotic stem cells (HSCs) and downstream progenitor populations.[44] These cells are responsible for regulating the production of immune cells such as monocytes, granulocytes, macrophages, natural killer cells, T cells, and B cells. In addition to these and other progenitor cells, the bone marrow also houses adipocytes and red blood cells.[45] One important factor to consider is the stromal environment of the bone marrow. On a macromolecular level, the bone marrow is comprised of mostly collagen and fibronectin secreted by fibroblasts.[46] In addition to progenitor and hemopoietic stem cells, mesenchymal stem cells in the bone marrow assist in regulating the vasculature and depositing matrix.[42] There are a variety of biophysical factors, such as stiffness and pressure within the bone marrow, that can assist cause breast cancer progression after metastasis.

Stresses in the Bone Marrow Environment

There are a variety of stresses within the bone marrow microenvironment. The bone marrow's mechanical factors can be categorized as internal and external forces. Internal forces consist of hydrostatic pressure and stiffness of the bone marrow while the external forces are due to physical loading.[47] Although these forces are different, each of them affects biological processes in the bone marrow including the production of osteoactive agents such as TGF-β.[48] Studies have shown that the stiffness of the bone marrow microenvironment influences the progenitor cells that are responsible for the development of certain cells such as HSCs.[49] With bone marrow spanning from 0.5 kPa to 40 kPa in stiffness, these biophysical changes within the bone environment can potentially affect the metastatic capability and proliferation of cancer cells and how they interact with the ECM.[50] (**Fig. 1.3**)

1.5 Bone Microenvironmental Influences on Breast Cancer Progression

Bone Marrow Microenvironment and Breast Cancer Progression

One of the most common sites of breast cancer metastasis is the bone marrow.[51] There are several factors that make this an optimal premetastatic niche. The high vascularity of the bone marrow increases the likelihood of hematogenous dissemination.[52] Additionally, cytokines within the environment such as stromal cell-derived factor 1-alpha (SDF1α) attract cancer cells to the metastatic sites since the binding partner (CXCR4) is highly expressed on cancer cells.[53,54] These cancer cells can also express genes such as Runt-related transcription factor 2 (RUNX2) and glioma 2 (Gli2) that help the survival of tumor cells in the bone microenvironment.[55]

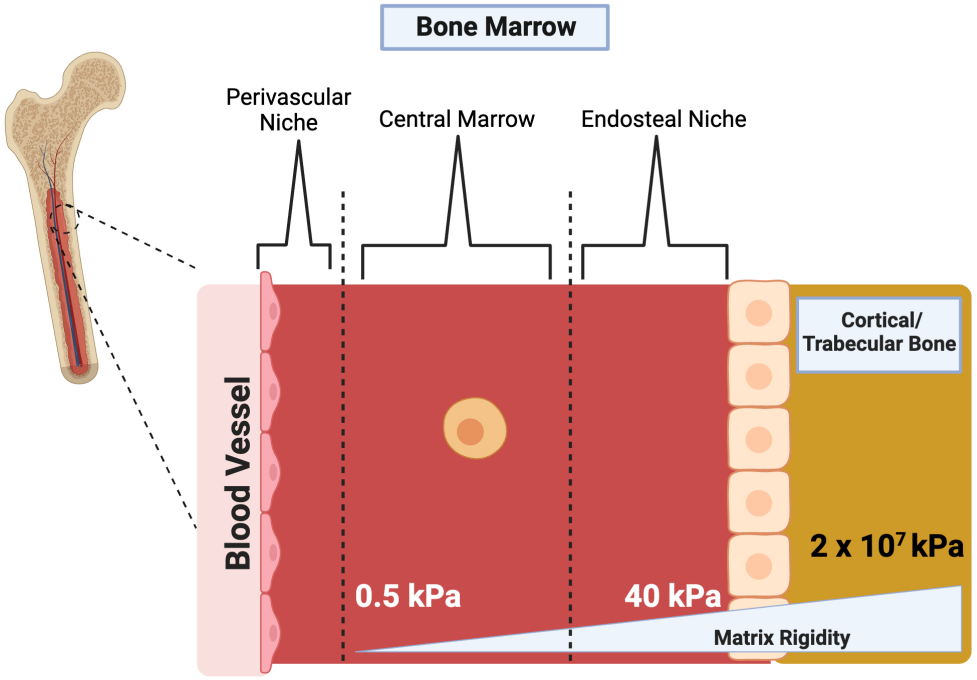

Figure 1.3. Matrix rigidities of the bone marrow and cortical bone. The rigidity of the bone marrow microenvironment increases from the central marrow (0.5 kPa), to the endosteal niche (40 kPa), and tis highest at the cortical bone (2 x 10^7 kPa). Figure created in BioRender.

Some studies showed that the adhesion to vascular cell adhesion molecule (VCAM-1) in the bone marrow can cause multiple myeloma to promote osteoclastogenic activity.[56] This process of the vicious cycle is mostly perpetuated by factors such as extracellular vesicles, extracellular calcium, and TGF-β, present within the environment and their signaling to other cells directly in the vicious cycle.[57] Other stromal interactions and processes in the extracellular matrix can cause breast cancer cells to metastasize and proliferate. These include key factors such as mesenchymal stem cells and CAFs that can deposit matrix into the environment. Few studies have been conducted to understand how the physical properties of the bone marrow and bone microenvironment can affect the metastatic niche and the propensity for cells to metastasize and proliferate. Some studies have shown that that increased stiffness of the bone marrow microenvironment causes upregulation of PTHrP, a key molecule in the vicious cycle of bone destruction.[58] Follow-up studies have shown that the expression of PTHrP and Gli2, the transcription factor for PTHrP, is regulated by Integrin Beta -3 (Iβ3) colocalized with TGF-β Receptor Type II (TGF-β RII).[59] Studies have shown that the bone microenvironment can increasingly become remodeled as cancer cells promote the production of thicker and denser collagen I.[60] With this knowledge, it may be plausible that vicious cycle may be a mechanotransducive process that is perpetuated not only through molecules from the bone matrix but biophysical changes through the ECM.

1.6 Hormone Status and Microenvironmental Influences

Hormonal Status and Metastasis in Breast Cancer

Hormonal status is highly important for diagnostic and treatment efforts for breast cancer. Breast cancer is typically evaluated by its expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). This can be further categorized by combinations of receptors such as luminal A (ER+, PR+, HER2-, and low Ki67), luminal B (ER+, PR+, HER2+/-, and high Ki67), HER2+ (ER-, PR-, and HER2+) and triple-negative breast cancer (ER-, PR-, and HER2-) (TNBC). Most breast cancer patients, 60-70%, have hormone receptor-positive breast cancer.[61] These receptors and their subsequent pathways are associated with development growth for normal epithelial cells.[62] However, these receptors can be mutated or overexpressed for continued proliferation in cancerous epithelial cells.[63,64] Depending on the subtype, many patients can receive hormonal therapy (for ER+ or PR+ cases), targeted therapy (typically for HER2+ cases) along with other combination therapies. For hormonal therapies, many of these cancers require an aromatase inhibitor, a selective estrogen/progesterone receptor degrader (SERD), or a selective estrogen/progesterone receptor modulator (SERM). Many of these therapies have been used for several years and are typically first line treatments for cancer, although there is debate about which ones are most effective.[65,66] Additionally, new hormonal therapies are being generated to target ER through a variety of mechanisms.[67]

Although these treatments are effective, breast cancer can metastasize to surrounding tissues and distant organs. In breast cancer patients who present metastases, HR-positive patients report more metastases to the bone while HR-negative breast cancer patients tend to have metastases to the brain, lymph nodes, and lung.[61] (**Fig. 1.4**) Currently, it is unclear why these patterns exist based on breast cancer

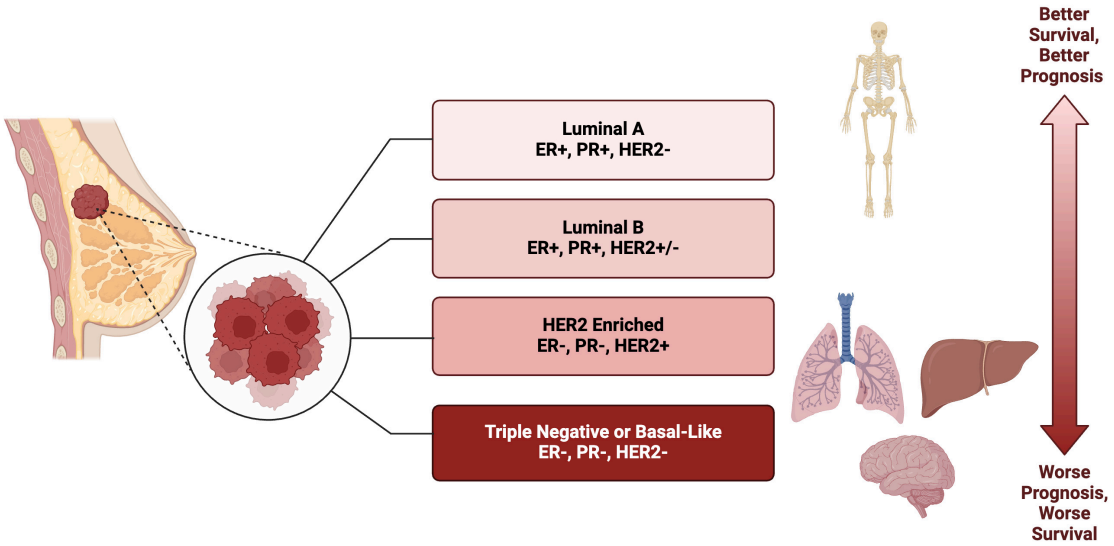

Figure 1.4. Breast cancer subtyping and common sites of metastasis based on receptor status. When breast cancer cells metastasize, the subtypes that express hormonal receptors show higher propensity to the bone. Whereas breast cancer subtypes that have lower hormone receptor status tend to metastasize to visceral organs such as the lung, liver and brain. Figure created in BioRender.

subtype; however, there are some working hypotheses. Some researchers believe that the bone microenvironment provides a hospitable environment for cells and can induce dormancy, while others also hypothesize that hormonal negative breast cancer does not have enough time to metastasize to the bone since it is more aggressive at other sites that cause immediate pathological issues for patients. Most breast cancer patients are less responsive to hormonal therapies after tumor metastasis and have a shorter life expectancy.

There is minimal understanding of what hormonal changes occur during the metastatic cascade. For tumor cells to undergo metastasis, cells must undergo epithelial-to-mesenchymal transition (EMT). Even though there is not a large body of work looking at the metastatic cascade though the lens of hormonal responses, there is some information that links HR status to EMT. Saleh and colleagues showed silencing of ERα using siRNAs induced a vimentin-based cytoskeleton in MCF7s.[68] Other studies also show that the deletion of ESR1, the gene that encodes for ERα, in MCF7s increased growth rates and expression of known EMT markers such as ZEB1, Snail2/Slug, and fibronectin. They also showed that ERα loss can decrease the expression of other hormone receptors.[69] Ma and coworkers also showed that the activated YAP can repress transcriptional expression of ESR1.[70] These processes of ER being decreased and cells changing their behavior have been seen at metastatic sites as well. Bado and colleagues show that in PDX and immortalized ER+ models, there is a decrease in ER in micrometastases in the bone, and tumor cells regain ER expression when they form overt tumors.[71] They also show that bone micrometastases of ER+ tumors cells are resistant to endocrine therapies. On the contrary, Clements and

colleagues showed that in MCF7 bone clones (MCF7b), nuclear and cytoplasmic ERα was not changed. However, there was a change in proliferation and invasion in MCF7b compared to parental MCF7 lines.[72]

This work shows that the microenvironment that the cells experience may contribute to how ER+ tumor cells behavior in a metastasis, However, it is unclear what exact factors of the microenvironment could contribute to those differences in behavior.

Physical Factors and Hormonal Statuses in Breast Cancer

Although there are several studies evaluating breast cancer response to different physical factors, there is little understood about how the physical factors influence hormonal signaling in breast cancer. Based on the current literature, there is not a definitive understanding of how HR-positive breast cancer cells respond to varying physical stimuli, but HR-negative tend to respond to the environment more readily. Current immortalized breast cancer models with a positive hormonal status, such as MCF7s and T47Ds, typically have non-invasive characteristics in both 2D and 3D cultures.[73] However, when the microenvironments change, the cell lines can change their behavior and signaling can change.

In a novel alginate-Matrigel system that mimics the stiffness of the bone marrow (0.5 kPa – 16 kPa), MCF7s embedded in the hydrogel at 16 kPa show similar morphologies as more invasive 4T1s. Others have identified that ER signaling can be activated when compressed by stiffnesses at 37 kPa, like that of dense primary tumors. They show that p38p and MAPK increased in both normal patient cells and breast cancer derived from patients.[74] Additionally, this concurs with other studies that show other hormone dependent cancers are modulated at different stiffnesses. One study

showed that MCF7 and T47Ds (PR+ cell lines) can modulate PR modulators at different stiffnesses related to tumorigenic environments.[75] Spencer and colleagues also showed that although both MCF7s and T47Ds did not increase proliferation at higher stiffnesses, there was a decrease in drug responses and lower proliferation, which is a hallmark of EMT.[76] However, Kloxin and colleagues also show that T47Ds, an ER+ breast cancer line, do not adjust their shape factors when embedded in a PEG hydrogel with various binding peptides associated with laminin, fibronectin and collagen.[77] It is also unclear how these breast cancer cells behave at stiffnesses related to metastatic sites. Ruppender and colleagues showed that in bone mimetic models, MCF7s did not respond to stiffness in terms of their morphologies and osteolytic factors compared to MDA-MB-231s in polyurethane bone-mimetic models.[78]

1.7 Dissertation Focus

My dissertation focuses on two projects related to understanding how the stiffness of the bone marrow contributes to breast cancer cell progression after metastasis. First, I will discuss a project where we developed a 3-D bone marrow mimetic hydrogel system and evaluated breast cancer cell behavior. Here, we were able to identify phenotypic changes to breast cancer cells that mimic an invasive phenotype. Secondly, I will present our findings on how stiffnesses of the bone marrow changes the transcriptional and molecular phenotypes of hormone positive breast cancer cells that can potentially contribute to changes in the bone microenvironment.

Chapter 2

Emerging Biomimetic Materials for Studying Tumor and Immune Cell Behavior

The work in this chapter is published and adapted from: Northcutt L*, Suarez-Arnedo A*, and Rafat M. Emerging Biomimetic Materials for Studying Tumor and Immune Cell Behavior. Ann Biomed Eng. 2020 Jul;48(7): 2064-2077.doi: 10.1007/s10439-019-02384-0. Epub 2019 Oct 15. (*both authors contributed equally to this work)

Abstract

Cancer is one of the leading causes of death both in the United States and worldwide. The dynamic microenvironment in which tumors grow consists of fibroblasts, immune cells, extracellular matrix (ECM), and cytokines that enable progression and metastasis. Novel biomaterials that mimic these complex surroundings give insight into the biological, chemical, and physical environment that cause cancer cells to metastasize and invade into other tissues. Two-dimensional (2D) cultures are useful for gaining limited information about cancer cell behavior; however, they do not accurately represent the environments that cells experience *in vivo*. Recent advances in the design and tunability of diverse three-dimensional (3D) biomaterials complement biological knowledge and allow for improved recapitulation of *in vivo* conditions. Understanding cell-ECM and cell-cell interactions that facilitate tumor survival will accelerate the design of more effective therapies. This review discusses innovative materials currently being used to study tumor and immune cell behavior and interactions, including materials that mimic the ECM composition, mechanical stiffness, and integrin binding sites of the tumor microenvironment.

2.1 Introduction

The ability of tumor cells to metastasize beyond the primary tumor and invade into new tissues is one highly-studied hallmark of cancer.[79] To gain more information about this phenomenon, physicians and researchers use techniques such as incisional biopsies to extract tumor samples. These specimens are helpful for gaining information such as expression of genes and prevalent proteins that are associated with malignant phenotypes and cancer cell proliferation.[80] Although informative and extracted from patients, one disadvantage of direct evaluation of primary tissues is the large degree of variability in each sample. Another popular methodology in studying cancer *in vitro* is two-dimensional (2D) tissue culture. One key limitation of this method is that 2D culture does not recapitulate physiologically relevant three-dimensional (3D) microenvironments, which typically results in distinct outcomes from *in vivo* conditions.[81] Another drawback of this technique is primarily the lack of integrin binding sites present in the tumor microenvironment. In addition, the complexity and tunability of 2D environments are severely limited.[82]

While much information has been gained from monolayer systems, there has been a recent push to develop tunable, 3D cell-culturing systems to better mimic the tumor microenvironment, which includes fibroblasts, immune cells, soluble factors, and extracellular matrix (ECM) proteins (**Fig. 2.1**). One of the most sought-after approaches to address these issues is the use of biomimetic materials that model specific *in vivo* characteristics, including chemical composition or stiffness.[83] Biomimetic materials have been developed for applications ranging from the regeneration of cartilage to wound

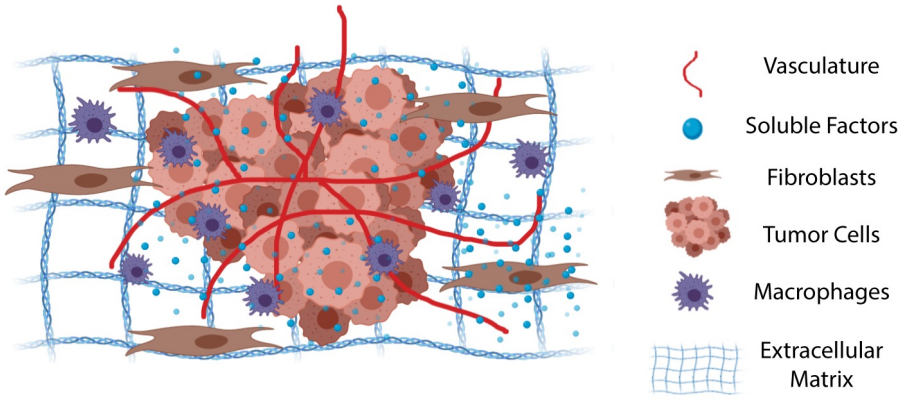

Figure 2.1. The tumor microenvironment and its components. Tumor cells, immune cells, stromal cells, ECM, and soluble factors. Figure created in BioRender.

healing.[84] Currently, these materials have also been used to create synthetic environments that can be used to study cell morphology as well as the underlying molecular causes of malignant cell morphology and invasion.

In addition to tumor cells alone, incorporating multiple cell types such as immune and stromal cell populations have allowed researchers to better model the *in vivo* microenvironment, cell-cell interactions, and cell-ECM interactions. This has led to an increased understanding of tumor cell behavior and improved tumor targeting therapeutic strategies. The immunological response is also an important factor when developing 3D microenvironments. Tumor-promoting inflammation is an additional hallmark of cancer, so understanding how immune cells interact with tumor cells in the microenvironment is imperative for determining the causes behind tumor progression and metastasis.[79,85,86] In this review, we will outline the emerging materials utilized in designing biomimetic microenvironments for evaluating tumor and immune cell behavior.

2.2 The Extracellular Matrix (ECM)

Defining the ECM

The ECM is a 3D network that consists of various proteins and macromolecules necessary for providing cellular, biochemical, and structural support. All tissues in the body contain ECM components but vary in their composition. Most mammalian ECM consists of collagen, fibronectin, and laminin, along with glycosaminoglycans that form proteoglycans.[24,87] **Table 1** lists the key components present in the ECM. In cancer studies, cell-ECM interactions are commonly studied (**Fig. 2.2**), which facilitate changes in

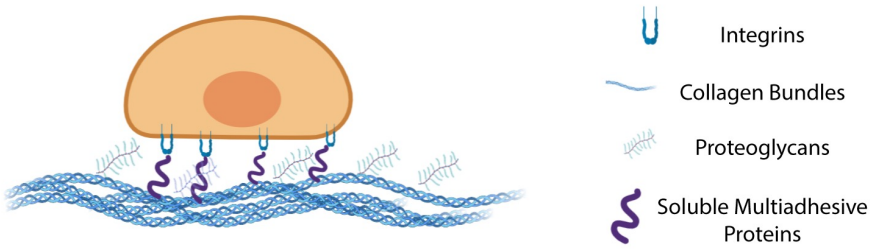

Figure 2.2. Extracellular matrix (ECM)-cell interactions. Figure created in BioRender.

Table 1. Components of the extracellular matrix (ECM).

Main Components	Macromolecules	Function
Highly Viscous Proteoglycans[88]	Aggrecan Decorin Lumican Perlecan Syndecan Versican	Interact with integrins Hydration buffer Mitigate shear stress
Insoluble Collagen Fibers[89,88]	Collagen I Collagen II Collagen III Collagen IV Collagen V	Provide structure Cell adhesion
Soluble Multiadhesive Proteins[90,91,88]	Laminin Fibronectin	Integrin binding Cell growth Cell adhesion

cytoskeletal actin filaments, upregulation or downregulation of oncogenes, and expression of proteins associated with cancer cell invasion and proliferation.[82,92]

ECM and the Tumor Microenvironment

The ECM is a prominent factor in cancer progression in the tumor microenvironment.[93] There has been extensive research into how the chemical and physical properties of the ECM can affect the proliferation and migration of cancer and immune cells as the composition and structure of the ECM is integral to the regulation of cell growth, phenotype, and organization.[94] Many recent studies have attempted to mimic particular ECM properties and structures to study cancer progression. For example, Narkhede et al. developed hyaluronic acid (HA)-based hydrogels since HA is a key component of the brain ECM and has similar viscoelastic properties to the ECM.[95] The HA hydrogel stiffness was varied from 0.2 kPa to 4.5 kPa to understand how changes in mechanical cues can affect the behavior of brain metastatic breast cancer cells. According to their results, breast cancer cells proliferate and adhere to a greater extent in stiffer microenvironments, and this was mediated by the focal adhesion kinase (FAK) - phosphoinositide-3 kinase (PI3K) pathway.[95] Another example is the work by Kim et al., where they incorporated collagen I and Matrigel into a microfluidic device to study how the recruitment of immune cells alters the invasiveness of cancer cells. This work demonstrated monocyte and macrophage-induced remodeling of the ECM, which enhanced the invasiveness of cancer cells.[96] ECM components have also been utilized as bioinks in 3D printing technologies.[97,98] For instance, Duarte Campos et al. used a type I collagen-based bioink to make a 3D mini-model of neuroblastoma. These models were

able to mimic *in vivo* characteristics of neuroblastoma such as proliferative potential and the formation of Homer Wright-like rosettes.[99,100]

Integrin Binding Patterns

One of the most important drivers of communication between cells and the ECM is the presence of integrins. Integrins have key functions in multiple processes that facilitate tumorigenesis, progression, and metastatic potential of cancer cells.[101] These cellular receptors are implicated in signaling molecules, cell migration machinery, the epithelial-to-mesenchymal transition (EMT), and mechanotransduction.[102,103] For instance, integrins allow for the activation of epidermal growth factor receptor (EGFR) and the modulation of epithelial differentiation in 3D microenvironments. EGFR also promotes the formation of focal adhesions, structures that bind integrins to the cytoskeleton and organize the conformation, tension, and morphology of cells.[104] Integrin αvβ6 is the binding receptor in tumorigenic cells while α5β1 is a constitutively expressed integrin that plays the same role in healthy cells.[105,106] Many studies have identified the role of specific integrin subtypes in cancer progression mediated by tissue stiffness using diverse biomaterials. Using alginate-Matrigel interpenetrating networks, Chaudhuri et al. proposed a mechanism where junction α6β4 integrins and laminin play an important role in the development of malignant phenotypes in breast cancer, which is dependent on ECM stiffness. If the stiffness of the ECM is in the normal range (30 Pa), the α6β4 integrin–laminin units overlap and allow for hemidesmosome formation. However, high ECM stiffness (310 Pa) reduces hemidesmosome formation, and the free β4 can lead to Rac1 signaling and PI3K activation for phosphorylation by receptor tyrosine kinases.[82]

Moreover, it was shown using 2D tunable collagen I-coated polyacrylamide (PA) hydrogels that an increase in stiffness and availability of collagen binding sites leads to the upregulation of integrin β1 in hepatocellular carcinoma (HCC) that promotes cancer progression by the activation of transforming growth factor beta-1 (TGFβ-1).[107]

2.3 Biomimetic Tumor Microenvironments

To study various organs and diseases, biomimetic environments have typically been designed to provide structure as a scaffold for cell growth and adhesion. More recently, many emerging biomaterials have been used to evaluate EMT, cell-ECM interactions, and immune cell infiltration, including organoids, decellularized ECM, natural materials, and synthetic materials. These materials and applications are summarized in **Table 2**.

2.4 Organoid and Spheroid Models

Organoid and spheroid systems are useful models for biomimetic microenvironments. Organoids are organ-like structures and are derived from cells and tissues ranging from murine to human species (**Fig. 2.3**). Organoids recapitulate key structural and functional components of various sites of interest from the brain to retinal systems.[108–110] Organoids have been used extensively in studying multiple cancer models. [111–114] For example, CRISPR/Cas9 gene editing was performed to introduce KRAS, SMAD4, APC, and TP53 mutations into normal human intestinal organoids to form adenocarcinomas in mouse models during xenograft transplantation.[115] Organoid models have been developed to mimic drug resistant tumors and to evaluate therapeutic efficacy.[108,116,117] Sun et al. reported the formation of organoids from reprogrammed

hepatocytes to initiate HCC through c-Myc expression.[118] It was shown that these organoid models can form tumors *in vivo*, making them a useful system for generating mouse xenografts to study drug response.

