The role of contact guidance on cancer metastasis

Jacob Allan Mebus Nuhn
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd
Part of the Chemical Engineering Commons

Recommended Citation
Mebus Nuhn, Jacob Allan, "The role of contact guidance on cancer metastasis" (2018). Graduate Theses and Dissertations. 17281.
https://lib.dr.iastate.edu/etd/17281
The role of contact guidance on cancer metastasis

by

Jacob Allan Mebus Nuhn

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

Program of Study Committee:
Ian Schneider, Major Professor
Kaitlin Bratlie
Surya Mallapragada
Maura McGrail
Long Que

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

Copyright © Jacob Allan Mebus Nuhn, 2018. All rights reserved.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTER 1: GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2 References</td>
<td>4</td>
</tr>
<tr>
<td>CHAPTER 2: LITERATURE REVIEW</td>
<td>8</td>
</tr>
<tr>
<td>2.1 What is Contact Guidance?</td>
<td>9</td>
</tr>
<tr>
<td>2.2 How Intracellular Properties Affect Contact Guidance</td>
<td>10</td>
</tr>
<tr>
<td>2.3 Role of Stromal Cells in Shaping the Contact Guidance Cue.</td>
<td>14</td>
</tr>
<tr>
<td>2.4 The Role of Contact Guidance in Tumor Progression and Metastasis.</td>
<td>15</td>
</tr>
<tr>
<td>2.5 The Role of Pancreatic Stellate Cells and Relationship with Pancreatic Cancer Cells</td>
<td>18</td>
</tr>
<tr>
<td>2.6 The Role of Hyaluronan in Tumor Progression and Metastasis</td>
<td>20</td>
</tr>
<tr>
<td>2.7 How Can You Model Contact Guidance Cues In Vitro</td>
<td>21</td>
</tr>
<tr>
<td>2.8 Conclusions</td>
<td>23</td>
</tr>
<tr>
<td>2.9 References</td>
<td>24</td>
</tr>
<tr>
<td>CHAPTER 3: CONTACT GUIDANCE DIVERSITY IN ROTATIONALLY ALIGNED COLLAGEN MATRICES</td>
<td>36</td>
</tr>
<tr>
<td>Abstract</td>
<td>36</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>37</td>
</tr>
<tr>
<td>3.2 Material and Methods</td>
<td>41</td>
</tr>
<tr>
<td>3.2.1 Culturing Cells</td>
<td>41</td>
</tr>
<tr>
<td>3.2.2 Sample Preparation for Confocal Microscopy</td>
<td>41</td>
</tr>
<tr>
<td>3.2.3 Confocal Microscopy and Analysis</td>
<td>42</td>
</tr>
<tr>
<td>3.2.4 Cell Migration Sample Preparation</td>
<td>43</td>
</tr>
<tr>
<td>3.2.5 Collagen Glycation Procedure</td>
<td>43</td>
</tr>
<tr>
<td>3.2.6 Contractility Inhibition</td>
<td>44</td>
</tr>
<tr>
<td>3.2.7 Cell Migration Analysis</td>
<td>44</td>
</tr>
<tr>
<td>3.2.8 Statistical Methods</td>
<td>45</td>
</tr>
</tbody>
</table>
3.3 Results 45
3.3.1 Rotation of Acupuncture Needles Align Collagen Fibers 45
3.3.2 MDA-MB-231 Cells Respond to 3D Contact Guidance Cues, but MTLn3 Cells Do Not 49
3.3.3 Role of Extracellular Stiffness and Intracellular Contractility in Driving 3D Contact Guidance 54
3.4 Discussion 58
3.5 Conclusions 63
3.6 Acknowledgements 64
3.7 References 64

CHAPTER 4: PANCERATIC CANCER AND STELLATE COMMUNICATION RESULTS IN ALTERED CONTACT GUIDANCE AND COLLAGEN FIBER ALIGNMENT 69

Abstract 69
4.1 Introduction 70
4.2 Material and Methods 74
4.2.1 Culturing Cells 74
4.2.2 Assembling 3D Collagen Structures 75
4.2.3 Assembling 2D Collagen Substrates 76
4.2.4 Assessing Collagen Alignment 76
4.2.5 Assessing Directed Cell Migration 78
4.2.6 MMP Activity and Inhibition Assays 81
4.2.7 Fluorescence Staining 83
4.3 Results 84
4.3.1 Rotation of Acupuncture Needles Align Collagen Fibers 84
4.3.2 Co-culturing PS and PC Cells Increases Speed While Knocking out the MUC4 gene affects PS Directionality 85
4.3.3 PS and PC Cells Increase the Alignment of the Fibers Except when MUC4 is Eliminated in PC Cells 90
4.3.4 MMP Activity Goes up in Co-cultured Conditioned Media and 3D9 Partially Inhibits MMP-14 Activity 92
4.4 Discussion 95
4.5 Conclusions 101
4.6 References 102
CHAPTER 5: INVESTIGATING THE ROLE OF HYALURONAN ON PANCREATIC STELLATE AND CANCER CELL MIGRATION

Abstract 109

5.1 Introduction 110
5.2 Materials and Methods 113
5.2.1 Culturing Cells 113
5.2.2 Assessing Directed Migration 114
5.2.3 Cell Extension Analysis 118
5.2.4 Statistical Methods 119
5.3 Results 119
5.4.1 Hyaluronan Results in Faster Cells with Similar Cell Morphology 119
5.3.2 Hyaluronan Results in More Directional Migration from Cancer Cells 121
5.3.3 Pancreatic Cancer Cells showed more Fan Extensions than PS cells 122
5.3.4 ECM Type had Little Effect on Extension Parameters 124
5.3.5 Lower Molecular Weight Hyaluronan has a Similar Effect on Migration 126
5.4 Discussion 128
5.5 Conclusions 131
5.6 References 131

CHAPTER 6: GENERAL CONCLUSIONS 136

CHAPTER 7: FUTURE DIRECTIONS AND IMPACT ON ONCOLOGY 141

7.1 Impact of my Work on the Cancer Field 141
7.2 Impact of my Work on Therapeutics 142
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Schematic of aligned collagen gels</td>
<td>46</td>
</tr>
<tr>
<td>3.2</td>
<td>Confocal reflectance microscopy images of the alignment of the collagen gel under different needle conditions</td>
<td>48</td>
</tr>
<tr>
<td>3.3</td>
<td>Confocal reflectance microscopy images of the alignment of the collagen gel under different numbers of rotations</td>
<td>49</td>
</tr>
<tr>
<td>3.4</td>
<td>Aspect ratios of MDA-MB-231 cancer cells</td>
<td>50</td>
</tr>
<tr>
<td>3.5</td>
<td>Cell migration analysis of MDA-MB-231 cells</td>
<td>52</td>
</tr>
<tr>
<td>3.6</td>
<td>Comparing contact guidance of MDA-MB-231 and MTLn3 cells</td>
<td>53</td>
</tr>
<tr>
<td>3.7</td>
<td>Comparing MDA-MB-231 cells and MTLn3 cells in glycated collagen matrices</td>
<td>55</td>
</tr>
<tr>
<td>3.8</td>
<td>Examining the effect of contractility inhibitors on MDA-MB-231 cells</td>
<td>57</td>
</tr>
<tr>
<td>3.9</td>
<td>Compilation of speed, directionality and aspect ratio across all conditions</td>
<td>58</td>
</tr>
<tr>
<td>4.1</td>
<td>Determining Alignment of Collagen Fibers</td>
<td>85</td>
</tr>
<tr>
<td>4.2</td>
<td>Comparing Contact Guidance of Pancreatic Stellate and Pancreatic Cancer Cells</td>
<td>87</td>
</tr>
<tr>
<td>4.3</td>
<td>Investigating the effect of Co-culturing Cells on Migration</td>
<td>88</td>
</tr>
<tr>
<td>4.4</td>
<td>Investigating the Role Mucin 4 has on PS and PC Cells</td>
<td>90</td>
</tr>
<tr>
<td>4.5</td>
<td>Analysis of Fiber Orientation after 24 hrs Containing Cells Using Second Harmonic Generation</td>
<td>91</td>
</tr>
<tr>
<td>4.6</td>
<td>MMPs Activity and Inhibition in PS and PC Cells</td>
<td>93</td>
</tr>
<tr>
<td>4.7</td>
<td>Investigating the role of MMP inhibition in Co-culture Contact Guidance Conditions</td>
<td>94</td>
</tr>
<tr>
<td>4.8</td>
<td>F-actin Fluorescence Levels in 3D Monoculture and Co-culture Aligned Conditions</td>
<td>95</td>
</tr>
<tr>
<td>5.1</td>
<td>Hyaluronan Effects Cellular Speed in Aligned Collagen:Hyaluronan Composite Gels</td>
<td>120</td>
</tr>
</tbody>
</table>
Figure 5.2: Hyaluronan Effects Cellular Directionality in Aligned Collagen:Hyaluronan Composite Gels ................................................................. 122

Figure 5.3: Examining How the Presence of Hyaluronan Effects Cellular Extensions Morphology in Aligned Gels ................................................................. 124

Figure 5.4: Examining How the Presence of Hyaluronan Effects Cellular Extension Dynamics in Aligned Gels ................................................................. 126

Figure 5.5: Changes in Migration Characteristics in Response to Lower Molecular Weight Hylauronan in Aligned Gels ........................................................................ 127
ACKNOWLEDGMENTS

First and foremost, I would like to thank Professor Dr. Ian Schneider for his support and guidance over the course of this project. Ian’s mentorship and invaluable insight have helped me grow both professionally and as a person. I will be eternally grateful. I would also like to thank the many students at Iowa State, IOWA, and Kansas with whom I have worked alongside. There are too many to mention individually but a special thanks is owed to the Schneider research group. I would also like to thank Iowa State and NIH for supporting my Ph.D. research project.

I would like to thank my friends who have supported me through each walk of life, my teachers who always believed in me academically and as a person, as well as the students and teachers at Perry High School who so graciously welcomed me into their classroom and reminded me not to lose focus on what is important.

Finally, I would like to thank my amazing family and wonderful fiancé. In particular, thank you to my mother, Robin Mebus, for guiding me as a person and showing me every day what it takes to find joy in life. Thank you to my brother, Charles Nuhn, for always being a light in my life and my best friend. Thank you to my sister, Rachel Nuhn, for always being willing to listen and reminding me what matters most in life. Thank you to my fiancé, Jenna Bellinder, who has shown me endless love and is willing to move across the country to help me achieve my dreams. I love you all. Without everyone’s endless encouragement and support, this dissertation would not be possible.

Thank you.
ABSTRACT

Cancer cell metastasis is responsible for approximately 90% of deaths related to cancer. The migration of cancer cells away from the primary tumor and into healthy tissue is driven in part by contact guidance, or directed migration in response to aligned extracellular matrix. While contact guidance has been a focus of many studies, much of this research has explored environments that present 2D contact guidance structures. Contact guidance environments in 3D more closely resemble *in vivo* conditions and model cell-ECM interactions better than 2D environments. We have developed a simple rotational alignment technique which allows for the study of cancer cells response to contact guidance in collagen fiber gels.

MDA-MB-231 (mesenchymal) and MTLn3 (amoeboid) cells. MDA-MB-231 cells migrate with high directional fidelity in aligned collagen gels, while MTLn3 cells show no directional migration. The collagen stiffness was increased through glycation, resulting in decreased MDA-MB-231 directionality in aligned collagen gels. Interestingly, partial inhibition of cell contractility dramatically decreased directionality in MDA-MB-231 cells. The directionality of MDA-MB-231 cells was most sensitive to ROCK inhibition, but unlike in 2D contact guidance environments, cell directionality and speed are more tightly coupled. Modulation of the contractile apparatus appears to more potently affect contact guidance than modulation of extracellular mechanical properties of the contact guidance cue.

Pancreatic cancer has one of the lowest 5 year survival rates (3-5%), which is, in part, due to poor diagnostics and treatment options. Recently, aligned fibers have been seen oriented away from the edge of the tumor within the pancreas. This suggests that contact guidance, directional migration in response to aligned ECM fibers, may be critical to the early stages of metastasis. Communication between pancreatic cancer (PC) and pancreatic stellate (PS) cells...
under contact guidance were chosen to study in the aligned fiber field. PS cells migrated directionally in monoculture, however when co-cultured the directionally vanished. PC cells never showed contact guidance. Mucin 4 is a large glycoprotein which is a known communication modulator overexpressed in pancreatic cancer. The MUC4 gene was knocked out of the PC cells (PC muc4 KO) which resulted a return of directionality to the PS cells but still did not produce contact guidance within the PC cells when co-cultured. Co-culturing PS and both PC cells always resulted in faster cell speeds. Second Harmonic Generation was used to investigate collage reorganization. PS and PC cells, when monocultured and co-cultured with each other, resulted in enhanced collagen fiber alignment. PC muc4 KO, when monocultured or co-cultured with PS cells, did not alter the alignment or disrupted it and returned it to an unaligned state. F-actin intensity and MMP activity were shown to be higher in co-cultured systems. When F-actin was inhibited in co-culture conditions, alignment was neither enhanced nor disrupted. When MMP-14 was inhibited in PS+PC conditions alignment, was disrupted and returned to an unaligned state. When MMP-14 was inhibited in PS+PC muc4 KO conditions, alignment was neither enhanced nor disrupted.

Hyaluronan (HA), a polysaccharide found to be overproduced within the pancreatic tumor, was added to the collagen gels to study how the presence of HA effects contact guidance in pancreatic cancer (PC) and pancreatic stellate (PS) cells. Mucin 4 is a large glycoprotein which is can block binding of cells to HA and is overexpressed in pancreatic cancer. The MUC4 gene was knocked out of the PC cells (PC muc4 KO) to investigate how mucin 4 may affect HA bonding. In the presence of HA, PS cells showed a slight increase in speed and directionality. Surprisingly, PC and PC muc4 KO cells showed directional migration in response to aligned fibers, which has not previously been seen. Further protrusion analysis revealed that the ratio of I
to Y extensions was inversely proportional to cellular speed. Low molecular weight HA (LMW HA) is believed to be important during the early stages of invasion within the pancreases, so the molecular weight was decreased but concentration kept the same. Migration speed and directionality remained consistent while motility coefficient ratio median increased significantly. This indicates persistence time may be important and regulated by LMW HA.
CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

The World Health Organization (WHO) estimates that in 2015, 8.8 million people were killed by cancer worldwide with more than 1,600 deaths a day in the United States [1-3]. This accounts for nearly 1 in 6 deaths across the globe and has brought cancer up to the second leading cause of death. Even more concerning is that fatalities due to cancer are expected to rise 70% over the next twenty years [4]. With the economic burden related to cancer already in the hundreds of billions worldwide, and projected to hit 458 billion by 2030, it is imperative that we move forward in our understanding of cancer [5].

Metastasis is the complex process by which cancer cells leave the primary tumor, invade into local tissue, travel through the blood stream or lymphatic system, and seed a secondary location in the body [6-8]. This process accounts for about 90% of the morbidity and mortality associated with cancer, and this number has only decreased slightly in the last 50 years [7]. The reason for this lies, in part, with the poor treatment options once the disease has progressed to this point. Radiation leaves the patient immunocompromised and often the five year survival rate is still low for most types of cancers [9]. Research into cancer metastasis is wide ranging, including the epithelial-mesenchymal transition (EMT), cancer stem cells, origin of metastatic tumor cells, expression of genes within metastatic cancer cells, circulating tumor cells (CTCs), and resistance to pharmaceuticals to name a few [10-15].

Another aspect of metastasis which has emerged over the last 25 years, is the interaction between cells and the external signals which direct metastasis [16-19]. Migration of cancer cells occurs in all stages of metastasis, however there is a surprising gap of knowledge when it comes
to understanding the role the extracellular matrix (ECM) plays in the initial migration out of the primary tumor. Directed migration occurs when an extracellular cue is interpreted and the cell migrates in response [10]. Understanding the role the ECM plays in metastasis will allow better models to be constructed and may give alternative options to slow or cease metastasis. This can be seen in the growing push to develop therapeutic targets focused on the tumor microenvironment (TME) along with the genetics of the cell [20].

The TME is made up of many cell types and structural components which are constantly moving and being rearranged [10, 21-23]. Communication between cells occurs through soluble chemicals and ECM reorganization and is important in metastasis [24]. The organization of structural fibers have long been known to act as a path for certain types of cells, including cancer cells. Cells align fibers by secretion or degradation of the ECM, using matrix metalloproteinases (MMPs), and reorganizing it [25-27]. Cells adhere to the fibers through surface proteins, called integrins, and can move along the fibers. This process is called contact guidance [28-30]. Both cancer cells as well as normal cells, such as stromal cells, have also been shown to reorient these fiber perpendicular to the surface of the tumor, creating an avenue for metastasis [31, 32]. Understanding how different types of cells organize these fibers, we can further understand how metastasis occurs and discover new ways to combat it.

An important discussion in the field of cancer revolves around differences between 2D vs 3D cell models. While 2D models are often simple, easily controlled, and imaged easily due to the cells residing on the same plain, there is debate of the applicability of 2D models due to the incongruity when compared to in vivo studies. A 3D model more closely mimic in vivo environment characteristics including topography, confinement and mechanical properties while still allowing for manipulation of variables. Through the development of a novel approach
utilizing the rotation of an acupuncture needle, alignment of 3D collagen gels can be achieved allowing for investigation of how contact guidance cues direct migration in 3D environments. Along with understanding how cells engage in contact guidance, this approach will allow for the investigation of how cells reorganize or disorganize already aligned fiber networks, which is not routinely done in 3D. Quantitative characterization of the alignment within the fiber network can be assessed to determine how cancer cells and stromal cells respond to aligned fibers.

Because of the established role of stromal cells in regulating cancer, understanding their interaction with cancer cells is paramount. This allows natural cell-cell communication to occur and has the power to give greater insight to what influences cancer metastasis [33, 34]. By inclusion of stromal cells in the collagen network we can examine other important physical constructs and proteins within the cell, which allow the cells to interact within the ECM.

While mimicking the TME using collagen is the norm throughout much of the field, there are other ECM components found in the tumor which may important when investigating migration [35]. One such component is hyaluronan (HA). HA is a nonsulfated glycosaminoglycan which has been linked to cell proliferation, migration, and involved in tumor progression [36-38]. It is a particularly interesting ECM component when discussing cancer as it has been shown to collapse blood vessels and inhibit drug movement to the TME, as well as high levels being linked to poor prognosis [39-41]. Enzymes which degrade hyaluronan are being paired with drugs to improve the efficacy. However, though there is little known about how hyaluronan cooperates with collagen [42]. By including this polysaccharide into the ECM, it will better mimic the TME and may allow for a better insight to how cells respond to alignment.

Knowing this information and having identified the gaps in the field of cancer metastasis, the work that has been completed towards my thesis focuses on these three specific aims:
1. Develop a system which produces aligned fibers in collagen gels which can be used to model alignment seen in the pancreatic TME.

2. Investigate the role of cancer-stromal cell communication on contact guidance and reorganization or disorganization of ECM fibers.

3. Examine the role that hyaluronan has on pancreatic cancer and stromal cell migration characteristics and extension dynamics.

By utilizing the simple concept of mechanical stress to align fibers and exposing them to multiple cell types with molecular perturbations these specific aims will be investigated.

1.2 References


CHAPTER 2: LITERATURE REVIEW

With cancer being responsible for more than 8.8 million deaths a year, it is understandable that we are devoting so many resources towards finding better diagnostics and treatment options [1]. Modeling the metastatic aspects of cancer can be difficult and we are only now beginning to understand what directs the process [2]. Metastasis is the process of cancer cells migrating from the primary tumor into the surrounding tissue, circulating through the blood or lymphatic systems, and seeding a secondary tumor elsewhere within the body [3]. Once this process has occurred, the long-term survival rate drops from 90% to 15% and treatment options dwindle [4]. One avenue of investigation focused towards understanding and combating metastasis is cancer invasion, or the initial migration of cells from the primary tumor towards the blood or lymphatic systems. While random migration can result in invasion, many forms of directed migration are important. Migration can be directed by chemical, ionic, and stiffness gradients, but over the past 10 years appreciation of another extracellular cue, aligned extracellular matrix fibers like collagen, has grown. Migration along aligned fibers is called contact guidance. Aligned collagen is a feature of the extracellular matrix at the tumor-stromal interface. Collagen has been observed to direct cancer cells along the fibers forming a highway leading directly out of the tumor and into the surrounding tissue. While collagen alignment around the tumor has been seen in vivo, it is unclear to what degree other environmental cues tune the effectiveness of this directional migration cue. Communication between cancer and stromal cells or cancer and other matrix components, such as hyaluronan, may be important in the interpretation of this cue. Due to contact guidance’s unique nature and the role that the extracellular matrix plays with regards to both cancer metastasis and regulation, understanding this cue may lead to alternative diagnostics and therapeutics. Identifying what interactions and
signals allow cellular interpretation of the alignment may lead to ways to block directed migration. Another potential therapeutic involves identifying therapeutics to, not only limit, but disrupt the alignment present which may result in the tumor regressing.

2.1 What is Contact Guidance?

Metastasis, as previously described, occurs whenever a cell migrates away from the primary tumor, invades into the surrounding tissue, and travels to distant parts of the body. This can be accomplished through one of two types of cell migration: random and directed [2]. Random migration occurs when there is a lack of external cue or the inability to recognize an external cue. This type of migration is often modeled using a persistent random walk model which operates by defining a speed and persistence time of the cell [2, 5]. The speed is the displacement of a cell divided by time. Persistence time is how long a cell migrates without changing either speed or direction. Random cell motility can be tuned by introducing migration cues, such as uniformly altering the ECM in which or on which the cell is migrating, or by introducing a uniform dose of a growth factor or chemokine. The random migration strategy can often be found within the body, and is utilized by cells which are best served by exploring the environment, such as immune and cancer cells in the absence of directional external cues [6].

When a cell is able to sense the presence of a spatially inhomogeneous cue, it may respond by biasing its migration causing directed migration. These external cues can be grouped into the categories of ECM and soluble molecule cues [2]. Extracellular matrix cues include aligned fibers, stiffness gradients, and ECM-bound chemical gradients. The cell must interpret these cues by interacting directly with the ECM. The soluble molecule cues include soluble chemical and electrical gradients and are detected using receptors along the cell’s body [7].
Contact guidance is the migration of cells along aligned fibers. This type of external cue is bidirectional meaning that the cell can migrate along the fiber in both directions equally [2]. Soluble chemical gradients result in a monodirectional cue which leads the cell to migrate to the source. This is chemotaxis. A cell which is migrating in response to a directional cue may migrate either slower or faster and the persistence time may be longer than a cell under the similar conditions migrating randomly.

The term contact guidance was first coined by Weiss in 1934 when studying nerve fiber regeneration [8]. The strength of the contact guidance cue depends on the density of the fibers and the degree of alignment [9]. If there is too much variation in fiber orientation within the fiber field, then the cell will not be able to discern the mean direction of the cue and the resulting migration will be random. It should also be noted that the more directional the fiber field, the higher degree of persistence by which the cell migrates, however perfect fiber alignment in vivo is not common. Cells use complex signaling pathway to interpret and respond to aligned fibers which are crucial in understanding contact guidance.

### 2.2 How Intracellular Properties Affect Contact Guidance

To understand how cells respond and interpret contact guidance cues, it is important that we first consider basic cell migration. Eukaryotic cell migration follows a cyclic pattern whereby polarization of the cell results in protrusions along the direction of the migration [7]. These protrusions interact with the extracellular matrix (ECM) through transmembrane proteins called integrins [10-12]. This interaction allows for the cells to exert contractile forces by pulling on the fibers located in the ECM, moving the cell forward while the rear edge of the cell retracts [7, 13]. The five steps of 3D migration have been identified as 1) pseudopod elongation, 2) adhesion,
force generation, 3) focalized proteolysis, 4) acto-myosin contraction, and 5) rear retraction and path release [13, 14].

The cell achieves this migration through a series of steps which involve proteins found both within the cell and attached to its membrane. After the initial response to a migration-promoting agent, such as growth factors or a physical cue, the cell extends protrusions in the direction of migration through actin polymerization [15]. Key proteins, such as Rho GTPases including Rho, Rac, and Cdc42 are responsible for conveying signals from the membrane to the cytoskeleton which initiates adhesion [7, 16-18]. These protrusions are furthered anchored to the ECM through transmembrane proteins which are connected to intracellular actin filaments which can produce the required traction force. There are a number of key adaptor proteins which connect the actin bundles to the integrins such as talin, kindlin, vinculin, and paxillin [19]. The interaction of FAK and Src, along with adapter proteins Cas and Crk, regulate the turnover of adhesions which is an important step as cells which struggle to retract their rear are poor migrators [7].

Once the cells have attached to the ECM, polymerization of globular actin monomers to filamentous actin (F-actin) at the leading edge drives the cell forward [20]. The process which actin monomers polymerize is well known and includes actin nucleation being regulated by formins and the Arp2/3 complex [21, 22]. Formins are responsible for regulating the growth of the linear actin filaments while the Arp2/3 complex forms branches which are necessary to produce the dendritic actin network which stabilizes protrusions. Contraction of the actin filament once formed is generated by myosin II motor proteins. Myosin II is responsible for promoting retrograde flow away from the leading edge which enhances the protrusion rate as well as producing contractions in the rear and sides of the cell which drive the cell forward [23-
Regulation of actin-myosin contraction is mediated by the phosphorylation of a myosin light chain. This process is regulated by calcium ions binding to myosin light-chain kinase. ROCK and MRCK are regulated by Rho and Cdc42 respectively which are also important [26].

Another important aspect of cell migration is the role surface proteins called glycoproteins play. Glycosylation is the process of enzymatically connecting saccharides to other saccharides, proteins, or lipids and is known to help stabilize the cell membrane as well as aid in cell-cell communication [27]. One family of glycoproteins which are upregulated in cancer, are mucins and are responsible for forming a physical barrier of mucous gel which protects the cell membrane [28]. Mucins-assigned Muc1 to Muc21- are large glycoproteins which can be secreted or attached to the membrane and form a protective barrier. This barrier can block cell-ECM attachments due to mucins being long and having many branches which can block an integrin. As previously discussed, integrins are responsible for adhesion and allow the cell to pull on the matrix using contractile forces. Fewer and weaker connections to the surrounding matrix resulting in a faster migration. The extent to which Mucins affect cellular migration, especially with regards to cancer, is still under investigation and is a hot topic of therapeutic interest.

While there are many ways to classify cell types, one method is to group them based on their migration type: amoeboid and mesenchymal [29-32]. Cellular migration is governed by cytoskeleton growth (polymerization and protrusion) and the shrinkage of the intracellular network (contraction) [30]. Cells on 2D surfaces have shown to exclusively use actin polymerization to extend the leading edge of cell migration and rely on lamellipodia to migrate, however this represents only one of several ways which cells migrate in 3D [33, 34]. Migration modes in 3D include lobopodial, amoeboid, and filopodial. Amoeboid cells in 3D matrices, being surrounded by an entrapping matrix, rely less upon surface attachments to the ECM and
use traction forces to migrate. Typical hallmarks to amoeboid cell invasion are a loss of cell polarity and a loose attachment to the ECM and due to this, amoeboid cells do not undergo a retraction step [35]. Amoeboid cells exhibit a round/ellipsoid shape with low to high migration velocity. This migration is reliant on non-focalized integrins and proteases which is the result of fast receptor assemblies along the cell membrane [36]. These cells overcome barriers within the matrix, not by degradation, but by squeezing through spaces within the matrix [36, 37]. Due to low adhesion to the matrix, single cell migration assays have shown cells with this migration mode to have a low propensity to migrating along contact guidance cues but do well responding to chemotaxis (chemical gradients) [35, 38, 39].

Mesenchymal cells typically have an elongated morphology while being more reliant on strong matrix attachment [36]. This affects cellular migration as the rearrangement of F-actin leads to polarized cells with focal adhesions in the front and a tail which contracts as structural proteins are degraded [35]. This strong attachment to the matrix and polarization is achieved, in part, through high surface expression of α2β1 integrins, which is how the cell attaches to the matrix [40]. Mesenchymal cells also have a propensity to degrade and remodel the matrix through metallomatrix proteins (MMPs) which is their primary method of overcoming barriers within the matrix [37, 41]. These cells have been shown to have high directionality in response to both contact guidance and chemotaxic gradients due to their strong attachments to the ECM [39, 42]. Common mesenchymal cells within the body are stromal cells which are responsible for shaping fiber alignment.
2.3 Role of Stromal Cells in Shaping the Contact Guidance Cue.

Stromal cells are connective tissue cells located in any organ and support the function of the parenchymal cells [43, 44]. The most common stromal cells include pericytes and fibroblasts. It is believed that these contractile cells are responsible for alignment throughout the body, including near the tumor. There are three main ways which stromal cells induce contact guidance naturally within the body: deposition, rearrangement, and MMP activity. Though contact guidance within the body can be found naturally from wound healing to embryogenesis, it is still an area which is not well understood [45]. What makes it an area of interest for cancer research is that high alignment has been seen in multiple cancers. Pancreatic and breast cancer, in particular, have a growing body of work which highlights the importance of contact guidance with high alignment seen in vivo and directional migration due to aligned physical constructs seen in vitro in 2D and 3D [24, 46-50].

One method by which cells have been shown to create organized matrices within the body is by deposition of new matrix which enhances the existing aligned cue [51]. This can be seen especially in corneal fibroblast cells which have been shown to produce aligned collagen in both organized and unorganized matrices [52, 53]. This type of fibroblast produces a highly organized matrix in the stroma which is the supporting structure for the cornea allowing sight. Though the mechanism by which the cells are able to produce this matrix is unknown, the enhancement of aligned fibers matrices is confirmed.

Another method by which matrix can be remodeled and create aligned ECM fibers is by physically moving the matrix. There are a few methods which can produce aligned fibers in this way, however they all include either pushing or pulling. Cell migrate in vivo in a number of different ways, however one common method is by following existing tracks within the matrix
Cells which do not attach very strongly to the matrix will find holes and paths which are large enough to push through which can align the gel. These gaps can be created by migration of other cells [55]. Another method is by highly contractile cells attaching to the matrix and dragging it along with the cell [45, 56]. In this scenario, the cell exerts enough force to pull the fibers behind as they migrate creating alignment.

The final method of alignment and most commonly associated with cancer involve activation of Matrix metalloproteinases (MMPs). MMPs are a family of more than 21 zinc dependent endopeptidases whose primary role is the degradation of ECM proteins [57]. There are eight distinct structural classes of MMPs which are comprised of 5 soluble MMPs (S-MMP) and 3 membrane tethered MMPs (MT-MMP). Cells regulate MMP activity through natural inhibitors called TIMPs, though this process is typically downregulated in cancerous cells, increasing overall MMP activity within the TME. MMPs can be furthered grouped based on their substrate specificity and location: collagenases, gelatinases, stromelysins, and MT-MMPs. Alignment occurs through the degradation of linkages between collagen fibers allowing the cell to actively rearrange the matrix locally. MMP activity has been found to be higher in amoeboid migrating cells as they must cut through dense matrix to migrate, however mesenchymal do utilize MMPs to align the matrix [58, 59]. The increase in MMP activity and concentration within the TME is important for more than just an increase in migration and metastasis, but have been linked to cell proliferation, angiogenesis, cancer initiation, and fiber alignment linked to contact guidance [57, 60].

2.4 The Role of Contact Guidance in Tumor Progression and Metastasis.

While originally thought of as a simple mass of homogenous cancer cells, the tumor microenvironment (TME) is, in fact, an incredibly complex combination of matrix fibers, cancer
stem cells, cancer cells, immune inflammatory cells, cancer-associated fibroblasts, endothelial cells, and pericytes [61, 62]. Each of these cells play a role in furthering the growth and spread of the disease. The immune inflammatory cells, typically associated with removing necrotic debris after apoptosis, and cancer-associated fibroblasts are recruited to the TME and promote cancer cell proliferation, angiogenesis, and remodel the matrix as previously discussed as a means to promote invasiveness [61, 63-65]. Endothelial cells and pericytes make up the blood vessels which bring oxygen to the cells and remove waste which is crucial to the growth of a tumor [61, 66-68]. Extracellular matrix components such as fibronectin, collagen, hyaluronan, and laminin can also be found in and around the TME [10, 69]. These proteins play a role in signal regulation, pathway activation, direction of metastasis, as well as many other aspects of cell survival [70, 71]. The ECM has been linked to cell proliferation, enabling replicative immorality, resisting cell death, and the activation of invasion and metastasis [61, 72].

As mentioned above, collagen is extremely important in the progression and metastasis of cancer. Collagen is an extracellular matrix protein which are made up of three polypeptide alpha chains organized into a triple helix [73-75]. This protein can be found, in its various forms, in the extracellular matrix, cartilage, reticular fibers, basement membrane, and even on cell surfaces [76, 77]. Members of the collagen family are the most abundant proteins in the ECM with fibril type 1 collagen giving support to the matrix and creating attachment points. Polymerized collagen 1 is commonly used for in vitro research to model the ECM and create gels which are used to study migration [78, 79]. Another option commonly used to model the TME in vitro is Matrigel [80, 81]. Matrigel is a protein mixture which has been extracted from a muse sarcoma which is rich in tumor extracellular matrix proteins which includes collagen, laminin, and entactin. This has been used in place of collagen gels to represent a more complex environment.
Hyaluronan, a negatively charged polysaccharide, has been shown to be overproduced in some cancers and the presence has been inversely linked to prognosis [82]. It is been shown to decrease the density and alter the viscoelasticity of the matrix.

The physical properties along with the compositional properties of the matrix have a direct effect on the cell and its ability to migrate in 3D. There are four physical variables which influence cell migration: rigidity of the matrix, adhesion to the matrix, confinement within the matrix, and topology of the matrix [83]. The rigidity of the matrix is an important factor as the stiffness of the matrix can both affect tactile forces which generate movement and have been shown to initiate metastasis within cancerous cells [84, 85]. Cells sense the stiffness of the matrix around them through a process called mechanotransduction [11, 86, 87]. When a cell is attached to the matrix via multiple points it can exert a force through actomyosin cables within the cell, resulting in the movement of the cell or reorganization of the matrix [7, 23]. Adhesion to the matrix through integrins have been discussed previously in this chapter. Suffice it to say the types of attachments and the stiffness are connected and have been shown to change focal adhesions which correlate to contact guidance alone the ECM fibers [12].

Confinement in 3D matrices and the topology of the matrix are also very important in cancer migration. Cells either cut the matrix to create a path for migration or find channels which they already can fit into [88]. Density, which creates confinement, and organization, which accounts for topology, of the matrix has been shown to increase the importance of actomyosin contractility which increases the cells likelihood to migrate instead of reorient the matrix inducing a contact guidance cue [84, 89]. This idea of confinement and topology is also an important concept when discussing collective migration of tumor cells [90]. In collective
migration, as opposed to single cell migration, the cells migrate in sheets and follow the tracks formed by cells at the leading edge of the formation [91].

Cancer cells connection to the extracellular matrix is extremely important as it not only receives signals from the ECM which affect migration, but can actively reshape the matrix to foster metastasis. Collagen rearrangement perpendicular to the tumor edge has been seen in both in vivo, ex vivo, and in vitro models [46, 92-96]. This ability allows cells to develop their own migratory cue and create an efficient highway to direct metastasis. The development of this cue is unique as it can be produced by cells actively reshaping the matrix as well as by cells producing new matrix which reinforces the existing cue. The process by which cells, typically believed to be stromal, are able to reorient the matrix is through the use of MMPs.

2.5 The Role of Pancreatic Stellate Cells and Relationship with Pancreatic Cancer Cells

Pancreatic stellate (PS) cells are myofibroblast-like stromal cells which play a critical role in the formation and production of the stroma within the pancreas. In a normal physiological state, PS cells are quiescent and contain retinoids and intracellular fat droplets, which distinguishes them from normal fibroblasts [97]. In the quiescent state, PS cell’s primary role is focused on maintenance of the pancreatic tissue and act as immune, progenitor, and intermediary cells. Quiescent PS cells become active PS cells through pathogenic factors such as TGFβ1, fibroblast growth factor, and plate-derived growth factor [98]. Activated PS cells function to contribute to fibrosis and hypoxic TME as well as being involved in angiogenesis. Once activated, the cell goes into an active mode producing ECM proteins, such as collagen, laminin, and fibronectin at an increased rate. Along with the ECM production, cell proliferation and migration also increase [97, 99]. The change can also be seen as activated PS cells lose their
balance between MMPs and TIMPs, producing more MMPs, and secrete more growth factors and chemokines [97].

Activation may occur naturally during times of injury which requires a more contractile cell and the production of new ECM proteins, however it also occurs during tumor development and growth [99]. The role of the PS cell is still being investigated, but there is plenty of evidence which suggests that they play a critical role in the progression and subsequent metastasis of pancreatic cancer (PC). Co-culture between PS and PC cells and PC conditioned media have resulted in a higher degree of migration for the cancer, suggesting that there is a soluble molecule signaling connection between the cells [97, 100]. This is not surprising as the activated PS cell shows an increase in soluble molecule secretion. Studies have also shown that the size and metastasis of the tumor corresponds to the amount of PS cells present [100].

While communication through soluble molecule is one way which these cells interact, another is through the ECM. As previously stated, activated PC cells show increased production of ECM proteins and higher contractility. This results in alignment in and around the TME which has been seen in vivo [47, 101]. This alignment is believed to result in directed migration of the PC cells and may initiate metastasis. Recently, the relationship between PC and PS cells have been an area of focus for the field [102-107]. Drifka et. al. found that PC cells are responsible for alignment and direct contact between PC and PS cells are critical for invasion [108]. Expanding on this idea, Koikawa et. al. molded tumor-like clumping, and identified the PS cells as, not only reorganizing the matrix, but acting as leading cells which allowed the PC cells to follow [109]. The evidence of a role that the PS cell plays in PC cell metastasis is only growing. As we continue learning about this relationship, new therapeutic targets may begin targeting the
PC or PS cells to limit metastasis directly or indirectly through their ability to communicate through ECM components.

2.6 The Role of Hyaluronan in Tumor Progression and Metastasis

While collagen has been the ECM component which has been focused on in this review, there is another which has been identified as playing a large role in tumor progression [82, 110]. Hyaluronan (HA), also known as hyaluronate or hyaluronic acid, is a negatively charged linear polysaccharide which has a high molecular mass and is known for having interesting viscoelastic properties [111, 112]. HA is produced as an unmodified polysaccharide within the cell and secreted through the plasma membrane into the ECM. HA plays an important role in cell migration, and cancer metastasis in particular, as it has interesting hydrodynamic characteristics which allows it to retain water and regulate the porosity and malleability of the matrices [112]. These are important factors as they determine whether cells invades tissues and reorient matrix, possibly resulting in contact guidance. Cells can sense and interact with HA in two ways. CD44 and RHAMM are specific cell-surface receptors which bind to HA and can cause a cascade of intracellular signals. The other way is through retention in which the cells essentially coat themselves, although the result of this is not well understood and will not be covered in this review. As previously stated, HA is important in metastasis as shown in numerous studies [113-115]. HA has been shown to be overproduced in tumors to a point that HA levels can be used as a diagnostic marker, and in some cases, is directly associated with low survival rates [116, 117]. A production increase of HA in the TME has also been shown to result in drug resistance making it a harder to treat the cancer [112, 118].

Due to HAs importance in cancer metastasis, its receptor, CD44, was identified as a therapeutic target in the early 1990’s [119-122]. The research quickly identified CD44v6
isoforms as crucial to the metastatic process in rat pancreatic tumor cells [123, 124]. CD44 antibodies inhibiting the CD44-HA bonding has been shown to block metastasis in matrigel (a basement membrane matrix mix) [125]. Research is also looking at using the HA itself to act as a delivery method to cancer cells. Due to the higher concentration of HA in the TME, it was targeted as a potential bioconjugate which could be paired with cytotoxic drugs such as butyric acid, paclitaxel, and doxorubicin. The cells attach to the HA, internalize the drugs through receptor-mediated endocytosis, and a release the active drug [111, 126]. The act of breaking up HA using hyaluronidase to enhance tumor permeability and allow better diffusion of nanovaccines has also been identified as a potential therapeutic option [127]. More recent studies, including one from Cheng Et. Al. in 2017, has linked HA production in pancreatic cancer and showed that 4-MU, an HA synthesis inhibitor, limits migration in co-culture systems [128]. This paper also indicated that when stromal cells and pancreatic cancer cells are co-cultured there is a drastic increase in HA production. Other groups have linked CD44 to pancreatic cancer indicating the important role of HA in this particular type of cancer [129-133]. The importance of HA within the TME is just starting to be realized and will be a focus for therapeutics in the coming years.

2.7 How Can You Model Contact Guidance Cues In Vitro

Studying contact guidance on 2D surfaces is not a new concept and can be accomplished by a number of surface treatment assays including electromicrospinning, microcontact printing, epitaxial grown collagen, or generating grooves or gratings [24, 48, 49, 134-137]. These produce assays which allow for a simple study of cancer cells migration; however they have drawbacks [2]. Modeling contact guidance in 2D may not capture the complex interactions happening on all
sides of the cell and within an ECM [138]. Since the contact guidance cue is only found on the bottom of the cell and is typically designed to be extremely directional, the cell is able to interpret and respond to the cue much easier than in 3D gels. While this can be useful for identifying cells which have a predilection to responding to the contact cue, it does not accurately model what happens in vivo [13, 139]. It is for this reason that a more complex, 3D models are required.

It is only recently that the field has begun moving towards using 3D models to study cancer’s response to external cues [2, 81]. This is because, with a more complex system, comes a higher degree of difficulty. Imaging, for instance, can be an issue as migration can now occur in three dimensions and the thickness of the gel can impede the use of lenses with higher magnification [140, 141]. A common assumption when analyzing cell migration in 3D gels is that most of the migration occurs on a 2D plane and models used to fit parameters will utilize this [142]. Some groups track the 3D migration through the use of a z-stack. This technique takes images in the same x-y location but will vary the z position within the gel allowing for tracking of nucleus in all three dimensions. This can be difficult, however, because the working distance of higher magnitude lenses means that you can image only so far into the sample. The focus in a z-stack is another issue as, for more detailed studies of the cell, the cell and its extensions may not be within the same z-plane. Another issue which occurs in 3D gels is the optimal way to align the fibers. Techniques used to align gels in the past include the use of magnetic beads, cell-based, and flow-based systems [93, 143-146]. While sometimes effective, they each have their own drawbacks. Magnetic bead alignment requires a strong magnetic field and the beads themselves may interact with the cells affecting migratory behavior. In addition, it is hard to get reproducible alignment. Cell-based alignment of 3D gels requires a decellurization step and is
difficult to tune the alignment [2]. Flow based systems, while popular for examining the migratory response to a chemotactic cue, require microfluidic devices and typically small length scales over which the contact guidance cue occurs.