Table 2. Common biomimetic materials and their applications.

	Biomaterial	Cancer	Application
Organoids	Mammary epithelial cells	Breast	Normal tissue radiation effects on tumor-stromal and immune interactions[119]
	Pancreatic cancer cells	Pancreatic	Tumor-stromal and tumor-immune interactions[120]
Decellularized ECM	Bladder-derived	Skin, colon, breast	Tumor growth delay and immune signatures[121,122]
	Mammary fat pad-derived	Breast	Radiation effects on tumor cell behavior, breast cancer proliferation, and invasion[123]
	Colorectal cancer-derived	Colon	Establishing the colorectal tumor microenvironment influence on macrophage-mediated cell invasion[124,125]
	Breast cancer-derived	Breast	Evaluation of tumor development and progression[126–129]
	Liver-derived	Liver	Determining how the mechanical properties of cirrhotic liver tissue alter tumor cell behavior[130]
	Human tumor-derived	Colon	Studying the mechanisms of chemotherapy resistance at different stages of malignancy[131,132]
	Brain tissue-derived	Brain	Investigating glioblastoma cell invasion[133]
	Colon tumor-derived	Colon	Evaluating tumor progression at different stages of tumorigenesis [134]
	Xenograft-derived	Breast, liver, lung	Studying the behavior of cancer cells in crosslinked dECM [135]
Natural materials	HA hydrogel	Breast	Stiffness effects on metastasis to the brain[95]
	Collagen I, Matrigel	Breast	Recruitment of immune cells and their effect on the invasiveness of cancer cells[96]
	Porous chitosan-alginate scaffolds	Prostate, breast, liver, brain	Evaluating cancer cell behavior on scaffolds mechanically mimicking cancer progression; Investigating cancer stem cell enrichment[136,137]
	Alginate-Matrigel	Breast	Effect of stiffness and ligand ratio on cell morphology and invasion[92,138]
	Alginate-based	Lung, breast, pancreatic, neuroblastoma, pituitary	Co-culture model for a drug screening platform[97–99,139,140]
Synthetic materials	PEG-b-poly(L-alanine) hydrogels	Breast	Linking chemical and mechanical ECM characteristics to tumor cell behavior [141]
	PA hydrogels	Thyroid, renal, breast	Stiffness and topography effects on normal and cancer cells[142–144]
	Alginate-PCL nanofibers	Liver	Cancer stem cell enrichment[145]

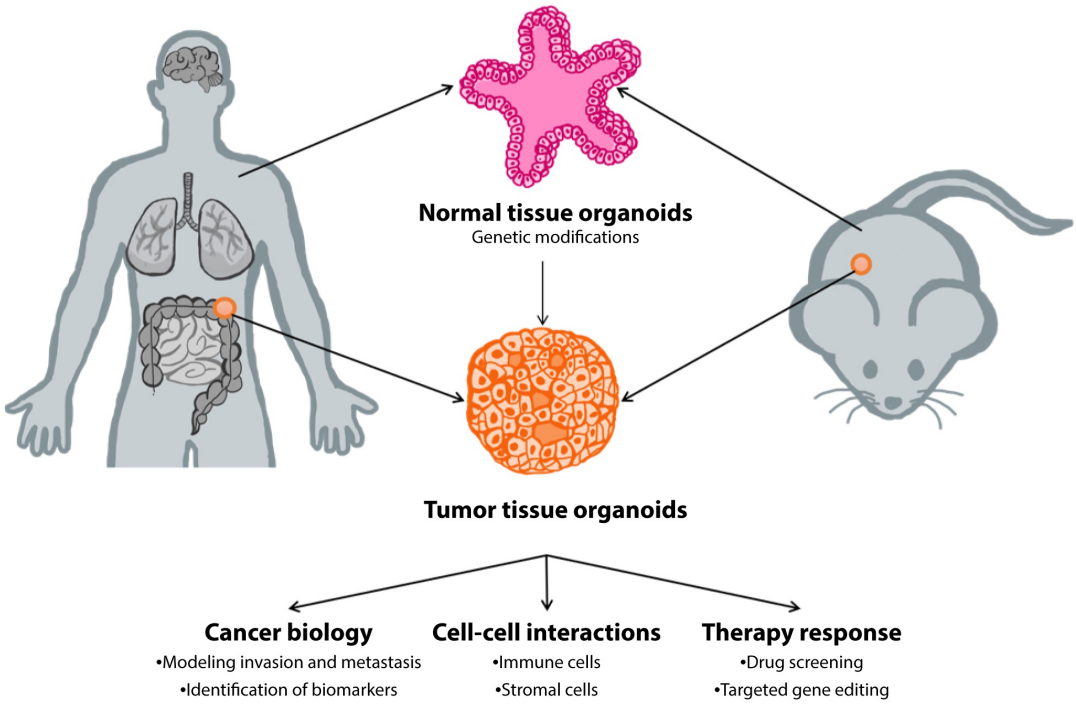

Figure 2.3. Normal tissue and tumor organoid model systems. Tumor organoid model systems can be produced from tumors or by genetic modification of organoids generated from normal tissues. Organoids can be used to answer questions about cancer biology, cell-cell interaction studies, and therapy response mechanisms.

In addition to tumor systems, organoids have been used as normal tissue models to evaluate tissue development as well as to study how normal tissues interact with tumor and immune cells.[119,146] For example, Hacker et. al derived mammary epithelial organoids from irradiated and normal murine mammary glands.[119] These organoids are being used to study the effects of normal tissue radiation damage on breast tumor-stromal and immune interactions, which may elucidate cancer recurrence mechanisms. Patient-derived organoids are also being explored to develop platforms for studying novel biomarkers and drug targets as well as tumor-immune and tumor-stromal interactions. [147,148,149, 150,120]

An additional approach to studying 3D tumor models is developing spheroids or micro-sized cell aggregates that function as *in vitro* models of the behavior of different tumor types.[151] Limitations in the use of tumor spheroids include the lack of ECM interactions and heterogeneity in the size and shape of the aggregates. To address that variability, Pradhan et al. designed and modeled 3D tumor microspheres formed by encapsulating MCF7 breast cancer cells in a PEG-fibrinogen hydrogel. This new model presented more cancer hallmarks compared to classic spheroids, such as increased disorganization, loss in apicobasal polarity, elevated nuclear-cytoplasmic ratio, nuclear volume density, and the reduction of cell-cell junction length.[152] Recently, alginate-based bioinks have been used to form 3D tissue spheroids of bone, cartilage, vascular tissue, and diverse tumors through bioprinting.[98,139,140,153–155] Compared to previous spheroid formation techniques, this method has been shown to improve cell viability, function, and architecture. 3D printing can form a ready-to-use tumor model that can mimic interactions with the ECM and with other cells in a co-culture system. Additionally, compared to self-

assembled spheroids using Matrigel, this method does not require complex components such as binding sites, growth factors, diverse extracellular fibers to promote tumor formation.[156] In their work, Swaminathan et al. not only studied the pre-spheroid formation of the MDA-MB-231 and MCF7 human breast cancer cells lines but also a non-tumorigenic MCF10A breast epithelial cell line. They performed a co-culture with vascular endothelial cells to examine drug response using Matrigel, gelatin-alginate, and collagen-alginate as bioinks. Laminin was found to be a key factor in the spheroid formation of breast epithelial cells, the spheroid structure was distinct when comparing tumorigenic and non-tumorigenic cell lines, and these morphologies were conserved post-bioprinting.[156]

2.5 Decellularized ECM (dECM)

Decellularization is a process that removes cellular material from tissues while leaving the majority of the ECM structure intact.[157] Since the 1990s, decellularized ECM (dECM) has been used for wound healing and regeneration but has been variable in its success.[158] dECM can be reconstituted into hydrogels and used for *in vitro* and *in vivo* experiments to study cancer progression (**Fig. 2.4**). These dECMs consist of structural and functional molecules that assist in the 3D organization of encapsulated cells. The organs from which these matrices are derived can produce distinct ECM components, and these differences alter cell-ECM interactions that serve to influence tumor cell proliferation and adhesion.[159] dECM derived from tumors has been shown to promote angiogenesis, the EMT response, and MMP-9 production.[132] Many studies use ECM obtained from tumor tissue to establish the effect of the microenvironment on the behavior and progression of various types of cancer, including colorectal[124,125,131,132,134], breast[126–

[129], liver[130], brain[133], and lung.[135] This approach allows for the evaluation of dECM obtained at different stages of tumorigenesis[134] and cancer cell interactions with immune cells.[124,125] One of the most important advantages of dECM is recapitulating the ECM microenvironment of specific tissues. For example, murine mammary fat pads were decellularized and exposed to radiation *ex vivo.*[123] Using these dECM hydrogels allowed for studying the effect of radiation on breast cancer proliferation and invasion in the context of recurrence after therapy. Wolf and coworkers also showed that the ECM derived from urinary bladder can support 3T3 fibroblast growth and proliferation.[160] Another notable characteristic of dECM is that it can promote a pro-regenerative immune phenotype and thus has been considered to combat tissue loss and promote wound healing following tumor resection.[121]

2.6 Naturally-Derived Materials

Matrigel

Matrigel is a solubilized basement membrane matrix that is derived from murine EHS sarcomas, murine tumors rich with ECM proteins such as laminin, collagen IV, and other growth factors.[161] Matrigel has mostly been used as a 3D matrix for cell growth and adhesion in culture systems due to relevant cell adhesion sites. Matrigel has also been combined with other 3D systems such as alginate hydrogels as will be described below.[82,92,138,162,163] Although abundantly examined, Matrigel can vary from batch to batch, and its components are undefined. Using Matrigel is nonetheless advantageous for studying how biological, chemical, and mechanical characteristics of the ECM affect the development and progression of cancer.

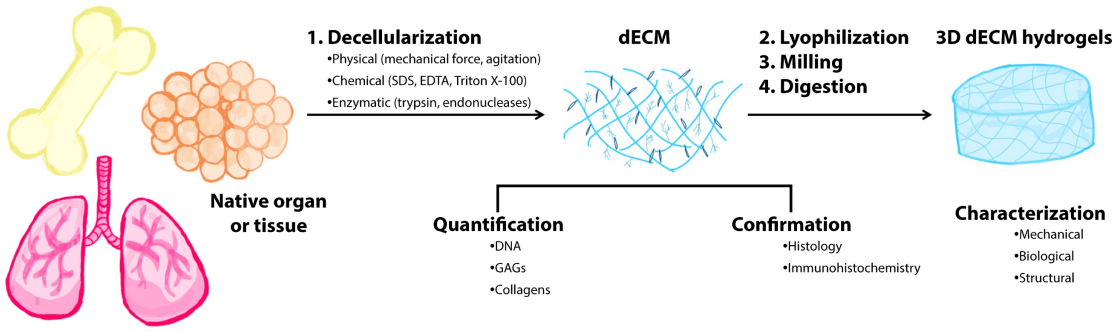

Figure 2.4. The process of deriving decellularized ECM from various organs for hydrogel formation.

Alginate

Alginic acid, or alginate, is an anionic polysaccharide derived from brown algae. It is mostly comprised of α-d-mannuronic acid and β-l-guluronic acid and can form crosslinked hydrogel networks of tunable mechanical properties through crosslinking using divalent ions such a calcium and manganese.[164] Alginate lacks cell adhesion binding sites and often requires modification with peptides or combination with collagen or Matrigel to form interpenetrating networks.[82] Read et. al showed that human colorectal cancer cells were viable when encapsulated within unmodified alginate hydrogels to study metabolic effects after irradiation.[165] However, not having binding sites available for the cells reduces the accuracy of the biomimetic environment since the progression of cancer is connected to cell-ECM dynamics.[94] In order to mitigate this, Cavo et al. developed an alginate hydrogel conjugated with RGD peptides that bind to integrins such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$.[92] Other peptide sequences such as DGEA and YIGSR, which are involved in cell adhesion to the ECM, have been used in conjunction with alginate to further produce biomimetic environments. It has been shown that the expression of these peptides can control EMT.[164] Alginate has also been used with collagen for immunotherapy treatments in mouse studies by Wei and coworkers. They show that the alginate-collagen matrix along with the TLR7 agonist R837 and radiation decreases 4T1 primary tumor growth and reduces the volume of distal tumors.[166] Alginate-chitosan scaffolds have been designed to enhance cell adhesion. These scaffolds have been used to mimic stages of cancer progression and metastasis in prostate adenocarcinoma and to examine cancer stem-like enrichment in prostate, breast, liver, and brain cancer.[136,137]

Since alginate can be easily tuned through the addition of divalent cations, it is widely used for evaluating biomimetic stiffnesses of the tumor microenvironment. Stiffness is an important factor when studying the tumor microenvironment as it has been shown that matrix rigidity can cause cells to undergo EMT that is characteristic of invasive phenotypes (**Fig. 2.5A**).[167,168,169] It is also important to tune biomaterials to mimic the vast range of mechanical properties experienced by cells in various organ microenvironments, including breast tissue (0.5-1 kPa), the intestines (20-40 kPa), and bone (15,000-20,000 kPa) (**Fig. 2.5B**). Alginate-Matrigel hydrogels have been used for breast cancer dormancy exit studies in relation to microenvironmental stiffness. Chaudhuri et al. fabricated alginate-Matrigel interpenetrating networks to evaluate breast cancer cell behavior in environments ranging from 30-300 Pa to mimic progressive tumors. Using the MCF10A breast cancer cell line, they observed that increased stiffness can promote the transition from epithelial to mesenchymal phenotypes, which is observed when dormant tumor cells exit dormancy.[82] This alginate-Matrigel model has also been used to study how mechanotransduction pathways such as YAP/TAZ signaling relate to breast cancer progression.[163] These models for integrin binding have also been used to study the invasiveness of tumor cells. Cavo et al. used alginate-Matrigel hydrogels to understand how stiffness can cause malignant progression as it has been shown that the biomechanical properties directly affect neoplastic disease.[170] MCF-7 ER+ breast cancer cells were shown to exhibit a rounded morphology in microenvironments of 5 kPa compared to MDA-MB-231 cells, an ER- cancer cell line with higher metastatic and invasive potential.[92,138]

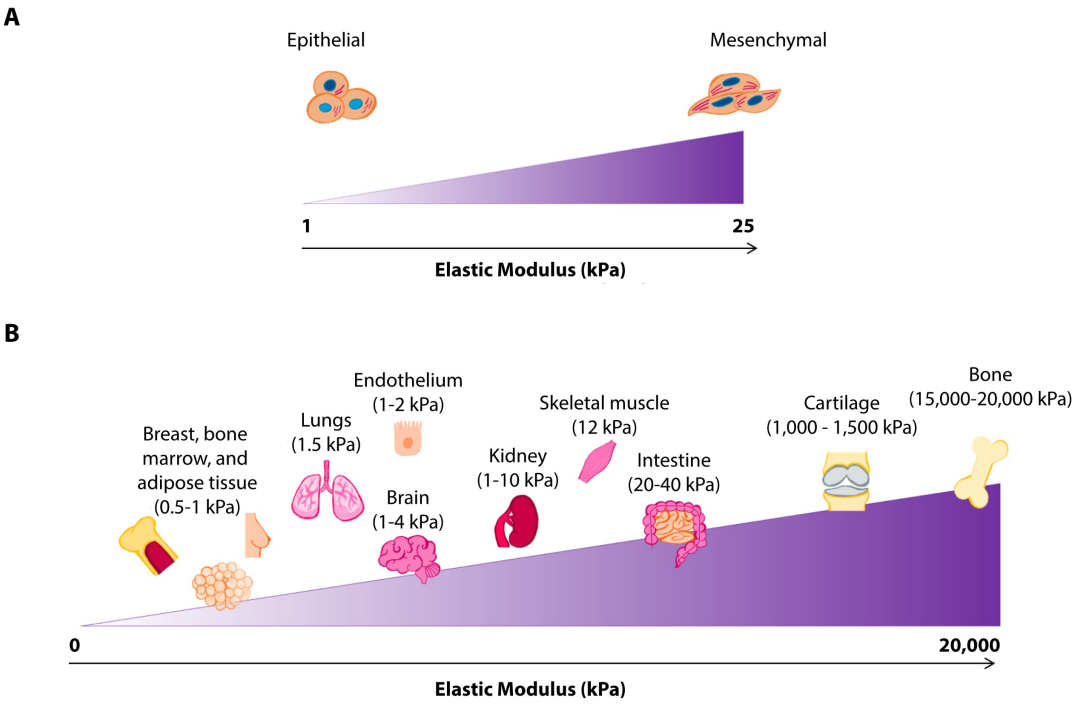

Figure 2.5. Biomimetic materials to model different physiological stiffness ranges. Biomimetic materials have been developed to span the stiffness range observed in (A) the epithelial-to-mesenchymal transition (EMT) and (B) commonly studied tissues and organs. Figure created in BioRender.

2.7 Synthetic Materials

Polyethylene Glycol (PEG)

PEG is a synthetic polymer that is used in a variety of biomedical applications.[171] PEG, when modified with methacrylate and dimethacrylate, is photocrosslinkable under UV light and can easily be conjugated with active protein sites for cell adhesion to better evaluate cell morphology and production of growth factors.[172,173] This material has been used to fabricate biologically active microenvironments by conjugating PEG to different protein binding sites such as RGD, IKVAV, and GFOGER to mimic the cellular binding sites in the fibrous structure of the ECM.[174–176] Gill and coworkers used PEG conjugated with RGDS (PEG-RGDS) for cell adhesion and GGGPQGIWGQGK (PQ) for degradation by matrix metalloproteinases. In this model, lung adenocarcinoma cells were used to understand morphological and epithelial changes in response to microenvironmental stiffness, ligand adhesion concentration, and TGFβ.[177] Stiffnesses were varied from 21 kPa to 55 kPa in order to study the effect of stiffness on the morphology of lung adenocarcinoma cells. Higher stiffnesses promoted an increase in mesenchymal phenotypes. In addition, cells interacting with larger amounts of RGDS peptide presented an epithelial phenotype. Sawicki and coworkers varied the concentration of the peptides GFOGER, RGDS, and IKVAV that bind to collagen, fibronectin, and laminin, respectively.[174] These concentrations were changed to mimic peptide concentrations associated with cancerous tissue, including increased collagen I binding sites.[178]

Polyacrylamide (PA)

Acrylamide-based hydrogels are widely used to model the stiffness range present in biological matrices, typically from 0.1 kPa to 119 kPa.[179] PA can be functionalized and coated with ECM components such as fibronectin, vitronectin, and collagen to encourage cell adhesion.[180] A major challenge, however, in using this material is the cytotoxicity of acrylamide monomers, which skews studies toward 2D models.[181] Currently, PA hydrogels are being deigned to understand how mechanical cues such as stiffness, topography, and geometry alter gene expression[144], EMT, drug resistance[169], migration[182], and tumor progression.[142–144] To evaluate mechanotransduction in gradient hydrogels, Hadden et al. fabricated PA-derived gels to create cell culture surfaces that have tunable stiffnesses.[183] Using human adipose-derived stem cells (hASCs), it was reported that cells experienced durotaxis on fibronectin-coated PA gels with a steep 8.2 kPa/mm gradient while cells did not migrate in response to a more shallow gradient of 2.9 kPa/mm. This indicates that conditions separating the effects of durotaxis from stiffness response should be considered in studying tumor cell behavior. PA hydrogels have also been used to understand cancer stem cell (CSC) plasticity. Tian et al. used PA hydrogels to understand whether matrix rigidity has an effect on the phenotype of CSCs.[184] It was reported that HCCs had stem cell-like properties and poor spreading at 5.9 kPa corresponding to normal liver stiffness compared to stiffnesses in the range of cancerous tissues (48.1 kPa). PA surfaces have also been utilized to study the response of human primary thyroid cells (S747) and anaplastic thyroid carcinoma cells (S277) when sensing a range of mechanical environments.[143] It was found that normal cells adapt their viscoelastic properties in response to stiffness changes while the carcinoma cell viscoelasticity remained constant.

Other Synthetic Biomaterials

Other commonly studied synthetic biomimetic materials are polydimethylsiloxane (PDMS), polycaprolactone (PCL), and polyurethane (PUR) films.[145,185,186] All of these materials allow for a higher degree of stiffness matching of tissues and organs compared to natural biomaterials, and they can also be functionalized or mixed with ECM components to form hybrid materials that can model the tumor microenvironment.[186] One notable example evaluates the development of porous and tunable alginate-PCL nanofiber scaffolds for investigating cancer stem-like cell enrichment, EMT, and cell distribution.[145]

2.8 Immune Cell Behavior

Studying the Immunological Response in Cancer Microenvironments

There are several components of the microenvironment that influence tumor growth, including cancer-associated fibroblasts, tumor associated neutrophils, and tumor associated macrophages (TAMs).[187,188] It has been shown that these cellular and immune populations, or lack thereof, can contribute to cancer progression and metastasis. For example, triple negative breast cancer tumor cell recruitment after radiation therapy was enhanced due to reduced lymphocytes and excess macrophages.[189] In addition, incorporating chemokines and cytokines into models are important as they enable pro-inflammatory responses that affect the growth of tumors and give signals to immune cells to encourage malignant behavior.[187,190]

One overarching goal in designing 3D biomimetic systems is to understand how tumor cells evade the immune response.[191] TAMs are associated with poor prognosis in patients and promote tumor growth and migration.[122,192] A biomimetic materials approach

has been undertaken to evaluate immune cell interactions with stromal and tumor cells. Wolf et al. used urinary bladder matrix (UBM) scaffolds embedded with three different cancer cells, melanoma (B16-F10), colorectal carcinoma (CT26), and mammary carcinoma (4T1) to evaluate how the ECM microenvironment influenced tumor progression. UBM is a decellularized scaffold composed of basal lamina and lamina propria of the porcine urinary bladder that contains collagen I, ECM-associated factors, glycoproteins, and proteoglycans.[193] The UBM scaffold inhibited and delayed tumor formation while promoting a distinct immune signature that was dependent on CD4+ T-cell and macrophage infiltration.[121] In addition, Zhu and co-authors have shown that cellular phenotypes and their responses can change within dECM hydrogels. dECM hydrogels were found to play a role in macrophage activation and polarization that was dependent on the organ from which the ECM was derived.[122] Keane et al. demonstrated that decellularized matrices can promote anti-inflammatory responses by reducing inflammatory macrophage infiltration.[194] The mechanisms behind how dECM can activate these macrophages warrant further study in order to evaluate polarization that mimics the tumor microenvironment.

3D bioprinted tumor models have also been used to study the interactions between different cell types, including immune, cancer, endothelial, and fibroblast cells *in vitro.*[97,98] Gelatin and gelatin methacrylate have been used for this application.[195] In their work, Heinrich et al. fabricated a mini-brain with encapsulated glioblastoma-associated macrophages (GAMs) and glioblastoma multiforme (GBM) tumor cells to study cell crosstalk *in vitro*. This work demonstrated how the presence of GAMs promotes the

invasion and proliferation of GBM as well as how GBM leads to GAM recruitment and proliferation.[97]

An important application of biomimetic materials is to study the behavior of cancer cells treated with novel therapies.[123] One approach is local cancer immunotherapy, a treatment intended to stimulate the immune system of cancer patients commonly achieved through T-cell infusion, vaccines, or antibodies to inhibit proteins produced by cancer cells using macroscale biomaterials.[196,197] The principal advantage of local immunotherapy is specific immunomodulation at the tumor site, which prevents systemic toxicity. Injectable biomaterials can include cargo such as immunomodulators, immune cells, or cancer vaccines. Mesoporous silica microrods, PEG with polypeptide blocks, synthetic peptides with specific motifs, and DNA-scaffolded biomaterials are being developed for this purpose.[198–201] Additionally, natural biopolymers such as alginate, chitosan, gelatin, and HA have been explored.[199,202] Lee et al. used DNA polyaptamer hydrogels with Cas9/sgRNA to promote the controlled delivery of immune checkpoint inhibitors for cancer immunotherapy. The hydrogels are composed of two templates that contain PD-1 aptamers and prepared by rolling circle amplification containing complementary sequences of sgRNA to be cleaved by Cas9. The release of a programmed death receptor (PD-1) DNA aptamer promotes the activation of T-cells and reduces tumor growth.[203] Furthermore, immune checkpoint inhibitors can be released with a hydrogel-based polypeptide vaccine to improve tumor immunotherapy. Song et al. developed an injectable PEG-b-poly(L-alanine) hydrogel with encapsulated granulocyte-macrophage colony-stimulating factor and anti-CTLA-4/PD-1 antibodies. The injectable hydrogel promoted the recruitment and activation of dendritic and T-cells. Moreover, the

hydrogel vaccine led to the secretion of specific cytokines that reduced the growth of B16 melanoma tumors. These injectable biomaterials can be used as a sustained delivery platform with co-delivery of immunotherapeutics to promote immune cell activation and eliminate tumors.[141]

Cell Membrane-Mimicking Nanoparticles

Currently, one of the major limitations in the use of drug delivery nanoparticles in chemotherapy is low therapeutic efficacy and limited tumor penetration.[204] For that reason, developing biomimetic nanoparticles as drug delivery vehicles to promote an immunomodulatory response has been explored. These nanoparticles are composed of a cell membrane coating and can mimic specific cell types due to the surface functionalization of the originating cell's membrane proteins.[205–212] An innovative system for targeting tumors is the design of paclitaxel-loaded polymeric nanoparticles coated with the cell membrane of a specific tumor cell. For example, Sun et al. fabricated cancer cell biomimetic nanoparticles from 4T1 murine triple negative breast cancer cell membrane-derived vesicles and paclitaxel-loaded polymeric nanoparticles composed of PCL. In contrast with other drug delivery systems, this class of biomimetic nanoparticle has a high specificity for targeting primary tumors.[207] In conjunction with other materials, PEG has also been used to help create immunological responses in a drug delivery system. Lai and coworkers fabricated DSPE-PEG nanoparticles coated with macrophage plasma membranes as a functional macrophage mimic.[213] This approach showed minimal cytotoxicity and successfully allowed the nanoparticles to cross the blood brain barrier to

selectively accumulate at glioblastoma sites, making this an optimal delivery strategy for reducing tumor growth.[122]

2.9 Conclusion

The development of biomimetic materials offers promising avenues of understanding tumor and immune cell behavior *in vitro* and *in vivo*. In this review, we have demonstrated how various materials and techniques are used to evaluate the phenotypic and functional changes in tumor and immune cells. Models composed of common biomaterials such as alginate and PEG, novel materials including DNA hydrogels and dECM, and new technologies like 3D bioprinting allow for recapitulating the complex tumor microenvironment and analyzing multiple variables that promote tumor progression.