Another method of aligning gels for cell migration analysis, introduced in this thesis, is by twisting an acupuncture needle which results in radial alignment emanating from the needle surface. This has the advantage of a simple setup and the alignment is tunable through the degree of rotation and through chemical modifications to the ECM. It is important to note that within the matrix, the collagen is connected via crosslinking and entanglement which increases the strain modulus greatly. When looking at stress strain curves of skin it was suggested that the spring constant for collagen is about 4.4 GPa within the connective tissue [147].

Upon insertion, collagen fibers attach to the needle allowing for the transfer of mechanical force across the collagen network. The rotation pulls the matrix inward, wrapping some fibers around the needle. This aligns the fibers circumferentially close to the needle. As the matrix is pulled inward, the fibers will begin to align radially further away. This results in radial alignment in the matrix not wrapped around the needle [148, 149]. Prior to the twisting of the needle it is hypothesized that the collagen fibers can bind loosely to the needle due to an electrostatic attraction between the negatively charged matrix and the metal needle [150]. While weak, this attraction allows for enough traction of the matrix on the needle to cause deformation. Once the rotation begins, the collagen fibers wrap around the needle allowing the compressive force of the collagen to gain a firmer hold around the needle.

2.8 Conclusions

Cancer invasion is complex and multifaceted. While there are multiple known cues which direct migration, recent evidence suggests that contact guidance, migration in response to aligned
fibers, may be critical in pancreatic and breast cancer. Stromal cells are believed to be responsible for alignment as well as promote tumor progression. One way alignment is achieved is through the use of MMPs which are overproduced within the TME. Cellular communication between cancer and stromal cells is believed to be key in the beginning stages of metastasis. Mucins, a family of glycoproteins, have been identified as communication modulators and overexpressed in certain cancers. One aspect not well understood however, is how cells enhance or disrupt alignment once it has been established. Other ECM components, such as hyaluronan, have been identified as important to structural properties of the ECM and are beginning to be the focus for therapeutic targeting. We have developed and characterized a simple rotational alignment technique which can be used to study how these parameters affects contact guidance which mimics alignment seen in vivo.

2.9 References


[33] Petrie RJ, Yamada KM. At the leading edge of three-dimensional cell migration. Journal of Cell Science 2012, 125: 5917-5926


[38] Smirnova T, Segall JE. Amoeboid Chemotaxis Future Challenges and Opportunities. Cell Adhesion & Migration 2007, 1: 165-170


[68] Ribeiro AL, Okamoto OK. Combined Effects of Pericytes in the Tumor Microenvironment. Stem Cells International 2015,


[95] Perry SW, Schueckler JM, Burke K, Arcuri GL, Brown EB. Stromal matrix metalloprotease-13 knockout alters Collagen I structure at the tumor-host interface and increases lung metastasis of C57BL/6 syngeneic E0771 mammary tumor cells. Bmc Cancer 2013, 13


Drifka CR, Loeffler AG, Esquibel CR, Weber SM, Eliceiri KW, Kao WJ. Human pancreatic stellate cells modulate 3D collagen alignment to promote the migration of pancreatic ductal adenocarcinoma cells. Biomedical Microdevices 2016, 18


Misra S, Hascall VC, Markwald RR, Ghatak S. Interactions between hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer. Frontiers in Immunology 2015, 6


Toole BP, Slomiany MG. Hyaluronan: A constitutive regulator of chemoresistance and malignancy in cancer cells. Seminars in Cancer Biology 2008, 18: 244-250


CHAPTER 3: CONTACT GUIDANCE DIVERSITY IN ROTATIONALLY ALIGNED COLLAGEN MATRICES

A paper published in Acta Biomaterialia

Jacob A.M. Nuhn\textsuperscript{a}, Anai M. Prez\textsuperscript{c}, Ian C. Schneider\textsuperscript{a,b,*}

\textsuperscript{a} Department of Chemical and Biological Engineering, Iowa State University, United States

\textsuperscript{b} Department of Genetics, Development and Cell Biology, Iowa State University, United States

\textsuperscript{c} Department of Chemistry and Physics, Grand View University, United States

*Corresponding author

Abstract

Cancer cell metastasis is responsible for approximately 90% of deaths related to cancer. The migration of cancer cells away from the primary tumor and into healthy tissue is driven in part by contact guidance, or directed migration in response to aligned extracellular matrix. While contact guidance has been a focus of many studies, much of this research has explored environments that present 2D contact guidance structures. Contact guidance environments in 3D more closely resemble \textit{in vivo} conditions and model cell-ECM interactions better than 2D environments. While most cells engage in directed migration on potent 2D contact guidance cues, there is diversity in response to contact guidance cues based on whether the cell migrates with a mesenchymal or amoeboid migration mode. In this paper, rotational alignment of collagen gels was used to study the differences in contact guidance between MDA-MB-231 (mesenchymal) and MTLn3 (amoeboid) cells. MDA-MB-231 cells migrate with high directional fidelity in aligned collagen gels, while MTLn3 cells show no directional migration. The collagen stiffness was increased through glycation, resulting in decreased MDA-MB-231 directionality in
aligned collagen gels. Interestingly, partial inhibition of cell contractility dramatically decreased directionality in MDA-MB-231 cells. The directionality of MDA-MB-231 cells was most sensitive to ROCK inhibition, but unlike in 2D contact guidance environments, cell directionality and speed are more tightly coupled. Modulation of the contractile apparatus appears to more potently affect contact guidance than modulation of extracellular mechanical properties of the contact guidance cue.

3.1 Introduction

Cancer cell metastasis is responsible for 90% of fatalities related to cancer [1]. Mimicking metastasis in engineered environments to separate or expand patient-derived cells, identifying metastatic diagnostic signatures and designing therapeutics that target metastasis is crucial to limiting cancer’s effects on public health. The complex and diverse migratory behavior that leads to metastasis has begun to be described by a reduced set of cell phenotypes called modes of migration. There are two main migration modes which have been identified as important in the metastasis of cancer cells: mesenchymal and amoeboid [2]. The mesenchymal mode of migration is typified by a spindle-like morphology, where cells protrude and retract multiple simultaneously existing extensions. Mesenchymal mode migrators seem to be highly adhesive and contractile, remodeling matrix by exerting large traction forces. These cells also rely on matrix degradation that is coupled to matrix remodeling through traction forces. The amoeboid mode of migration is typified by a rounded morphology, where cells generally extend one pseudopod-like structure. Amoeboid mode migrators are less adhesive and the contractility is not devoted to traction force generation, so much as it is to cytoskeletal-mediated squeezing, producing movement of the cell body toward the extended pseudopod [3]. Contact guidance, or directed migration along aligned fibers, has been shown to play a large role in some metastatic
cancers. For instance, in breast cancers [4], stromal and cancer cells cooperate to orient collagen fibers perpendicular to the tumor margin and cancer cells use this directional cue to migrate from the primary tumor. *In vitro* models of tumors also show radial fiber alignment [5]. It is becoming more appreciated that cells with different migration modes may respond to contact guidance cues with much different fidelities. Cell type differences in contact guidance have been observed for quite some time. More recently, we and others have shown that motility mode can predict the fidelity of contact guidance, even in situations where migration speed is similar [6-8]. This suggests that metastasis as driven by structural changes in the collagen fiber orientation may only be potent for certain cell phenotypes.

In addition to structural organization of collagen fibers, the tumor microenvironment tends to be stiffer in highly invasive cancers as compared to normal tissue [9, 10]. It has long been known that the stiffness of the extracellular matrix (ECM) can have a profound influence on cell morphology and migration [11-14]. Model 2D flexible substrates including polyacrylamide and polydimethylsiloxane have been used frequently to uncover the effects of stiffness on cell function. Controlling stiffness in 3D environments like collagen gels is a bit more difficult. Increasing collagen concentration results in stiffer gels, but the ligand density for receptor binding is also different, convoluting chemical and physical cues. Collagen gels can also be crosslinked by chemicals or enzymes; however this crosslinking is frequently done in the presence of cells and can present some practical difficulties. Recently, glycation has been used to increase the stiffness of collagen gels [15]. Collagen can be non-enzymatically functionalized with ribose, resulting in a stiffer gel, while keeping the collagen concentration and consequently, ligand density the same. This approach has been used frequently to assess the role of the mechanical properties of the collagen gel in controlling cell function including cell migration.
While the role of stiffness in controlling cell migration is relatively well-known, it is unknown how stiffness affects contact guidance. Do networks with the same collagen structure, but different stiffness result in different contact guidance?

Predicting how a cell’s migratory mode as well as how the ECM stiffness affects migration behavior requires understanding how a cell’s cytoskeletal structures function. Cells adhere to collagen fibers using integrins and discoidin domain receptors on the surface of the cell. Receptor binding leads to focal adhesion assembly that is linked to a contractile F-actin cytoskeletal network, allowing for the cell to transmit force to the surroundings [16, 17]. Mesenchymal cells have shown a propensity to form strong bonds with their surroundings, allowing them to remodel the matrix while they migrate [18]. Amoeboid cells bind the ECM with less force and use a number of physical mechanisms such as contraction-based blebbing or squeezing [19]. These differences between the two modes lead mesenchymal cells to form much stronger attachments to the ECM and allow them to respond more robustly to directional cues from aligned fibers. Contractility is generated through myosin II-mediated contraction of the F-actin cytoskeleton. Several signaling proteins including kinases such as Rho kinase (ROCK) can dynamically regulate contractility through phosphorylation of myosin II regulatory light chain and we have shown this to be important in contact guidance on 2D substrates [6]. Others have shown contractility to be important in 3D contact guidance environments [20].

In vivo, the ECM environment is compositionally, structurally and mechanically complex. This complexity has forced many simplifications in in vitro systems. For instance, most of the research conducted with regards to contact guidance has focused on 2D models. 2D models provide finer and more reproducible control than 3D models over structural properties of the contact guidance cue including fiber size and orientation. The most common 2D systems for
studying contact guidance include gratings coated with ECM, microcontact printed lines of ECM and epitaxial grown collagen fibers [7, 21-23]. 3D systems are more difficult to control and image through, but several have been devised including cell-based, flow-based and magnetic orientation of contact guidance cues [8, 20, 24-27]. Cell-based systems provide little tunable control over the orientation of the ECM and require decellularization. Flow-based systems commonly require microfluidic devices and small length scales over which ECM orientation occurs. Magnetic fields alone or in combination with iron oxide particles can generate aligned collagen networks. However, these require either large magnetic fields or particles that could perturb cell behavior. Another intriguing approach is to use rotational alignment imparted by an acupuncture needle [28, 29]. When an acupuncture needle is inserted into a collagen gel, collagen fibers adhere to the surface. During rotation collagen fibers are wrapped around the needle generating alignment of the fiber network. This approach has been used to both assess the role of acupuncture in eliciting cell biological responses [30] and has been used to show directional orientation of fibroblasts in oriented collagen gels [28, 29]. In this paper, we use rotational alignment of collagen fibers to assess contact guidance. We quantify the alignment of collagen fiber networks and use them to assess how mesenchymal and amoeboid cells differ in their ability to engage in contact guidance. Furthermore, we examine the effects of tuning extracellular stiffness and intracellular contractility on contact guidance fidelity and attempt to parse different contributions of these perturbations to both random and directional aspects of cell migration.
3.2 Material and Methods

3.2.1 Culturing Cells

MDA-MB-231 cells (human mammary basal/claudin low carcinoma cells, ATCC, Manassas, VA, USA) were cultured using Dulbecco’s Modified Eagles Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 2% glutamax (Gibco) and 1% penicillin/streptomycin (Gibco). Imaging media used for experiments consisted of DMEM lacking phenol red, supplemented with 10% FBS, 2% Glutamax, 1% penicillin/streptomycin, 1% sodium pyruvate (Gibco), and 12 mM HEPES (Sigma Aldrich).

MTLn3 cells (rat mammary basal adenocarcinoma cells, Jeffrey Segall, Albert Einstein College of Medicine and authenticated using IDEXX BioResearch, Westbrook, Maine, USA) were cultured using MEMα media (Sigma Aldrich) supplemented with 5% FBS and 1% penicillin/streptomycin. Imaging media used for experiments consisted of MEM alpha lacking phenol red, supplemented with 5% FBS, 1% penicillin/streptomycin, and 12 mM HEPES. Mycoplasma was tested using a MycoFluor mycoplasma detection kit (Invitrogen, Carlsbad, CA, USA) and no mycoplasma were detected.

3.2.2 Sample Preparation for Confocal Microscopy

Confocal experiments were prepared by mixing imaging media and non-pepsin-treated rat collagen type I solution (Corning, Corning, NY, USA) to a concentration of 2 mg ml⁻¹. A volume of collagen solution (125 µl) was pipetted into a MatTek dish. MatTek dishes are conventional 35 mm tissue culture dishes with a hole in the bottom that is covered with a glass coverslip. This coverslip is glued to the bottom, creating a shallow well (MatTek Corporation,
Ashland, MA, USA). Collagen is spread evenly on the bottom of the well, and allowed to polymerize for 45 mins. A 0.25 x 25 mm stainless steel acupuncture needle with a tapered tip (Hwato, Weymouth, MA, USA) was inserted through a 5 mm thick pad of polydimethylsiloxane (PDMS) (Dow Corning Corporation, Midland, MI, USA) to the depth of the well and placed on top of the collagen gel, sealed with food grade lubricant (The McGlaughlin Oil Company, Columbus, OH, USA). The needle was then rotated the required degrees slowly to limit the amount of gel tearing and cut with wire cutters.

### 3.2.3 Confocal Microscopy and Analysis

Confocal reflectance microscopy was used to determine the degree of collagen fiber alignment. At least four samples of each condition were imaged on a Leica SP5 X MP confocal/multiphoton microscope system with a 40X (NA = 1.25) oil immersion objective. Images with significant tearing, due to the needle tip dragging when it was cut were removed from analysis. In addition, images were discarded if the ripped area was larger than 25% of the total image area due to needle rotation. The images were then averaged using a Gaussian blur filter with a standard deviation of 2 and analyzed with the ImageJ plugin OrientationJ [31] with a cubic spline gradient and a Gaussian window of 4 pixels. The coherency and orientation images were then saved and used later in the analysis. A Matlab (Mathworks, Natick, MA, USA) script was written which identifies average collagen fiber orientation and builds a circumferential vector field. The coherency and orientation images were then used to compare the fiber orientation to a circumferential vector field using coherency cutoff of 0.2. The angle between the local collagen fiber orientation vector and a circumferential vector field is \( \theta \). The directionality of the fiber field was defined in the following way.
When the fiber orientation is circumferential, the $DI_f$ is 1 and when the fiber orientation is radial, the $DI_f$ is 0.

3.2.4 Cell Migration Sample Preparation

Cell migration experiments were conducted by mixing trypsinized cells at a concentration of at 600,000 cells ml$^{-1}$ in a 2 mg ml$^{-1}$ non-pepsin-treated rat collagen type I solution (Corning, Corning, NY, USA). The protocol is similar to the confocal experiments, however after polymerization the well is filled with 125 ml of imaging media specific to the cell type. The needle is either not rotated or rotated one full rotation (2$\pi$) and then cut by wire cutters. The samples are then placed in a 37 °C incubator. After 24 hr the samples are moved to a heating stage to keep the samples at 37 °C and imaged every 2 min for 8 hr. At least 3 samples over at least 2 different days compiled a complete data set.

3.2.5 Collagen Glycation Procedure

Collagen fiber stiffness was controlled by glycating collagen. Non-pepsin-treated rat collagen type I solution was diluted with ribose dissolved in nanopure water at a concentration of 200 mM, resulting in a collagen concentration of 7 mg ml$^{-1}$ and lowering the acetic acid concentration which the collagen is diluted in from 0.02 N to 0.015 N. This solution was allowed to react for 5 days at 4 °C. The glycation occurs through the reduction of sugar, crosslinking collagen fibers which increases the stiffness of the gel by enhancing collagen-collagen interactions without changing other important properties such as pore size or collagen density.
This collagen was then used in place of non-glycated collagen and the procedure was completed as previously described with alignment following collagen polymerization.

### 3.2.6 Contractility Inhibition

Contractility was inhibited by subjecting cells to Y-27632 (Calbiochem, Billerica, MA, USA) and blebbistatin (Sigma). Cells were suspended in a 1 µM solution of Y-27632 or 3 µM solution of blebbistatin prior to the 24 hr incubation. We have shown these concentrations to partially block contact guidance on 2D surfaces [6].

### 3.2.7 Cell Migration Analysis

Analysis of cell migration was conducted using the MTrackJ plugin in ImageJ (National Institutes of Health, Bethesda, MD, USA) which allows the tracking of cells on an image-by-image basis. The result of this is the $x$-$y$ coordinates of the cell at each time point. Two migration characteristics were calculated. The first was a random migration parameter, cell speed. The second was a directed migration parameter and quantifies the projection of the migration direction towards the collagen alignment, cell directionality. These points are then analyzed using a custom Matlab script which calculates the quotient of the distance the cell moves between each image and the time interval. When this is averaged over the entire timelapse, an average cell speed is calculated. A Matlab script was written which determines the cell directionality using a similar approach to measuring collagen fiber directionality. A cell’s movement direction over a time interval of 24 min was compared to the direction of a radial vector field originating from the needles center to calculate directionality using the following equation.
Aspect ratio was calculated by dividing the length of a cell by the width of that same cell.

### 3.2.8 Statistical Methods

Confidence intervals (95%) were calculated using Matlab. A two tailed $t$-test, $p \leq 0.05$, was conducted for experiments which only compared two conditions while an Anova test, $p \leq 0.05$ was conducted for experiments which had more than two conditions. These are used to identify statistical differences which were depicted with connecting bars over the particular conditions. Details of the number of samples and experiments are included in the figure legends.

### 3.3 Results

#### 3.3.1 Rotation of Acupuncture Needles Align Collagen Fibers

We have used a method which allows for direct manipulation of the alignment of collagen gels through the rotation of an inserted acupuncture needle resulting in a fibrous radial pattern extending from the needle surface to investigate the role of contact guidance on metastatic cancer cells. To achieve radial alignment of the collagen fibers an acupuncture needle is inserted into a gel and rotated clockwise (Figure 1A). Collagen adheres to the needle surface, creating mechanical attachment for force transfer between the needle and the gel. After needle rotation, the fibers should no longer exhibit a random orientation, but instead show directional orientation (Figure 1B&C). Fibers immediately near the needle should show circumferential alignment as the fibers are wound around the needle. Fibers further from the needle should show a radial
alignment with slight directional variations caused by the entangled, but incompletely mechanically coupled gel.

**Figure 3.1: Schematic of aligned collagen gels.** (A) An acupuncture needle is inserted into a collagen gel and rotated. A top view of the needle in the gel (B) before rotation and (C) after rotation. The scale bars are 1 cm.

Confocal reflectance microscopy was used to image the collagen fibers and gain an understanding of how the insertion of the needle and the time at which the needle is rotated affects the formation of the oriented collagen fiber field. We collected a series of images of gels without any needle insertion, with a needle, but without any rotation, with rotation before the collagen has fully polymerized into a gel, and with rotation after 45 mins of polymerization (Fig 2A-D). The images are taken within approximately 100 μm of the glass-gel interface. The needle is often shown as a bright white spot, although at times a dark hole is present, due to small movement by the needle in the z-direction away from the surface. Small changes in fiber directionality are difficult to discern visually, so fiber directionality ($DI_f$) was calculated using the cosine of the angle between the circumferential direction and fiber direction as a function of
distance from the center of the needle and averaged over several experiments. When fiber directionality has a value of 1, fibers are oriented circumferentially. When fiber directionality has a value of 0, fibers are oriented radially. The fiber directionality as a function of distance from the needle center for two conditions (no needle and a rotated needle before collagen is fully polymerized) is shown in Figure 2E. Fiber directionality changes as a function of the distance from the needle center, where regions close to the needle have higher fiber directionality (circumferential orientation) than regions further away from the surface (radial orientation). The surface of the needle falls within the dotted line in Figure 2E, which represents the average needle radius +/- the standard deviation. Fiber directionality to the left of this dotted box was ignored, because this represents a region within the needle and not actual collagen fibers. A slope can be used to characterize the change in fiber directionality as a function of distance from the needle center. When the needle is not inserted the slope is approximately 0 (Figure 2F). Needle insertion does not change the slope. However, needle rotation, even before full collagen polymerization results in a non-zero, negative slope, indicating some alignment. Furthermore, rotation after 45 min of polymerization results in a large negative slope (Figure 2F). In addition to slope, the fiber directionality at 100 μm from the needle center (DI_{100}) was calculated. Conditions that showed rotation resulted in the lowest DI_{100} values (Figure 2G). Next, we wanted to determine how different degrees of rotation affected collagen alignment (Figure 3A-F). When the needle was rotated one full rotation (2π), it resulted in both higher circumferential alignment closer to the needle (higher DI_f) and higher radial alignment away from the needle as compared to the no rotation control (higher DI_r, Figure 3G). All rotation magnitudes that we probed resulted in a large negative slope (Figure 3G). The DI_{100} values were much more variable and closer to the zero rotation condition, but also decreased (Figure 3I). However, while large
degrees of rotation showed dramatic radial alignment (Figure 3F), we observed more tearing of the gel either proximal or distal to the needle under this condition, so an intermediate level of rotation (2\(\pi\)) was used for the cell studies.

Figure 3.2: Confocal reflectance microscopy images of the alignment of the collagen gel under different needle conditions. Image of a fully polymerized collagen gel with (A) no needle, (B) a needle which has been inserted but not rotated (0\(\pi\)), (C) a needle which has been rotated (2\(\pi\)) prior to complete collagen polymerization (0 min) and (D) a needle which has been rotated (2\(\pi\)) at 45 min after the start of collagen polymerization. The scale bar is 50 \(\mu\)m. (E) Fiber directionality as a function of distance from the needle center for a gel with no needle (black circles) and a needle that has been rotated prior to complete collagen polymerization (grey squares). A linear fit of the fiber directionality from 25-140 \(\mu\)m is shown as a solid line, yielding a slope used in (F). An average fiber directionality centered 100 \(\mu\)m from the center of the needle (\(D_{100}\)) is marked with an arrow and used in (G). The dotted line represents the average needle radius +/- one standard deviation. The (F) slope of the fiber directionality curve for each conditions and (G) average fiber directionality centered 100 \(\mu\)m from the center of the needle (\(D_{100}\)) for the conditions shown in (A-D). The error bars represent 95% confidence intervals of the mean (\(N_{gels} > 4\)). Significance bars represent non-overlapping confidence intervals.
Figure 3.3: Confocal reflectance microscopy images of the alignment of the collagen gel under different numbers of rotations. Image of a fully polymerized collagen gel rotated (A) $0\pi$, (B) $\pi$, (C) $2\pi$, (D) $3\pi$, and (E) $4\pi$. The scale bar is 50 $\mu$m. A zoomed region in (E) is shown in (F). (G) Fiber directionality as a function of distance from the needle center for a gel rotated $0\pi$ (black circles) and $2\pi$ (grey squares). A linear fit of the fiber directionality from 25-140 $\mu$m is shown as a solid line, yielding a slope used in (H). An average fiber directionality centered 100 $\mu$m from the center of the needle ($D_{100}$) is marked with an arrow and used in (I). The dotted line represents the average needle radius +/- one standard deviation. The (H) slope of the fiber directionality curve for each conditions and (I) average fiber directionality centered 100 $\mu$m from the center of the needle ($D_{100}$) for the conditions shown in (A-E). The error bars represent 95% confidence intervals of the mean ($N_{gels} = 5$). Significance bars represent non-overlapping confidence intervals.

3.3.2 MDA-MB-231 Cells Respond to 3D Contact Guidance Cues, but MTLn3 Cells Do Not

It was important to identify the time over which cells attach and engage in contact guidance in the radially aligned gels. MDA-MB-231 cells were seeded in the gels and imaged prior to, immediately after, and at ~24 hr after rotation (Figure 4A-C). Elongation, as measured by cell aspect ratio, only occurred after ~24 hr after rotation (Figure 4G). This confirms that the act of rotating the needle isn’t responsible for the elongation of the cells within the gel. To further
confirm the claim that the needle rotation had no effect on cell morphology, a needle was inserted and left unspun for 24 hours allowing the cells to interact with the ECM. The cells were then imaged prior to, immediately after, and at ~24 hr after rotation (Figure 4D-F). Little difference is seen in the aspect ratio after cells have attached to the ECM, indicating that the mechanical movement of collagen fibers does not act to elongate cells, even when they are well-attached to the ECM (Figure 4G).

**Figure 3.4: Aspect ratios of MDA-MB-231 cancer cells.** Phase contrast images of MDA-MB-231 cells (A) before $2\pi$, (B) after $2\pi$, (C) at $\sim 24$ hr after $2\pi$ needle rotation at $t = 0$, (D) before $2\pi$, (E) after $2\pi$, and (F) at $\sim 24$ hr after $2\pi$ needle rotation at $t = 24$ hr. The scale bar is 50 $\mu$m. (G) Aspect ratios of conditions shown in (A-F). The experimental scheme is shown below. The error bars represent 95% confidence intervals of the mean ($N_{\text{experiments}} = 3$, $N_{\text{cells}} > 300$). Lines represent statistical significance using an Anova test, $p \leq 0.05$. 


Armed with the information that the collagen gel can be aligned by rotating an acupuncture needle and that cell elongation occurred ~24 hr after rotation, we next wanted to see if the collagen alignment induced contact guidance. We measured contact guidance by analyzing the directionality of cells using live cell imaging. Figure 5A&B show the cell tracks from three different experiments after $0\pi$ and $2\pi$ needle rotations. Cells migrate under both conditions, but the tracks are in the direction of the needle in the gels where needles have been rotated $2\pi$ and where radial collagen alignment with respect to the needle occurs (Figure 5B, black arrowhead). Tracks of cells in gels can be persistent, but not directed to the needle with no radial collagen alignment (Figure 5A, black arrowhead). Furthermore, while cells are elongated when the collagen gel is not radially aligned (Figure 5C-F), radial collagen alignment produces persistent migration toward or away from the needle (Figure 5G-J). There appears to be only a slight bias for migration towards the needle. Cell speed was quantified and calculated under both conditions (Figure 5K). Cell migration speed was over 2-fold faster under radial alignment conditions (Figure 5K). In addition to changes in cell speed, cell directionality was dramatically increased when the needle was rotated $2\pi$ and the collagen gel was radially aligned (Figure 5L). The directionality when the needle was not rotated was not statistically different from 0, a value of directionality for random migration. We have shown contact guidance differences between MDA-MB-231 and MTLn3 cells on 2D collagen fibril forming substrates, attributed this in part to their different migratory modes (mesenchymal vs. amoeboid) [7]. Given this difference seen in 2D, we were interested in whether this relationship was seen in 3D, so we probed contact guidance in MTLn3 cells.
Figure 3.5: Cell migration analysis of MDA-MB-231 cells. MDA-MB-231 cell tracks for (A) \(0\pi\) and (B) \(2\pi\) needle rotations. White circles indicate needle locations, while different colors (green, red and yellow) represent different experiments. The diameter of the circle is about 60 \(\mu\)m. Phase contrast images of MDA-MB-231 cells migrating in a collagen gel rotated by (C-F) \(0\pi\) or (G-J) \(2\pi\) taken at time 0, 2, 4, and 6 hr and outlined with a yellow dashed line. The scale bar is 50 \(\mu\)m. (K) Cell speed and (L) directionality of the cells in collagen gels rotated \(0\pi\) and \(2\pi\). Error bars are 95% confidence intervals of the mean (\(N_{\text{experiments}} = 3\) and \(N_{\text{cells}} > 105\)). Lines represent statistical significance using a two tailed t-test, \(p \leq 0.05\).

Amoeboid MTLn3 cells were subjected to the same conditions as the mesenchymal MDA-MB-231 cells (Figures 6A-D). The differences in morphology are easy to see. MDA-MB-231 cells are spindle-shaped with multiple extensions in the front and usually one tail (Figure 6A&C). MTLn3 cells on the other hand are relatively round with few long extensions, even in the presence of radially aligned collagen fibers (Figure 6B&D). Two possibilities could explain this. First, MTLn3 cells could be non-migratory within the collagen gel. However, MTLn3 cells did migrate and at speeds around those seen for MDA-MB-231 cells (Figure 6E). Second, MTLn3 cells could be highly directional even though they are not well attached to the collagen matrix. However, the directionality of MTLn3 cells was not sensitive to whether the collagen gel was radially aligned and the directionality was similar to that seen for MDA-MB-231 cells in
unaligned collagen gels (Figure 6F). The value for directionality of the MTLn3 cells is not statistically different from 0, which is the value of directionality for random migration. Aspect ratios of the MDA-MB-231 and MTLn3 cells also point to the differences in migration mode (Figure 6G). MTLn3 cells have aspect ratios around 1, which correspond to a circular geometry, whereas MDA-MB-231 cells tend to be about 4-6 times longer than they are wide.

**Figure 3.6:** Comparing contact guidance of MDA-MB-231 and MTLn3 cells. Phase contrast images of (A&C) MDA-MB-231 and (B&D) MTLn3 cells in a collagen gel rotated by (A&B) $0\pi$ or (C&D) $2\pi$. The scale bar is 50 µm. Cell (E) speed, (F) directionality, and (G) aspect ratio. Error bars are 95% confidence intervals of the mean ($N_{\text{experiments}} = 3$ and $N_{\text{cells}} > 105$). Lines represent statistical significance between conditions with at least one common condition parameter using an Anova test, $p \leq 0.05$. 
3.3.3 Role of Extracellular Stiffness and Intracellular Contractility in Driving 3D Contact Guidance

We next wanted to investigate how ECM stiffness affects contact guidance of MDA-MB-231 and MTLn3 cells. To achieve this end we used non-enzymatic glycation to stiffen collagen gels [32, 33]. This allowed us to use the same collagen concentration while increasing the collagen stiffness independently. Furthermore, collagen glycation did not seem to alter the structural alignment caused by needle rotation (Figure 7E&F). Given the results mentioned above, we were curious as to whether increases in stiffness affected migration in both cell types. Changes in stiffness through glycation did not seem to alter the morphology greatly (Figure 7A-D). Cell migration speed in MDA-MB-231 cells increased in unaligned and glycated collagen networks as compared to unaligned networks alone, but decreased in aligned and glycated collagen networks as compared to aligned networks alone (Figure 7G). On the other hand, glycation resulted in increasing MTLn3 cell speed in both unaligned and aligned collagen networks (Figure 7J). Interestingly, this effect on speed looks to be approximately additive, where the increase in speed in glycated or aligned collagen networks can be summed to predict the speed for glycated and aligned collagen networks. Both directionality and elongation were diminished in MDA-MB-231 cells with collagen glycation and increased stiffness, indicating that the directional aspect of contact guidance is sensitive to network stiffness (Figure 7H-I&K-L).
After examining how cells respond to a stiffer collagen matrix, we wanted to determine if internal changes in myosin II-mediated contractility mirrored changes in contact guidance seen with external changes in matrix stiffness. Indeed, we and others have shown that myosin inhibition alters directional migration on 2D contact guidance cues [6]. To achieve this, MDA-MB-231 cells were exposed to two contractility inhibitors, blebbistatin (a myosin II inhibitor) and Y-27632 (a Rho kinase inhibitor) and seeded in unaligned and aligned collagen gels (Figure 8A-D). We chose inhibitor concentrations that are known to only partially block contractility [6].
Both drugs eliminated the speed differences seen between unaligned and aligned collagen networks, although Rho-kinase inhibition resulted in lower speeds (Figure 8E). However, the most striking result of limiting the contractility is the marked decrease in directionality in response to Y-27632 (Figure 8F). Y-27632 almost completely transformed the response of MDA-MB-231 cells to an aligned collagen network to that of an unaligned collagen network (Figure 8F). Blebbistatin on the other hand was much less effective in decreasing both speed and directionality. The response of aspect ratio to contractility inhibitors was similar to directionality, indicating that elongation in aligned collagen networks is a likely proxy for directionality (Figure 8G). This can be more easily shown when aspect ratio and directionality for all conditions probed above are plotted against each other for both unaligned (grey) and aligned (black) collagen gels (Figure 9A). A clear linear and increasing relationship is seen in aligned collagen networks, whereas no linear relationship is seen in unaligned collagen networks across all conditions.
Figure 3.8: Examining the effect of contractility inhibitors on MDA-MB-231 cells. Phase contrast images of MDA-MB-231 cells in unaligned (A&B) and aligned (C&D) collagen networks treated with 3 μM Blebbistatin (A&C) or 1 μM Y-27632 (B&D). The scale bar is 50 μm. Cell (E) speed, (F) directionality, and (G) aspect ratio. Error bars are 95% confidence intervals of the mean (N_experiments = 3 and N_cells > 105). Lines represent statistical significance between conditions with at least one common condition parameter using an Anova test, p ≤ 0.05.
Figure 3.9: Compilation of speed, directionality and aspect ratio across all conditions. These data are replicated from previous figures and presented differently. (A) Directionality and aspect ratio for aligned (black) and unaligned (grey) across control, glycated collagen and contractility inhibited MDA-MB-231 and MTLn3 cells. (B) Directionality and speed for MTLn3 cells under glycation (g) and aligned (2π) conditions. (C) Directionality and speed for MDA-MB-231 cells under glycation (g) and aligned (2π) conditions. (D) Directionality and speed for MDA-MB-231 cells under blebbistatin (b), Y-27632 (Y) and aligned (2π) conditions. Error bars are 95% confidence intervals of the mean (N_{experiments} = 3 and N_{cells} > 105).

3.4 Discussion

In this paper we present results assessing contact guidance in a 3D ECM environment. 2D systems, while shedding some light on how cells engage in contact guidance, neglect many important characteristics observed in vivo. Rotational alignment of the ECM using acupuncture needles has several advantages [28, 29]. First, rotational alignment is relatively simple to achieve, requiring only acupuncture needles. Second, while we did not demonstrate quantitative tuning here, the degree of alignment might be controlled through the number of rotations or mechanical attachment to the collagen. The alignment relies heavily on adhesion of collagen fibers onto the acupuncture needle. Future work might include optimizing the needle surface in
order to tune the mechanical linkage between the needle and the collagen network. This linkage may be dependent on size, material, and geometry of the needle which has yet to be studied with respect to alignment of collagen networks. Third, this approach allows for local manipulation of the collagen fiber alignment. Many of the other techniques mentioned in the introduction result in global alignment of collagen or other ECM. Several of these advantages can be leveraged to understand or control the behavior of cells in ECM. For instance, in the tumor microenvironment (TME), collagen is frequently present in combination with other ECM components such as fibronectin and hyaluronan. Since it is relatively easy to simultaneously image the collagen field during rotation, propagation lengths of collagen alignment in these composite environments with various types and degrees of crosslinking would give a clearer picture of the micromechanical properties of the ECM in the TME. An important note to make is that this method does have limitations as a model. The ECM within the tumor is much more complex and includes a number of cellular and ECM species, which are not currently addressed. Having said this, the contact guidance behavior we see does correlate with invasion and metastasis seen in side-by-side comparisons of the cell lines used here [34]. Since we use a PDMS pad to steady the needle during rotation, it is likely that this approach could be easily combined with microfluidic chambers that present gradients across 3D gels, migration responses to multi-cue environments like those present in the TME could be assessed [21]. Because of the local nature of this alignment approach, different combinations of directional cues (parallel, orthogonal, etc.) could be probed simultaneously.

Along with the potential to use this contact guidance system in other contexts, we show in this paper that MDA-MB-231 cells sense contact guidance cues and MTLn3 cells do not. Several previous papers have indicated that different cell types sense 2D contact guidance cues
differently. We and others have attributed this to motility mode, particularly in the context of cancer cell migration [6-8, 35]. Our previous work conducted using epitaxially grown collagen fibrils, a 2D contact guidance cue, resulted in MDA-MB-231 cells, a mesenchymal mode migrator, showing a high directionality while MTLn3 cells, an amoeboid mode migrator, showed low directionality [7]. Directionality measured in the 2D system is about 2 fold higher than that measured in the 3D system here. Two possible explanations seem reasonable. First, the contact guidance cue is only presented to the ventral plasma membrane, allowing for a more robust cytoskeletal organization along the fiber alignment direction [36]. Second, the degree of fiber alignment in 3D is less than that in 2D. Recently, another group has indicated that motility mode difference explains variation in 3D contact guidance fidelity [8]. Cancer stem cells with more amoeboid like morphology poorly follow contact guidance cues, whereas mesenchymal mode cells follow the directional cue well. What causes this difference between motility modes? It is likely the adhesion characteristics. Mesenchymal cells which adhere to the ECM more firmly can extend protrusions along collagen fibers and stay in physical contact with the ECM for longer times. Amoeboid cells on the other hand follow the pore structure. However, it is not obvious if there is anisotropy of pore orientations or sizes in aligned collagen fiber networks. Cells are known to move in paths where matrix has been degraded [37], but in the absence of mesenchymal cells that actively degrade the matrix and that engage in contact guidance, it is unlikely that these directional pores exist. This diversity in response of mesenchymal and amoeboid cells likely impacts our understanding of metastasis. Because cells don’t respond similarly to the same aligned ECM cue, predicting metastasis may depend on knowing the motility mode. Appropriate adhesion, contraction or matrix degradation biomarkers of motility mode could be used. However, it has also been shown that cancer cells are relatively plastic and
can switch motility modes [37]. Consequently, ECM alignment may select from one or several motile populations of cancer cells for cells that use the mesenchymal mode of motility [3]. This selection results in a different population of cells as secondary sites and might require different therapeutic approaches.

When plotting cell migration speed vs. directionality, one can see that speed and directionality are not necessarily coupled processes as has been observed in 1D migration [38]. We see similar effects in 2D, where directionality and speed can be jointly or individually controlled depending on the perturbation [6]. The directionality of MTLn3 cells did not increase upon radial fiber alignment (2π). However, the speed did (Figure 9B). Furthermore, glycation (g) produced about the same speed increases as radial fiber alignment. Both the mechanical properties of the aligned fiber field as well as the local density could be higher. Others have reported about a 5-fold increase in Young’s modulus in aligned fiber fields [20]. In addition, we noticed slight increases in the signal of collagen close to the needle placement. This agrees with previous reports that indicate that radial alignment by acupuncture needles increases local collagen density [28]. The change in elastic or compressive modulus seen with glycation is on the order of 3-10 fold [32], so speed changes with collagen alignment could be associated with changes in elastic or compressive modulus. It is interesting to note that the changes in speed appear to be additive as glycated (g), aligned collagen fibers (2π) result in the largest speed (Figure 9B). Consequently, MTLn3 cells under our control conditions likely reside on the upslope of the biphasic migration speed curve. MDA-MB-231 cells increase their speed in both glycated and aligned collagen networks, however the response is not additive (Figure 9C). Perhaps MDA-MB-231 cells are closer to the migration maximum, so increasing the stiffness by
both glycating and aligning the collagen network actually results in a lower migration speed due to crossing over the maximum on the biphasic migration speed curve.

While collagen glycation acts to increase speed, it also has interesting effects on directionality. Aligned collagen fibers that are glycated (g) and that are consequently stiffer act to diminish contact guidance fidelity when compared to collagen alone (Figure 9C). Why do stiffer environments result in a directionality decrease? Cells appear to locally organize collagen fibers when migrating through collagen networks [39-41]. This may act as an amplification mechanism turning a modestly aligned collagen network into highly aligned cell migration, resulting in high directionality. If the environment is stiffer, this amplification mechanism may be diminished leading to lower directionality. In addition, matrix degradation can act to soften the local environment [42]. Consequently, inhibiting matrix degradation would act to decrease the ability of cells to locally rearrange collagen fibers resulting in lower directionality. Indeed, we have seen that the addition of TIMPs act to degrade directionality in 3D with perhaps more modest effects on 2D contact guidance cues [43].

Finally, as in 2D environments, it appears that myosin II regulatory light chain phosphorylation through Rho kinase has a substantial role in tuning contact guidance fidelity. When myosin II is partially inhibited with sub-maximal doses of blebbistatin [6], there is a small decrease in both speed and directionality (Figure 9D). However when Rho kinase is partially inhibited with sub-maximal doses of Y-27632 [6], directionality is dramatically altered. This may indicate that Rho kinase is critically important during migration of mesenchymal cells in 3D. Contractility, through Rho kinase has long been known to be important in migration [44-47]. Myosin contractility is not required for the initial stages of spreading [48] and other cytoskeletal proteins that govern protrusion can modulate contact guidance [49]. However, we and others have recently shown...
Rho-kinase’s role in modulating directionality in contact guidance separate from migration speed [6, 20]. Rho kinase inhibition decreases contact guidance fidelity [6, 20], likely by altering adhesion through focal adhesions [35]. This is potentially interesting as contractility is likely modulated by soluble factors in the TME, creating the possibility that contact guidance response can be attenuated. Thus, contact guidance depends intimately on the degree of alignment of the ECM, however different cells respond differently to contact guidance cues and both extracellular factors like ECM stiffness as well as intracellular factors like Rho kinase-mediated contractility can modulate the contact guidance response.

3.5 Conclusions

We have investigated contact guidance in 3D using an effective method of radially aligning collagen fibers towards a rotated acupuncture needle. MDA-MB-231 (mesenchymal) cells show a high degree of directional response to the contact guidance cue while MTLn3 (amoeboid) cells do not migrate directionally, behavior that is seen in 2D contact guidance systems. However, MTLn3 cells were not immune to changes in migration. Cell speed increased when the collagen network was aligned or when the stiffness was increased through glycation, suggesting additional changes in the collagen network occur separate from collagen fiber alignment after acupuncture needle rotation. In fact, changes in migration speed brought on by collagen alignment seemed to be additive with respect to changes brought on by glycation. Increasing the stiffness of the aligned collagen network by through glycation marginally decreases directionality in MDA-MB-231 cells. However, lowering intracellular contractility, particularly through Rho kinase drastically decreases directionality in MDA-MB-231 cells, but unlike in 2D contact guidance environments cell directionality and speed are more tightly coupled. This
suggests modulation of the contractile apparatus more potently affects contact guidance than modulation of extracellular mechanical properties of the contact guidance cue.