New biomimetic materials and synthetic environments are being designed in order to replicate complex microenvironments of interest and answer relevant biological questions. Some biomimetic models, such as organoids, are being implanted *in vivo* to encourage a vascularized environment.[214] Other studies have also begun to incorporate hydrogels into bioreactors to better mimic physiological conditions.[215–217] Despite the development of these biomimetic materials and new, innovative ways of using them, not all of the factors that influence tumor progression can be tested simultaneously. This necessitates the continued use of *in vivo* models to validate *in vitro* observations. The complexity of design and how closely these models will resemble living systems remains to be seen. Nonetheless, these biomimetic materials allow for the study of tumor cell crosstalk with immune cells, mechanotransduction, ligand density, and cell-ECM

interactions, which can advance the development of more effective therapies leading to improved patient outcomes.

Chapter 3

Development of an alginate-Matrigel hydrogel system to evaluate cancer cell behavior in the stiffness range of the bone marrow

The work in this chapter is published and adapted from: Northcutt L, Questell A, Rhoades J, Rafat M. Development of an alginate-Matrigel hydrogel system to evaluate cancer cell behavior in the stiffness range of the bone marrow. Front Biomater Sci. 2023:2:1140641.doi: 10.3389/fbiom.2023.1140641. Epub 2023 Jun 2.

Abstract

Bone metastasis is highly prevalent in breast cancer patients with metastatic disease. These metastatic cells may eventually form osteolytic lesions and affect the integrity of the bone, causing pathological fractures and impairing patient quality of life. Although some mechanisms have been determined in the metastatic cascade to the bone, little is known about how the mechanical cues of the bone marrow microenvironment influence tumor cell growth and invasion once they have homed to the secondary site. The mechanical properties within the bone marrow range from 0.5 kPa in the sinusoidal region to 40 kPa in the endosteal region. Here, we report an alginate-Matrigel hydrogel that can be modulated to the stiffness range of the bone marrow and used to evaluate tumor cell behavior. We fabricated alginate-Matrigel hydrogels with varying calcium sulfate ($CaSO_4$) concentrations to tune stiffness, and we demonstrated that these hydrogels recapitulated the mechanical properties observed in the bone marrow microenvironment (0.7 kPa to 16 kPa). We encapsulated multiple breast cancer cell lines into these hydrogels to assess growth and invasion. Tumor cells in stiffer hydrogels exhibited increased proliferation and enhanced elongation compared to lower stiffness hydrogels, which suggests that stiffer environments in the bone

marrow promote cellular invasive capacity. This work establishes a system that replicates bone marrow mechanical properties to elucidate the physical factors that contribute to metastatic growth.

3.1 Introduction

Metastasis, the spread of cancer cells from the primary tumor site to a distant site in the body, is a well-studied hallmark of cancer and is associated with higher death rates in patients. [4,5] In breast cancer, distant recurrence is common in sites such as the brain, bone, liver, and lungs [6]. It has been shown that bone metastases are correlated with lower incidences of death in breast cancer patients, but 70% of patients who succumb to the disease have bone metastases after autopsy.[11] The bone marrow microenvironment is highly dynamic and composed of many fibrous macromolecules and progenitor cells. [42,218] The bone marrow is highly variable in terms of biophysical properties such as stiffness, three-dimensional (3D) architecture, and fluid flow. The process by which mechanical cues, such as matrix stiffness and porosity, can affect biophysical and biochemical responses is called mechanotransduction and is highly integral to tumor cell progression in the primary site. The mechanical forces of the environment can affect biological processes in the bone marrow such as the production of osteoactive agents for tumor-induced bone disease. [48] Studies have shown that the stiffness of the bone marrow microenvironment can influence progenitor cells that are responsible for the development and prevalence of hemopoietic stem cells. [49] Additionally, previous work has shown that the increased stiffness in the primary breast tumor microenvironment alters cell behavior, leading to more mesenchymal phenotypes and enhanced proliferation. [219] However, many sites of metastasis are ten-fold stiffer

than the breast primary site, and how physical factors at metastatic sites influence tumor cell behavior is not well-studied [49]. We therefore hypothesized that the stiffness changes within the bone marrow environment, which spans 0.3 kPa to >35 kPa, will affect the behavior of cancer cells. [50]

Like the breast microenvironment, systems to mimic the bone microenvironment can be synthetic or naturally derived. [220] Cancer-related bone pathologies in both the marrow and bone are typically evaluated with *in vivo* models, which can be costly and can take time to see osteolytic effects, using techniques such as intratibial injections to study established tumors in bone as well as potential treatments to inhibit tumor-induced bone disease. [221–223] Synthetic hydrogels are highly tunable but often require UV-crosslinking that may reduce the viability of encapsulated cells. Naturally-derived hydrogel systems can also be utilized for *in vitro* studies as they may better replicate the range of proteins and binding sites in tissues. In addition, engineered systems such as microfluidic devices have been used to study the metastatic properties of breast cancer to the bone matrix along with mineralized osteoblastic bone tissue. [224,225]

Although many studies attempt to model the bone marrow and its surrounding environment, few systems can replicate its stiffness without changing the number of biological binding sites. [226] Many of these materials evaluate stiffness in 2D environments, which limits studying the forces that surround the cell and potential interactions with the microenvironment. [227,228] 3D hydrogels typically do not incorporate both mechanical properties and biological complexity. Recently, Jansen et al. designed synthetic polyethylene glycol hydrogels with a bone marrow-specific protein signature to mimic the bone marrow microenvironment. This novel work combined relevant, tunable

mechanical properties and chemical extracellular matrix (ECM) cues. [229] However, the study focused on cell behavior in an environment that matched the average marrow modulus. Here, we present an alginate-Matrigel hydrogel system as a bone marrow model with varied crosslinking through calcium sulfate ($CaSO_4$) to allow for changes in stiffness alone. While stiffness does not necessarily drive cell behavior in 3D, we are interested in probing how 3D environmental stiffness directly influences breast cancer cells, which may give insight into how sites of metastasis promote tumor growth and invasion. [230,231] Indeed, the tunability of alginate and the ECM proteins that Matrigel provides allow for evaluating cellular mechanotransduction in 3D [232–234]. We found the stiffness of our hydrogels can span two orders of magnitude within the range of the bone marrow microenvironment, which can alter tumor cell proliferation and morphology. Overall, we show how our tunable system may be used to understand how the stiffness of the bone marrow affects metastatic progression.

3.2 Materials and Methods

IPN Formulation. Hydrogels were developed as previously described.[235] The hydrogels consisted of high G alginate (PRONOVA) 5 – 10 mg/mL and growth-factor reduced Matrigel (4.5 mg/mL, Corning). Calcium sulfate ($CaSO_4$) was used for alginate crosslinking starting at a stock concentration at 1.22 M and diluted to 122 mM in the appropriate media. For gel crosslinking, the volume of 122 mM was adjusted for each gel concentration. To form hydrogels, two 1 mL syringes were connected via a Luer lock with alginate with Matrigel in one and $CaSO_4$ in a separate syringe, and the solutions were mixed back and forth 10 times (**Fig. 3.1 A**).

Rheology Measurements. Stiffness measurements of the hydrogels were conducted using a rheometer (AR 2000 Ex, TA Instruments) with a 25 mm top and bottom plate. The plate was rotationally mapped. 500 µL hydrogel solution was added to the plate, and a disk was formed by lowering the plate head. The plate was warmed to 37^{o}C, and mineral oil was used to coat the edges of plate to prevent dehydration of the gel. The resulting plate separation was between 500-1000µm. Gel characteristics were measured over time until the storage modulus reached equilibrium (between 1 to 2.5 hours depending on the crosslinking density) with 0.5% applied strain and strain frequency of 1Hz. The average storage and loss modulus of the last 3 points were averaged and calculated using the Young's Modulus (E) equation in units of Pascals (Pa):

$$E = 2\,G\,(1 + \nu),$$

where the ν is Poisson's Ratio and assumed to be 0.5. [226]

G (bulk modulus) is calculated using G = (G' + G''), where G' is the storage modulus and G'' is the loss modulus.

Culturing of Cancer Cells in Hydrogels. 4T1 murine triple-negative breast cancer cells (ATCC) were cultured in RPMI media, supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and 1% penicillin-streptomycin. MCF7 human estrogen receptor-positive breast cancer cells (gifted from Dr. Rachelle Johnson) were cultured in DMEM media, supplemented with 10% HI-FBS and 1% penicillin-streptomycin. Cells were embedded at a concentration of 1.0 x 10^5 cells/mL for each condition and incorporated in the hydrogel (**Fig. 3.1 B**). This resulted in

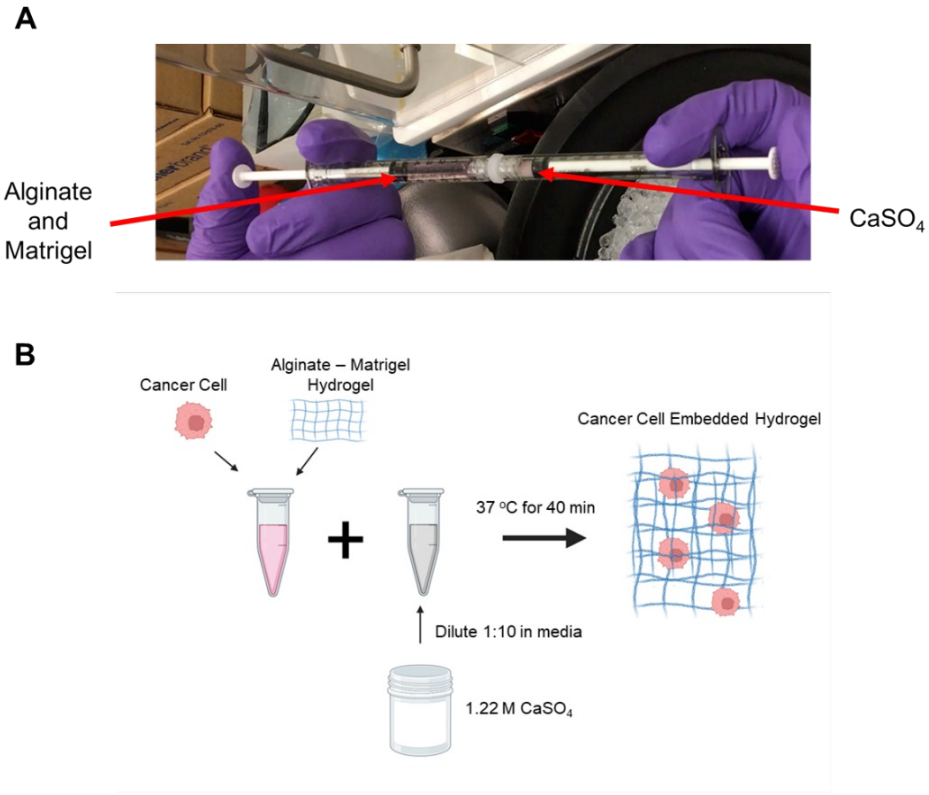

Figure 3.1. Development of Crosslinked Alginate-Matrigel Hydrogels. (A) Image of component mixing to form hydrogels. (B) Schematic of cell encapsulation within hydrogels. Figure created in BioRender.

an evenly distributed single cell suspension. A MatTek dish with a No.1 glass slide was used for culturing cells in hydrogels, and 100 µL of the cell-pre-gel solution was added to the wells for complete coverage (MatTek, P35G-0-10-C). Hydrogels were allowed to get for 45 minutes at 37°C before adding 3 mL of media to the wells. The cells formed clusters and were grown in the hydrogels for either 2 or 7 days in complete media. The media was changed every 2 days.

Fluorescence Staining. Following culture, the gels were fixed in 10% formalin for 15 minutes and washed with phosphate-buffered saline (PBS) 3 times. The cells were permeabilized with 0.1% Triton in PBS and blocked with 5% normal goat serum (NGS) in PBS for 1 hour. After blocking, 1000X Phalloidin (Phalloidin-iFluor 594 Reagent, Abcam) was diluted to 1X in 5% NGS and incubated for 1.5 hours in the dark. After staining the actin cytoskeleton, the gels were mounted with Antifade Diamond Mount with NucBlue overnight. The gels were imaged using a DMi8 fluorescence microscope.

Cluster Morphology Analysis. 10-15 images (0.045 mm^2 field) per independent replicate were acquired using a Leica DMi8 inverted microscope. Using ImageJ, multicellular clusters were traced using the "Freehand Selection Tool" to measure the major and minor axis dimensions. The elongation index (EI) was calculated using the following equation [236]:

$$EI = Major\ Axis/Minor\ Axis$$

Statistics. Data are presented as the standard deviation. Data were analyzed using analysis of variance (ANOVA) to determine statistical significance ($p < 0.05$) after confirming normality. All analyses were performed in GraphPad Prism 9.

3.3 Results and Discussion

3.1 Alginate-Matrigel Hydrogels Crosslinked with $CaSO_4$ Replicate the Stiffness of the Bone Marrow

In previously published studies using the alginate-Matrigel system, the stiffness of the breast tumor microenvironment has been mimicked. Here, we intended to extend previously published methods by increasing $CaSO_4$ concentrations up to 50 mM to achieve stiffnesses within the bone marrow microenvironment range of 0.5 - 40 kPa.[50] In hydrogels with 5 mg/mL alginate and 4.5 mg/mL Matrigel, Young's moduli ranged from approximately 0.7 to 7 kPa when varying $CaSO_4$ concentrations between 10 to 50 mM (**Fig. 3.2 A,C**). To increase the stiffness range, the alginate concentration was increased to 10 mg/mL, and the Young's moduli range expanded to 16 kPa when using 5-50 mM $CaSO_4$ (**Fig. 3.2 B, D**). Although this system did not exceed 35 kPa, this system was able to achieve a stiffness range of more than two orders of magnitude, which to our knowledge has not been shown in similar alginate-Matrigel hydrogel systems. We continued to use the 5 mg/mL alginate formulation in our proof-of-concept studies, which spanned one order of magnitude within the stiffness range of the bone marrow, as the 10 mg/mL formulation showed reduced viability in our cell lines. Future

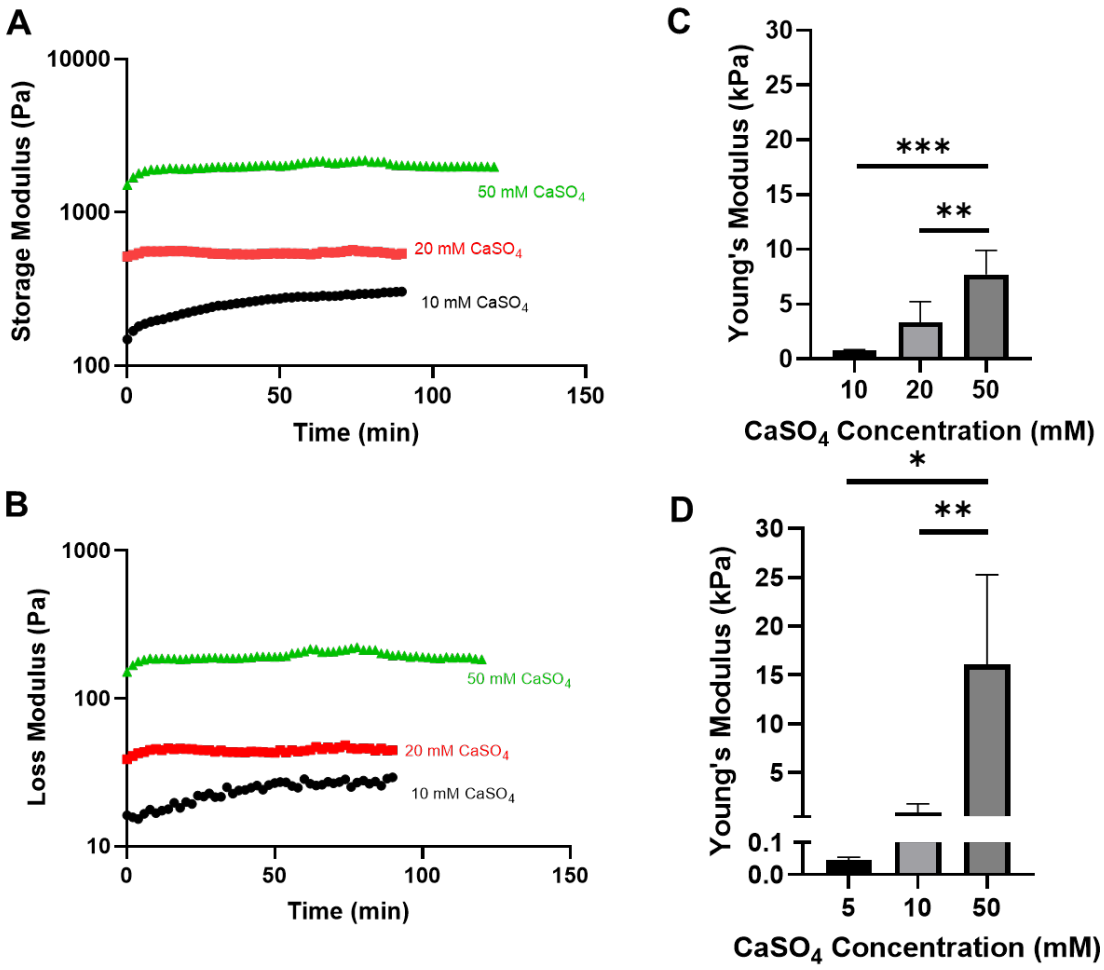

Figure 3.2. Evaluation of alginate-Matrigel hydrogel stiffness with varying alginate and calcium sulfate ($CaSO_4$) concentrations. Hydrogel mechanical properties were analyzed using rheology. The timecourse of gelation is shown in (A-B). Young's moduli were calculated for (C) 5 mg/mL and (D) 10 mg/mL alginate-Matrigel hydrogels. Results represent n = 3-9 independent replicates. Error bars are standard deviation. Statistical significance was determined by One Way ANOVA with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.0001$.

studies will explore composite hydrogels and the addition of relevant peptides to the bone marrow microenvironment that may better support cell growth.

3.2 Evaluating Tumor Cell Proliferation

After developing hydrogels within the stiffness range of the bone marrow microenvironment, we then evaluated the proliferative response of tumor cells encapsulated in the hydrogels by counting the nuclei in cell clusters. 4T1 and MCF7 cells were seeded in 1 kPa (10 mM $CaSO_4$) and 7 kPa (50 mM $CaSO_4$) hydrogels up to 7 days. Fluorescence images from nuclear staining demonstrate an increasing proliferation trend in 4T1 cells (**Fig. 3.3 A, 3.3 C**) but not MCF7 cells (**Fig. 3.3 B, 3.3 D**) after 7 days. Both 4T1 and MCF7 cells are known to grow in clusters in 3D [237–239]. Nuclei counts were used as a proxy for proliferation in this study, and we therefore need to further validate the results. Future studies will confirm proliferative capacity through Ki67 staining as well as additional proliferation assays that directly measure DNA synthesis. In addition, we will evaluate cell proliferation beyond 7 days, which may reveal larger differences in cell subtype.

3.3 Increased Stiffness Enhances Elongation in Triple Negative Breast Cancer Cell Clusters

Cell morphology has been shown to correlate with the ability of tumor cells to become invasive and motile. [240] Additionally, alginate-Matrigel systems mimicking breast tissue environments display increased cell elongation and expression of epithelial-to-mesenchymal transition markers. [219] However, this system has not been shown to mimic

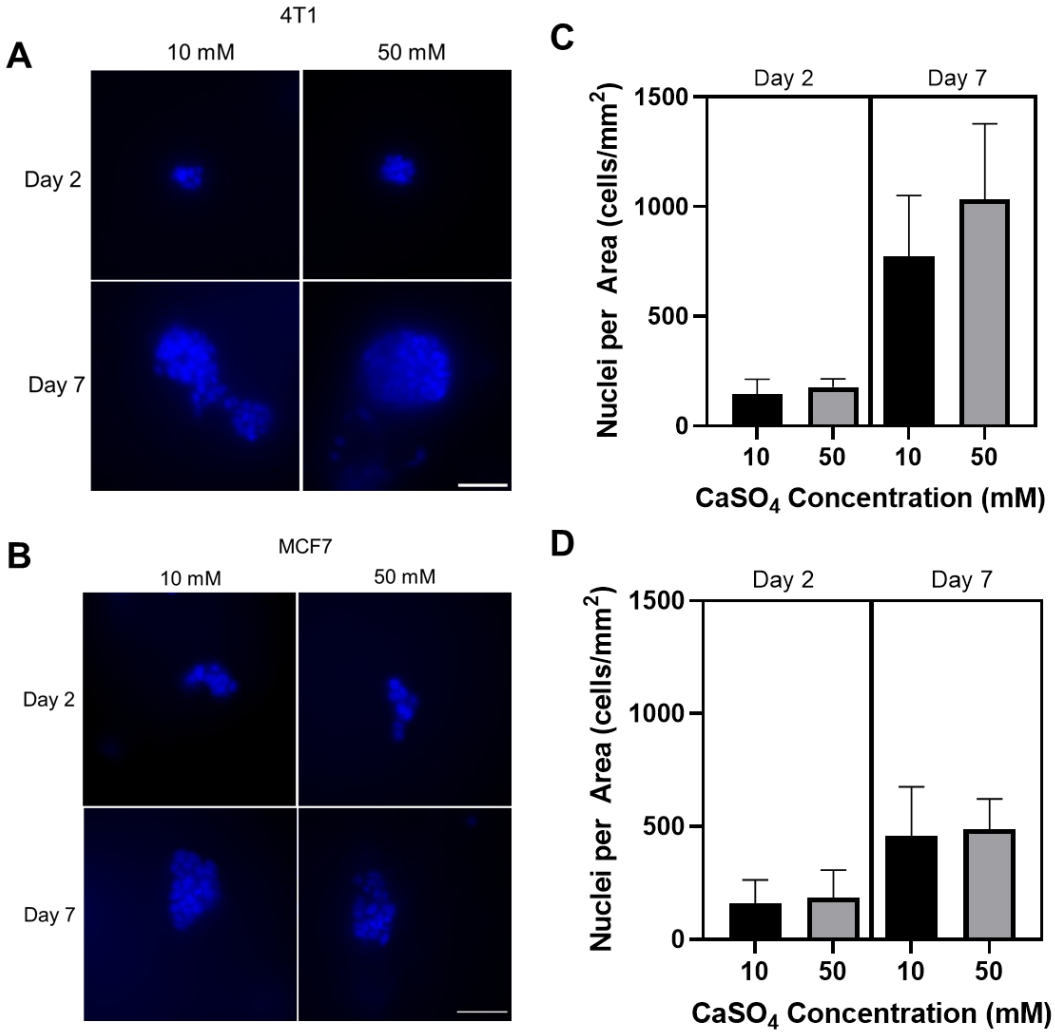

Figure 3.3. Determination of the effect of stiffness on breast cancer cell proliferation. Breast cancer cells (mouse 4T1; human MCF7) were embedded into alginate (5 mg/mL)-Matrigel hydrogels crosslinked with 10 mM and 50 mM $CaSO_4$ up to 7 days, and stained nuclei (blue) were counted. Representative (A) 4T1 and (B) MCF7 images are shown. Quantification of nuclei for 4T1 (C) and MCF7 (D) using ImageJ. 10-15 fields of 0.045 mm^2 were taken per independent hydrogel replicate (n = 3) to determine nuclei counts. Cells were seeded at a concentration of $1x10^5$ cells/mL. Scale bar is 50 μm. Error bars are standard deviation.

the bone marrow microenvironment. Here, we evaluated the morphology of encapsulated cell clusters by determining the EI following F-actin staining (**Fig. 3.4 A-B**). EI showed statistically significant increases after 2 and 7 days in both cell lines in stiffer hydrogels (**Fig. 3.4 C-D**). 4T1 cells showed a greater increase in EI following 7d compared to MCF7s with an approximately 40% increase in EI compared to a 20% increase in MCF7 cells in stiffer hydrogels. Taken together, we have shown the feasibility of studying breast cancer cell invasive properties and cytoskeletal dynamics in a biomimetic bone marrow hydrogel system and that there may be a differential response according to subtype. We measured cell cluster elongation consistent with invasion, but additional work must be done to evaluate cellular invasion beyond correlative properties. We will evaluate movement through the gel, invadopodia through cortactin-actin co-localization, and invasion gene signatures in the future.