3.6 Acknowledgements

We acknowledge Margaret Carter at the Confocal and Multiphoton Facility at Iowa State University with help in imaging samples with confocal reflectance microscopy. Anai Perez was supported by an NSF-REU [1560012]. This work was supported by the National Institutes of Health/National Cancer Institute [R03CA184575] and National Institutes of Health/National Institute for General Medical Sciences [R01GM115672]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

3.7 References


CHAPTER 4: PANCERATIC CANCER AND STELLATE COMMUNICATION RESULTS IN ALTERED CONTACT GUDANACE AND COLLAGEN FIBER ALIGNMENT

Abstract

Pancreatic cancer has one of the lowest 5 year survival rates (3-5%), which is, in part, due to poor diagnostics and treatment options. Recently, aligned fibers have been seen oriented away from the edge of the tumor within the pancreas. This suggests that contact guidance, directional migration in response to aliened ECM fibers, may be critical to the early stages of metastasis. In this paper, rotational alignment of collagen gels was used to study communication between pancreatic cancer (PC) and pancreatic stellate (PS) cells under contact guidance. PS cells migrated directionally in monoculture, however when co-cultured the directionally vanished. PC cells never showed contact guidance. Mucin 4 is a large glycoprotein which is a known communication modulator overexpressed in pancreatic cancer. The MUC4 gene was knocked out of the PC cells (PC muc4 KO) which resulted a return of directionality to the PS cells but still did not produce contact guidance within the PC cells when co-cultured. Co-culturing PS and both PC cells always resulted in faster cell speeds. Second Harmonic Generation was used to investigate collage reorganization. PS and PC cells, when monocultured and co-cultured with each other, resulted in enhanced collagen fiber alignment. PC muc4 KO, when monocultured or co-cultured with PS cells, did not alter the alignment or disrupted it and returned it to an unaligned state. F-actin intensity and MMP activity were shown to be higher in co-cultured systems. When F-actin was inhibited in co-culture conditions, alignment was neither enhanced nor disrupted. When MMP-14 was inhibited in PS+PC conditions alignment, was
disrupted and returned to an unaligned state. When MMP-14 was inhibited in PS+PC muc4 KO conditions, alignment was neither enhanced nor disrupted. This simple rotational alignment technique allows for easy studying of fiber reorganization and study of therapeutics on ECM alignment. Inhibition of MMPs, highlighting MMP-14, seems to be a promising therapeutic target to disrupt alignment around the tumor.

4.1 Introduction

Pancreatic cancer has one of the lowest 5 year survival rates (3-5%), which is, in part, due to poor diagnostics and treatment options [1]. Plaguing this, as with most types of cancers, is the lack of understanding between how the stromal compartment changes over time and its effect on cancer cell invasion [2]. It is believed that pancreatic cancer and stellate cells communicate, effecting migration characteristics and the ability to reorient the matrix, two functions related to metastasis [3, 4]. Reorientation of collagen fibers perpendicular to the tumor interface by pancreatic stellate cells may lead to directed migration [5]. These two types of cells are so closely tied that some have proposed targeting stellate cells within the tumor with therapies as a means to combat the metastatic nature of the cancer [6]. However, how these cells communicate and through which pathways are currently unclear. Recently, the mucin family, particularly mucin 4 (muc4), has been identified as highly expressed in pancreatic tumors which has many focusing on them as a way to detect the presence of a tumor[7, 8]. Another way cells communicate is through reorganization of the extracellular matrix (ECM), mediated by matrix metalloproteinases (MMPs). The remodeling can result in dense ECM composed of aligned collagen fibers.
There are a few ways by which aligned fibers can be shaped *in vivo*. These include fiber assembly and deposition, reorganization, and degradation through the use of matrix metalloproteinases (MMPs) [9-12]. MMPs are calcium dependent zinc-containing endopeptidases [13, 14]. There are 23 human MMPs, of which 17 are soluble, and are responsible for degrading the ECM. The fact that MMPs are used to help reorganize the matrix makes them an area of interest with regards to cancer research, as the production of MMPs are typically upregulated in cancer cells [15, 16]. MT1-MMP, or MMP-14, is a membrane bound collagenase which plays an important role in metastasis, as it supports invasiveness and enhances cell speed [17, 18]. Cell-cell communication, such as between stellate and cancer, has been shown to also enhance MMP activity which further indicates that increasing the complexity of *in vitro* models is paramount [19, 20]. MMP-14 has also been shown in some studies to induce significant collagen remodeling by stromal cells in collagen-rich peritoneal microenvironment of ovarian tumors, which has been linked to markers for the metastatic progression of cancer [37, 38]. MMP-14 has also been shown to influence TGF-β signaling in stellate cells, as well as the expression of alpha smooth muscle actin, both of which caused up-regulation of collagen deposition [39]. In cases of chronic obstructive pulmonary disease, upregulated MMP-14 was linked to gel-forming mucin 5AC which causes aggravation of respiratory difficulties in patients. These studies were shown by observing how acrolein binds to specific binding sites in the MMP-14 protein instigating cell signaling cascades that result in increased goblet like mucus in the airways of the animal subjects [40]. In contrast, mucin-1 shedding by MMP-14 was observed in studies where human epithelial uterine cells were found to be exposed to tyrosine phosphatase inhibitor treatments like pervanadate stimulation [41]. To this end co-culturing cells has been
shown to affect not only MMP activity but migration characteristics, proliferation, and cell viability [21-23].

A known biomarker of pancreatic cancer, mucin 4, is a large polysaccharide which has been identified as being overexpressed in pancreatic cancer and may be responsible for modulating cancer and stellate cell communication [7]. Mucins are known to regulate the detachment of cells from the primary tumor and play a large role in adhesion at metastatic sites. Their large size blocks integrin-mediated adhesion to the ECM, which creates cells with lose attachments to the ECM and fast migratory behavior. It is believed that knocking out the MUC4 gene may limit the metastatic ability of pancreatic cancer cells as it will result in stronger attachments to the ECM [24-26]. There is also evidence which suggests that mucins activate MMPs, in particular MMP-9 and MMP-7 [27]. There is some indication of MMP-14 upregulation through ROCK which occurs coincidently with MUC4 [28].

Aligned collagen fibers are a marker for poor prognosis in PC [29]. Within or near the tumor, aligned collagen provides an external cue which can be sensed by cells and leads to migration in the direction of the long axis of the fibers, a process called contact guidance [30-33]. This process relies on the cell’s ability to adhere to the extracellular matrix (ECM) and generate contractile forces [34]. There has been much work done on understanding how cells interpret contact guidance cues on 2D surfaces, [35-42] however there is much less work done in 3D systems. While basic concepts, such as identifying what cells have a predilection to migrating in response to a contact guidance cue can be discovered in 2D systems, 2D environments do a poor job in modeling a complex tumor microenvironment (TME). This is due to the fact that 2D models often deal with monolayers while in vivo conditions are typically spheroid. 2D cultures also struggle with mimicking cell-cell and cell-ECM contact, because they differ inherently in
topography, confinement and frequently in mechanical properties. Other common techniques are beginning to emerge as reliable methods to produce aligned fibers in 3D gels and include magnetic bead alignment, cell-based and flow based systems, and mechanical stress [43-47]. While these are reasonably effective, they each have downsides. It is difficult to achieve alignment using magnetic beads. Cell-mediated is also difficult to use as it relies on cells migration into the matrix. Microfluidics have been gaining popularity, however they require complicated setups. A stromal rearrangement assay could be very useful in studying the potential for tumor stromal alignment regression. A novel method was presented in the previous chapter, which uses acupuncture needles to transmit a force to the collagen network by twisting aligned fibers forming a radial pattern of collagen alignment. This technique provides a simple method for inducing a contact guidance cue reliably in 3D.

Anchoring these topics is the need for understanding how PS and PC cells communicate and how muc4 modulates that communication to promote metastasis. Mucin 4 is known to be important in migration but its role in contact guidance is unknown. Pancreatic stellate and cancer cells communicate, but how this affects contact guidance and ECM remodeling is unknown. MMPs are important, but how mucin 4 controls them to regulate contact guidance and ECM remodeling is now known. We begin to answer these questions through the use of a simple fiber aligning technique to see how cancer and stromal cells interpret a contact guidance cue in aligned ECM.
4.2 Material and Methods

4.2.1 Culturing Cells

Cells used were human pancreatic stellate cells (PS) and Capan-1 human pancreatic cancer (PC) cells (ATCC® HTB-79™) [48]. Genetic editing using CRISPR-Cas9 to knock out the MUC4 gene in the PC resulting in a cell which does not produce mucin 4 (PC muc4 KO). Cells were cultured using Dulbecco’s Modified Eagles Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 2% glutamax (Gibco) and 1% penicillin/streptomycin (Gibco) referenced in the future as complete DMEM. Imaging media used for experiments consisted of DMEM lacking phenol red, supplemented with 10% FBS, 2% Glutamax, 1% penicillin/streptomycin, and 12 mM HEPES (Sigma Aldrich).

The MUC4 expression in the pancreatic cancer cells were silenced using Crisper-cas9 gene editing system. The guide RNA sequence (GCGAGTGGCACAACCTTC) targeting exon 2 was cloned in pSpCas9BB-2A-GFP (PX458) vector. The insertion of the gRNA was confirmed by sequencing. The Capan1 pancreatic cancer cells were transfected with PX458 vector containing gRNA using lipofectamine 2000. After 48h post-transfection, the GFP positive cells were sorted in 96 well plate as a single clone. The clones were subsequently sub-cultured and probe for MUC4 expression. The clones negative for MUC4 expression (due to change in the reading frame during DNA repair) were selected and used for the study as a single clone or pooled population.
4.2.2 Assembling 3D Collagen Structures

To achieve alignment in 3D collagen gels, samples were prepared by mixing imaging media, with suspended cells if the sample required cells, and non-pepsin-treated rat collagen type I solution (Corning, Corning, NY, USA) to a concentration of 2 mg ml\(^{-1}\). A volume of solution (125 µl) was pipetted into a MatTek dish (MatTek Corporation, Ashland, MA, USA), spread evenly on the bottom of the well, and allowed to polymerize for 45 mins. A 0.25 x 25 mm stainless steel acupuncture needle (Hwato, Weymouth, MA, USA) was inserted through a 5 mm thick pad of polydimethylsiloxane (PDMS) (Dow Corning Corporation, Midland, MI, USA) to the depth of the well and placed on top of the collagen gel, sealed with high vacuum grease (Dow Corning, Midland, MI, USA). The needle was then rotated the required degrees slowly to limit the amount of gel tearing and cut with wire cutters.

Cell migration experiments were conducted by mixing trypsinized cells at a concentration of at 600,000 cells ml\(^{-1}\), with co-culture experiments using 300,000 cells ml\(^{-1}\) of each cell line, in a 2 mg ml\(^{-1}\) non-pepsin-treated rat collagen type I solution (Corning, Corning, NY, USA). The protocol is similar to the SHG experiments, however after polymerization the well was filled with 125 ml of imaging media specific to the cell type. The needle is either not rotated or rotated one full rotation (2π) and then cut by wire cutters. The samples are then placed in a 37 °C incubator. After 24 hr, the samples were moved to a heating stage to keep the samples at 37 °C and imaged every 2 min for 8 hr. At least 3 samples over at least 2 different days compiled a complete data set.

Inhibition assays were prepared the same way as described, except the inhibitor was added to the mixture prior to polymerization. To inhibit MMP-14 an antigen-binding fragment (FAB) called 3D9 was added to the mixture of cells, media, and collagen prior to polymerization.
at a working concentration of 500 nM [49]. To inhibit contractility the myosin II inhibitor blebbistatin (Sigma Aldrich) was used at a working concentration of 30 μM was added [40].

4.2.3 Assembling 2D Collagen Substrates

Collagen fibrils were epitaxially grown on 15 mm x 15 mm pieces of muscovite mica (highest grade VI, Ted Pella, Redding, CA, USA) that were freshly cleaved using tape [41]. Rat tail collagen type I was diluted (BD Bioscience, 10 μg ml⁻¹) in the buffer solution consisted of 50 mM Tris-HCl (Fisher Scientific) and 200 mM KCl (Fisher Scientific) at pH 9.2. After incubation of 18 h the collagen solution was washed with deionized water, the mica was laid against the edge of a tissue culture dish and the mica was allowed to dry overnight and was used the next day.

Each cell line was plated at 40,000 cells ml⁻¹ in 2 ml of media in 35 mm dishes and incubated for 4 hours on organized type I collagen substrates in imaging media. Mica with type I collagen fibrils and cells attached to the fibrils were inverted onto two strips of double sided tape attached to a microscope slide to generate a flow chamber. The chamber was filled with imaging media and sealed with VALAP. Chambers were imaged by phase contrast microscopy on a heated stage at 37 °C every 2 minutes for 12 hours. Images were captured at 10× (NA 0.50, Nikon) with a charge-coupled device (CoolSNAP HQ2, Photometrics) attached to an inverted microscope (Ti-E, Nikon) that was driven by μManager [50].

4.2.4 Assessing Collagen Alignment

Second harmonic generation (SHG) was used to determine the degree of collagen fiber alignment. The samples were imaged using a mode-locked Ti:Sapphire laser (100 fs pulse width, 1 kHz repetition rate, Libra, Coherent, Santa Clara, CA) that produces an 800 nm fundamental.
The average power at the sample image plane was controlled using a combination of a half-wave plate and a Glan-Thompson polarizer (Thorlabs, Newton, NJ). Second harmonic signal was collected in the transmission mode. For this setup, an inverted microscope (AmScope, Irvine, CA) and Nikon Plan Fluorite objective (20 x, 0.50 NA, 2.1 mm WD, Nikon, Melville, NY) was used to focus the beam and the SHG transmission was collected with a Nikon water immersion objective (40 x, 0.8 NA, 3.5 mm WD, Nikon, Melville, NY). The transmitted SHG signal was reflected by a dichroic mirror (FGB37M, Thorlabs, Newton, NJ) and separated from the fundamental beam with a short pass filter < 450nm (FGB37M, Thorlabs, Newton, NJ) and 808 nm notch filter (NF-808.0-E-25.0M, Melles Griot, Rochester, NY), before detection by an intensified CCD (iCCD, iStar 334T, Andor, Belfast, UK). Polarized SHG imaging was conducted using a Glan-Thompson polarizer and a half-wave plate mounted on a motor driven rotational stage (Thorlabs Newton, NJ) to achieve linear polarization. Images of the samples were collected every 10° from 0° to 350°. A minimum of three images for each experimental condition was taken. From this collection of images, regions of interest (ROI) were fit using the following equation:

\[
I_{\text{SHG}} = c \cdot \left\{ \sin^2(\theta_e - \theta_o) + \left( \frac{\chi_{xxx}}{\chi_{zzz}} \right) \cos^2(\theta_e - \theta_o) \right\}^2 + \left( \frac{\chi_{xxz}}{\chi_{zzz}} \right)^2 \sin^2(2(\theta_e - \theta_o)), \tag{1}
\]

where \( \frac{\chi_{xxx}}{\chi_{zzz}} \) and \( \frac{\chi_{xxz}}{\chi_{zzz}} \) are second-order susceptibility tensor element ratios, \( \theta_e \) and \( \theta_o \) are incident polarization angle and collagen fiber angle, respectively. The orientation angle of collagen in each ROI was calculated and a histogram was generated. Collagen organization was calculated using the full-width at half-maximum (FWHM) of a Gaussian fit to the histogram. The different ROI were classified as ordered, non-ordered and non-collagen, to evaluate the micro-scale ordering of the collagen fibers in the samples.
4.2.5 Assessing Directed Cell Migration

Analysis of cell migration was conducted using the MTrackJ plugin in ImageJ (National Institutes of Health, Bethesda, MD, USA) which allows the tracking of cells on an image-by-image basis [51]. A total of at least 90 cells over 3 experiments were chosen randomly from the monoculture samples. PC cells were labeled with Cell Tracker Green (CTG) (ThermoFisher Scientific, Waltham, MA) at a dilution of 1.5:1000 in imaging media and incubated for 3 hr before being trypsinized and mixed with one of the PC cell lines for the co-culture samples. A total of at least 27 cells of each cell type over 3 experiments for more than 81 cells were chosen randomly from the co-culture samples. The result of this is the \(x-y\) coordinates of the cell at each time point. These points are then analyzed using a custom Matlab script which calculates the mean square displacement using the following equation:

\[
< MSD > = (x_i - x_{i+1})^2 + (y_i - y_{i+1})^2
\]  \hspace{1cm} (2)

where \(<MSD>\) is the mean square displacement, \(x_i\) and \(y_i\) are the x and y displacements between two times, and \(i\) is the image number [52]. The mean square displacement can be used to calculate the average speed using the following equation:

\[
S_i = \frac{1}{N} \sum_{l}^{N} \frac{\sqrt{<MSD>}}{\tau}
\]  \hspace{1cm} (3)
Where $S_i$ is the instantaneous speed, $N$ is the number of time intervals for each cell and $<\text{MSD}>$ is the mean square displacement, and $\tau$ is the time step ($t_{i+1} - t_i = \tau$). For these experiments, each $i$ step is a time interval of 24 mins. To evaluate the motility coefficient, which was only done in 3D, we first had to decompose the migration vector into two vectors, one parallel with the fiber field and one orthogonal to the first. This allowed us to look at the motility coefficient in the direction of the alignment vs perpendicular at any location within the matrix. A program in Matlab was written which took the cell steps, every 24 mins, and broke them up into a parallel and orthogonal step and exported them to excel. Next, a model based speed and persistence time were calculated for the new parallel or orthogonal cell tracks using a 1D model and the mean square displacement using the following equation:

$$<\text{MSD}> = n S_m^2 \left[ P_m t - P_m^2 \left( 1 - e^{-\frac{t}{P_m}} \right) \right]$$

(4)

where $<\text{MSD}>$ is the means square displacement, $n$ is the number of dimensions, $S_m$ is the model based speed for the parallel or orthogonal steps, $P_m$ is the model persistence time for the parallel and orthogonal steps, and $t$ is the time step. At this point we wanted to account for poor fits of the model so a power law relationship model was fitted the model persistence time to the ratio of the model speed and the instantaneous speed binned based on persistence times between 2 to 80. This bin was chosen as it was the smallest time step and the largest time step used to calculate the model parameters and values outside of these parameters had high error. The ratio was calculated using the following equation:
\[ \frac{S_m}{S_i} = aP_m^{-b} \]  \hspace{1cm} (5)

where \( S_m \) was the model based speed for the parallel or orthogonal steps, \( S_i \) is the instantaneous speed for the parallel or orthogonal steps, \( a \) and \( b \) are model constants, and \( P_m \) is model based persistence time for the parallel or orthogonal steps. A new speed (\( S^* \)) was calculated for parallel or orthogonal steps based rearranging Equation 4 and using the model persistence time. A model motility coefficient was calculated using model speed and persistence time using the following equation:

\[ \mu_m = S_m^2 \times P_m \]  \hspace{1cm} (6)

where \( \mu_m \) is the model based motility coefficient, \( S_m \) is the model speed for the parallel or orthogonal steps, and \( P_m \) is the model persistence time for the parallel or orthogonal steps. This model motility coefficient was then combined with the new speed (\( S^* \)) and a rearranged Equation 5 to calculate a new persistence time (\( P^* \)). At this time the final motility coefficient was calculated using the following equation:

\[ \mu = S_i^2 \times P^* \]  \hspace{1cm} (7)

where \( \mu \) is the motility coefficient, \( S_i \) is the instantaneous speed for the parallel or orthogonal steps, and \( P^* \) is the new persistence time speed for the parallel or orthogonal steps. To calculate the directionality in 2D, a vector was identified in the direction of the uniform alignment and
inputted into the same program which calculated instantaneous speed, model based speed, and model based persistence time but the equation used was the same which will be described as 3D alignment. 3D alignment was more complicated as the directionality was not uniform within the gel. A radial vector mask was created from the center of the needle which mimicked the fiber alignment with the same conditions as previously published [51]. The cell migration vector could be compared to the radial vector field to get directionality using the following equation:

\[
DI_c = \cos 2\theta_c
\]  

(8)

where \( DI_c \) is the directionality of the cell and \( \theta_c \) is the angle between the cell step vector and a radial vector from the center of the needle.

### 4.2.6 MMP Activity and Inhibition Assays

Quantifying MMP activity in mono and co-culture environments was accomplished by seeding 1.5 million cells in a 60 mm tissue culture dish in complete DMEM. After 24 hrs the media was replaced with 1 ml of complete DMEM minus FBS. After 24 hr the complete DMEM was removed and put on ice, and the cells were trypsinized and counted. The media was then run through a Fluromax-4 Spectrofluorometer (Horiba Scientific, Kyoto, Japan) with a broad-spectrum MMP-substrate M-2305 fluorescence substrate (Mca-PLGL-Dpa-AR-NH\textsubscript{2}) Bachem, Bubendorf, Switzerland) at a working concentration of 10 µM. The wavelength and slit width were 328 nM and 3 nM respectively for the excitation scan and 397 nM and 3 nM respectively for the emission scan. The time increment for the scans was 10 s for a total time of 20 min. The integration time was 0.1 s and the laser was shuttered when the signal was not detected. Controls
consisted of the following conditions: non-conditioned complete media without FBS, non-conditioned complete media without FBS with the MMP-substrate, non-conditioned complete media without FBS with MMP-14 head domain, and non-conditioned complete media without FBS with the MMP-substrate and MMP-14 head domain. MMP-14 head domain was stored at 14 µM in 50 mM HEPES, 150 mM NaCl, 5 mM CaCl₂, and 0.5 mM ZnCl₂ at a working concentration of 23.3 nM received from collaborators from UC Riverside as controls.