3.3 Limitations

Although our work is useful for understanding cellular responses within the stiffness of the bone marrow, using confocal or two-photon microscopy will enhance the cellular image quality. Our study evaluates single stiffness gels, which do not capture the complex physical properties of the bone marrow. Future studies will expand this work to incorporate a stiffness gradient to replicate the bone marrow microenvironment more accurately. It is also necessary to study multiple microenvironmental factors in the bone marrow, including other mechanical cues. While this system directly evaluates stiffness, the primary components of Matrigel are laminin and collagen IV whereas the bone marrow is mainly comprised of collagen I.[241] Characterizing and mimicking the

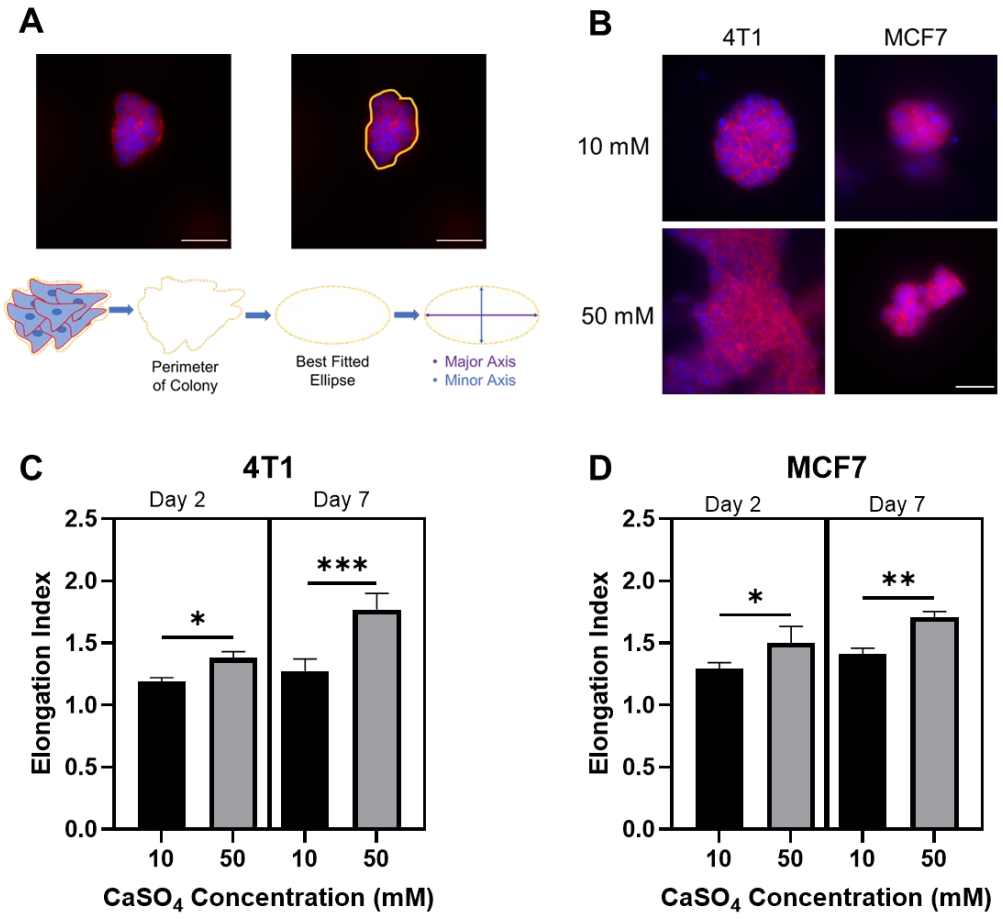

Figure 3.4. Investigating breast cancer cell cluster morphology to determine invasive capacity. (A) Example analysis of cell cluster elongation index. Representative images of (B) F-actin (red) and nuclei (blue) staining of 4T1 and MCF7 cells following 7 days incubation in alginate (5 mg/mL)-Matrigel hydrogels crosslinked with 10 mM and 50 mM $CaSO_4$. Quantification of elongation index in (C) 4T1 and (D) MCF7 cell clusters following incubation for 2 and 7 days. Scale Bar is 50 μm. Results represent n = 3 independent hydrogel replicates with a minimum of 10 colonies evaluated for the 2 day incubation or 5 colonies for the 7 day incubation per replicate. Statistical significance was determined by ANOVA with *$p<0.05$. Error bars are standard deviation.

bone marrow ECM, controlling and modulating other physical properties such as degradability, and including additional relevant cell types will increase the impact of this system. Lastly, we visualized cells using fluorescent markers. Future studies will explore cell extraction from hydrogels to examine specific molecular mechanotransduction pathways involved in promoting metastasis in the bone marrow microenvironment.

3.4 Conclusion

Currently, there are limited systems that allow for varying environmental stiffnesses without changing the number of biological binding sites. We have developed alginate-Matrigel hydrogels that replicate the stiffness within the bone marrow microenvironment by changing only crosslinker concentration and leaving binding sites constant. This stiffness range has not been previously studied. Additionally, we have shown the possibility of studying proliferation and invasive capacity in this system. This proof-of-concept work will be expanded in the future to evaluate additional cell types, time points, and gene expression to better understand the role of mechanical properties in influencing metastatic potential in breast cancer.

Chapter 4

Increased Matrix Rigidities Modulate Interleukin Gene Signatures in ER+ Breast Cancer

Abstract

Metastases of breast cancer is a prevalent problem with over 25% of patients suffering with metastatic disease. Of the patients that present with metastatic disease, the majority are estrogen receptor positive (ER+) and have skeletal metastases. These skeletal metastases can cause several issues including pathological fractures, chronic pain, and hypercalcemia. Currently, there are not any cures for metastatic breast cancer in bone and only therapies to mediate the osteolysis caused by breast cancer. The bone microenvironment presents various physical forces that can act on the tumor cell that has been studied in ER- cells, however, the physical forces on ER+ tumor cells have not been widely evaluated. In this study, we explored the transcriptional changes that occur at different matrix rigidities associated with the bone microenvironment (bone marrow: 0.5 kPa to 32 kPa; cortical and trabecular bone – 2 x 10^7 kPa). We observed that lower stiffnesses contributed to increased gene signatures associated with interleukin signaling. Additionally, we observed that downstream estrogen signaling outputs were modified. These interesting findings give us insights on what may be changing when ER+ tumor cells encounter rigidities associated with the bone microenvironment.

4.1 Introduction

Physical factors of the tumor microenvironment can play a role in the aggressiveness of breast cancer cell and transition normal cells into more cancerous

phenotypes.[242,243] Recent studies have shown that increased rigidity of the microenvironment can cause elongated morphologies and increased proliferation, which are characteristics of more aggressive phenotypes due to their abilities to become more motile and degrade the matrix.[235,244] These changes associated with the physical microenvironment are typically driven signaling changes that are mechanically regulated such as yes-associated protein signaling and activation of focal adhesion kinases. [245–247] Additionally, cell regulation processes and epigenetic modifications can occur at different stiffnesses and promote tumor initiation processes.[248,249] As tumor cells become motile and begin to metastasize, mechanical environments can still modify their behaviors and can prime them for colonization and survival in the secondary sites. Triple negative breast cancer cells and prostate cancer can produce more osteolytic factors when seeded at higher stiffnesses towards the cortical bone.[78,250] RUNX2, a transcription factor that regulates bone metastasis, is also mechanically regulated in SUM159 cells.[251] Interestingly, tumor cells that are subtyped differently on their hormonal status, can respond differently to rigid environments and have differing propensity for metastasis to certain sites.

In breast cancer, the subtyping of cells contributes to therapeutic options such as aromatase inhibitors or chemotherapy options.[252–255] Breast cancer typically categorized as hormone receptor positive; expressing estrogen receptor (ER) and progesterone receptor; or hormone receptor negative. Interestingly, there has been a growing body of work that evaluates how the tumor cell subtyping is associated with mechano-responsiveness. At stiffnesses akin to the primary site, studies suggest that there seems to be an increase in activated ERα and proliferation in hormonally positive

cells.[74] However, it is unclear what happens to the signaling processes of ER+ cells, particularly in the bone. Some studies suggest that hormonal signaling remains intact while other studies suggest that tumor cells dysregulate their signaling after dissemination.[256–258] There is some work to suggest how ER+ cells respond to primary microenvironmental stiffness, but not in the ranges akin to metastatic sites such as the lymph nodes, liver and bone which are typically stiffer than the primary sites. At the moment, we do not have a clear understanding of what happens to ER+ tumors when metastasis occurs to the bone due to model limitations as most ER+ breast cancer cell models do not have characteristics leading to metastasis. Furthermore, gaining information about metastatic disease of ER+ breast cancer patients remains difficult due to the limitations of these models and timeliness to gain metastases *in vivo*.

Here we sought to explore how the softer matrix rigidities of the bone environments contribute to tumor progression in ER+ breast cancer cells. We report that ER+ breast cancer cells increased gene signatures associated with interleukin signaling in softer environments compared to stiffer environments. We also show that ER+ cells decrease their estrogen signaling at lower stiffnesses compared to higher stiffnesses, inhibiting signaling needed for survival in ER+ breast cancers and potentially causing decreasing in efficacy of aromatase inhibitors. Overall, this works provides an analysis how the mechanical microenvironment can contribute to changes in ER+ breast cancer.

4.2 Materials and Methods

Culturing of Cancer Cells on Rigidity Plates

CytoSoft® Rigidity Plates were purchased from Advanced BioMatrix. The plate wells were coated with 4 ug/mL of fibronectin (Advanced Biomatrix) for 1 hour before use. MCF7s and T47Ds (human estrogen receptor positive breast cancer positive cells; ATCC) were cultured in phenol-red free DMEM (Gibco) and phenol-red free RPMI (Gibco), respectively, and supplemented with 10% charcoal-stripped fetal bovine serum and 1% penicillin-streptomycin. After 24 hours in culture, cells were plated at 0.2 x 10^6 cells per well (6-well plate) and supplemented with 10 nM 17-β estradiol (E2) (Invitrogen). Cells were cultured at 37^o C for 48 hours.

RNA Sequencing and Data Analysis

mRNA samples were harvested from cells using QIAzol (Qiagen). RNA samples for MCF7 cells were sequenced by VANTAGE. The Vanderbilt Creative Data Solutions Shared Resource (RRID:SCR_022366) performed the RNA-Seq data processing, differential gene expression analysis, GSEA and data deposition.

Western Blotting

Cells cultured on the CytoSoft® Rigidity Plates were harvested for protein using RIPA buffer. Protein concentration was determined by BCA Assay and 20 ug of protein was loaded onto an SDS-Page Gel, transferred onto PVDF and blocked with 5% BSA in TBS for 2 hours. Membranes were probed with antibodies against Calnexin (Abcam), estrogen receptor α (Invitrogen), growth regulating estrogen receptor binding 1 (GREB1) (Cell Signaling Technologies), and trefoil factor 1 (TFF1) (Cell Signaling Technologies).

Statistical Analysis

Data is presented as standard deviation. Data was analyzed using analysis of variance (ANOVA) to determine statistical significance ($p < 0.05$). Analyses were performed in GraphPad Prism 10.

4.3 Results

The Vicious Cycle of Bone Destruction is Not Mechanically Modulated at Stiffnesses in the Bone Marrow in ER+ Breast Cancer Cells

To mimic the bone marrow microenvironment, we plated MCF7s and T47Ds on CytoSoft® Rigidity Plates from Advanced BioMatrix ® coated with 4 ug/mL fibronectin. Plates with 0.5 kPa was used to recapitulate the sinusoidal region, 32 kPa was used to recapitulate the endosteal region and normal tissue plates were used to mimic the stiffness of the cortical and trabecular bone (**Fig. 4.1A**) and cell lysates were collected for RNA and protein after 48 hours (**Fig.4.1B**). Gli2 and PTHrP are responsible for activating osteolytic behavior of tumor cells in ER- cells at various stiffnesses associated with the bone and bone marrow, but it is unclear if this takes place in ER+ cell types. Here, we aimed to evaluate if the varying stiffnesses of the bone marrow can modulate the expression of Gli2 and PTHrP in ER+ cell lines. Using qPCR, did not identify any significant changes in *Gli2* or *PTHLH* expression in MCF7s and T47Ds (**Fig. 4.2A-D**). This implies that stiffness of the bone marrow does not modulate PTHrP in ER+ breast cancer. However, we sought to evaluate other cellular processes that could potentially be modulated by bone marrow stiffnesses. From this study, we looked to

analyze other factors that could contribute to osteolysis or bone remodeling for ER+ breast cancer cells.

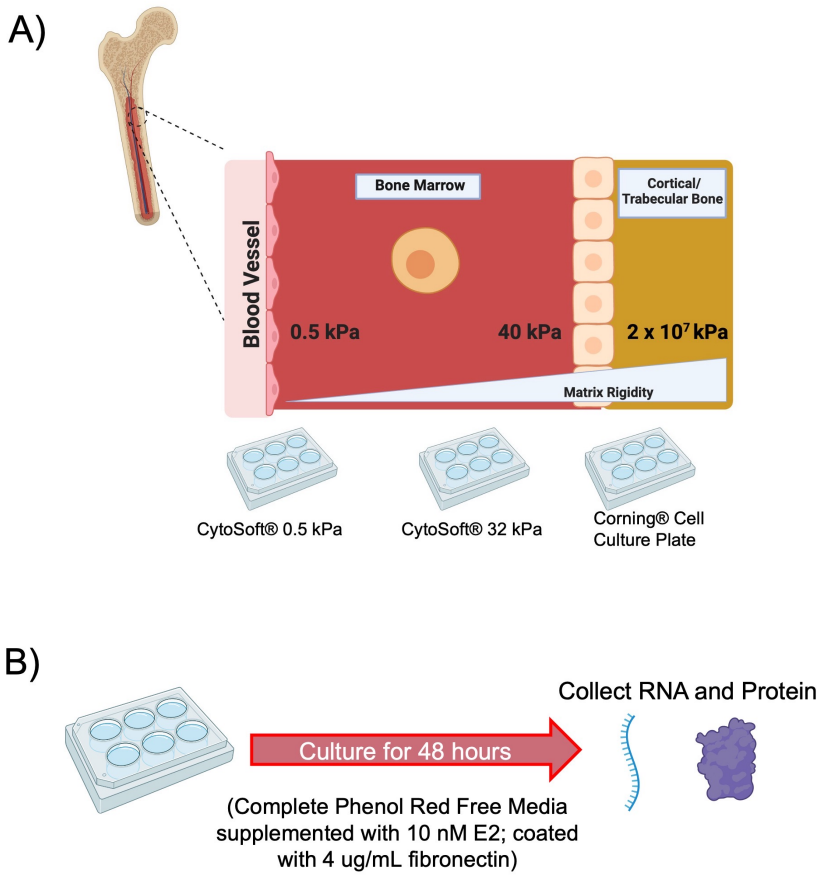

Figure 4.1. Workflow of evaluating ER+ cell behavior on bone marrow mimetic stiffnesses. Created in BioRender.

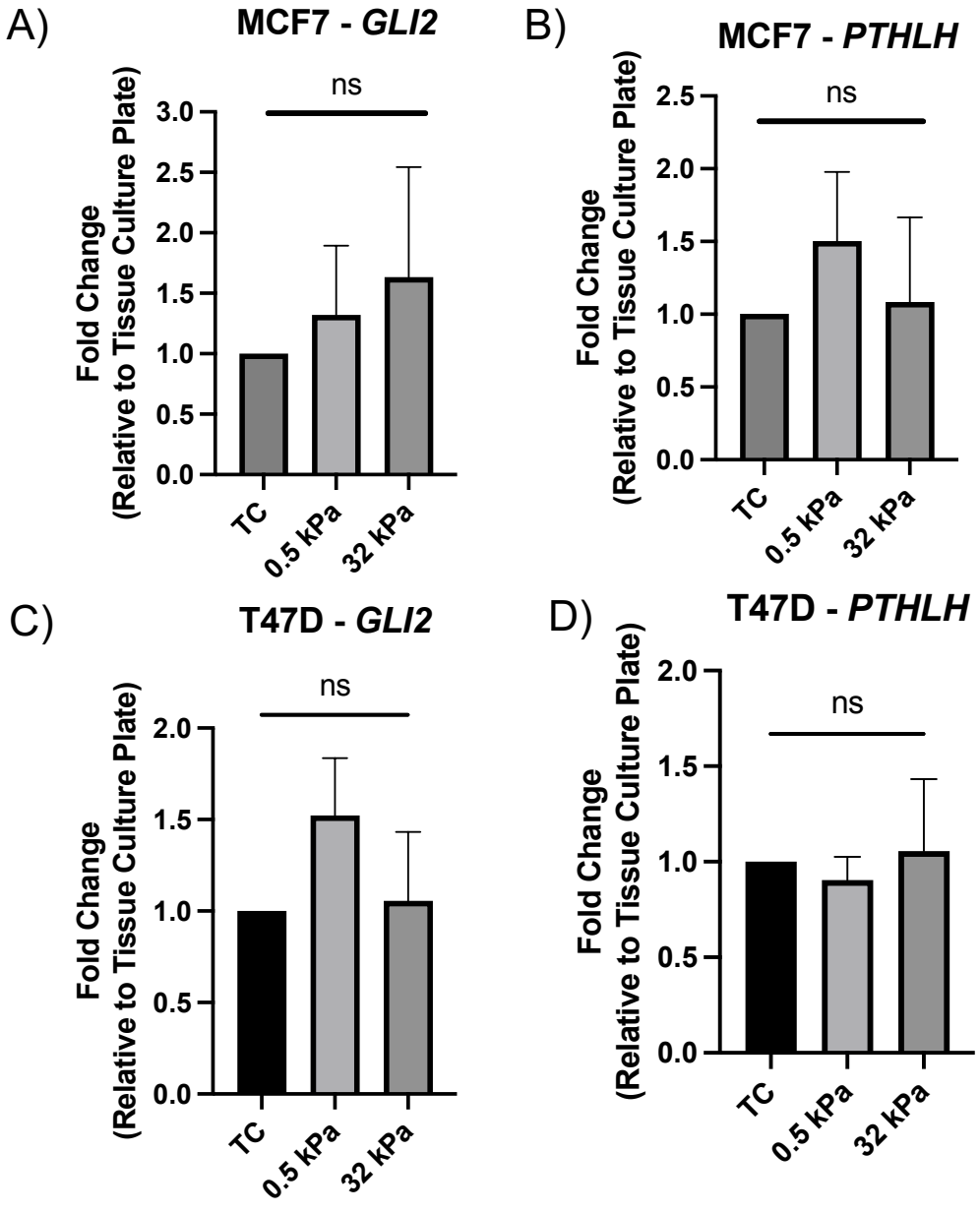

Figure 4.2. Bone marrow mimetic stiffnesses does not change *GLI2* and *PTHLH* expression in ER+ breast cancer cells compared to normal tissue culture plates. qPCR of *GLI2* and *PTHLH* in at different stiffnesses in MCF7s and T47Ds. n = 3; ANOVA.

RNA Sequencing Suggests Increased Changes in Interleukin Secretion at Bone Marrow Mimetic Stiffnesses

To gain an understanding of how ER+ cells are changing at stiffnesses relative to the bone marrow, we conducted RNA sequencing to evaluate transcriptional changes in the cells. Interestingly, there were not any differentially expressed genes between 0.5 kPa and 32 kPa (**Fig. 4.3C**), differing from what we originally hypothesized. However, there were differentially expressed genes between the 0.5 kPa vs. TC (**Fig. 4.3A, 4.3D**) and 32 kPa vs. TC (**Fig 4.3B, 4.3E**). This leads us to believe that stiffnesses of the sinusoidal and endosteal niche are not different enough to cause transcriptional changes in breast cancer cells although previous studies have shown that morphology changes occur at these stiffnesses. It is more likely that any major transcriptional changes occur near the cortical and trabecular bone after metastasis. In further analysis of this data, we compared the top 50 upregulated (**Fig. 4.3F**) and downregulated (**Fig. 4.3G**) genes in the 0.5 kPa vs. TC and the 32 kPa vs. TC to evaluate general changes that are occurring in the softer environments compared to the stiffer environments.

Gene Set Enrichment Analysis Suggests Increased Interleukin Associated Gene Signature Changes

Using differentially expressed genes; a gene set enrichment analysis (GSEA) was conducted to analyze biological processes that would be changed at different stiffnesses. Interestingly, several processes associated with immune activation and interleukin production were upregulated in softer environments compared to stiffer ones (**Fig. 4.4A-B)**. IL-1 and IL-6 are known to activate osteoclastogenesis and are not normally secreted at baseline in most studies in MCF7s.[259–262] It is also understood that

A) **0.5 kPa vs. TC**

Differentially expressed genes where ***padj < 0.05***

Regulation	$Log_2FC > 0$	$Log_2FC > 1$	$Log_2FC > 2$	$Log_2FC > 3$
down	371	31	6	2
up	814	361	11	NA

B) **32 kPa vs. TC**

Differentially expressed genes where ***padj < 0.05***

Regulation	$Log_2FC > 0$	$Log_2FC > 1$	$Log_2FC > 2$	$Log_2FC > 3$
down	23	13	3	2
up	38	13	NA	NA

C) **0.5 kPa vs. 32 kPa**

No differentially expressed genes

Figure 4.3. RNA Sequencing shows transcriptional changes between marrow mimetic stiffnesses and normal tissue culture plates, but not between 0.5 kPa and 32 kPa in MCF7s. Numbers of differentially expressed genes comparing (A) 0.5 kPa and TC, (B) 32 kPa vs. TC, (C) 0.5 kPa vs. 32 kPa.

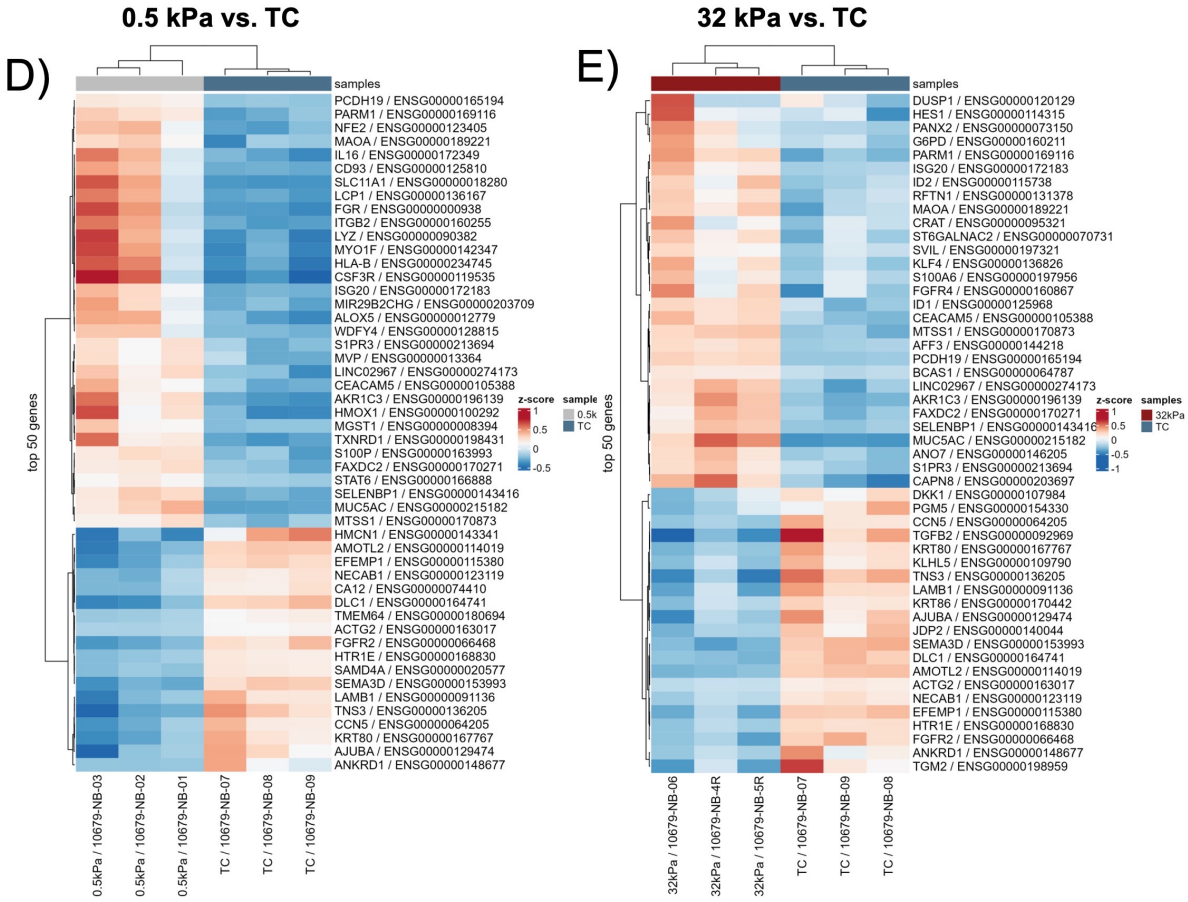

Figure 4.3 Continued. Heat Maps of top 50 genes by p-value (D) 0.5 kPa vs. TC and (E) 32 kPa vs. TC.