The MMP activity in mono and co-culture environments were quantified by calculating the slope of the fluorescence between 200 and 1200 s. This slope was normalized on a per cell basis obtained from counting the number of cells in the dish at the time the conditioned media was removed using a hemocytometer. The data was collected over at least two separate days with a total of three replicates.

A similar protocol was used to quantify the effectiveness of the function blocking antibody for MMP-14 (MMP-14 antigen-binding fragment (FAB): 3D9). MMP-14 head domain at concentration of 23.3 nM was added to the complete DMEM with the 3D9 inhibitor at a concentration of 500 nM and put on ice for 30 minutes. The MMP substrate (10 µM) was added to the enzyme mix and an excitation/emission scan using identical parameters was run for 300 s. Controls included excitation/emission scans of the complete DMEM alone, with the MMP-substrate only, with the MMP-14 head domain only, and with the MMP-substrate and MMP-14 head domain together without inhibitor.

Analysis of the effectiveness of the MMP-14 inhibition was conducted by obtaining the initial slope of the fluorescence correlating to the time between 0 and 50 s. The initial rates for the inhibited and negative control samples were divided by the positive control, which is defined as the sample with the complete DMEM, MMP-14 substrate, and MMP-14 head domain, for
each day that was run to give an average percent inhibition. The data was collected over at least three separate days with a total of five replicates.

4.2.7 Fluorescence Staining

Cells were embedded in collagen as previously discussed but with a concentration of 300,000 cells ml\(^{-1}\) resulted in less crowded images. After an incubation time of 24 hr, the cells were fixed with 4% paraformaldehyde (Fisher), permeabilized with 0.5% triton-X (Fisher) and stained for F-actin using alexa 488-phalloidin (Molecular Probes). For co-culture samples the 300,000 cells ml\(^{-1}\) were split between the two cell types at 150,000 cells ml\(^{-1}\). The PS cells were stained with Cell Trace Far Red Cell Proliferation Kit (ThermoFisher) at a dilution of 1:1000 of 10 mM stock for 45 minutes prior to trypsinizing the cells. This allowed for the PS and PC cells to be identifiable and analyzed independently.

4.2.8 Statistical Methods

Confidence intervals (95%) were calculated using Matlab. A two tailed \(t\)-test, \(p \leq 0.05\), was conducted for experiments which only compared two conditions while an Anova test, \(p \leq 0.05\) was conducted for experiments which had more than two conditions. These are used to identify statistical differences which were depicted with connecting bars over the particular conditions. Conditions sharing at least 1 common perturbation were tested. Details of the number of samples and experiments are included in the figure legends.
4.3 Results

4.3.1 Rotation of Acupuncture Needles Align Collagen Fibers

We have used a mechanical method of collagen alignment which produces radially aligned collagen gels which induce a contact guidance cue. Second harmonic generation (SHG) can be used to measure the local alignment of the collagen fibers. SHG reveals the changes in the orientation of the collagen gel under four distinct conditions: native collagen, $0\pi$ rotation, $2\pi$ rotation, and $4\pi$ rotation. Consequently, SHG and was used to validate the alignment of the method. Collagen gels, which were polymerized and not disturbed with a needle (Figure 1A&D) give a baseline with a full width half maximum (FWHM) of $63 \pm 5.0$. Inserting a needle but not twisting (Figure 1B&E) results in a FWHM of $33.6 \pm 1.8$. Examining how twisting affects the alignment of the gel, we see that one twist of the needle give a FWHM of $21.6 \pm 6.2$ (Figure 1C&F). The FWHM of one twist was not statistically significant which indicates that alignment is the same (Figure 1G). The alignment for one twist and two twist also was not statistically significant from 2D alignment making the 3D and 2D aligned conditions comparable. It was important to have a baseline to compare the FWHM for each of the gels, so the FWHM was measured far from the needle (1 cm). As expected, the orientation far from the needle roughly matches that for an undisturbed gel (Figure 1G). Since one rotation results in alignment with less visual tearing we used one rotation for the remainder of the study. This condition also matched the alignment seen when we self-assemble collagen onto 2D substrates, allowing us to more properly compare 2D and 3D. (Figure 1G).
Figure 4.1: Determining Alignment of Collagen Fibers. Sample SGH images with analysis for collagen gel (A), zero twist (B), and one twist (C). Histogram of the orientation angle with model fit from second harmonic generation analysis with corresponding full width half max of native collagen gel (D), zero twist (E), and one twist (F). Full width half maximum graph for 2D and 3D conditions (G).

4.3.2 Co-culturing PS and PC Cells Increases Speed While Knocking out the MUC4 gene affects PS Directionality

Given our ability to organize collagen into aligned or unaligned gels, the collagen gels were seeded with PC and PS cells separately. Figure 2A-F shows how the morphology of PC and PS cells are affected by the lack or presence of alignment and that PS cells exhibit a mesenchymal cell morphology while PC cells exhibit an amoeboid cell morphology. Comparing Figure 2A to 2C-D, we see that PS cell morphology is similar in 2D and 3D conditions. Comparing Figure 2B and 2E-F show similar PC cell morphology, except the 2D condition resulted in slightly a less rounded cell morphology. PS cells seem to show cell polarization in both aligned and unaligned gels, while PC cells do not. Directionality of PS cells increases in aligned gels (Figure 2G), however the directionality was lower in 3D than it was in 2D. On the other hand, PC cell migration was not directional at all in either 2D or 3D. This is not unexpected as PS cells migrate using a mesenchymal mode while the PC cells migrate using an amoeboid
mode. Across the board, cell speed seems to remain relatively similar when comparing the aligned condition with the unaligned condition (Figure 2H) with 2D showing a higher migration speed as compared to 3D. Weak correlation between cell directionality and speed indicates that collagen alignment may result in either faster or slower cell migration (Figure 2I). This is further supported by Figure 2J which shows the directional cells having a higher motility coefficient ratio. Motility coefficient is a term that combines speed and persistence time. Each cell step was broken up into two parts. The first was projected radially from the center of the needle and the second orthogonal to the projected. The ratio of the motility coefficients parallel and perpendicular to the collagen alignment was computed forming a motility coefficient ratio, which describes cell migration in the parallel direction vs. perpendicular. Motility coefficient ratio, a measure of which shows that the aligned PS cells have the largest median motility coefficient. These results are not surprising as the contractile PS cells responded to the contact guidance cue while the more amoeboid cells did not.
While these cells responded as expected to aligned fibers while on their own, it is unknown how PS and PC cells communicate during contact guidance. It is known that they regulate each other’s migration [53, 54]. Consequently, we measured the contact guidance response of both PC and PS cells in co-culture. Interestingly, a decrease in PS directionality when co-cultured with PC cells in aligned gels is seen in 3D (Figure 3A). This was also observed in 2D, although it was not as pronounced. PC cells differed in their response. In 2D, PC cells showed an increase in directionality and a decrease in speed when co-cultured with PS cells (Figure 3B&3D). However, PC cells do not show any increase in directionality when co-cultured

**Figure 4.210: Comparing Contact Guidance of Pancreatic Stellate and Pancreatic Cancer Cells.** Phase contrast images of 2D PS (A) and PC (B) cells, 3D PS (C-D) and PC (E-F) cells in a collagen gel. 2D conditions are aligned while 3D conditions were rotated by (C&E) 0\(\pi\) or (D&F) 2\(\pi\). The scale bar is 50 \(\mu\)m. Cell directionality (G), speed (H). Trend comparisons for speed and directionality (I) and motility coefficient ratio (J) for 3D conditions. Stars indicate outliers outside frame. Error bars are 95% confidence intervals of the mean (N_{experiments} = 3 and N_{cells} > 105). Lines represent statistical significance using a two tailed t-test, \(p \leq 0.05\). Anova test only done on samples with similar conditions.
with the PS cells in 3D. On the other hand, speed increases in both aligned and unaligned conditions for both cell types in 3D (Figure 3C&3D). This indicates that the presence of another cell type has a tangible effect on both cell speed and directionality, where PS cell directionality decreases, whereas both PS and PC cell migration speed increases. The PC and PS cell motility coefficient ratio medians indicates that the overall migration was hindered (Figures 3E&3F). Interestingly, cell communication between PS and PC cells resulted less directional migration in response to aligned fibers.

Figure 4.3: Investigating the effect of Co-culturing Cells on Migration. Cell directionality (A-B), speed (C-D) and motility coefficient ratio (E-F) for PS (A, C, E) and PC (B, D, F) cells in 2D and 3D in monoculture and co-culture conditions. Stars indicate outliers outside frame. Error bars are 95% confidence intervals of the mean ($N_{\text{experiments}} = 3$ and $N_{\text{cells}} > 30$). Lines represent statistical significance using a two tailed t-test, $p \leq 0.05$. Anova test only done on samples with similar conditions.
With co-culture resulting in a surprising lack of directionality in either cell, it was decided to take a look at what is important in pancreatic cancer communication. MUC4 has been known to disrupt cell-cell and cell-ECM adhesion forces as well as promote cell survival so as to bypass apoptosis [55, 56]. In addition, it can modulate interactions between PS and PC cells. By knocking this gene out, it is possible to examine its role in directing contact guidance as well as communication between PS and PC cells during contact guidance. MUC4 knockdown was verified using western blotting (Figure 4G). MUC4 did not seem to affect either directionality or speed of PS cells in 2D. However, this differs in 3D. As expected there is no directional migration in unaligned gels. However, the absence of MUC4 in PC Muc4 KO cells restores the directionality of the PS cells in 3D (Figure 4A). In addition, the speed of the PS cells increase even more when co-cultured (Figure 4C). The average cell speed of the PC muc4 KO cells was lower than the speed for the PC cells (Figure 4H). However, the co-culture conditions eliminate this difference (Figure 4I). What we see is that co-culturing the cells together dramatically increases the PC speed by 2-3 times whether the MUC4 gene is present or not. Taken together, this suggests that MUC4 acts to diminish PS cell migration in co-culture in 3D, even though it has no effect on PC directional migration. Motility coefficient ratio also matches the directionality trends which shows increases from low back to high when MUC4 is knocked out (Figure 4E). The motility coefficient ratio medians for the PS muc4 KO cells did not increase when co-cultured (Figure 4F). This is interesting as it indicates that eliminating MUC4 from the PC cells affects the migration of PS cells, further confirming MUC4’s importance as a communication modulator.
Figure 4.4: Investigating the Role Mucin 4 has on PS and PC Cells. Cell directionality (A-B), speed (C-D) and motility coefficient ratio (E-F) for PS (A, C, E) and PC muc4 KO (B, D, F) cells in 2D and 3D in monoculture and co-culture conditions. SDS-PAGE results showing lack of KO gene in muc4 KO cells without affecting actin (G). Speeds of PC and PC muc4 KO monoculture (H) and co-culture (I) are provided for ease of comparison. Stars indicate outliers outside frame. Error bars are 95% confidence intervals of the mean (N_{experiments} = 3 and N_{cells} > 30). Lines represent statistical significance using a two tailed t-test, p ≤ 0.05. Anova test only done on samples with similar conditions.

4.3.3 PS and PC Cells Increase the Alignment of the Fibers Except when MUC4 is Eliminated in PC Cells

Armed with the knowledge that co-culturing PC and PS cells affects migration characteristics we next wanted to see how the PC and PS cells remodel the collagen gels. This
prealigned environment gives us the unique opportunity to examine collagen fiber disorganization and reveal potential routes for normalizing the stroma around pancreatic cancer cells. Using SHG, we investigated how the cells remodeled the ECM in 3D. Aligned collagen gels, where seeded with PC and PS cells, in either monoculture or co-culture. No conditions resulted in measurable differences in the collagen. Recall, large FWHM corresponds to disordered collagen fibers whereas small FWHM corresponds to ordered collagen fibers. Both PC and PS cells showed an increased alignment of the gels. Surprisingly, PC cells produced the most aligned collagen gel with a FWHM of 12.15 ± 2.04 (Figure 5). Interestingly, the PC muc4 KO cells did not enhance the alignment of the collagen gels, but rather blocked any increase in the alignment (Figure 5). This is surprising since the co-culture of PS and PC muc4 KO cells resulted in a return to directional migration.

Figure 4.5: Analysis of Fiber Orientation after 24 hrs Containing Cells Using Second Harmonic Generation. Analysis includes collagen signal and full width half max values for each sample, along with the zero and one twist conditions for reference. Lines represent statistical significance using a two tailed t-test, p ≤ 0.05.
4.3.4 MMP Activity Goes up in Co-cultured Conditioned Media and 3D9 Partially Inhibits MMP-14 Activity

Due to the dramatic reorganization of the matrix that was seen, producing both more and less aligned gels, we wanted to investigate how co-culturing these cells affected the tools which cells use to degrade and align the matrix. Cells generate force through contraction of F-actin fibers which are necessary for reorganization. Degradation of the ECM fibers can also occur through secreted proteinases called matrix metalloproteinases (MMPs). Using fluorescent phalloidin, F-actin was stained in each of the cells in monoculture and co-culture conditions. Sample images of the fluorescence can be found in Figure 6A-G. Fluorescence intensity was measured and showed an increase in F-actin fluorescence when co-cultured in each condition (Figure 6H). MMP activity is an absolute requirement for ECM remodeling and is involved heavily in causing alignment in fibrosis as well as disorganization of collagen in normal wound healing [14, 15]. By culturing these cells alone and with one another on 2D culture tissue dishes, we were able to condition media and measure, on a per cell basis, how the MMP activity changed when the two cell types were allowed to interact. Both PS and PC cells have low MMP activity while the PC muc4 KO cells show high MMP activity (Figure 6I). When the cancer cells were co-cultured with the stellate cells we see, not just an additive affect, but an amplification of the response. We would expect to see an average of the response from two cell types, however, we see the new average MMP activity to be around two times higher. F-actin intensity and MMP activity were shown to be higher in co-culture for each cell line, indicating that communication is indeed occurring and affecting cellular contractility and protease activity.
Figure 4.6: MMPs Activity and Inhibition in PS and PC Cells. Rate of fluorescence per cell linking to MMP activity in conditioned media from monoculture and co-cultured 2D dishes (A). Percent inhibition of initial rate using 3D9 FAB inhibitor (B). Example graph illustrating initial rates over 5 minutes (C). Error bars are 95% confidence intervals of the mean (N_experiments = 3). Lines represent statistical significance using a two tailed t-test, p ≤ 0.05. Anova test only done on samples with similar conditions.

Showing that the MMP activity was increased in co-cultured systems, we wanted to see how the cells would respond when MMP-14, an MMP known to regulate collagen homeostasis, was inhibited. An example trial of fluorescence intensity shows that using the inhibitor 3D9 resulted in a much slower growth of intensity (Figure 7A). 3D9 was seen to inhibit MMP activity nearly 70% (Figure 7A). This is an acceptable level of inhibition as we wanted to lower this cells ability to use MMP-14 not completely block it. Now that we had evidence to suggest that the MMP activity in the co-cultured systems was upregulated and a way to inhibit an important collagenase, we examined how blocking this would affect the migratory characteristics in the co-culture systems. When looking at the PS cells when co-cultured with either pancreatic cancer cells, we see no directionality and a marked decrease in speed, indicating that the cells themselves are hardly migrating (Figure 7C-D&7G-H). The native pancreatic cancer cell showed
a slight increase in directionality but a decrease in speed, again indicating that the cells were hardly migrating (Figure 7E&7I). The pancreatic cancer muc4 KO cells showed no directional migration and a much lower speed as well (Figure 7F&7J). Inhibition of MMP-14 resulted in significantly slower cells for each of the three cell types, and a lack of directionality in almost every condition.

**Figure 4.7: Investigating the role of MMP inhibition in Co-culture Contact Guidance Conditions.** Cell Directionality (A-D) and Speed (E-H) under co-culture conditions for PS (A&B, E&F), PC (C&G), and PC muc4 KO (D&H). Collagen orientation under co-culture conditions with drugs (I). Error bars are 95% confidence intervals of the mean (N\textsubscript{experiments} = 3 and N\textsubscript{cells} > 30). Lines represent statistical significance using a two tailed t-test, p ≤ 0.05.

After identifying that both MMP activity and F-actin intensity were increased in co-culture systems, and that inhibition of MMP activity resulted in very different migration characteristics in co-cultured systems, we wanted to see how the cells reorganized the matrix when we inhibited MMPs or contractility (Figure 8). First, examining the PS and PC conditions, we see that using either inhibitor resulted in an increased FWHM which indicates a less aligned matrix. Inhibiting MMP activity in these cells resulted alignment similar to the collagen 0\pi condition indicating that the alignment was disrupted. When contractility was inhibited, using
blebbistatin, the resulting alignment was similar to the collagen $2\pi$ indicating that there was no disruption or enhancement of the preexisting alignment. Moving onto the PS co-cultured with the muc4 KO cells, inhibition of both MMP and contractility resulted in alignment similar to the collagen $0\pi$ condition indicating no enhanced or disrupted alignment. In general, inhibition of MMP-14 and contractility never resulted in enhanced alignment which may indicate certain therapeutic targets for the future.

**Figure 4.8: F-actin Fluorescence Levels in 3D Monoculture and Co-culture Aligned Conditions.** Sample fluorescence images of PS cells (A), PS co-cultured with PC cells (B), PS co-cultured with PC muc4 KO cells (C), PC (D), PC co-cultured with PS cells (E), PC muc4 KO cells (F), and PC muc4 KO co-cultured with PS cells (G). Scale bar is 25 $\mu$m. F-actin intensity values minus background for each condition (H). Error bars are 95% confidence intervals of the mean ($N_{\text{experiments}} = 3$ and $N_{\text{cells}} > 55$). Lines represent statistical significance using a two tailed t-test, $p \leq 0.05$.

### 4.4 Discussion

In this chapter we present results addressing contact guidance in a 3D collagen environment. First, it was furthered confirmed that as the number of rotations of an acupuncture needle increases, the degree of local alignment in collagen gels increases. One rotation results in the same degree of alignment as twisting a second time and was similar to alignment seen in 2D.
Second, it was established that PS and PC (both with and without MUC4) cells exhibit mesenchymal and amoeboid cell morphologies respectively, and that only the PS cells respond to contact guidance cue. Third, co-culturing the cancer and stellate cells resulted in increased speed across cell lines and affected the directionality of the PS cells while not resulting in a difference in either PC cell line. Fourth, monocultured and co-cultured gels resulted in a higher degree of alignment compared to native gels. Fifth, PC cells with the MUC4 gene knocked out responded in unexpected ways compared to our understanding of MUC4’s role in pancreatic cancer. This includes similar speed in both the PC and PC muc4 KO cells, as well as the fact the lack of mucin 4 in PC muc4 KO cells resulted in a more directional PS cell compared to when co-cultured with native PC cells. Finally, while myosin is required in co-culture for organization and disorganization MMP-14 inhibition can actually result in collagen fiber disorganization.

While the protocol of using acupuncture needles to align collagen gels was discussed previously in another chapter, there was still some doubt about the effectiveness of producing aligned gels. While there was a directional migration of cells in gels that had undergone a twist in the previous chapter, the confocal reflectance microscopy only indicated, but did not confirm the desired alignment. In addition, non-large differences could be seen between twists and a comparison between alignment in 2D and 3D was not conducted. For this reason, we probed alignment of the collagen gels using SHG, which verified that rotating the needle produced a higher degree of local alignment in the gels. This can be seen by the halving of the FWHM after one twist of the needle compared to native collagen gels. While this is not a direct indication of the degree of alignment, it does show that the act of the twist does affect and produce a more directionally uniform gel, which supports the data seen using confocal microscopy. Interestingly, the act of inserting a needle does seems to increase the alignment. This is believed to be due to
drag caused by the needle passing through the gel, pulling fibers in the z-direction. This would indicate that in the absence of rotation, slight directional migration could be induced, which is indeed seen in some PS cells in some co-culture conditions. Though this does occur, it seems as the incidental alignment only translates into a meager contact guidance cue for the cells.

It is been hypothesized that cells with different migration modes respond to external cues with varying degrees of intensity [57, 58]. There has been plenty of work in 2D which supports this and it is believed that the same results occur in 3D, as seen in the previous chapter [35, 59-63]. The cell lines within this paper, while not both cancerous, can be classified into the two migration modes: mesenchymal and amoeboid. PS cells are pancreatic stellate cells which show mesenchymal characteristics when migrating while PC cells exhibit amoeboid migration characteristics. This is further supported by the directional response to the contact guidance cue of almost 0.32 seen only in the PS cell which is comparable to the MDA-MB-231 human breast cancer cell line which exhibits the mesenchymal cell migration mode and a directionality of just under 0.40. While the original hypothesis of this work was that co-culturing PS and cells may produce directional migration for the PC cells under a contact guidance cue was only partly realized. The PC cell line only showed a directional response when co-cultured on aligned 2D substrates, but never in 3D. We know that aligned fibers are seen in vivo near the tumor-stromal interface in metastasizing pancreatic cancer suggesting that contact guidance does play a role in this type of metastasis [32, 33]. There may be a number of possible answers for this including the need for another type of ECM protein or component within the aligned gels, stiffness or degree of alignment need to be higher, or perhaps the aligned fibers serve another purpose other than providing a contact guidance cue. What we can conclude is that under an aligned fiber condition the PC cells did not migrate directionally in 3D.
Increasing the complexity of the *in vitro* model is important as the TME is a complex environment which cells are constantly interpreting cues from the matrix, interstitial fluid, and surrounding cells [64, 65]. It is because of this that we wanted to look at how the migration characteristics of the highly metastatic PC cell changed when co-cultured with PS cells in the 3D collagen gels. As shown in other work, co-culture or the use of conditioned media led to an increase in instantaneous speed for both cell lines [66-68]. Pancreatic stellate cells have been shown to be important in tumor metabolism and depositing ECM which can lead to higher levels of growth factors. Some have even proposed that pancreatic stellate cells are used as a therapeutic target due to their symbiotic nature [69, 70]. Seeing an increase in speed further indicates that the relationship between these two cells is important. Interestingly, we showed a decrease in directionality in PS cells when co-cultured with PC cells. Perhaps the PC cells are not responding to the contact guidance cue, so aligned deposition of new ECM may not be as important as the composition diversity of ECM fibers. Also worthy of note is that when the MUC4 gene is knocked out in the PC we see a return to PS directionality and increased PS cell speed which might indicate that some aspects of the communication are broken in the absence of MUC4.

Observing changes in cell directionality and speed change in co-culture systems led us to wonder what the cells were doing to the ECM. What was surprising was that the cells actually increased the alignment of the gels significantly. When the individual cells PC and PS cells were seeded it resulted in an increase of alignment of around 40% and 25% respectively. This would indicate that both cells types are interacting and reorganizing the matrix, however we see hardly any directed migration from the PC cells. It is also of note that it is commonly assumed remodeling is done by the contractile PS cells, however we see enhancement of the aligned fibers.
in both cell types. This could be due to force exerted by the cell as it migrates. When these cells were co-cultured, resulting in an increase of alignment of about 40%, we see hardly any directed migration for either cell. This would suggest that the alignment of the collagen may serve another purpose other than providing a contact guidance cue for metastasis or that the alignment of collagen does not depend on aligned migration within the collagen. This data would also suggest that the PS cells are ignoring the cue which was shown to lead to directed migration when monocultured.

Mucin 4 is a glycoprotein found on the cell surface which has found to be overexpressed in pancreatic cancers aiding in tumor growth and metastasis, though the mechanism by which it does so is still unknown [7, 71]. By knocking out the MUC4 gene in the PC cells we would expect to see altered migration characteristics which would limit metastatic potential. When compared to the PC cells under aligned and unaligned gel conditions we did not see a change, nor did we see any change when co-cultured with PS cells. What did stand out was PS directionality when co-cultured with the PC muc4 KO cells. The PS cells showed a return of directional migration, comparable to the monoculture condition. We had expected to see the PC cell migration characteristics change when the MUC4 gene was knocked out, however the PS cells were the ones that showed a response, indicating once again that there is a symbiotic relationship between the pancreatic cancer cells and the healthy stellate cells in the TME. What was not expected was the way the two cells interacted with the ECM. The PC muc4 KO cell did not seem to remodel the ECM at all, resulting in no statistical difference between the native aligned collagen and the aligned collagen seeded with the PC muc4 KO cells. However, when we examined PC muc4 KO cells co-cultured with the PS cells, the gels were actually disorganized. This is unexpected and hard to explain as the condition returned the PS condition
to directional. This would indicate that the directional migration was either not in response or differentially sensitive to the contact guidance cue, as expected, but due to something else. It is possible that the PS cells were responding to a change in local density caused by the wrapping of the fibers. This is an aspect that needs to be investigated further. Overall, this data suggests that when muc4 decreases, it appears to disorganize the stroma, potentially providing a route for stromal reprogramming through muc4 inhibition. This coupled with the decreased migration speed, even in the face of no change in directional migration, might represent a good therapeutic path.

Another aspect that we felt was important to probe was MMPs role in the co-culture system and being a potential target for future therapeutics. Due to the fact that we see an increase in speed through the gels in co-culture systems, as well as rearrangement of the matrix, it was hypothesized that increased MMP activity was at least partially responsible. The cells were plated and conditioned media was allowed to sit for 24 hrs. Using a broad-spectrum MMP-substrate we were able to see how MMP activity was affected in monoculture and co-culture systems; the results supported our hypothesis. When PS and PC cells were plated separately they had about 2-3 times less activity than when they were plated together. An important note is that the total cell number plated on day 1 was the same. Although the co-cultured dish had two cell lines plated onto the dish the total cell number was 1.5 million cells. The fact that the activity is 2-3 times larger than the individual cells indicates that there is an amplification effect. This is not completely unexpected as MMP activity increases in co-culture environments has been seen before, however this does indicate that the communication between the cells will result in the capability to alter the matrix at a higher rate [72-75]. This amplification was also seen in the PC muc4 KO co-culture, however not to the same extent. When MMP-14 (a common collagenase,
was blocked, speed decreased and directionality decreased in the PS co-culture cells while for both co-culture cancer cells the speed decreased and directionality remained at 0. This indicates that the MMPs upregulation is an important part in the symbiotic relationship between these two cells. By looking at how the collagen organization was effected by inhibition of contractility and MMP-14, we are able to see some interesting trends which we may be able to use for therapeutic targets. Inhibiting MMP-14 resulted in a disruption of previously enhanced alignment. This inhibition was more effective than inhibiting contractility, through inhibiting myosin II, which resulted in no enhancement or disruption. This would indicate that MMP inhibition may limit metastatic propensity and should be investigated further as a therapeutic target for pancreatic cancer.

4.5 Conclusions

We have successfully mimicked alignment of fibers which has been seen in vivo, oriented away from the edge of the tumor within the pancreas. By examining pancreatic stellate (PS) and pancreatic cancer cells with (PC) or without mucin 4 (PC muc4 KO) in aligned fiber networks, we are able to study the relationship that communication has on contact guidance. PS cells showed directional migration in response to aligned fibers until they were co-cultured with PC cells. When PS cells were co-cultured with PC muc4 KO cells directionality returned, indicating that muc4 is important in PS-PC communication. Using our alignment technique to study reorganization of the fibers, we saw that PS and PC cells monocultured or co-cultured together will enhance the alignment. PC muc4 KO cells when monocultured won’t reorganize the fibers at all and when co-cultured will actively disrupt the alignment. We also showed that F-actin intensity and MMP activity were shown to be higher in co-cultured systems. When F-actin was inhibited in co-culture conditions, alignment was neither enhanced nor disrupted. When MMP-
14 was inhibited in PS+PC conditions alignment, was disrupted and returned to an unaligned state. When MMP-14 was inhibited in PS+PC muc4 KO conditions, alignment was neither enhanced nor disrupted. This simple rotational alignment technique allows for easy studying of fiber reorganization and study of therapeutics on ECM alignment. Inhibition of MMPs, highlighting MMP-14, seems to be a promising therapeutic target to disrupt alignment around the tumor.

4.6 References


X. H. Wang et al., "Endothelial Cells Enhance Prostate Cancer Metastasis via IL-6 -&gt; Androgen Receptor -&gt; TGF-beta -&gt; MMP-9 Signals," Molecular Cancer Therapeutics, vol. 12, no. 6, pp. 1026-1037, Jun 2013.


Abstract

Pancreatic cancer has one of the lowest 5 year survival rates (3-5%), which is, in part, due to poor diagnostics and treatment options. Recently, aligned fibers have been seen oriented away from the edge of the tumor within the pancreas. This suggests that contact guidance, directional migration in response to aligned ECM fibers, may be critical to the early stages of metastasis. In this paper, hyaluronan (HA), a polysaccharide found to be overproduced within the pancreatic tumor, was added to the collagen gels to study how the presence of HA effects contact guidance in pancreatic cancer (PC) and pancreatic stellate (PS) cells. Mucin 4 is a large glycoprotein which is can block binding of cells to HA and is overexpressed in pancreatic cancer. The MUC4 gene was knocked out of the PC cells (PC muc4 KO) to investigate how mucin 4 may affect HA bonding. In the presence of HA, PS cells showed a slight increase in speed and directionality. Surprisingly, PC and PC muc4 KO cells showed directional migration in response to aligned fibers, which has not previously been seen. Further protrusion analysis revealed that the ratio of I to Y extensions was inversely proportional to cellular speed. Low molecular weight HA (LMW HA) is believed to be important during the early stages of invasion within the pancreases, so the molecular weight was decreased but concentration kept the same. Migration speed and directionality remained consistent while motility coefficient ratio median increased significantly. This indicates persistence time may be important and regulated by LMW HA.
5.1 Introduction

Pancreatic cancer is consistently one of the deadliest forms of cancer with a 5 year survival rate of 3-5% [1]. Metastasis is the migration of cancer cells from the primary tumor to other regions of the body where they can seed and form secondary tumors [2]. An important but not well understood step of metastasis involves cancer cells invading into the local tissue surrounding the tumor. This invasion can be directed by the extracellular matrix (ECM) in the form of aligned fibers, a process called contact guidance. What makes the ECM particularly interesting and important is that it not only directs invasion in certain circumstances, but in other situations it can increase the difficulty in delivering drugs. ECM is produced, degraded and remodeled by both cancerous and healthy, stromal cells in the tumor microenvironment (TME). However, the density as well as the structure of the ECM in the TME is distinct from healthy tissues, forming a barrier which blocks therapeutic delivery [3, 4]. Therefore, understanding how structure and density affect cancer and stromal cell function are important aspects of uncovering mechanisms of tumor invasion and metastasis.

Aligned fibers in the pancreatic cancer TME can direct migration of cancer cells into the surrounding tissue in a process called contact guidance [5-7]. ECM fibers in vivo are most often collagen fibers, though there are other important ECM components that drive or inhibit cell migration and block drug development [8]. This type of alignment has been seen in vivo around the pancreatic TME indicating that it plays an important role in tumor development [9]. This process could also be used to direct stromal cells inward toward the cancer. Indeed, pancreatic cancers contain a large number of stromal cells, primarily pancreatic stellate cells, which constitute about 4-7% of the total cell mass in the gland [10]. Aligned collagen fibers constitute an important marker for poor prognosis in pancreatic cancer [9]. As mentioned above, directed
migration in response aligned fibers is called contact guidance. Contact guidance on collagen fibers proceeds through cell adhesion to the collagen and generation of contractile forces which move the cell along the alignment [11].

Along with collagen, another important TME component is hyaluronan (HA) which is overproduced in pancreatic cancer [12]. Hyaluronan is also referred to as hyaluronate and hyaluronic acid and is used interchangeably within in literature. HA is produced by hyaluronan synthases within the Golgi network and degraded by hyaluronidases [13, 14]. The misbalanced activity of these result in overproduction of HA. This polysaccharide acts to collapse vessels and promote tumor growth and survival through effecting numerous signaling pathways [15]. HA is a negatively charged molecule of high molecular mass and imparts interesting viscoelastic properties on the ECM which results in increasing compressive resistance and decreasing in shear modulus [16]. The interesting viscoelastic properties are due to its ability to retain water and regulate the porosity and malleability of the ECM, which has been shown to increase cell motility due to a less dense matrix [17, 18]. Cells have the ability to interact with HA through CD44 and RHAMM which bind to HA, making them a key focus in cancer therapeutic research. An increase in the production of HA in the TME has also been linked to drug resistance making it harder to treat cancer [19, 20]. High HA levels have been seen in pancreatic cancer which correlate to poor prognosis [12]. The importance of both aligned collagen and HA suggest that models that mimic the physical cues in the PC TME, particularly in the context of contact guidance, should include both aligned collagen and HA.

Collagen and HA work together to affect physical properties of the matrix. An important note to make is that there are crosslinked HA networks that are used in regeneration studies which are different than collagen:HA composite matrices with uncrosslinked HA. Studies
involving the types of composite matrices with uncrosslinked HA have shown that adding HA to collagen gels alters mechanical properties and structures of the gel [21]. Kreger and Voytik-Harbin combined high molecular weight (MW) hyaluronan with collagen gels and observed significant alterations to the viscoelastic properties, in particular, an increasing in the compressive resistance and decreasing in the shear storage modulus [16]. Along with altering the mechanical properties of the matrix, the combination of collagen and HA have resulted in interesting cellular responses. Adding HA to collagen gels have resulted in increased cell proliferation, thicker actin filaments, and increased matrix contraction in fibroblasts [22-25].

There has also been a growing understanding that the size of HA matters. Low molecular weight (LMW) HA has been linked to affect both mechanical properties as well as increased tumor progression and cellular response [26-29]. However, HA’s role on contact guidance is not known. Alignment in collagen:HA has been studied, however it focused on the impact that the alignment had on diffusion of therapeutics [30]. Using a rotational alignment technique, pancreatic cancer and stellate cell migration characteristics in aligned collagen:HA composite matrices will be studied. This will further our understanding of the importance of HA in the pancreatic TME and evaluate its place in models. The importance of the collagen:HA composite matrix on pancreatic cancer has been identified, however its roles on contact guidance are not well understood.

Our lab has developed and characterized a rotational alignment technique which has resulted in predictable and reliable collagen fiber alignment in 3D [31]. In this paper we will examine cellular migration and extension characteristics in collagen and collagen:HA composite matrices. We will also investigate how altering the MW of the HA affects migration characteristics. Other factors which have also been identified as important to pancreatic tumor
progression include activated pancreatic stellate cells and the surface glycoprotein mucin-4, however their interactions with collagen:HA composite matrices are not well understood [32-34]. While there are a few common methods to induce alignment in fibrous gels, our lab has developed

5.2 Materials and Methods

5.2.1 Culturing Cells

Cells used were human pancreatic stellate cells (PS) and Capan-1 human pancreatic cancer (PC) cells (ATCC® HTB-79™) [35]. Genetic editing using CRISPR-Cas9 to knock out the MUC4 gene in the PC resulting in a cell which does not produce mucin-4 (PC muc4 KO). were cultured using Dulbecco’s Modified Eagles Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 2% glutamax (Gibco) and 1% penicillin/streptomycin (Gibco) referenced in the future as complete DMEM. Imaging media used for experiments consisted of DMEM lacking phenol red, supplemented with 10% FBS, 2% Glutamax, 1% penicillin/streptomycin, 1% sodium pyruvate (Gibco), and 12 mM HEPES (Sigma Aldrich).

The MUC4 expression in the pancreatic cancer cells were silenced using Crisper-cas9 gene editing system. The guide RNA sequence (GCGAGTGGCACACACCTTC) targeting exon 2 was cloned in pSpCas9BB-2A-GFP (PX458) vector. The insertion of the gRNA was confirmed by sequencing. The Capan1 pancreatic cancer cells were transfected with PX458 vector containing gRNA using lipofectamine 2000. After 48h post-transfection, the GFP positive cells were sorted in 96 well plate as a single clone. The clones were subsequently sub-cultured and probe for MUC4 expression. The clones negative for MUC4 expression (due to change in
the reading frame during DNA repair) were selected and used for the study as a single clone or pooled population.

### 5.2.2 Assessing Directed Migration

Cell migration experiments were conducted by adding 600,000 cells ml\(^{-1}\) to 2 mg ml\(^{-1}\) non-pepsin-treated rat tail collagen type I solution (Corning, Corning, NY, USA) and, if required, 2 mg ml\(^{-1}\) Hyaluronan (LifeCore Biomedical, Chaska, MN, USA). The Hyaluronan was designated as high MW (HA) at 1.0-1.8 MDa and LMW HA at 100-150 kDa. A volume of solution (125 μl) of imaging media was pipetted into a MatTek dish (MatTek Corporation, Ashland, MA, USA), spread evenly on the bottom of the well, and allowed to polymerize for 45 mins at room temperature. Imaging media (125 μl) was then added to the top of the gel and a 0.25 x 25 mm stainless steel acupuncture needle (Hwato, Weymouth, MA, USA) was inserted through a 5 mm thick pad of polydimethylsiloxane (PDMS) (Dow Corning Corporation, Midland, MI, USA) to the depth of the well and placed on top of the collagen gel, sealed with high vacuum grease (Dow Corning, Midland, MI, USA). The needle was then rotated one full rotation to achieve alignment. The samples are then placed in a 37 °C incubator. After 24 hr the samples are moved to a heating stage to keep the samples at 37 °C and imaged every 2 min for 8 hr at 10x (NA 0.50, Nikon) with a charge-coupled device (CoolSNAP HQ2, Photometrics) attached to an inverted microscope (Ti-E. Nikon) that was driven by μManager. At least three samples over at least two different days compiled a complete data set.

Analysis of cell migration was conducted using the MTrackJ plugin in ImageJ (National Institutes of Health, Bethesda, MD, USA) which allows the tracking of cells on an image-by-image basis. A total of at least 70 cells over 3 experiments were chosen randomly from the
images. The result of this is the x-y coordinates of the cell at each time point. These points are then analyzed using a custom Matlab script which calculates the mean square displacement using the following equation:

\[ < MSD > = (x_i - x_{i+1})^2 + (y_i - y_{i+1})^2 \]  

(1)

where \(<MSD>\) is the mean square displacement, \(x_i\) and \(y_i\) are the x and y displacements between two times, and \(i\) is the image number [36]. The mean square displacement can be used to calculate the average speed using the following equation:

\[ S_i = \frac{1}{N} \sum_i^N \frac{\sqrt{<MSD>}}{\tau} \]  

(2)

Where \(S_i\) is the instantaneous speed, \(N\) is the number of time intervals for each cell and \(<MSD>\) is the mean square displacement, and \(\tau\) is the time step \((t_{i+1} - t_i = \tau)\). For these experiments, each \(i\) step is a time interval of 24 mins. To evaluate the motility coefficient, we first had to decompose the migration vector into two vectors, one parallel with the fiber field and one orthogonal to the first. This allowed us to look at the motility coefficient in the direction of the alignment vs perpendicular at any location within the matrix. A program in Matlab was written which took the cell steps, every 24 mins, and broke them up into a parallel and orthogonal step and exported them to excel. The number of positive and negative steps could be evaluated to determine any bias towards or away from the needle. Next, a model based speed and persistence time were calculated for the new parallel or orthogonal cell tracks using a 1D model. This was combined with the instantaneous speed for the parallel or orthogonal cell tracks using Equation 1 to get a mean square displacement using the following equation:
\[ <MSD> = n S_m^2 [P_m t - P_m^2 \left( 1 - e^{-\frac{t}{P_m}} \right)] \]  

(3)

where \(<MSD>\) is the means square displacement, \(n\) is the number of dimensions, \(S_m\) is the model based speed for the parallel or orthogonal steps, \(P_m\) is the model persistence time for the parallel and orthogonal steps, and \(t\) is the time step. At this point we wanted to account for poor fits in the model so a power law relationship model was fitted the model persistence time to the ratio of the model speed and the instantaneous speed binned based on persistence times between 2 to 80. This bin was chosen as it was the smallest time step and the largest time step used to calculate the model parameters and values outside of these parameters had high error. The ratio was calculated using the following equation:

\[ \frac{S_m}{S_i} = a P_m^{-b} \]  

(4)

where \(S_m\) was the model based speed for the parallel or orthogonal steps, \(S_i\) is the instantaneous speed for the parallel or orthogonal steps, \(a\) and \(b\) are model constants, and \(P_m\) is model based persistence time for the parallel or orthogonal steps. A new speed \((S^*)\) was calculated for parallel or orthogonal steps based on the power law model constants and the model persistence time. A motility coefficient was calculated using model speed and persistence time using the following equation:

\[ \mu_m = S_m^2 \times P_m \]  

(5)
where $\mu_m$ is the model based motility coefficient, $S_m$ is the model speed for the parallel or orthogonal steps, and $P_m$ is the model persistence time for the parallel or orthogonal steps. This motility coefficient was then combined with the new speed ($S^*$) and a rearranged Equation 5 to calculate a new persistence time ($P^*$). At this time the final motility coefficient was calculated using the following equation:

$$\mu = S_i^2 \ast P^*$$

(6)

where $\mu$ is the motility coefficient, $S_i$ is the instantaneous speed for the parallel or orthogonal steps, and $P^*$ is the new persistence time speed for the parallel or orthogonal steps. To calculate the directionality, a radial vector mask was created from the center of the needle which mimicked the fiber alignment with the same conditions as previously published [31]. The cell migration vector could be compared to the radial vector field to get directionality using the following equation:

$$DI_c = \cos 2\theta_c$$

(3)

where $DI_c$ is the directionality of the cell and $\theta_c$ is the angle between the cell step vector and a radial vector from the center of the needle.
5.2.3 Cell Extension Analysis

Cell extension experiments were prepared in the same way as migration experiments, which resulted in a sample at 600,000 cells ml−1 sealed in a MatTek dish. After 24 hours of incubation, the samples were removed from the incubator and placed on a heating stage set at 37° Celsius. The samples were imaged in a z-stack of 10 μm every 30 s for 90 min at 20x (NA = 0.45, WD = 8.2-6.9, Nikon) using the same microscope described above. Cells in focus were identified. The number of extensions at time zero were counted and the length of each were measured. Each extension was also categorized as being an I (no secondary branches), Y (one secondary branch), or FAN (having multiple connected branches). Next, the images were moved forward in time until a new extension was identified and that image number was recorded. The images were moved forward until the extension fully retracted or the stack of images ended. The last image was recorded and the lifetime of the extension was calculated. If the stack ended before the end of the extension lifetime the first image was recorded but the lifetime was not calculated. The stack was then moved back and the longest length of the extension was measured. The extension was also categorized as previously described. Once the extension was measured and categorized, the stack was returned to the first image which had the extension and moved forward looking for another new extension. This was repeated until all the extensions of the cell were measured and categorized. Once this was done, the length and width of the cell at the first image was measured and an aspect ratio was calculated. Then this would be repeated on other cell in the z-stack from other samples.