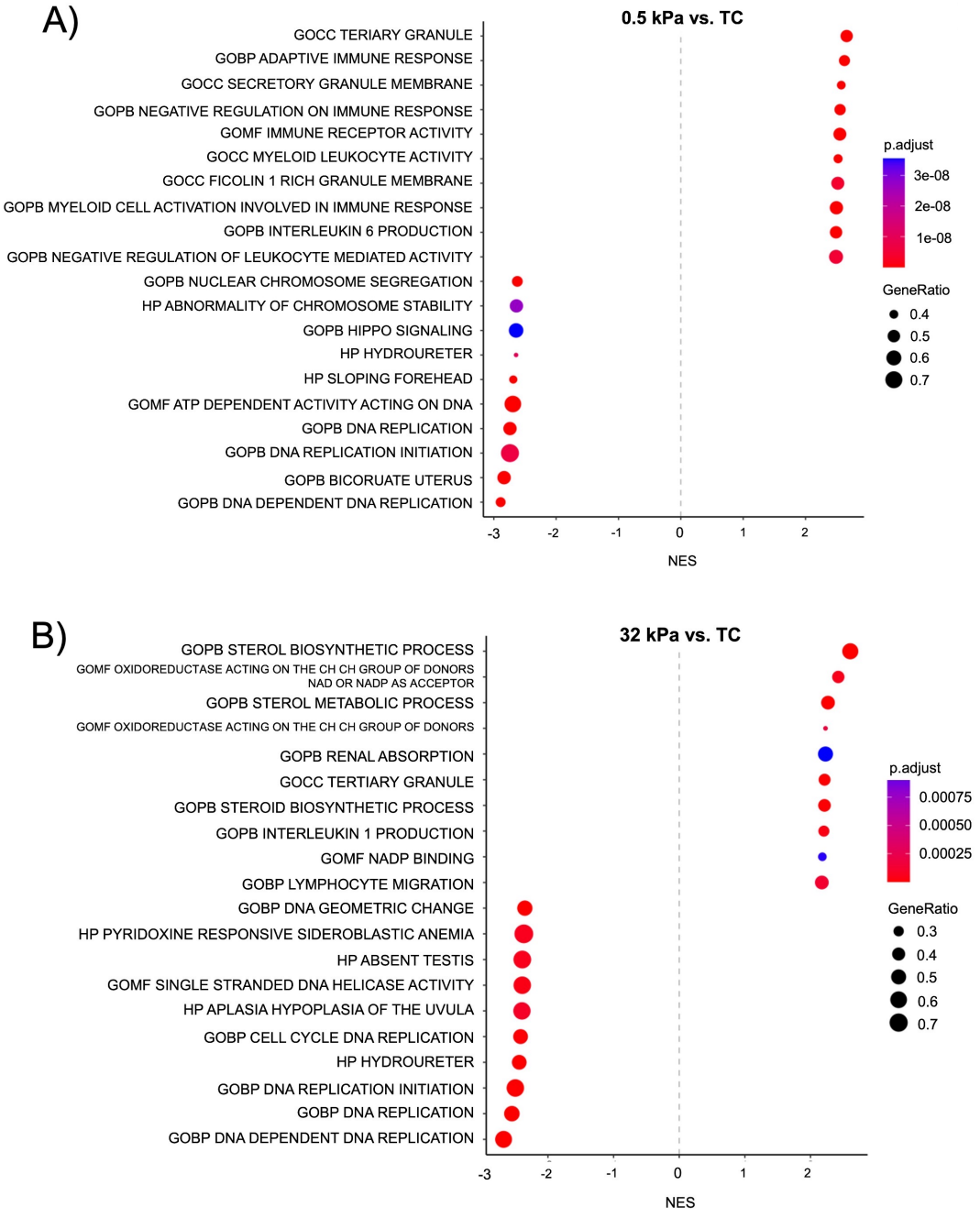

Figure 4.4. Gene Set Enrichment Analyses shows increased expression of genes associated with Interleukin-1 and Interleukin-6 Production Pathway in MCF7s at bone marrow related stiffnesses. Gene ontology analyses comparing (A) 0.5 kPa vs. TC and (B) 32 kPa vs. TC.

increased IL-1b and IL-6 can promote tumor cell invasion and metastasis.[263–266] Additionally, increased interleukin signaling can also change the differentiation of naïve macrophages into pro-tumorigenic macrophages.[267,268] We saw that there were not any changes in secreted IL-6 and IL-1b in conditioned media collected from MCF7s in Luminex. (**Fig. 4.5)** This change in gene associations but not in protein secretion led us to believe that the interleukin association processes are changing at rigidities without the distinct expressions of interleukins.

Softer Environments of the Bone Marrow Show Decrease in Estrogen Signaling Compared to Stiffer Environments

While evaluating changes in interleukin signaling, some literature suggested that IL-6 and its downstream targets can co-opt enhancers of estrogen signaling.[269] Estrogen signaling is an important signaling pathway for ER+ tumor cell progression and a common target of systemic treatment of ER+ breast cancer. [253,270–272] Some work has evaluated increased physical factors that can cause activated nuclear localization of estrogen receptor α in systems that mimic the primary site.[74] We evaluated if there are changes in estrogen signaling at different stiffnesses associated with the bone marrow. Using RNA sequencing data, we conducted gene set enrichment analyses for processes associated with 'ESTROGEN' and found that in 0.5 kPa vs. TC that there was a downregulation in the 'ESTROGEN RESPONSE EARLY' Hallmark Pathway (**Fig. 4.6A**). In the 32 kPa vs. TC, there was an in increase in the 'ESTROGEN RESPONSE EARLY' and 'ESTROGEN RESPONSE LATE' Hallmark Pathways (**Fig. 4.6B**). This led us to evaluate downstream outputs of estrogen signaling to see if they were changing.

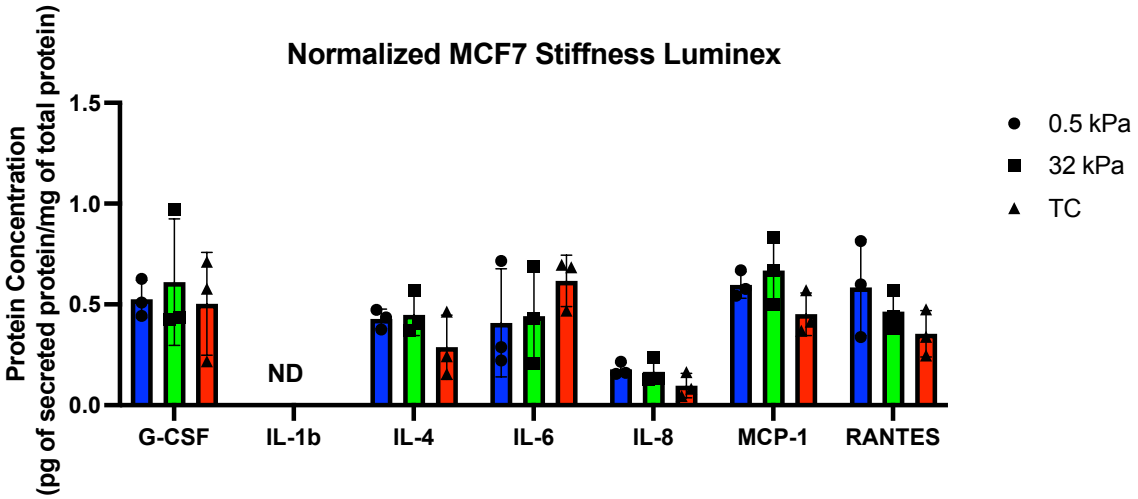

Figure 4.5 Increasing rigidities does not modulate cytokine secretion in MCF7s. MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel results of tumor conditioned media collected after culturing MCF7 cells for 48 hours in phenol-red free media supplemented with E2. Statistical significance was determined using one-way ANOVA. (*$p < 0.05$)

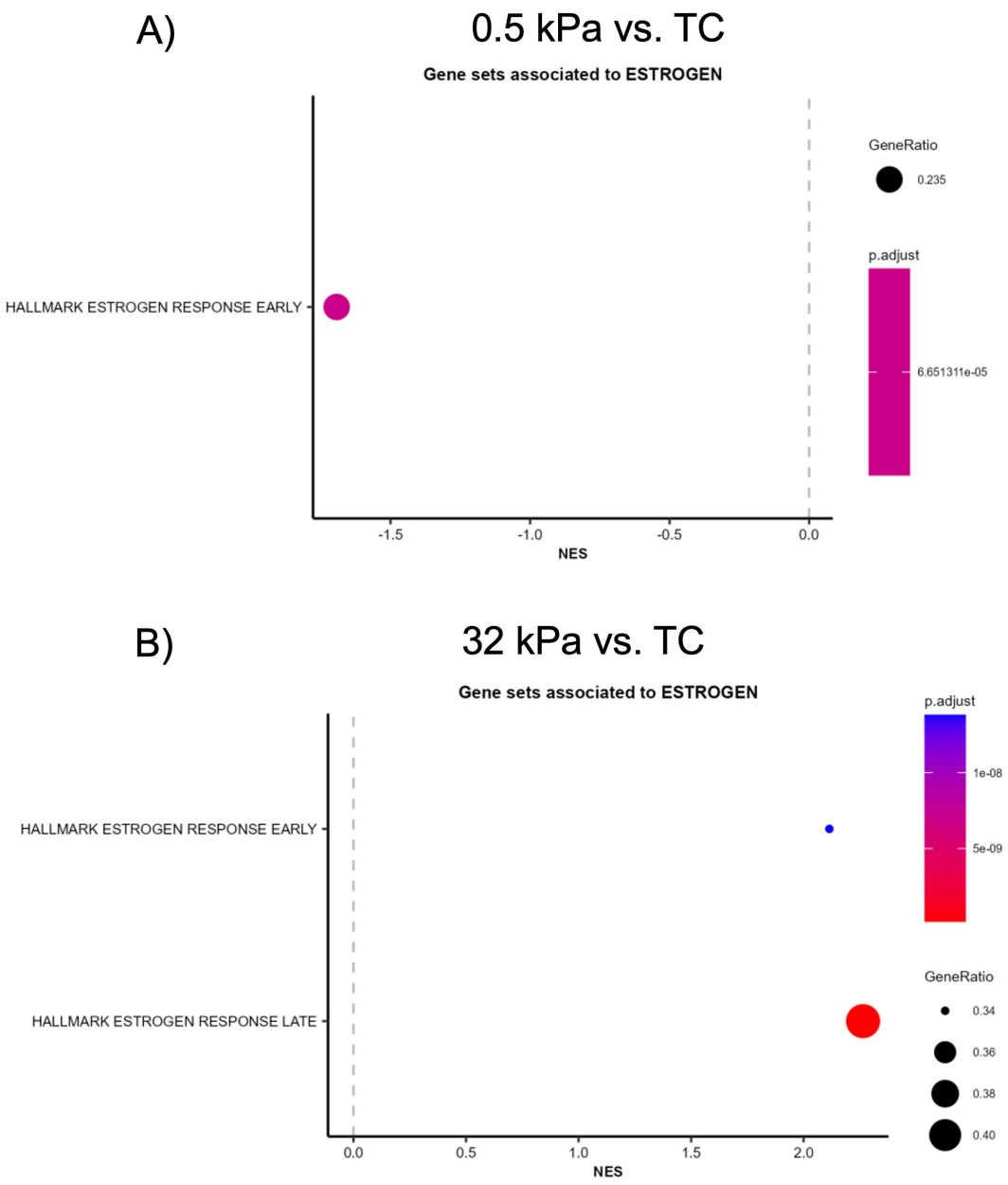

Figure 4.6. RNA Sequencing from MCF7s show higher estrogen signaling gene set enrichments at lower stiffnesses compared to higher stiffnesses. Gene ontology analyses with 'ESTROGEN' as a key GO Term.

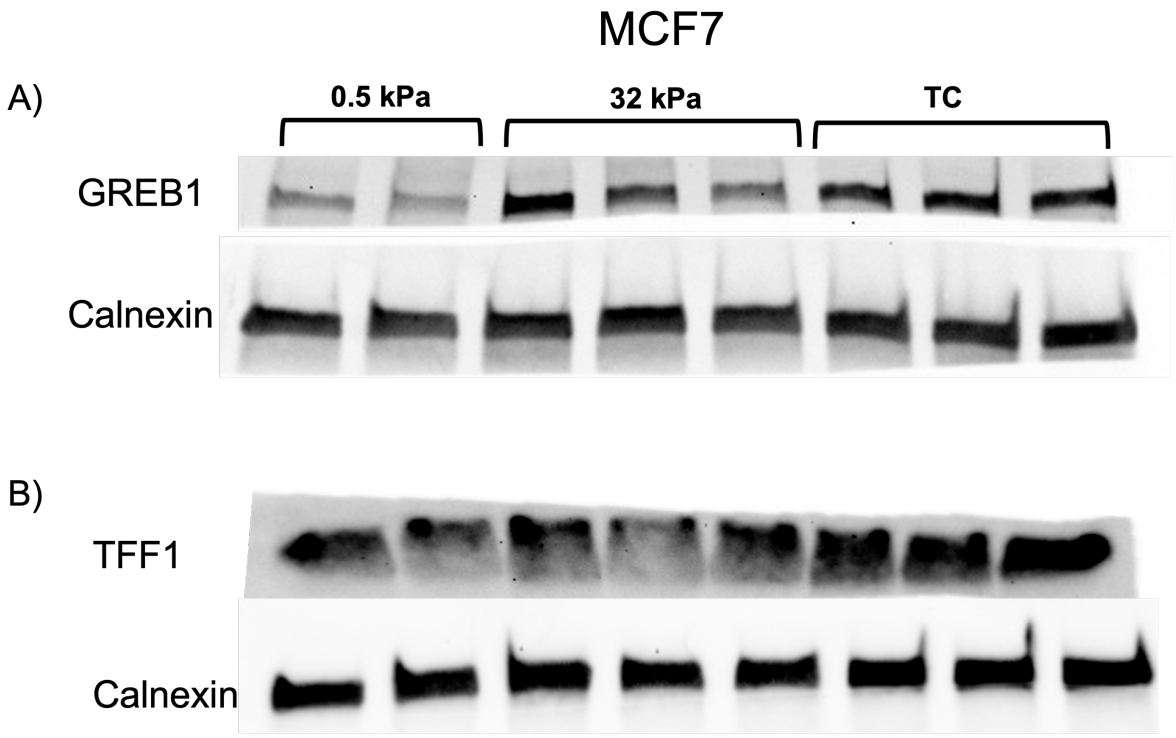

Figure 4.7. ER+ breast cancer cells show decreased expression of downstream estrogen outputs at protein level at lower stiffnesses. Western blot of GREB1 and TFF1 at 0.5 kPa, 32 kPa and TC. Calnexin used as loading control.

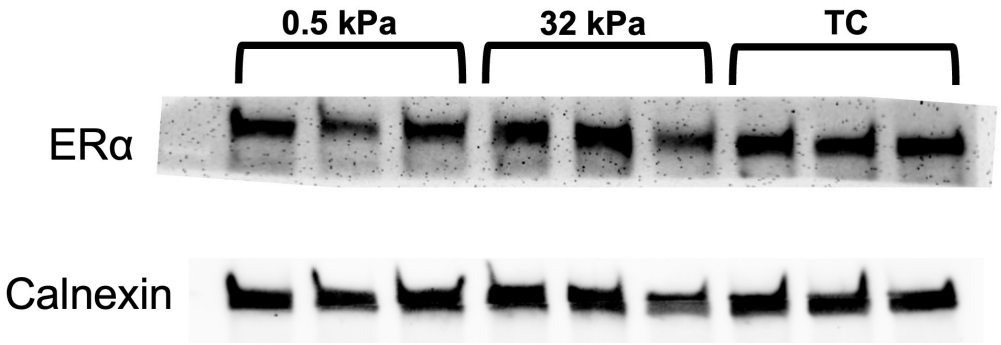

Figure 4.8. Increasing rigidities does not modulate ERα in MCF7s.
Western blot for ERα in MCF7s after 48 hours in phenol-red free media supplemented with E2.

Using GREB1 and TFF1, common downstream output of estrogen signaling, we saw that GREB1 and TFF1 expression was less expressed in 0.5 kPa compared to 32 kPa or TC in MCF7s (**Fig. 4.7A-B**). Interestingly, total ERα was not changed at different stiffnesses, leading us to believe that changes are happening after dimerization (**Fig. 4.8)**.

4.4 Discussion

Physical factors of the primary tumor microenvironment are an important aspect consider for cancer cell progression.[76] However, it is unclear how the physical factors of the secondary site contribute to metastasis in ER+ breast cancer. In this work, we sought to determine how the stiffnesses associated with the bone marrow can change the transcriptional phenotypes of breast cancer cells. Here, we used ER+ breast cancer cells as they are understudied in the context of bone metastasis models and not as highly evaluated in mechanotransduction models.

In our studies, we used CytoSoft Rigidity Plates to mimic the sinusoidal region and the endosteal region of the bone marrow. Physiologically, this would mimic the physical environment after breast cancer colonization in the bone. Using RNA Sequencing, we analyzed the transcriptional changes in the ER+ cells at different stiffnesses. We have shown that in previous studies, increased stiffnesses are associated with tumor cell morphology and proliferation changes. Here, we hypothesized that there would be large transcriptional changes between 0.5 kPa and 32 kPa. Interestingly, there were not any transcriptional changes between 0.5 and 32 kPa. However, there were changes between 0.5 kPa vs. TC and 32 kPa vs. TC, indicating

that larger transcriptional changes in ER+ breast cancer cells may not occur until reaching higher stiffnesses.

In our analysis of the RNA Sequencing, we observed gene signature changes in interleukin production and immune associated changes. This led us to evaluate production of interleukins, particularly IL-1b and IL-6; cytokines that could lead to a tumor promoting environment by macrophage populations. Further analysis by Luminex and show that there is no significant change in interleukin production. To see if there were any changes to the myeloid cell populations, we treated monocytes with conditioned media collected from tumor cells showed no immediate changes in populations. This could be due to the shorter culturing times at the different stiffnesses. Also, there is work to suggest at IL-1 and IL-6 may not be stable in culture media without protease inhibition. In the future, we plan to incorporate longer culturing times for these studies.

Lastly, we analyzed estrogen signaling in the ER+ tumor cells at varying stiffnesses. This became of interest to us as interleukin signaling has been shown to have a negative correlation to estrogen signaling.[273–275] Additionally, aromatase inhibitors are less effective after metastases to metastatic sites. On our study, we observed through RNA sequencing that estrogen signaling may be decreased at softer environments compared to stiffer ones. We observed though downstream outputs of estrogen signaling, GREB1 and TFF1, were decreased in the softer environment compared to stiffer ones. This leads us to believe that softer environments of the bone marrow may contribute to decreased effectiveness in hormonal drug targets. In the future, we plan to evaluate ER signaling with luciferase reporter cell lines of estrogen

response elements to further evaluate estrogen signaling. We also plan to conduct drug studies with aromatase inhibitors to evaluate drug efficacy at different stiffnesses.

4.5 Conclusions

Currently, there is limited understanding in how ER+ breast cancer cells respond to the physical factors of the metastatic microenvironment of the bone. In our studies, we find that ER+ breast cancer cells on softer environments show propensity to modify the bone microenvironment via interleukin signaling and downregulation in estrogen signaling. Overall, this work can give us insight on the mechanoregulation ER+ breast cancer cells when experiencing the metastatic microenvironment and potential considerations when treating breast cancer metastases at different metastatic regions.

Chapter 5

Summary and Future Directions

The physical characteristics of the metastatic microenvironment are important aspects to consider when treating metastatic disease. Even though some work has investigated how physical forces of the cancer microenvironment contribute to progression at the primary sites, there is not a consensus on how it contributes to overall growth, colonization, and survival to other sites.

The overall goal of this dissertation was to evaluate how one of the differing physical factors of the bone marrow, matrix rigidity, can contribute to breast cancer cell behavior. Utilizing 2D and 3D approaches, we identified that increasing rigidities relative to the bone marrow can cause breast cancer cells to change their morphologies to those seen in invasive phenotypes. We also probed the transcriptional changes that occurred in ER+ breast cancer cells. Here, we determined that interleukin production gene signature changes occurred in rigidities associated with softer areas bone marrow and by proxy can cause changes in the downstream target proteins associated with estrogen signaling. These findings gave us insights into potential ways that breast cancer cells behave at rigidities associated with their metastatic sites.

5.1 Synopsis of Dissertation Work

When first embarking on this project, we were interested in understanding how the differing matrix rigidities of bone marrow contribute to phenotypic changes in breast cancer cells. This was primarily influenced by the work conducted by Chadhuri and

colleagues that used alginate-Matrigel IPNs probe MCF10A cell behaviors at rigidities mimicking normal and tumorigenic breast microenvironments.[235,248] We sought to understand how tumor cells responded to rigidities associated within the ranges of the bone marrow microenvironments, which is reflected in Chapter 3 of this dissertation. First, we needed to recapitulate the rigidity of the bone marrow microenvironment which ranges from 0.5 kPa to 40 kPa.[276] To create these systems, we modulated the stiffnesses of the microenvironments using $CaSO_4$ ranging from 5 mM to 50 mM. We were able to gain a range from 0.5 kPa to 16 kPa, which to our knowledge, was the first to achieve this stiffness range using this alginate-Matrigel system. After finalizing the stiffness ranges, we encapsulated tumor cells in the hydrogels and analyzed their proliferation and morphology using immunofluorescence. Here, we used murine 4T1s and human MCF7s. We were able to quantify nuclei counts using DAPI staining and observed that there were not any statically significant changes in nuclei counts at 2 days and 7 days in culture, but we recognize a positive correlation between increasing matrix rigidity and proliferation. Using Phalloidin staining, we were able to quantify the morphology of the cell colonies at differing matrix rigidities using an elongation index outlined in Chapter 2. We observed that higher stiffnesses caused an increase in the colony EI at 2 days and 7 days in culture. These results were intriguing to us due to the epithelial-like morphology in 4T1s in the softer environments and the mesenchymal-like morphology of the MCF7s in the stiffer environment. Both observations that are different from their baseline morphologies in normal tissue culture plates.

During the time of completing Chapter 3 of this thesis, the COVID-19 pandemic halted our acquisition of materials needed for further analysis using the alginate-

Matrigel systems. However, we became intrigued in further evaluating changes in the ER+ cell lines that we observed. This was primarily because most breast cancer patients who have metastases to the bone are ER+. We sought probe these changes using a commercially available system with differing rigidities.

In Chapter 4 of my thesis, we sought to evaluate if the different matrix rigidities of the bone and bone marrow caused ER+ breast cell lines to change their baseline phenotypes into osteolytic ones. This was developed from literature suggesting that ER+ cell lines decreased their estrogen signaling when encountering the bone microenvironment during colonization.[257] However, bone metastatic clones of MCF7s when cultured on tissue culture plates did not have differences in estrogen signaling.[258] We also reviewed literature that suggested that decreases in estrogen signaling also increased transcription factors associated with osteolysis. We hypothesized that lower rigidities would increase osteolytic factors. We cultured cells on CytoSoft® Rigidity Plates at 0.5 kPa, 32 kPa and normal tissue culture (TC), mimicking the sinusoidal marrow, endosteal marrow and cortical bone, respectively. We cultured MCF7s and T47Ds for 48 hours with phenol-red free media supplemented with 10 nM E2. Subsequently, we extracted mRNA and protein lysates for analysis. RNA Sequencing from MCF7s showed that in the lower stiffnesses, there were increased gene signatures associated with IL-1 and IL-6 production, both of which are associated with increased metastatic potential of BC cells and osteoclastogenesis. It is also shown in literature that IL-6 is a known inhibitor of estrogen signaling, a signaling pathway that is associated with proliferation in ER+ cells. We became interested if estrogen signaling was downregulated in ER+ cells at lower stiffnesses. We then probed GREB1 and TFF1,

downstream outputs of estrogen signaling. There was an observed decrease in GREB1 and TFF1 at lower rigidities compared to higher rigidities. This correlates to the inverse interleukin activity, although Luminex shows that there is not a significant increase in IL-1b or IL-6. This work interest us as this shows a potential mechanism for ER+ cells to promote bone destruction since ER+ cells do not highly express PTHrP or transcription factors associated with osteolysis. Furthermore, this can also give us insight into how ER+ cells change the immune microenvironment of the bone marrow to promote tumorigenesis.

5.2 Limitations

Although this work was completed with much consideration for proper experimental set-up, there were limitations to this work that is outlined below:

5.2.1 Chapter 3 Limitations:

Conducting the development of the alginate-Matrigel hydrogels was a novel undertaking for both labs that yielded interesting results outlining breast cancer cell morphologies at stiffnesses mimicking the bone marrow. However, there are limitations to this work. One of the major limitations of this work was access to materials to conduct gene expression and protein expression analyses. Matrigel® became less available to purchase in proper quantities to conduct western blot and qPCR due to the COVID-19 pandemic. This led us to use immunofluorescence as a major means to quantify cell behavior. Secondly, due to BSL2 restrictions, we were not able to analyze the rigidity of the materials after culturing of the BC cells. This would be of interest to us because the degradation caused by BC cells could influence the stiffness of the materials over time.

In the future, we hope to use atomic force microscopy to evaluate the rigidity of the materials after BC cell culture. Another limitation of this work was analyzing how cells modify their behavior when exposed to varying stiffnesses. The stiffnesses analyzed in this model were discrete whereas the stiffnesses of the bone marrow are structured as a gradient.[276] We have developed model systems to mimic this gradient, however, more optimization is needed to find compatible materials and proper time frames for culturing. Lastly, another limitation was the cell lines that were tested. Although 4T1s and MCF7s were useful models for our experiments, they are also immortalized cell lines from different species. MDA-MB-231, a human TNBC cell line, were used but overproliferated after 7 days, not allowing for proper evaluation of cell morphology. In the future, we will use other ER- cells lines to evaluate morphologies.

5.2.2 Chapter 4 Limitations:

Evaluating ER+ BC cell changes at different rigidities relative to the bone marrow was a novel direction for both labs, although osteolytic behaviors of ER+ cells at cortical bone marrow stiffnesses have been evaluated.[78,250] In this system, we were able to gain insights into how ER+ BC cells respond to different rigidities of the bone marrow and how these rigidities may contribute to osteolysis. However, there are caveats associated with this work. One caveat was the proliferation of the cells in the softer microenvironment. In comparison to TC, BC cells did not proliferate as quickly. In the RNA sequencing data, several gene signatures associated with DNA replication and proliferation associated processes were downregulated in the softer environment. This led us to believe that BC cells may undergo processes that induce dormancy in softer

environments compared to stiffer ones. Subsequent experiments where confluency of cells is a factor (cytokine expression, etc.) may be difficult to achieve convincing results. Another limitation is directly translating this work *in vivo*. Most studies have not directly outlined how the metastatic clones in BC cells from *in vivo* experiments mimic the changes seen at different rigidities. We believe that this can be remedied with meta-analyses of sequencing data comparing *in vivo* experiments with studies analyzing stiffness changes. Moving forward, we are planning on collaborating with computational scientists to compare data sets from *in vivo* to other stiffness studies.

5.3 Future Directions

The projects that I conducted were novel projects that utilized several disciplines to elucidate our questions. However, there are more questions that we want to evaluate in the future:

5.3.1 Does changes in the molecules that develop the ECM have an effect along with the difference in stiffnesses of the bone marrow?

In the first project analyzing tumor cell morphologies at different rigidities relative to the bone marrow, we utilized an alginate-Matrigel® interpenetrating network crosslinked with $CaSO_4$. We mostly used Matrigel® as our integrin-binding molecule due to previously published works with the system. However, it is known that Matrigel® is primarily composed of laminin, an ECM protein that is not widely expressed in the bone microenvironment. Other proteins such as fibronectin and collagens are more widely present in the bone marrow at different areas.[277] In the future, we plan to remodel the alginate network with varying ECM molecules. This can be done by

substituting the Matrigel® for other molecules more relevant to the bone microenvironment such as fibronectin and collagen. We plan to make the same formulations conducted in Chapter 3. Using rheology, the rigidity of the hydrogels would be calculated. We would also evaluate morphological and proliferation changes to see if they are similar or different than our findings.

5.3.2 Does the stiffness of the bone marrow microenvironment cause changes in immune cell populations in the microenvironment?

In our studies evaluating ER+ breast cancer cell responses to varying stiffnesses, we observed that softer stiffness ranges elicited a transcriptional change that increased gene ontology processes that dealt with interleukin signaling and increased immune activation. It would be of interest for us to evaluate changes in immune populations at different rigidities. We would allow the cells to seed for 48 – 72 hours and collect tumor conditioned media (TCM) from the samples. We would then treat immortalized monocytes (THP-1) with the TCM for 5 days. Using flow cytometry, we would then analyze the changes in the immune populations. Considering that the myeloid derived cell lineages are the most involved in the metastatic cascade at the bone, we plan to use a pro- and anti-tumorigenic flow cytometry macrophage panel. Additionally, we are interested in seeing if the treatment of the monocytes of the TCM pushed towards osteoclasts by collecting mRNA and using qPCR for pre-osteoclastic markers.

5.3.3 Does the stiffness of the bone marrow microenvironment cause changes in response to hormonal therapies used to treat metastatic breast cancer?

In our matrix rigidity ER+ breast cancer study, we found that the lower stiffness had less downstream outputs of GREB1 and TFF1, which are canonical estrogen signaling. This data correlates to previously published work that concludes there is a negative correlation between interleukin signaling and estrogen signaling.[269,273–275] With this data in mind, we are curious about how the matrix rigidity influences antiestrogen treatments for hormone – positive breast cancer. In this study, we will plate MCF7s and T47Ds at different stiffnesses associated with the bone marrow using the 96 Well CytoSoft® Matrix Rigidity Plate. We will then treat the cells with tamoxifen or fulvestrant for 48 hours and conduct viability assays. Here, we can gain a better idea of how efficacious the hormonal therapies are at different rigidities.

5.4 Concluding Remarks

Metastasis of breast cancer is a prevalent issue for patients and adds complexity to treatment options. The biological process of breast cancer metastasis involves several interactions with the primary and secondary extracellular matrixes, which vary in physical properties. Regarding breast cancer metastasis to the bone, we hypothesized that higher matrix rigidities associated with the bone marrow and bone microenvironment can cause cell behaviors to be more conducive for tumor growth.

In the first part of my dissertation, we evaluated how the differing matrix rigidities of the bone marrow can influence behaviors in different breast cancer cell lines. In the second part, we evaluated how ER+ breast cancer cell lines respond to different rigidities associated with the bone marrow and bone microenvironment, something that has not been widely studied in the context of tumor-induced bone disease. We hope

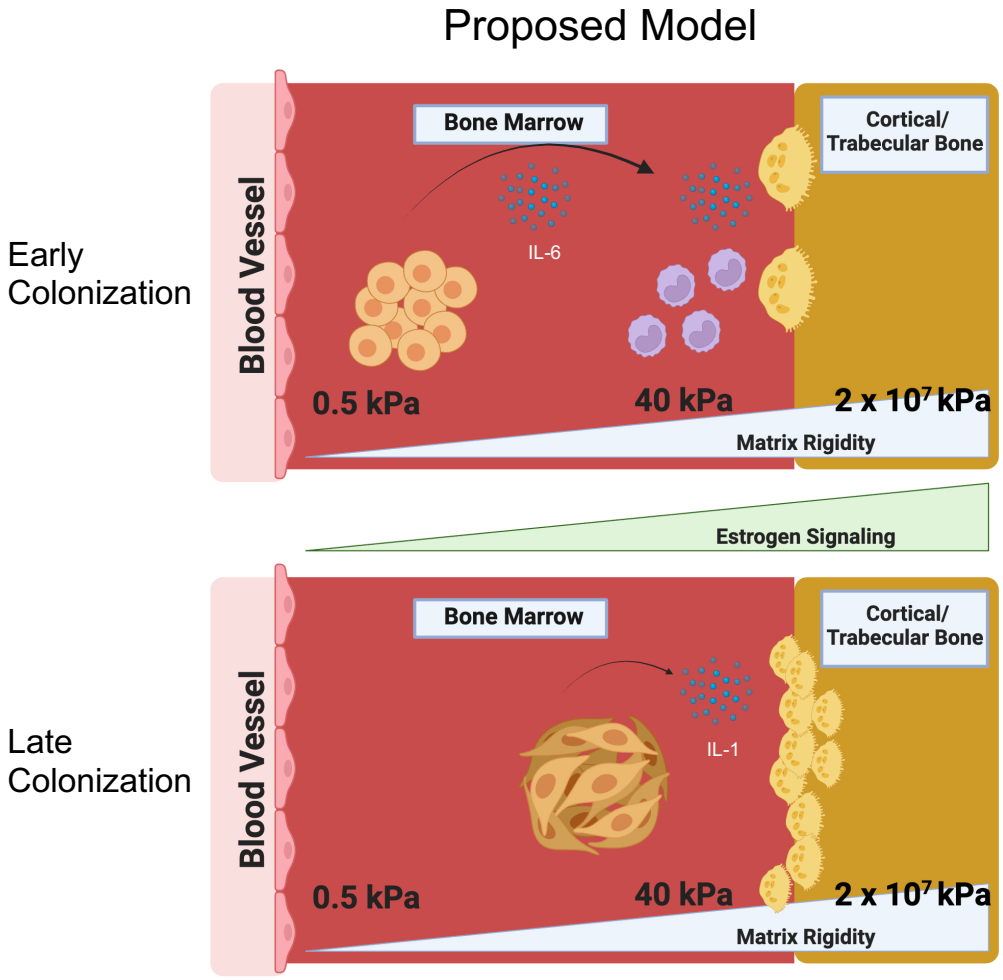

Figure 5.1. Potential model of interleukin secretion and bone microenvironment remodeling in ER+ breast cancer cells at different stiffnesses. As the tumor cells exit the vasculature in early colonization and interact with the softer parts of the marrow, the tumor cells may secrete IL-6 and promote osteoclast formation. In late state colonization, the tumor cell colonies change their morphology and increase IL-1 cytokine secretion, leading into increased osteoclast activity.

that this work promotes a general understanding of how the metastatic microenvironment influences cell behavior and informs considerations when treating metastatic breast cancer.

REFERENCES

1. Mariotto AB, Enewold L, Zhao J, Zeruto CA, Robin Yabroff K. Medical care costs associated with cancer survivorship in the United States. *Cancer Epidemiology Biomarkers and Prevention*. 2020;29(7):1304-1312. doi:10.1158/1055-9965.EPI-19-1534
2. Mariotto AB, Robin Yabroff K, Shao Y, Feuer EJ, Brown ML. Projections of the cost of cancer care in the United States: 2010-2020. *J Natl Cancer Inst*. 2011;103(2):117-128. doi:10.1093/jnci/djq495
3. Bluethmann SM, Mariotto AB, Rowland JH. Anticipating the “silver tsunami”: Prevalence trajectories and comorbidity burden among older cancer survivors in the United States. *Cancer Epidemiology Biomarkers and Prevention*. 2016;25(7):1029-1036. doi:10.1158/1055-9965.EPI-16-0133
4. Hanahan D, Weinberg RAA. Hallmarks of cancer: The next generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013
5. Chopra S, Davies EL. Breast cancer. *Medicine (United Kingdom)*. 2020;48(2):113-118. doi:10.1016/j.mpmed.2019.11.009
6. Berman AT, Thukral AD, Hwang WT, Solin LJ, Vapiwala N. Incidence and patterns of distant metastases for patients with early-stage breast cancer after breast conservation treatment. *Clin Breast Cancer*. 2013;13(2):88-94. doi:10.1016/j.clbc.2012.11.001
7. Mittal V. Epithelial Mesenchymal Transition in Tumor Metastasis. Published online 2017. doi:10.1146/annurev-pathol-020117

8. Chen W, Hoffmann AD, Liu H, Liu X. Organotropism: new insights into molecular mechanisms of breast cancer metastasis. *NPJ Precis Oncol*. 2018;2(1). doi:10.1038/s41698-018-0047-0

9. Langley RR, Fidler IJ. The seed and soil hypothesis revisited-The role of tumor-stroma interactions in metastasis to different organs. *Int J Cancer*. 2011;128(11):2527-2535. doi:10.1002/ijc.26031

10. Yip RKH, Rimes JS, Capaldo BD, et al. Mammary tumour cells remodel the bone marrow vascular microenvironment to support metastasis. *Nat Commun*. 2021;12(1). doi:10.1038/s41467-021-26556-6

11. Macedo F, Ladeira K, Pinho F, et al. Bone metastases: An overview. *Oncol Rev*. 2017;11(1). doi:10.4081/oncol.2017.321

12. Elkholi IE, Lalonde A, Park M, Côté JF. Breast Cancer Metastatic Dormancy and Relapse: An Enigma of Microenvironment(s). *Cancer Res*. 2022;82(24):4497-4510. doi:10.1158/0008-5472.CAN-22-1902

13. Bushnell GG, Deshmukh AP, den Hollander P, et al. Breast cancer dormancy: need for clinically relevant models to address current gaps in knowledge. *NPJ Breast Cancer*. 2021;7(1). doi:10.1038/s41523-021-00269-x

14. Sowder ME, Johnson RW. Bone as a Preferential Site for Metastasis. *JBMR Plus*. 2019;3(3). doi:10.1002/jbm4.10126

15. Zhang XHF, Giuliano M, Trivedi M V., Schiff R, Kent Osborne C. Metastasis dormancy in estrogen receptor-positive breast cancer. *Clinical Cancer Research*. 2013;19(23):6389-6397. doi:10.1158/1078-0432.CCR-13-0838

16. Yin JJ, Pollock CB, Kelly K. *Mechanisms of Cancer Metastasis to the Bone*. Vol 15.; 2005. www.cell-research.com|

17. Esposito M, Guise T, Kang Y. The biology of bone metastasis. *Cold Spring Harb Perspect Med*. 2018;8(6). doi:10.1101/cshperspect.a031252

18. Park JH, Lee NK, Lee SY. Current understanding of RANK signaling in osteoclast differentiation and maturation. *Mol Cells*. 2017;40(10):706-713. doi:10.14348/molcells.2017.0225

19. Sterling JA, Edwards JR, Martin TJ, Mundy GR. Advances in the biology of bone metastasis: How the skeleton affects tumor behavior. *Bone*. 2011;48(1):6-15. doi:10.1016/j.bone.2010.07.015

20. Huang J, Freyhult E, Buckland R, Josefsson A, Damber JE, Welén K. Osteoclasts directly influence castration-resistant prostate cancer cells. *Clin Exp Metastasis*. 2022;39(5):801-814. doi:10.1007/s10585-022-10179-2

21. Venetis K, Piciotti R, Sajjadi E, et al. Breast cancer with bone metastasis: Molecular insights and clinical management. *Cells*. 2021;10(6). doi:10.3390/cells10061377

22. Qiao RQ, Zhang HR, Ma RX, Li RF, Hu YC. Prognostic Factors for Bone Survival and Functional Outcomes in Patients With Breast Cancer Spine Metastases. *Technol Cancer Res Treat*. 2022;21. doi:10.1177/15330338221122642

23. Randles MJ, Humphries MJ, Lennon R. Proteomic definitions of basement membrane composition in health and disease. *Matrix Biology*. 2017;57-58:12-28. doi:10.1016/j.matbio.2016.08.006

24. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. *J Cell Sci*. 2010;123(24):4195-4200. doi:10.1242/jcs.023820

25. Barney LE, Hall CL, Schwartz AD, et al. Tumor cell-organized fibronectin maintenance of a dormant breast cancer population. *Sci Adv*. 2020;6(11):1-13. doi:10.1126/sciadv.aaz4157

26. Tang X, Hou Y, Yang G, et al. Stromal miR-200s contribute to breast cancer cell invasion through CAF activation and ECM remodeling. *Cell Death Differ*. 2016;23(1):132-145. doi:10.1038/cdd.2015.78

27. Mosier JA, Rahman-Zaman A, Zanotelli MR, et al. Extent of Cell Confinement in Microtracks Affects Speed and Results in Differential Matrix Strains. *Biophys J*. 2019;117(9):1692-1701. doi:10.1016/j.bpj.2019.09.024

28. Martino F, Perestrelo AR, Vinarský V, Pagliari S, Forte G. Cellular mechanotransduction: From tension to function. *Front Physiol*. 2018;9(JUL):1-21. doi:10.3389/fphys.2018.00824

29. Li X, Jin Y, Xue J. Unveiling Collagen's Role in Breast Cancer: Insights into Expression Patterns, Functions and Clinical Implications. *Int J Gen Med*. 2024;Volume 17:1773-1787. doi:10.2147/ijgm.s463649

30. Broders-Bondon F, Ho-Bouldoires THN, Fernandez-Sanchez ME, Farge E. Mechanotransduction in tumor progression: The dark side of the force. *Journal of Cell Biology*. 2018;217(5):1571-1587. doi:10.1083/jcb.201701039

31. Dupont S, Morsut L, Aragona M, et al. Role of YAP/TAZ in mechanotransduction. *Nature*. 2011;474(7350):179-184. doi:10.1038/nature10137

32. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol*. 2014;15(12):786-801. doi:10.1038/nrm3904

33. Ovijit Chaudhuri, Luo Gu, Darinka Klumpers, Max Darnell SA, Bencherif, James C. Weaver, Nathaniel Huebsch, Hong-pyo Lee EL, Mooney GND and DJ, et al. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat Mater*. 2016;15(3):10-11. doi:10.1038/nmat4489.Hydrogels

34. Adebowale K, Rafat M, Lee JY, et al. Matrix mechanical plasticity regulates cancer cell migration through confining microenvironments. *Nat Commun*. 2018;9(1). doi:10.1038/s41467-018-06641-z

35. Sawicki LA, Kloxin AM, Ross KE, et al. Tunable synthetic extracellular matrices to investigate breast cancer response to biophysical and biochemical cues. *APL Bioeng*. 2019;3(1):016101. doi:10.1063/1.5064596

36. Zhu T, Alves SM, Adamo A, et al. Mammary Tissue-Derived Extracellular Matrix Hydrogels Reveal the Role of the Irradiated Microenvironment in Breast Cancer Recurrence. doi:10.1101/2022.05.16.492117

37. He XT, Wu RX, Xu XY, Wang J, Yin Y, Chen FM. Macrophage involvement affects matrix stiffness-related influences on cell osteogenesis under three-dimensional culture conditions. *Acta Biomater*. 2018;71:132-147. doi:10.1016/j.actbio.2018.02.015

38. El-Mohri H, Wu Y, Mohanty S, Ghosh G. Impact of matrix stiffness on fibroblast function. *Materials Science and Engineering C*. 2017;74:146-151. doi:10.1016/j.msec.2017.02.001

39. Schwager SC, Bordeleau F, Zhang J, Antonyak MA, Cerione RA, Reinhart-King CA. Matrix stiffness regulates microvesicle-induced fibroblast activation. *Am J Physiol Cell Physiol*. 2019;317(1):C82-C92. doi:10.1152/ajpcell.00418.2018

40. Northcutt LA, Suarez-Arnedo A, Rafat M. Emerging Biomimetic Materials for Studying Tumor and Immune Cell Behavior. *Ann Biomed Eng*. 2019;48(7):2064-2077. doi:10.1007/s10439-019-02384-0

41. Buenrostro D, Park SI, Sterling JA. Dissecting the role of bone marrow stromal cells on bone metastases. *Biomed Res Int*. 2014;2014. doi:10.1155/2014/875305

42. Haider MT, Smit DJ, Taipaleenmäki H. The Endosteal Niche in Breast Cancer Bone Metastasis. *Front Oncol*. 2020;10(March):1-11. doi:10.3389/fonc.2020.00335

43. Travlos GS. Normal Structure, Function, and Histology of the Bone Marrow. *Toxicol Pathol*. 2006;34(5):548-565. doi:10.1080/01926230600939856

44. Birbrair A, Frenette PS, Biology C. Niche heterogeneity in the bone marrow Alexander. *Ann N Y Acad Sci*. 2017;1370(1):82-96. doi:10.1111/nyas.13016.Niche

45. Gurevitch O, Slavin S, Resnick I, Khitrin S, Feldman A. Mesenchymal progenitor cells in red and yellow bone marrow. *Folia Biol (Praha)*. 2009;55(1):27-34.

46. Kräter M, Jacobi A, Otto O, et al. Bone marrow niche-mimetics modulate HSPC function via integrin signaling. *Sci Rep*. 2017;7(1):1-15. doi:10.1038/s41598-017-02352-5

47. Gurkan UA, Akkus O. The mechanical environment of bone marrow: A review. *Ann Biomed Eng*. 2008;36(12):1978-1991. doi:10.1007/s10439-008-9577-x

48. Allen JL, Cooke ME, Alliston T. ECM stiffness primes the TGFβ pathway to promote chondrocyte differentiation. *Mol Biol Cell*. 2012;23(18):3731-3742. doi:10.1091/mbc.E12-03-0172

49. Choi JS, Harley BAC. Marrow-inspired matrix cues rapidly affect early fate decisions of hematopoietic stem and progenitor cells. *Sci Adv*. 2017;3(1):1-10. doi:10.1126/sciadv.1600455

50. Nelson MR, Roy K. Bone-marrow mimicking biomaterial niches for studying hematopoietic stem and progenitor cells. *J Mater Chem B*. 2016;4(20):3490-3503. doi:10.1039/c5tb02644j

51. Farach-Carson MC, Li SH, Nalty T, Satcher RL. Sex differences and bone metastases of breast, lung, and prostate cancers: Do bone homing cancers favor feminized bone marrow? *Front Oncol*. 2017;7(AUG):163. doi:10.3389/fonc.2017.00163

52. Kopp HG, Avecilla ST, Hooper AT, Rafii S. The bone marrow vascular niche: Home of HSC differentiation and mobilization. *Physiology*. 2005;20(5):349-356. doi:10.1152/physiol.00025.2005

53. Okuyama Kishima M, Oliveira CEC De, Banin-Hirata BK, et al. Immunohistochemical Expression of CXCR4 on Breast Cancer and Its Clinical Significance. *Analytical Cellular Pathology*. 2015;2015. doi:10.1155/2015/891020

54. Aravindan BK, Prabhakar J, Somanathan T, Subhadra L. The role of chemokine receptor 4 and its ligand stromal cell derived factor 1 in breast cancer. *Ann Transl Med*. 2015;3(2):1-10. doi:10.3978/j.issn.2305-5839.2014.12.13

55. Tan CC, Li GX, Tan LD, et al. Breast cancer cells obtain an osteomimetic feature via epithelial-mesenchymal transition that have undergone BMP2/RUNX2 signaling pathway induction. *Oncotarget*. 2016;7(48):79688-79705. doi:10.18632/oncotarget.12939

56. Abe M, Hiura K, Ozaki S, Kido S, Matsumoto T. Vicious cycle between myeloma cell binding to bone marrow stromal cells via VLA-4-VCAM-1 adhesion and macrophage inflammatory protein-1α and MIP-1β production. *J Bone Miner Metab*. 2009;27(1):16-23. doi:10.1007/s00774-008-0012-z

57. Cappariello A, Capulli M. The Vicious Cycle of Breast Cancer-Induced Bone Metastases, a Complex Biological and Therapeutic Target. *Curr Mol Biol Rep*. 2018;4(3):123-131. doi:10.1007/s40610-018-0099-5

58. Ruppender NS, Merkel AR, Martin TJ, Mundy GR, Sterling JA, Guelcher SA. Matrix rigidity induces osteolytic gene expression of metastatic breast cancer cells. *PLoS One*. 2010;5(11). doi:10.1371/journal.pone.0015451

59. Page JM, Merkel AR, Ruppender NS, et al. Matrix rigidity regulates the transition of tumor cells to a bone-destructive phenotype through integrin β3 and TGF-β receptor type II. *Biomaterials*. 2015;64:33-44. doi:10.1016/j.biomaterials.2015.06.026

60. Kolb AD, Bussard KM. The bone extracellular matrix as an ideal milieu for cancer cell metastases. *Cancers (Basel)*. 2019;11(7). doi:10.3390/cancers11071020

61. Chanda D, Morgan CJ, Siegal GP. Breast Cancer Subtypes Predispose the Site of Distant Metastases. Published online 2015:471-478. doi:10.1309/AJCPYO5FSV3UPEXS

62. Feng Y, Manka D, Wagner K uwe, Khan SA. Estrogen receptor- ␣ expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. Published online 2007.

63. Brett JO, Spring LM, Bardia A, Wander SA. ESR1 mutation as an emerging clinical biomarker in metastatic hormone receptor-positive breast cancer. *Breast Cancer Research*. 2021;23(1):85. doi:10.1186/s13058-021-01462-3

64. Gelsomino L, Panza S, Giordano C, et al. Mutations in the estrogen receptor alpha hormone binding domain promote stem cell phenotype through notch activation in breast cancer cell lines. *Cancer Lett*. 2018;428:12-20. doi:10.1016/j.canlet.2018.04.023

65. Lloyd MR, Wander SA, Hamilton E, Razavi P. Next-generation selective estrogen receptor degraders and other novel endocrine therapies for management of metastatic hormone receptor-positive breast cancer : current and emerging role. Published online 2022. doi:10.1177/17588359221113694

66. Johnston SRD. Endocrinology and hormone therapy in breast cancer Selective oestrogen receptor modulators and downregulators for breast cancer – have they lost their way ? Published online 2005:119-130. doi:10.1186/bcr1023

67. Patel R, Klein P, Tiersten A, Sparano JA. An emerging generation of endocrine therapies in breast cancer : a clinical perspective. Published online 2023:1-12. doi:10.1038/s41523-023-00523-4

68. Al Saleh S, Al Mulla F, Luqmani YA. Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. *PLoS One*. 2011;6(6). doi:10.1371/journal.pone.0020610

69. Bouris P, Skandalis SS, Piperigkou Z, et al. Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells. *Matrix Biology*. 2015;43:42-60. doi:10.1016/j.matbio.2015.02.008

70. Ma S, Tang T, Probst G, et al. Transcriptional repression of estrogen receptor alpha by YAP reveals the Hippo pathway as therapeutic target for ER+ breast cancer. *Nat Commun*. 2022;13(1):1-17. doi:10.1038/s41467-022-28691-0

71. Bado IL, Zhang W, Hu J, et al. The bone microenvironment increases phenotypic plasticity of ER+ breast cancer cells. *Dev Cell*. 2021;56(8):1100-1117.e9. doi:10.1016/j.devcel.2021.03.008

72. Clements ME, Johnson RW. PREX1 drives spontaneous bone dissemination of ER+ breast cancer cells. *Oncogene*. 2020;39(6):1318-1334. doi:10.1038/s41388-019-1064-3

73. Campbell JJ, Husmann A, Hume RD, Watson CJ, Cameron RE. Development of three-dimensional collagen scaffolds with controlled architecture for cell migration studies using breast cancer cell lines. *Biomaterials*. 2017;114:34-43. doi:10.1016/j.biomaterials.2016.10.048

74. Munne PM, Martikainen L, Räty I, et al. Compressive stress-mediated p38 activation required for ERα + phenotype in breast cancer. *Nat Commun*. 2021;12(1):1-17. doi:10.1038/s41467-021-27220-9

75. Grant E, Bucklain FA, Ginn L, Laity P, Ciani B, Bryant HE. Progesterone receptor expression contributes to gemcitabine resistance at higher ECM stiffness in

breast cancer cell lines. *PLoS One*. 2022;17(5 5). doi:10.1371/journal.pone.0268300

76. Spencer A, Sligar AD, Chavarria D, et al. Biomechanical regulation of breast cancer metastasis and progression. *Sci Rep*. 2021;11(1):1-15. doi:10.1038/s41598-021-89288-z

77. Sawicki LA, Kloxin AM, Ross KE, et al. Tunable synthetic extracellular matrices to investigate breast cancer response to biophysical and biochemical cues. *APL Bioeng*. 2019;3(1):016101. doi:10.1063/1.5064596

78. Ruppender NS, Merkel AR, Martin TJ, Mundy GR, Sterling JA, Guelcher SA. Matrix rigidity induces osteolytic gene expression of metastatic breast cancer cells. *PLoS One*. 2010;5(11). doi:10.1371/journal.pone.0015451

79. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013

80. Pfeifer CR, Alvey CM, Irianto J, Discher DE. Genome variation across cancers scales with tissue stiffness – An invasion-mutation mechanism and implications for immune cell infiltration. *Curr Opin Syst Biol*. 2017;2:103-114. doi:10.1016/j.coisb.2017.04.005

81. Luca AC, Mersch S, Deenen R, et al. Impact of the 3D Microenvironment on Phenotype, Gene Expression, and EGFR Inhibition of Colorectal Cancer Cell Lines. *PLoS One*. 2013;8(3). doi:10.1371/journal.pone.0059689

82. Chaudhuri O, Koshy ST, Branco Da Cunha C, et al. Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium. *Nat Mater*. 2014;13(10):970-978. doi:10.1038/nmat4009

83. Glaser DE, Viney C. Biomimetic Materials. *Biomater Sci*. Published online January 1, 2013:349-360. doi:10.1016/B978-0-08-087780-8.00033-4

84. Aljghami ME, Saboor S, Amini-Nik S. Emerging Innovative Wound Dressings. *Ann Biomed Eng*. 2019;47(3):659-675. doi:10.1007/s10439-018-02186-w

85. Teng MWL, Ngiow SF, Ribas A, Smyth MJ. Classifying cancers basedon T-cell infiltration and PD-L1. *Cancer Res*. 2015;75(11):2139-2145. doi:10.1158/0008-5472.CAN-15-0255

86. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: From tumor initiation to metastatic progression. *Genes Dev*. 2018;32(19-20):1267-1284. doi:10.1101/GAD.314617.118

87. Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK. Extracellular matrix structure. *Adv Drug Deliv Rev*. 2016;97. doi:10.1016/j.addr.2015.11.001

88. Yue B. Biology of the extracellular matrix: An overview. *J Glaucoma*. 2014;23(8):S20-S23. doi:10.1097/IJG.0000000000000108

89. Fang M, Yuan J, Peng C, Li Y. Collagen as a double-edged sword in tumor progression. *Tumour Biol*. 2014;35(4):2871-2882. doi:10.1007/s13277-013-1511-7

90. Akhavan A, Griffith OL, Soroceanu L, et al. Loss of cell-surface laminin anchoring promotes tumor growth and is associated with poor clinical outcomes. *Cancer Res*. 2012;72(10):2578-2588. doi:10.1158/0008-5472.CAN-11-3732

91. Han Z, Lu ZR. Targeting fibronectin for cancer imaging and therapy. *J Mater Chem B*. 2017;5(4):639-654. doi:10.1039/c6tb02008a

92. Cavo M, Caria M, Pulsoni I, Beltrame F, Fato M, Scaglione S. A new cell-laden 3D Alginate-Matrigel hydrogel resembles human breast cancer cell malignant morphology, spread and invasion capability observed “in vivo.” *Sci Rep*. 2018;8(1):1-12. doi:10.1038/s41598-018-23250-4

93. Bissell MJ, Radisky D. Putting tumours in context. *Nat Rev Cancer*. 2001;1(1):46-54. doi:10.1038/35094059

94. Walker C, Mojares E, Del Río Hernández A. Role of Extracellular Matrix in Development and Cancer Progression. *Int J Mol Sci*. 2018;19(10). doi:10.3390/ijms19103028

95. Narkhede AA, Crenshaw JH, Manning RM, Rao SS. The influence of matrix stiffness on the behavior of brain metastatic breast cancer cells in a biomimetic hyaluronic acid hydrogel platform. *J Biomed Mater Res A*. 2018;106(7):1832-1841. doi:10.1002/jbm.a.36379

96. Kim H, Chung H, Kim J, et al. Macrophages-Triggered Sequential Remodeling of Endothelium-Interstitial Matrix to Form Pre-Metastatic Niche in Microfluidic Tumor Microenvironment. *Advanced Science*. 2019;6(11):1900195. doi:10.1002/advs.201900195

97. Heinrich MA, Bansal R, Lammers T, Zhang YS, Michel Schiffelers R, Prakash J. 3D-Bioprinted Mini-Brain: A Glioblastoma Model to Study Cellular Interactions and Therapeutics. *Advanced Materials*. 2019;31(14):1806590. doi:10.1002/adma.201806590

98. Langer EM, Allen-Petersen BL, King SM, et al. Modeling Tumor Phenotypes In Vitro with Three-Dimensional Bioprinting. *Cell Rep*. 2019;26(3):608-623.e6. doi:10.1016/J.CELREP.2018.12.090

99. Duarte Campos DF, Bonnin Marquez A, O'Seanain C, et al. Exploring Cancer Cell Behavior In Vitro in Three-Dimensional Multicellular Bioprintable Collagen-Based Hydrogels. *Cancers (Basel)*. 2019;11(2):180. doi:10.3390/cancers11020180

100. Wippold FJ, Perry A. Neuropathology for the neuroradiologist: rosettes and pseudorosettes. *AJNR Am J Neuroradiol*. 2006;27(3):488-492.

101. Jin H, Varner J. Integrins: roles in cancer development and as treatment targets. *Br J Cancer*. 2004;90(3):561-565. doi:10.1038/sj.bjc.6601576

102. Hamidi H, Ivaska J. Every step of the way: integrins in cancer progression and metastasis. doi:10.1038/s41568-018-0038-z

103. Wei SC, Yang J. Forcing through Tumor Metastasis: The Interplay between Tissue Rigidity and Epithelial-Mesenchymal Transition. *Trends Cell Biol*. 2016;26(2):111-120. doi:10.1016/j.tcb.2015.09.009

104. Huang S, Ingber DE. Cell tension, matrix mechanics, and cancer development. *Cancer Cell*. 2005;8(3):175-176. doi:10.1016/J.CCR.2005.08.009

105. Jang I, Beningo KA, Jang I, Beningo KA. Integrins, CAFs and Mechanical Forces in the Progression of Cancer. *Cancers (Basel)*. 2019;11(5):721. doi:10.3390/cancers11050721

106. Elosegui-Artola A, Bazellières E, Allen MD, et al. Rigidity sensing and adaptation through regulation of integrin types. *Nat Mater*. 2014;13(6):631-637. doi:10.1038/nmat3960

107. Pang M, Teng Y, Huang J, Yuan Y, Lin F, Xiong C. Substrate stiffness promotes latent TGF-β1 activation in hepatocellular carcinoma. *Biochem Biophys Res Commun*. 2017;483(1):553-558. doi:10.1016/j.bbrc.2016.12.107

108. Clevers H. Modeling Development and Disease with Organoids. *Cell*. 2016;165(7):1586-1597. doi:10.1016/j.cell.2016.05.082

109. Rossi G, Manfrin A, Lutolf MP. Progress and potential in organoid research. *Nat Rev Genet*. 2018;19(11):671-687. doi:10.1038/s41576-018-0051-9

110. Fatehullah A, Tan SH, Barker N. Organoids as an in vitro model of human development and disease. *Nat Cell Biol*. 2016;18(3):246-254. doi:10.1038/ncb3312

111. Sachs N, Clevers H. Organoid cultures for the analysis of cancer phenotypes. *Curr Opin Genet Dev*. 2014;24(1):68-73. doi:10.1016/j.gde.2013.11.012

112. Chan AS, Yan HHN, Leung SY. Breakthrough Moments: Organoid Models of Cancer. *Cell Stem Cell*. 2019;24(6):839-840. doi:10.1016/j.stem.2019.05.006

113. Bleijs M, Wetering M, Clevers H, Drost J. Xenograft and organoid model systems in cancer research. *EMBO J*. 2019;38(15):1-11. doi:10.15252/embj.2019101654

114. Drost J, Clevers H. Organoids in cancer research. *Nat Rev Cancer*. 2018;18(7):407-418. doi:10.1038/s41568-018-0007-6

115. Drost J, Van Jaarsveld RH, Ponsioen B, et al. Sequential cancer mutations in cultured human intestinal stem cells. *Nature*. 2015;521(7550):43-47. doi:10.1038/nature14415

116. Duarte AA, Gogola E, Sachs N, et al. BRCA-deficient mouse mammary tumor organoids to study cancer-drug resistance. *Nat Methods*. 2018;15(2):134-140. doi:10.1038/nmeth.4535

117. Astashkina A, Grainger DW. Critical analysis of 3-D organoid in vitro cell culture models for high-throughput drug candidate toxicity assessments. *Adv Drug Deliv Rev*. 2014;69-70:1-18. doi:10.1016/j.addr.2014.02.008

118. Sun L, Wang Y, Cen J, et al. Modelling liver cancer initiation with organoids derived from directly reprogrammed human hepatocytes. *Nat Cell Biol*. 2019;21(August). doi:10.1038/s41556-019-0359-5

119. Hacker BC, Gomez JD, Batista CAS, Rafat M. Growth and Characterization of Irradiated Organoids from Mammary Glands. *Journal of Visualized Experiments*. 2019;(147). doi:10.3791/59293

120. Tsai S, McOlash L, Palen K, et al. Development of primary human pancreatic cancer organoids, matched stromal and immune cells and 3D tumor microenvironment models. *BMC Cancer*. 2018;18(1):335. doi:10.1186/s12885-018-4238-4

121. Wolf MT, Ganguly S, Wang TL, et al. A biologic scaffold–associated type 2 immune microenvironment inhibits tumor formation and synergizes with checkpoint immunotherapy. *Sci Transl Med*. 2019;11(477):eaat7973. doi:10.1126/SCITRANSLMED.AAT7973

122. Zhu Y, Hideyoshi S, Jiang H, et al. Injectable, porous, biohybrid hydrogels incorporating decellularized tissue components for soft tissue applications. *Acta Biomater*. 2018;73:112-126. doi:10.1016/j.actbio.2018.04.003

123. Alves SM, Zhu T, Shostak A, Rossen NS, Rafat M. Studying Normal Tissue Radiation Effects using Extracellular Matrix Hydrogels. *Journal of Visualized Experiments*. 2019;(149):e59304. doi:10.3791/59304

124. Pinto ML, Rios E, Silva AC, et al. Decellularized human colorectal cancer matrices polarize macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18. *Biomaterials*. 2017;124:211-224. doi:10.1016/J.BIOMATERIALS.2017.02.004

125. Piccoli M, D'Angelo E, Crotti S, et al. Decellularized colorectal cancer matrix as bioactive microenvironment for in vitro 3D cancer research. *J Cell Physiol*. 2018;233(8):5937-5948. doi:10.1002/jcp.26403

126. Jin Q, Liu G, Li S, et al. Decellularized breast matrix as bioactive microenvironment for in vitro three-dimensional cancer culture. *J Cell Physiol*. 2019;234(4):3425-3435. doi:10.1002/jcp.26782

127. Liu G, Wang B, Li S, Jin Q, Dai Y. Human breast cancer decellularized scaffolds promote epithelial-to-mesenchymal transitions and stemness of breast cancer cells in vitro. *J Cell Physiol*. 2019;234(6):9447-9456. doi:10.1002/jcp.27630

128. Mollica PA, Booth-Creech EN, Reid JA, et al. 3D bioprinted mammary organoids and tumoroids in human mammary derived ECM hydrogels. *Acta Biomater*. Published online June 21, 2019. doi:10.1016/j.actbio.2019.06.017

129. Hoshiba T, Tanaka M. Breast cancer cell behaviors on staged tumorigenesis-mimicking matrices derived from tumor cells at various malignant stages. *Biochem Biophys Res Commun*. 2013;439(2):291-296. doi:10.1016/j.bbrc.2013.08.038

130. Ma X, Yu C, Wang P, et al. Rapid 3D bioprinting of decellularized extracellular matrix with regionally varied mechanical properties and biomimetic microarchitecture. *Biomaterials*. 2018;185:310-321. doi:10.1016/j.biomaterials.2018.09.026

131. Hoshiba T, Tanaka M. Decellularized matrices as in vitro models of extracellular matrix in tumor tissues at different malignant levels: Mechanism of 5-fluorouracil resistance in colorectal tumor cells. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2016;1863(11):2749-2757. doi:10.1016/j.bbamcr.2016.08.009

132. Hoshiba T. Decellularized extracellular matrix for cancer research. *Materials*. 2019;12(8):1-16. doi:10.3390/ma12081311

133. Koh I, Cha J, Park J, Choi J, Kang SG, Kim P. The mode and dynamics of glioblastoma cell invasion into a decellularized tissue-derived extracellular matrix-based three-dimensional tumor model. *Sci Rep*. 2018;8(1):4608. doi:10.1038/s41598-018-22681-3

134. Romero-López M, Trinh AL, Sobrino A, et al. Recapitulating the human tumor microenvironment: Colon tumor-derived extracellular matrix promotes angiogenesis and tumor cell growth. *Biomaterials*. 2017;116:118-129. doi:10.1016/J.BIOMATERIALS.2016.11.034

135. Lü WD, Sun RF, Hu YR, et al. Photooxidatively crosslinked acellular tumor extracellular matrices as potential tumor engineering scaffolds. *Acta Biomater*. 2018;71. doi:10.1016/j.actbio.2018.03.020

136. Florczyk SJ, Kievit FM, Wang K, Erickson AE, Ellenbogen RG, Zhang M. 3D porous chitosan–alginate scaffolds promote proliferation and enrichment of cancer stem-like cells. *J Mater Chem B*. 2016;4(38):6326-6334. doi:10.1039/C6TB01713D

137. Xu K, Ganapathy K, Andl T, et al. 3D porous chitosan-alginate scaffold stiffness promotes differential responses in prostate cancer cell lines. *Biomaterials*. 2019;217. doi:10.1016/j.biomaterials.2019.119311

138. Cavo M, Fato M, Peñuela L, Beltrame F, Raiteri R, Scaglione S. Microenvironment complexity and matrix stiffness regulate breast cancer cell activity in a 3D in vitro model. *Sci Rep*. 2016;6(1):35367. doi:10.1038/srep35367

139. Diao J, Zhang C, Zhang D, et al. Role and mechanisms of a three-dimensional bioprinted microtissue model in promoting proliferation and invasion of growth-hormone-secreting pituitary adenoma cells. *Biofabrication*. 2019;11(2):025006. doi:10.1088/1758-5090/aaf7ea

140. Mondal A, Gebeyehu A, Subramanian R, Rishi A, Singh M. Abstract 5018: Bioprinted (3D) co-cultured spheroids with NSCLC PDX cells and cancer associated fibroblasts (CAFs) using alginate/gelatin hydrogel. In: *Tumor Biology*. Vol 78. American Association for Cancer Research; 2018:5018-5018. doi:10.1158/1538-7445.AM2018-5018

141. Song H, Yang P, Huang P, Zhang C, Kong D, Wang W. Injectable polypeptide hydrogel-based co-delivery of vaccine and immune checkpoint inhibitors improves tumor immunotherapy. *Theranostics*. 2019;9(8):2299-2314. doi:10.7150/thno.30577

142. Rianna C, Radmacher M. Influence of microenvironment topography and stiffness on the mechanics and motility of normal and cancer renal cells †. *Nanoscale*. 2017;9. doi:10.1039/c7nr02940c

143. Rianna C, Radmacher M. Comparison of viscoelastic properties of cancer and normal thyroid cells on different stiffness substrates. *European Biophysics Journal*. 2017;46(4):309-324. doi:10.1007/s00249-016-1168-4

144. Medina SH, Bush B, Cam M, et al. Identification of a mechanogenetic link between substrate stiffness and chemotherapeutic response in breast cancer. *Biomaterials*. 2019;202:1-11. doi:10.1016/j.biomaterials.2019.02.018

145. Hu WW, Lin CH, Hong ZJ. The enrichment of cancer stem cells using composite alginate/polycaprolactone nanofibers. *Carbohydr Polym*. 2019;206:70-79. doi:10.1016/J.CARBPOL.2018.10.087

146. Huch M, Koo BK. Modeling mouse and human development using organoid cultures. *Development*. 2015;142(18):3113-3125. doi:10.1242/dev.118570

147. Neal JT, Li X, Zhu J, et al. Organoid Modeling of the Tumor Immune Microenvironment. *Cell*. 2018;175(7):1972-1988.e16. doi:10.1016/j.cell.2018.11.021

148. Ramamoorthy P, Thomas SM, Kaushik G, et al. Metastatic tumor-in-A-Dish, a novel multicellular organoid to study lung colonization and predict therapeutic response. *Cancer Res*. 2019;79(7):1681-1695. doi:10.1158/0008-5472.CAN-18-2602

149. Saito Y, Muramatsu T, Kanai Y, et al. Establishment of Patient-Derived Organoids and Drug Screening for Biliary Tract Carcinoma. *Cell Rep*. 2019;27(4):1265-1276.e4. doi:10.1016/j.celrep.2019.03.088

150. Tian YF, Ahn H, Schneider RS, et al. Integrin-specific hydrogels as adaptable tumor organoids for malignant B and T cells. *Biomaterials*. 2015;73:110-119. doi:10.1016/j.biomaterials.2015.09.007

151. Costa EC, Moreira AF, de Melo-Diogo D, Gaspar VM, Carvalho MP, Correia IJ. 3D tumor spheroids: an overview on the tools and techniques used for their analysis. *Biotechnol Adv*. 2016;34(8):1427-1441. doi:10.1016/j.biotechadv.2016.11.002

152. Pradhan S, Clary JM, Seliktar D, Lipke EA. A three-dimensional spheroidal cancer model based on PEG-fibrinogen hydrogel microspheres. *Biomaterials*. 2017;115:141-154. doi:10.1016/J.BIOMATERIALS.2016.10.052

153. Axpe E, Oyen M. Applications of Alginate-Based Bioinks in 3D Bioprinting. *Int J Mol Sci*. 2016;17(12):1976. doi:10.3390/ijms17121976

154. Wang X, Zhang X, Dai X, et al. Tumor-like lung cancer model based on 3D bioprinting. *3 Biotech*. 2018;8(12):501. doi:10.1007/s13205-018-1519-1

155. Gopinathan J, Noh I. Recent trends in bioinks for 3D printing. *Biomater Res*. 2018;22:11. doi:10.1186/s40824-018-0122-1

156. Swaminathan S, Hamid Q, Sun W, Clyne AM. Bioprinting of 3D breast epithelial spheroids for human cancer models. *Biofabrication*. 2019;11(2):025003. doi:10.1088/1758-5090/aafc49

157. Saldin LT, Cramer MC, Velankar SS, White LJ, Badylak SF. Extracellular matrix hydrogels from decellularized tissues: Structure and function. *Acta Biomater*. 2017;49:1-15. doi:10.1016/j.actbio.2016.11.068

158. Hinderer S, Layland SL, Schenke-Layland K. ECM and ECM-like materials - Biomaterials for applications in regenerative medicine and cancer therapy. *Adv Drug Deliv Rev*. 2016;97:260-269. doi:10.1016/j.addr.2015.11.019

159. Beachley VZ, Wolf MT, Sadtler K, et al. Tissue matrix arrays for high-throughput screening and systems analysis of cell function. *Nat Methods*. 2015;12(12):1197-1204. doi:10.1038/nmeth.3619

160. Wolf MT, Daly KA, Brennan-Pierce EP, et al. A hydrogel derived from decellularized dermal extracellular matrix. *Biomaterials*. 2012;33(29):7028-7038. doi:10.1016/j.biomaterials.2012.06.051

161. Kleinman HK, Martin GR. Matrigel: Basement membrane matrix with biological activity. *Semin Cancer Biol*. 2005;15(5 SPEC. ISS.):378-386. doi:10.1016/j.semcancer.2005.05.004

162. Wisdom KM, Adebowale K, Chang J, et al. Matrix mechanical plasticity regulates cancer cell migration through confining microenvironments. *Nat Commun*. 2018;9(1):4144. doi:10.1038/s41467-018-06641-z

163. Lee JY, Chang J, Dominguez AA, et al. YAP-independent mechanotransduction drives breast cancer progression. *bioRxiv*. 2018;(2019):495499. doi:10.1101/495499

164. Kuen Yong Lee, David J. Mooney. Alginate: Properties and Biomedical Applications. *Prog Polym Sci*. 2000;37(1):106-126. doi:10.1016/j.progpolymsci.2011.06.003.Alginate

165. Read GH, Miura N, Carter JL, et al. Three-dimensional alginate hydrogels for radiobiological and metabolic studies of cancer cells. *Colloids Surf B Biointerfaces*. 2018;171(April):197-204. doi:10.1016/j.colsurfb.2018.06.018

166. Mei E, Li S, Song J, Xing R, Li Z, Yan X. Self-assembling Collagen/Alginate hybrid hydrogels for combinatorial photothermal and immuno tumor therapy. *Colloids Surf A Physicochem Eng Asp*. 2019;577(June):570-575. doi:10.1016/j.colsurfa.2019.06.023

167. Brabletz T, Kalluri R, Nieto MA, Weinberg RA. EMT in cancer. *Nat Rev Cancer*. 2018;18(2):128-134. doi:10.1038/nrc.2017.118

168. Dai J, Qin L, Chen Y, et al. Matrix stiffness regulates epithelial-mesenchymal transition via cytoskeletal remodeling and MRTF-A translocation in osteosarcoma cells. *J Mech Behav Biomed Mater*. 2019;90(October 2018):226-238. doi:10.1016/j.jmbbm.2018.10.012

169. Rice AJ, Cortes E, Lachowski D, et al. Matrix stiffness induces epithelial–mesenchymal transition and promotes chemoresistance in pancreatic cancer cells. *Oncogenesis*. 2017;6(7):e352-e352. doi:10.1038/oncsis.2017.54

170. Suresh S. Biomechanics and biophysics of cancer cells. *Acta Mater*. 2007;55(12):3989-4014. doi:10.1016/J.ACTAMAT.2007.04.022

171. Migonney V. History of Biomaterials. *Biomaterials*. Published online 2014:1-10. doi:10.1002/9781119043553.ch1

172. Hwang JW, Noh SM, Kim B, Jung HW. Gelation and crosslinking characteristics of photopolymerized poly(ethylene glycol) hydrogels. *J Appl Polym Sci*. 2015;132(22):1-6. doi:10.1002/app.41939

173. Smithmyer ME, Spohn JB, Kloxin AM. Probing Fibroblast Activation in Response to Extracellular Cues with Whole Protein- or Peptide-Functionalized Step-Growth Hydrogels. *ACS Biomater Sci Eng*. 2018;4(9):3304-3316. doi:10.1021/acsbiomaterials.8b00491

174. Sawicki LA, Kloxin AM, Ross KE, et al. Tunable synthetic extracellular matrices to investigate breast cancer response to biophysical and biochemical cues. *APL Bioeng*. 2019;3(1):016101. doi:10.1063/1.5064596

175. Macdougall LJ, Wiley KL, Kloxin AM, Dove AP. Design of synthetic extracellular matrices for probing breast cancer cell growth using robust cyctocompatible nucleophilic thiol-yne addition chemistry. *Biomaterials*. 2018;178:435-447. doi:10.1016/j.biomaterials.2018.04.046

176. Yom-Tov O, Seliktar D, Bianco-Peled H. PEG-Thiol based hydrogels with controllable properties. *Eur Polym J*. 2016;74:1-12. doi:10.1016/j.eurpolymj.2015.11.002

177. Gill BJ, Gibbons DL, Roudsari LC, et al. A synthetic matrix with independently tunable biochemistry and mechanical properties to study epithelial morphogenesis and EMT in a lung adenocarcinoma model. *Cancer Res*. 2012;72(22):6013-6023. doi:10.1158/0008-5472.CAN-12-0895

178. Tlsty TD, Coussens LM. Tumor Stroma and Regulation of Cancer Development. *Annual Review of Pathology: Mechanisms of Disease*. 2006;1(1):119-150. doi:10.1146/annurev.pathol.1.110304.100224

179. Chaudhuri PK, Low BC, Lim CT. Mechanobiology of Tumor Growth. *Chem Rev*. 2018;118(14):6499-6515. doi:10.1021/acs.chemrev.8b00042

180. Cretu A, Castagnino P, Assoian R. Studying the Effects of Matrix Stiffness on Cellular Function using Acrylamide-based Hydrogels. *Journal of Visualized Experiments*. 2010;(42):e2089. doi:10.3791/2089

181. Zamani E, Shokrzadeh M, Fallah M, Shaki F. A review of acrylamide toxicity and its mechanism. *Pharmaceutical and Biomedical Research*. 2017;3(1):1-7. doi:10.18869/acadpub.pbr.3.1.1

182. Kuo CHR, Xian J, Brenton JD, Franze K, Sivaniah E. Complex Stiffness Gradient Substrates for Studying Mechanotactic Cell Migration. *Advanced Materials*. 2012;24(45):6059-6064. doi:10.1002/adma.201202520

183. Hadden WJ, Young JL, Holle AW, et al. Stem cell migration and mechanotransduction on linear stiffness gradient hydrogels. *Proc Natl Acad Sci U S A*. 2017;114(22):5647-5652. doi:10.1073/pnas.1618239114

184. Tian B, Luo Q, Ju Y, Song G. A Soft Matrix Enhances the Cancer Stem Cell Phenotype of HCC Cells. *Int J Mol Sci*. 2019;20(11). doi:10.3390/ijms20112831

185. Lemma ED, Sergio S, Spagnolo B, et al. Tunable mechanical properties of stent-like microscaffolds for studying cancer cell recognition of stiffness gradients. *Microelectron Eng*. 2018;190:11-18. doi:10.1016/j.mee.2018.01.007

186. Page JM, Merkel AR, Ruppender NS, et al. Matrix rigidity regulates the transition of tumor cells to a bone-destructive phenotype through integrin β3 and TGF-β receptor type II. *Biomaterials*. 2015;64:33-44. doi:10.1016/j.biomaterials.2015.06.026

187. Kim J, Bae JS. Tumor-associated macrophages and neutrophils in tumor microenvironment. *Mediators Inflamm*. 2016;2016. doi:10.1155/2016/6058147

188. Monteran L, Erez N. The Dark Side of Fibroblasts: Cancer-Associated Fibroblasts as Mediators of Immunosuppression in the Tumor Microenvironment. *Front Immunol*. 2019;10(August):1-15. doi:10.3389/fimmu.2019.01835

189. Rafat M, Aguilera TA, Vilalta M, et al. Macrophages promote circulating tumor cell-mediated local recurrence following radiotherapy in immunosuppressed patients. *Cancer Res*. 2018;78(15):4241-4252. doi:10.1158/0008-5472.CAN-17-3623

190. Xuan W, Qu Q, Zheng B, Xiong S, Fan GH. The chemotaxis of M1 and M2 macrophages is regulated by different chemokines. *J Leukoc Biol*. 2015;97(1):61-69. doi:10.1189/jlb.1a0314-170r

191. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: From tumor initiation to metastatic progression. *Genes Dev*. 2018;32(19-20):1267-1284. doi:10.1101/GAD.314617.118

192. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol*. 2017;14(7):399-416. doi:10.1038/nrclinonc.2016.217

193. Sadtler K, Sommerfeld SD, Wolf MT, et al. Proteomic composition and immunomodulatory properties of urinary bladder matrix scaffolds in homeostasis and injury. *Semin Immunol*. 2017;29:14-23. doi:10.1016/j.smim.2017.05.002

194. Keane TJ, Dziki J, Sobieski E, et al. Restoring Mucosal Barrier Function and Modifying Macrophage Phenotype with an Extracellular Matrix Hydrogel: Potential Therapy for Ulcerative Colitis. *J Crohns Colitis*. 2017;11(3):360-368. doi:10.1093/ecco-jcc/jjw149

195. Meng F, Meyer CM, Joung D, Vallera DA, McAlpine MC, Panoskaltsis-Mortari A. 3D Bioprinted In Vitro Metastatic Models via Reconstruction of Tumor Microenvironments. *Advanced Materials*. 2019;31(10):1806899. doi:10.1002/adma.201806899

196. Eskiizmir G, Baskın Y, Yapıcı K. Graphene-based nanomaterials in cancer treatment and diagnosis. *Fullerens, Graphenes and Nanotubes*. Published online January 1, 2018:331-374. doi:10.1016/B978-0-12-813691-1.00009-9

197. Manmohan Singh, Salnikova M. *Novel Approaches and Strategies for Biologics, Vaccines and Cancer Therapies*.; 2015.

198. Huang X, Williams JZ, Chang R, et al. DNA-scaffolded biomaterials enable modular and tunable control of cell-based cancer immunotherapies. *bioRxiv*. Published online March 23, 2019:587105. doi:10.1101/587105

199. Lei K, Tang L. Surgery-free injectable macroscale biomaterials for local cancer immunotherapy. *Biomater Sci*. 2019;7(3):733-749. doi:10.1039/C8BM01470A

200. Ishii S, Kaneko J, Nagasaki Y. Development of a long-acting, protein-loaded, redox-active, injectable gel formed by a polyion complex for local protein

therapeutics. *Biomaterials*. 2016;84:210-218. doi:10.1016/j.biomaterials.2016.01.029

201. Yu S, Wang C, Yu J, et al. Injectable Bioresponsive Gel Depot for Enhanced Immune Checkpoint Blockade. *Advanced Materials*. 2018;30(28):1801527. doi:10.1002/adma.201801527
202. Xu K, Lee F, Gao SJ, Chung JE, Yano H, Kurisawa M. Injectable hyaluronic acid-tyramine hydrogels incorporating interferon-α2a for liver cancer therapy. *Journal of Controlled Release*. 2013;166(3):203-210. doi:10.1016/J.JCONREL.2013.01.008
203. Lee J, Le QV, Yang G, Oh YK. Cas9-edited immune checkpoint blockade PD-1 DNA polyaptamer hydrogel for cancer immunotherapy. *Biomaterials*. 2019;218:119359. doi:10.1016/j.biomaterials.2019.119359
204. Jin H, Zhu T, Huang X, et al. ROS-responsive nanoparticles based on amphiphilic hyperbranched polyphosphoester for drug delivery: Light-triggered size-reducing and enhanced tumor penetration. *Biomaterials*. 2019;211:68-80. doi:10.1016/J.BIOMATERIALS.2019.04.029
205. Jin J, Krishnamachary B, Barnett JD, et al. Human Cancer Cell Membrane-Coated Biomimetic Nanoparticles Reduce Fibroblast-Mediated Invasion and Metastasis and Induce T-Cells. *ACS Appl Mater Interfaces*. 2019;11(8):7850-7861. doi:10.1021/acsami.8b22309
206. Kroll A V., Jiang Y, Zhou J, Holay M, Fang RH, Zhang L. Biomimetic Nanoparticle Vaccines for Cancer Therapy. *Adv Biosyst*. 2019;3(1):1800219. doi:10.1002/adbi.201800219

207. Sun H, Su J, Meng Q, et al. Cancer-Cell-Biomimetic Nanoparticles for Targeted Therapy of Homotypic Tumors. *Advanced Materials*. 2016;28(43):9581-9588. doi:10.1002/adma.201602173

208. Chen Z, Zhao P, Luo Z, et al. Cancer Cell Membrane–Biomimetic Nanoparticles for Homologous-Targeting Dual-Modal Imaging and Photothermal Therapy. *ACS Nano*. 2016;10(11):10049-10057. doi:10.1021/acsnano.6b04695

209. Zhang Z, Qian H, Yang M, et al. Gambogic acid-loaded biomimetic nanoparticles in colorectal cancer treatment. *Int J Nanomedicine*. 2017;12:1593-1605. doi:10.2147/IJN.S127256

210. Tian H, Luo Z, Liu L, et al. Cancer Cell Membrane-Biomimetic Oxygen Nanocarrier for Breaking Hypoxia-Induced Chemoresistance. *Adv Funct Mater*. 2017;27(38):1703197. doi:10.1002/adfm.201703197

211. Yu W, He X, Yang Z, et al. Sequentially responsive biomimetic nanoparticles with optimal size in combination with checkpoint blockade for cascade synergetic treatment of breast cancer and lung metastasis. *Biomaterials*. 2019;217:119309. doi:10.1016/J.BIOMATERIALS.2019.119309

212. Díaz-Saldívar P, Huidobro-Toro JP. ATP-loaded biomimetic nanoparticles as controlled release system for extracellular drugs in cancer applications. *Int J Nanomedicine*. 2019;14:2433-2447. doi:10.2147/IJN.S192925

213. Lai J, Deng G, Sun Z, et al. Scaffolds biomimicking macrophages for a glioblastoma NIR-Ib imaging guided photothermal therapeutic strategy by crossing Blood-Brain Barrier. *Biomaterials*. 2019;211(February):48-56. doi:10.1016/j.biomaterials.2019.04.026

214. Mansour AA, Gonçalves JT, Bloyd CW, et al. An in vivo model of functional and vascularized human brain organoids. *Nat Biotechnol*. 2018;36(5):432-441. doi:10.1038/nbt.4127

215. Meinert C, Schrobback K, Hutmacher DW, Klein TJ. A novel bioreactor system for biaxial mechanical loading enhances the properties of tissue-engineered human cartilage. *Sci Rep*. 2017;7(1):1-14. doi:10.1038/s41598-017-16523-x

216. Novak CM, Horst EN, Taylor CC, Liu CZ, Mehta G. Fluid shear stress stimulates breast cancer cells to display invasive and chemoresistant phenotypes while upregulating PLAU in a 3D bioreactor . *Biotechnol Bioeng*. Published online 2019. doi:10.1002/bit.27119

217. Guller AE, Grebenyuk PN, Shekhter AB, Zvyagin A V., Deyev SM. Bioreactor-based tumor tissue engineering. *Acta Naturae*. 2016;8(3):44-58.

218. Buenrostro D, Park SI, Sterling JA. Dissecting the role of bone marrow stromal cells on bone metastases. *Biomed Res Int*. 2014;2014. doi:10.1155/2014/875305

219. Stowers RS, Shcherbina A, Israeli J, et al. Matrix stiffness induces a tumorigenic phenotype in mammary epithelium through changes in chromatin accessibility. *Nat Biomed Eng*. 2019;3(12):1009-1019. doi:10.1038/s41551-019-0420-5

220. Northcutt LA, Suarez-Arnedo A, Rafat M. Emerging Biomimetic Materials for Studying Tumor and Immune Cell Behavior. *Ann Biomed Eng*. 2019;48(7):2064-2077. doi:10.1007/s10439-019-02384-0

221. Rosol TJ, Tannehill-Gregg SH, LeRoy BE, Mandl S, Contag CH. Animal models of bone metastasis. *Cancer*. 2003;97(S3):748-757. doi:10.1002/cncr.11150

222. Blouin S, Baslé MF, Chappard D. Interactions between microenvironment and cancer cells in two animal models of bone metastasis. *Br J Cancer*. 2008;98(4):809-815. doi:10.1038/sj.bjc.6604238

223. Vanderburgh JP, Kwakwa KA, Werfel TA, et al. Systemic delivery of a Gli inhibitor via polymeric nanocarriers inhibits tumor-induced bone disease. *Journal of Controlled Release*. 2019;311-312(May):257-272. doi:10.1016/j.jconrel.2019.08.038

224. Dhurjati R, Krishnan V, Shuman LA, Mastro AM, Vogler EA. Metastatic breast cancer cells colonize and degrade three-dimensional osteoblastic tissue in vitro. *Clin Exp Metastasis*. 2008;25(7):741-752. doi:10.1007/s10585-008-9185-z

225. Bersini S, Jeon JS, Dubini G, et al. A microfluidic 3D invitro model for specificity of breast cancer metastasis to bone. *Biomaterials*. 2014;35(8):2454-2461. doi:10.1016/j.biomaterials.2013.11.050

226. Chaudhuri O, Koshy ST, Branco Da Cunha C, et al. Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium. *Nat Mater*. 2014;13(10):970-978. doi:10.1038/nmat4009

227. Xiao Y, McGuinness CAS, Doherty-Boyd WS, Salmeron-Sanchez M, Donnelly H, Dalby MJ. Current insights into the bone marrow niche: From biology in vivo to bioengineering ex vivo. *Biomaterials*. 2022;286(May):121568. doi:10.1016/j.biomaterials.2022.121568

228. Baruffaldi D, Palmara G, Pirri C, Frascella F. 3D Cell Culture: Recent Development in Materials with Tunable Stiffness. *ACS Appl Bio Mater*. 2021;4(3):2233-2250. doi:10.1021/acsabm.0c01472

229. Jansen LE, Kim H, Hall CL, McCarthy TP, Lee MJ, Peyton SR. A poly(ethylene glycol) three-dimensional bone marrow hydrogel. *Biomaterials*. 2022;280(November 2021):121270. doi:10.1016/j.biomaterials.2021.121270

230. Riaz N, Wolden SL, Gelblum DY, Eric J. Degradation-mediated cellular traction directs stem cell fate in covalently. 2016;118(24):6072-6078. doi:10.1038/nmat3586.Degradation-mediated

231. Zonderland J, Moroni L. Steering cell behavior through mechanobiology in 3D: A regenerative medicine perspective. *Biomaterials*. 2021;268:120572. doi:10.1016/j.biomaterials.2020.120572

232. Caliari SR, Burdick JA. A practical guide to hydrogels for cell culture. *Nat Methods*. 2016;13(5):405-414. doi:10.1038/nmeth.3839

233. Neves MI, Moroni L, Barrias CC. Modulating Alginate Hydrogels for Improved Biological Performance as Cellular 3D Microenvironments. *Front Bioeng Biotechnol*. 2020;8(June). doi:10.3389/fbioe.2020.00665

234. Cao H, Duan L, Zhang Y, Cao J, Zhang K. Current hydrogel advances in physicochemical and biological response-driven biomedical application diversity. *Signal Transduct Target Ther*. 2021;6(1):1-31. doi:10.1038/s41392-021-00830-x

235. Chaudhuri O, Koshy ST, Branco Da Cunha C, et al. Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium. *Nat Mater*. 2014;13(10):970-978. doi:10.1038/nmat4009

236. Cavo M, Caria M, Pulsoni I, Beltrame F, Fato M, Scaglione S. A new cell-laden 3D Alginate-Matrigel hydrogel resembles human breast cancer cell malignant

morphology, spread and invasion capability observed “in vivo.” *Sci Rep*. 2018;8(1):1-12. doi:10.1038/s41598-018-23250-4

237. Krause S, Maffini M V., Soto AM, Sonnenschein C. The microenvironment determines the breast cancer cells’ phenotype: Organization of MCF7 cells in 3D cultures. *BMC Cancer*. 2010;10. doi:10.1186/1471-2407-10-263
238. Perrin L, Belova E, Bayarmagnai B, Tüzel E, Gligorijevic B. Invadopodia enable cooperative invasion and metastasis of breast cancer cells. *Commun Biol*. 2022;5(1):758. doi:10.1038/s42003-022-03642-z
239. Li L, Lu Y. Optimizing a 3D culture system to study the interaction between epithelial breast cancer and its surrounding fibroblasts. *J Cancer*. 2011;2(1):458-466. doi:10.7150/jca.2.458
240. Anderson ARA, Weaver AM, Cummings PT, Quaranta V. Tumor Morphology and Phenotypic Evolution Driven by Selective Pressure from the Microenvironment. *Cell*. 2006;127(5):905-915. doi:10.1016/j.cell.2006.09.042
241. Boskey AL. Bone composition: relationship to bone fragility and antiosteoporotic drug effects. *Bonekey Rep*. 2013;2(July):1-11. doi:10.1038/bonekey.2013.181
242. Yamada KM, Collins JW, Cruz Walma DA, et al. Extracellular matrix dynamics in cell migration, invasion and tissue morphogenesis. *Int J Exp Pathol*. 2019;100(3):144-152. doi:10.1111/iep.12329
243. Kalli M, Stylianopoulos T. Defining the role of solid stress and matrix stiffness in cancer cell proliferation and metastasis. *Front Oncol*. 2018;8(MAR). doi:10.3389/fonc.2018.00055

244. Cavo M, Fato M, Peñuela L, Beltrame F, Raiteri R, Scaglione S. Microenvironment complexity and matrix stiffness regulate breast cancer cell activity in a 3D in vitro model. *Sci Rep*. 2016;6(1):35367. doi:10.1038/srep35367

245. Dupont S, Morsut L, Aragona M, et al. Role of YAP/TAZ in mechanotransduction. *Nature*. 2011;474(7350):179-184. doi:10.1038/nature10137

246. Rice AJ, Cortes E, Lachowski D, et al. Matrix stiffness induces epithelial–mesenchymal transition and promotes chemoresistance in pancreatic cancer cells. *Oncogenesis*. 2017;6(7):e352-e352. doi:10.1038/oncsis.2017.54

247. Polacheck WJ, German AE, Mammoto A, Ingber DE, Kamm RD. Mechanotransduction of fluid stresses governs 3D cell migration. *Proc Natl Acad Sci U S A*. 2014;111(7):2447-2452. doi:10.1073/pnas.1316848111

248. Stowers RS, Shcherbina A, Israeli J, et al. Matrix stiffness induces a tumorigenic phenotype in mammary epithelium through changes in chromatin accessibility. *Nat Biomed Eng*. 2019;3(12):1009-1019. doi:10.1038/s41551-019-0420-5

249. Lou J, Stowers R, Nam S, Xia Y, Chaudhuri O. Stress relaxing hyaluronic acid-collagen hydrogels promote cell spreading, fiber remodeling, and focal adhesion formation in 3D cell culture. *Biomaterials*. 2018;154:213-222. doi:10.1016/j.biomaterials.2017.11.004

250. Page JM, Merkel AR, Ruppender NS, et al. Matrix rigidity regulates the transition of tumor cells to a bone-destructive phenotype through integrin β3 and TGF-β receptor type II. *Biomaterials*. 2015;64. doi:10.1016/j.biomaterials.2015.06.026

251. Watson AW, Grant AD, Parker SS, et al. Breast tumor stiffness instructs bone metastasis via maintenance of mechanical conditioning. *Cell Rep*. 2021;35(13). doi:10.1016/j.celrep.2021.109293

252. Hancock GR, Young KS, Hosfield DJ, et al. Unconventional isoquinoline-based SERMs elicit fulvestrant-like transcriptional programs in ER+ breast cancer cells. *NPJ Breast Cancer*. 2022;8(1). doi:10.1038/s41523-022-00497-9

253. Hanker AB, Sudhan DR, Arteaga CL. Overcoming Endocrine Resistance in Breast Cancer. *Cancer Cell*. 2020;37(4):496-513. doi:10.1016/j.ccell.2020.03.009

254. Lloyd MR, Wander SA, Hamilton E, Razavi P. Next-generation selective estrogen receptor degraders and other novel endocrine therapies for management of metastatic hormone receptor-positive breast cancer : current and emerging role. Published online 2022. doi:10.1177/17588359221113694

255. Johnston SRD. Endocrinology and hormone therapy in breast cancer Selective oestrogen receptor modulators and downregulators for breast cancer – have they lost their way ? Published online 2005:119-130. doi:10.1186/bcr1023

256. Bado I, Gugala Z, Fuqua SAW, Zhang XHF. Estrogen receptors in breast and bone: From virtue of remodeling to vileness of metastasis. *Oncogene*. 2017;36(32):4527-4537. doi:10.1038/onc.2017.94

257. Bado IL, Zhang W, Hu J, et al. The bone microenvironment increases phenotypic plasticity of ER+ breast cancer cells. *Dev Cell*. 2021;56(8):1100-1117.e9. doi:10.1016/j.devcel.2021.03.008

258. Clements ME, Johnson RW. PREX1 drives spontaneous bone dissemination of ER+ breast cancer cells. *Oncogene*. 2020;39(6):1318-1334. doi:10.1038/s41388-019-1064-3

259. McGregor NE, Murat M, Elango J, et al. IL-6 exhibits both cis- And trans-signaling in osteocytes and osteoblasts, but only trans-signaling promotes bone formation and osteoclastogenesis. *Journal of Biological Chemistry*. 2019;294(19):7850-7863. doi:10.1074/jbc.RA119.008074

260. Kim JH, Jin HM, Kim K, et al. The Mechanism of Osteoclast Differentiation Induced by IL-1. *The Journal of Immunology*. 2009;183(3):1862-1870. doi:10.4049/jimmunol.0803007

261. Harmer D, Falank C, Reagan MR. Interleukin-6 interweaves the bone marrow microenvironment, bone loss, and multiple myeloma. *Front Endocrinol (Lausanne)*. 2019;10(JAN). doi:10.3389/fendo.2018.00788

262. Chang PY, Wu HK, Chen YH, et al. Interleukin-6 transiently promotes proliferation of osteoclast precursors and stimulates the production of inflammatory mediators. *Mol Biol Rep*. 2022;49(5):3927-3937. doi:10.1007/s11033-022-07243-1

263. Rašková M, Lacina L, Kejík Z, et al. The Role of IL-6 in Cancer Cell Invasiveness and Metastasis—Overview and Therapeutic Opportunities. *Cells*. 2022;11(22). doi:10.3390/cells11223698

264. Nisar MA, Zheng Q, Saleem MZ, et al. IL-1β Promotes Vasculogenic Mimicry of Breast Cancer Cells Through p38/MAPK and PI3K/Akt Signaling Pathways. *Front Oncol*. 2021;11. doi:10.3389/fonc.2021.618839

265. Tulotta C, Lefley D V., Freeman K, et al. Endogenous production of IL1B by breast cancer cells drives metastasis and colonization of the bone microenvironment. *Clinical Cancer Research*. 2019;25(9):2769-2782. doi:10.1158/1078-0432.CCR-18-2202

266. Tulotta C, Ottewell P. The role of IL-1B in breast cancer bone metastasis. *Endocr Relat Cancer*. 2018;25(7):R421-R434. doi:10.1530/ERC-17-0309

267. Watari K, Shibata T, Kawahara A, et al. Tumor-derived interleukin-1 promotes lymphangiogenesis and lymph node metastasis through M2-type macrophages. *PLoS One*. 2014;9(6). doi:10.1371/journal.pone.0099568

268. Magidey-Klein K, Cooper TJ, Kveler K, et al. IL-6 contributes to metastatic switch via the differentiation of monocytic-dendritic progenitors into prometastatic immune cells. *J Immunother Cancer*. 2021;9(6). doi:10.1136/jitc-2021-002856

269. Siersbæk R, Scabia V, Nagarajan S, et al. IL6/STAT3 Signaling Hijacks Estrogen Receptor α Enhancers to Drive Breast Cancer Metastasis. *Cancer Cell*. 2020;38(3):412-423.e9. doi:10.1016/j.ccell.2020.06.007

270. McDonnell DP, Wardell SE, Chang CY, Norris JD. Next-Generation Endocrine Therapies for Breast Cancer. *Journal of Clinical Oncology*. 2021;39(12):1383-1388. doi:10.1200/JCO.20.03565

271. Gu G, Tian L, Herzog SK, et al. Hormonal modulation of ESR1 mutant metastasis. *Oncogene*. 2021;40(5):997-1011. doi:10.1038/s41388-020-01563-x

272. Xiao T, Li W, Wang X, et al. Estrogen-regulated feedback loop limits the efficacy of estrogen receptor–targeted breast cancer therapy. *Proc Natl Acad Sci U S A*. 2018;115(31):7869-7878. doi:10.1073/pnas.1722617115

273. Wang LH, Yang XY, Mihalic K, Xiao W, Li D, Farrar WL. Activation of Estrogen Receptor Blocks Interleukin-6-inducible Cell Growth of Human Multiple Myeloma Involving Molecular Cross-talk between Estrogen Receptor and STAT3 Mediated by Co-regulator PIAS3. *Journal of Biological Chemistry*. 2001;276(34):31839-31844. doi:10.1074/jbc.M105185200

274. Galien R, Garcia T. *Estrogen Receptor Impairs Interleukin-6 Expression by Preventing Protein Binding on the NF-KB Site*. Vol 25. Oxford University Press; 1997.

275. Stein B, Yang MX. Repression of the Interleukin-6 Promoter by Estrogen Receptor Is Mediated by NF-κB and C/EBPβ. *Mol Cell Biol*. 1995;15(9):4971-4979. doi:10.1128/mcb.15.9.4971

276. Nelson MR, Roy K. Bone-marrow mimicking biomaterial niches for studying hematopoietic stem and progenitor cells. *J Mater Chem B*. 2016;4(20):3490-3503. doi:10.1039/c5tb02644j

277. Feng X. Chemical and Biochemical Basis of Cell-Bone Matrix Interaction in Health and Disease. *Curr Chem Biol*. 2009;3(2):189-196. doi:10.2174/187231309788166398