Once all of the cell extensions were analyzed the results were combined. Extensions per cell were calculated. To calculate the average length of an extension the average pixel length was converted into μm. To calculate the average lifetime of the extensions, only extensions which
began and ended within the 90 mins were calculated. Any extensions which were present at $t=0$
or were still present at the end of the stack of images were excluded. The time was then
calculated by taking the measured values and multiplying by 0.5 to account for a picture being
taken every 30 seconds. The extension types (I, Y, and FAN) were totaled and ratios between
types were calculated.

5.2.4 Statistical Methods

Confidence intervals (95%) were calculated using Matlab. A two tailed $t$-test, $p \leq 0.05$, was conducted for experiments which only compared two conditions while an Anova test, $p \leq 0.05$ was conducted for experiments which had more than two conditions. These are used to identify statistical differences which were depicted with connecting bars over the particular conditions. Details of the number of samples and experiments are included in the figure legends.

5.3 Results

5.4.1 Hyaluronan Results in Faster Cells with Similar Cell Morphology

As previously discussed, hyaluronan (HA) is abundant in pancreatic cancer making it a target of interest. We first evaluated the role of high molecular weight (MW) HA in modulating cell speed in collagen gels. Normal tissues include a MW range of HA which is $0.1-8 \times 10^7$ Da [18, 37]. Therefore, we decided to use a MW that was on the high end of the spectrum: $1.5 \times 10^7$ Da. We hypothesized that due to HA’s ability to change the viscoelasticity of the matrix, reducing the density, it would potentially increase migration motility, in particular speed. Therefore, we examined the role of HA in modulating pancreatic cancer as well as stellate cells.

In addition, because mucin 4 (muc4) has the ability to modulate adhesion to the matrix and CD44, we examined cell speed in muc4 deficient cells [38]. Cell morphology remained roughly
unchanged with the addition of HA to collagen for the three cell types (Figure 2A-2G).

Pancreatic stellate cells took on an elongated morphology, whereas both pancreatic cancer and pancreatic cancer muc4 KO cells took on a rounded morphology. The speed, however, increased for the cells in HA gels (Figure 2AG). PS cells showed an increase of nearly 10 μm/hr. PC cells did not show a statistical change in speed. PC muc4 KO showed an increase in speed of over 5 μm/hr. Given changes in speed, we were interested in whether other motility factors were affected by the addition of HA to collagen.

**Figure 5.1: Hyaluronan Effects Cellular Speed in Aligned Collagen:Hyaluronan Composite Gels.** Sample images of PS (A-B), PC (C-D) and PC muc4 KO (E-F) cells in collagen (A&C&D) and collagen: HA composites (B&D&F). Cell speed was measured and analyzed (G). Error bars are 95% confidence intervals of the mean ($N_{\text{experiments}} = 3$ and $N_{\text{cells}} \geq 75$). Scale bar is 50 μm.
Lines represent statistical significance using a two tailed t-test, \( p \leq 0.05 \). Anova test only done on samples with similar conditions.

### 5.3.2 Hyaluronan Results in More Directional Migration from Cancer Cells

Given that the addition of HA has an effect on migration speed, we moved on to examine the directional part of migration. PS cells are fibroblast in nature and likely to show directional migration when exposed to aligned fibers. Indeed they did. Collagen:HA composite matrices modestly, but not significantly increased cell directionality as compared to collagen matrices alone. PC cells are amoeboid-like and likely show no directional migration. Surprisingly, the PC cells did show significant directional migration in collagen:HA composite gels as compared to collagen gels. Similarly, when muc4 was knocked out from the cancer cells, it did not alter the directionality in collagen:HA gels as compared to the control cells, but like the control cells showed an increase in directionality in comparison to the collagen gels (Figure 2A). While DI measures directionality, it cannot discern the difference between a cell which migrates exclusively towards or away from the needle, and a cell which switches directions frequently. Aligned fibers are a bi-directional cue and migration towards and away from the needle are counted similarly. With the collagen wrapped around the needle after alignment, there was some concern that a potential change in stiffness or fiber density near the needle may bias migration towards the needle. Figure 2B reveals the motility coefficient ratios which is related to directionality through persistence time. As discussed in the material and methods section, the motility coefficient ratio is a parameter that combines the instantaneous speed with persistence time to give an understanding of how fast the cell is and how long it stays moving without changing speed or direction. It would be expected that a directional cell would have a higher motility coefficient ratio as the speed and persistence time would be higher in the direction of the
radial alignment while cells. Cells that do not migrate directionally would be expected to have a motility coefficient ratio closer to one. Indeed, Figure 2B shows that the conditions which showed directionality had higher motility coefficient ratios. Due to the fact that the motility coefficient increased in directional cells, it was decided to investigate if the cells migrated towards or away from the needle. Figure 2C shows that there is no large bias towards or away from the needle. The largest deviation from 50 in fact, is for the PS in collagen:HA composite matrices and it is biased away from the needle. We have shown that migration is not biased toward or away from the needle. The addition of HA resulted in pancreatic cancer cell contact guidance for the first time as well as increased motility coefficient ratio. This is the first time pancreatic cancer cells showed contact guidance in vitro.

**Figure 5.2: Hyaluronan Effects Cellular Directionality in Aligned Collagen:Hyaluronan Composite Gels.** Cell directionality (A), motility coefficient ratio B), and fraction of cell steps moving towards and away from the needle (C) was measured and analyzed. Error bars are 95% confidence intervals of the mean (N_experiments = 3 and N_cells ≥ 75). Significance bars represent non-overlapping confidence intervals. Lines represent statistical significance using a two tailed t-test, p ≤ 0.05. Anova test only done on samples with similar conditions.

### 5.3.3 Pancreatic Cancer Cells showed more Fan Extensions than PS cells

Given that HA increases speed and directionality, we examined whether the HA affected the extension. To analyze the extension characteristics of the three cell lines, the extensions were categorized as I, Y, or Fan (Figure 3A-3C). I and Y extensions would be expected to be the result
of high contractility and generate more force than FAN extensions. Migration linked with high contractility is associated with cells that migrate using contact guidance. The overall makeup of the extensions did not change much when comparing collagen gels with collagen:HA composite matrices. The PC cells in collagen:HA composite matrices did produce a higher ratio of I extensions (Figure 3E). However, the PC muc4 KO cells did not follow that trend, producing less I extensions in collagen:HA composites (Figure 3E). Overall, the largest difference can be seen, not between different ECM, but between different cell lines (Figure 3D). Overall the I extension seemed to be the most prevalent, though both cancer cells did have a significant Y fraction. The PS muc4 KO cells had a significant amount of FAN shaped extensions. Interestingly, it appears as if Y extension fraction correlates with speed for PS and PC muc4 KO. When comparing Figure 3E to Figure 1G, the conditions that had a lower number of Y extensions increased in speed. This fractional analysis of extension type showed an inverse relationship between number of Y extensions and cellular speed as well as indicate that morphology does not seem to correlate with directionality.
Figure 5.3: Examining How the Presence of Hyaluronan Effects Cellular Extensions Morphology in Aligned Gels. Sample images of I (A), Y (B), and FAN (C) extensions. Pie charts of each condition (D), along with ratio of extension comparisons of I and Y (E). Scale bar is equal to 10 μm ($N_{\text{experiments}} = 3$ and $N_{\text{cells}} \geq 15$). Lines represent statistical significance using a two tailed t-test, $p \leq 0.05$. Anova test only done on samples with similar conditions.

5.3.4 ECM Type had Little Effect on Extension Parameters

After seeing that the extension category mirrored the change in speed between the two matrices, we wondered if similar links could be seen from other whole cell or subcellular measurements. The aspect ratio of the length of the cell divided by the width and can give morphological information. Highly contractile cells tend to be elongated which would result in a high aspect ratio. We would expect the PS cells to have a higher aspect ratio than the PC and PC muc4 KO cells. Indeed this is true for the collagen condition (Figure 4A). For the collagen:HA composite matrices, the aspect ratio did not result in a statically significant difference in aspect
ratio between the stellate and either cancer cell. Also the addition of HA did not change the aspect ratio significantly.

Next it was decided to look at subcellular measurements. These included a more thorough extension analysis. It was hypothesized that the HA may impede extensions resulting in lower extension lifetimes, lower average lengths and potentially more extensions per cell. An important note to make is that extensions per cell refer to the base extension and each category is referred to as one extension. Figure 4B-D show that this was not the case. Both extension lifetime and average number of extensions did not change between the ECM conditions. In fact, there was no statistical difference between the average number of extensions for any condition (Figure 4B). The same can be said for the average lifetime of extensions (Figure 4C). The average length of extension did vary between cell types (Figure 4D). PS cells had the highest length which correlates with their higher aspect ratio. The PC and PC muc4 KO cells were not statistically different from one either in either the collagen or collagen:HA composite matrix. While we did see correlations between the differences in migration and extension morphology with the addition of HA, we did not see any differences in whole cell or subcellular measurements.
5.3.5 Lower Molecular Weight Hyaluronan has a Similar Effect on Migration

Hyaluronan is degraded through action of hyaluronidases. These can either be produced by cells in the TME or can be administered exogenously as a co-factor with cancer therapies. The action of hyaluronidases produces lower MW hyaluronan, thus affecting the MW distribution. As previously discussed, there is a wide size range of HA within the body and it is not clear if the MW can alter the sensing of collagen alignment in the PC TME. There are some reports that suggest that low molecular weight hyaluronan (LMW HA) accumulate in cancer tissues involved in tumor progression [39]. Therefore, we examined cell migration in response to LMW HA (100 kDa). Given the previous data, we hypothesized that the LMW HA would potentially allow for faster speeds due to the fact that LMW produces less viscous solutions and may not inhibit motility like high MW HA. However, this was not the case. Speed of the cells did not differ between collagen:HA composite matrices composed of different MWs (Figure 5A). Similarly it was hypothesized that the directionality may decrease with LMW HA due to smaller fragments within the gel. Again, this hypothesis was incorrect as directionality of the cells resulted in no statistical difference between the two weights of HA (Figure 5B). The median motility coefficients ratios did decrease for the PS cells, which was a result of more consistent migration speeds and slightly lower persistence times. The PC and PC muc4 KO cells showed an increase in motility coefficient ratio medians indicating that the persistence times increased since there was no change in speed seen (Figure 5C). The fraction of migration steps towards and
away from the needle within the LMW HA gels were even closer than the HMW HA to 0.5, indicating that there was no bias towards the needle (Figure 5D). Except for the PS motility coefficient, the LMW HA did not alter the migration characteristics measured here.

Figure 5.5: Changes in Migration Characteristics in Response to Lower Molecular Weight Hylauronan in Aligned Gels. Migration parameters including cell speed (A), cell directionality (B), fractional cell migration to and from needle (C), and motility coefficient ratio (D) were analyzed for gels containing hyaluronan and LMW hyaluronan. (N_experiments = 3 and N_cells ≥ 75). Significance bars represent non-overlapping confidence intervals. Lines represent statistical significance using a two tailed t-test, p ≤ 0.05. Anova test only done on samples with similar conditions.
5.4 Discussion

With more and more studies finding the importance of hyaluronan (HA) in pancreatic cancer, we wanted to understand if this polysaccharide plays an important role in metastasis through contact guidance. Using our alignment technique, HA was doped into the collagen gel and surprising results were achieved. The biggest surprise was that the pancreatic cancer cells began to migrate directionality within the gel, indicating that HA is an important ECM component with regards to migration. Another surprising result was that this shift in migration did not result in much difference of the cellular extensions of each cell. Finally, the HA MW did not affect the migration characteristics speed or directionally but did increase motility coefficient ratio, suggesting that this is more of a receptor-mediated processes and not biophysical processes that drives migration differences seen in the presence of HA. Perhaps the degradation of HA into smaller fragments resulting in this signaling is most important. These results are exciting as they further link HA to pancreatic cancer metastasis and demonstrates its role in contact guidance.

While there have been many studies which link HA to pancreatic cancer, there hasn’t been a model which shows pancreatic cancer undergoing contact guidance. Aligned fibers have been seen in vivo around the pancreas, however it has proven difficult to model the resulting migration in vitro [9, 40]. Both MWs of HA, when combined with collagen, resulted in significant directed migration. What is not clear is if this change in directionality is due to the biophysical or biochemical interactions. Migration due to the biophysical could be a result of the changes in shear modulus and compressive resistance of the matrix or a directing of the cells due to a more confined space. This is not believed to be the cause however, as lowering the molecular weight would have an effect on the physical parameters and HA has been shown to decrease the density of the matrix [18]. It is possible that during the incubation time the cells
produce hyaluronidases which degrades both sizes of HA into similar sized pieces which would result in similar migration. As previously discussed, pancreatic tumors have very LMW HA which would indicate higher hyaluronidase activity. Migration due to biochemical interactions would be the result of the HA interaction with the cells through CD44 and RHAMM. CD44, in particular, has been shown to influence adhesiveness, motility, matrix degradation, and proliferation [41]. This type of interaction could explain why both MWs of HA resulted in increased migration characteristics as the presence of HA would be what was required to get the result.

There were three distinct types of extensions produced by the cells. The more contractile PS cells tended to have many more I and the occasional Y shaped extensions. These extensions seemed more apt to pull on the fibers. The less contractile cancer cells still showed a significant proportion of I, however they also included FAN like extensions which were smaller and more lamellipodial-like. It was hypothesized that when the cells were exposed to HA, there would be a shift which would result in even less FAN shaped extensions, however this was not exactly the case. The PS cells still produced a majority of I extensions with a slight increase of Y extension. The PC cells did show a slightly higher I extensions while the Y and FAN extensions decreased. This could be what we hypothesis, a more contractile cell. What is interesting is that the PC muc4 KO cells actually increased in FAN extensions. The muc4 is a large polysaccharide which can block binding to the ECM [42, 43]. In particular, muc4 can shield CD44 which is a primary HA receptor resulting in less adhesive cells which may explain the increase in FAN extensions [38]. This indicates that the lack of mucin-4 may have affected the cells interaction with the matrix, and that extension analysis may be more complicated than simply seeing if a cell is more or less contractile.
Another interesting conclusion of this work is that the MW of the HA did not affect the speed at all and only the directionality slightly. Comparing our MWs to other papers, the LMW HA seems to fit in the low to medium-low MW while our high is definitely within the high category [44]. Within the body typical HA MWs are between $10^5$-$10^7$ Da which indicates that the weights that were used in this experiment are biologically valid [12]. There are two potential reasons that the MW of the HA did not affect migration speed or directionality. The first is that we reached a saturation concentration of HA within the gel. If this were the case than the cells could sense but the MW of the HA but it not matter because what was important was that the cell was sensing the presence of the HA at all. This would indicate that the receptors, CD44 and RHAMM, which interact with HA may be contractility regulators and not extension regulators as we saw no difference in extension characteristics. Another potential reason for what was seen was that the cells degraded the HA into smaller pieces which resulted in a similar cellular response. Hyaluronidases have been shown to be overproduced within the pancreatic TME. This, coupled with an increased production of HA, results in a large amount of LMW HA. This is important to consider as many studies have suggested that very LMW HA results in much more motility and invasion progress [16]. If these conditions are also present in our model, it is possible that the original MW of the HA is not important since it is being degraded into much smaller pieces. If we were to block hyaluronidases, the migration characteristics of the cells could potentially change. The results do not directly suggest that HAs ability to affect porosity or malleability of the ECM resulted in the change as different sized HA would have different effects on the ECM. Interestingly, the LMW HA resulted in much higher motility coefficient ratio medians indicating that persistence time was increasing since speed remained consistent. This may be important in local invasion as the cells may be traveling more consistently without
slowing or turning. Unless the two sizes resulted in a similar affect or they saturated the cells ability to tell the difference this is unlikely.

The final take away from this paper is that this alignment method did not result in significant migration toward or away from the needle. While there were slight biases for different conditions, they never even reached a 40/60 split. There was some concern that cell directed migration may have been the result of a higher concentration of collagen and stiffness near the needle. This would have been caused by the wrapping of the protein around the needle which the cells may have sensed. This study suggests that this is not the case and that the directed migration seen is due to the alignment of collagen by the rotated needle.

5.5 Conclusions

Using a simple alignment technique, we were able to study hyaluronas (HA) effect on pancreatic stellate (PS) and pancreatic cancer with (PC) and without (PC muc4 KO) mucin 4. In the presence of HA, PS cells showed a slight increase in speed and directionality. Surprisingly, PC and PC muc4 KO cells showed directional migration in response to aligned fibers, which has not previously been seen. Further protrusion analysis revealed that the ratio of I to Y extensions was inversely proportional to cellular speed. Low molecular weight HA (LMW HA) is believed to be important during the early stages of invasion within the pancreases, so the molecular weight was decreased but concentration kept the same. Migration speed and directionality remained consistent while motility coefficient ratio median increased significantly. This indicates persistence time may be important and regulated by LMW HA.

5.6 References


CHAPTER 6: GENERAL CONCLUSIONS

Cancer accounts for nearly 1 in 6 deaths across the globe with more than 1,600 deaths a day here in the United States. The economic burden of cancer is projected to hit 458 billion by 2030 which gives us both the moral imperative and economic basis to continue to both understand and defeat this disease. 90% of morbidity and mortality can be directly linked to metastasis, which is the spreading of cancer from a primary tumor around the body to form a secondary tumor. Once metastasis has begun it is incredibly difficult to eradicate and results in poor treatment options. There is a surprising lack of understanding in what directs cancer metastasis, particularly in the initial stages. There is ample research which correlates alignment of collagen fibers to poor prognosis and increased metastasis. In particular, as the degree of alignment of collagen fibers increases in pancreatic cancer, lifetime expectancy decreases. A cells response to aligned fibers is called contact guidance. This external cue has been studied in 2D extensively, however there is a lack of reliable methods to model this type of cellular migration in 3D.

To study contact guidance, it was imperative that a reliable method for generating aligned fibers be identified. A simple approach using mechanical rotation of an acupuncture needle in a collagen gel was identified and used. Confocal reflectance and second harmonic generation microscopy was used independently to verify that significant alignment could be achieved without disruption of the collagen gel. This simple method provided a method to study directed migration and ECM remodeling which was lacking in the field.

Once a reliably approach for aligning collagen in 3D was developed, breast cancer cells were embedded in the gel and subjected to the alignment. Cells which migrated using a mesenchymal migration mode were shown to move quickly and directionality in aligned gels,
while cells that migrated using an amoeboid migration showed no directional migratory response to the aligned fibers. This was not altogether surprising as cells which migrate using a mesenchymal mode of migration have been shown to attach and reorient the matrix more than amoeboid cells. When the matrix stiffness was increased, the migration speed in aligned gels decreased for mesenchymal migration while increasing for amoeboid migration. Directionality in aligned gels which were stiffer, decreased for cells which showed directional migration but did not alter directionality in cells that did not migrate directionally. Lowering intracellular contractility, especially through Rho kinase, lowered any directional migration that was seen. This suggests that the modulation of the contractile apparatus has potential as a therapeutic target for limiting contact guidance. Given that we developed a protocol to study directed cancer migration, we decided to focus on a particular type of cancer where alignment is a prominent feature of the tumor microenvironment. Consequently, we focused our attention on pancreatic cancer where collagen fibers are known to be highly organized near the tumor.

The initial findings of subjecting the pancreatic cancer to aligned fibers showed movement within the gel but no directional migration. This is surprising as aligned fibers have been observed around metastasizing pancreatic tumors, suggesting the importance of contact guidance to this particular type of cancer. When pancreatic cancer cells were co-cultured with pancreatic stellate cells, there was an increase in speed but the pancreatic cancer cells continued to lack directional migration. However, stellate cells, when monocultured, migrated directionally suggesting that collagen alignment in the pancreatic tumor microenvironment results in stellate cell contact guidance. In addition to probing contact guidance, the collagen fiber alignment technique can be used as a powerful tool for assessing ECM organization enhancement or disruption. In vivo, cells interact and reorganize the ECM, which is an aspect lost in many 3D
models. Rotational alignment of collagen fibers allows us to use SHG to study how the cells reshape the matrix. One way cells do this is through matrix metalloproteinases (MMPs). MMP activity was increased, when pancreatic cancer and stellate cells were co-cultured, resulting in an increased degree of matrix alignment. When MMP-14 was blocked, collagen fiber alignment was disrupted and returned to a more disorganized or random fiber alignment. This was different from blocking cellular contractility as that resulted in a lack of migration and no enhancement or disruption of the alignment. Co-culture of pancreatic cancer and stellate cells also resulted in a higher degree of internal cellular contractility. When the communication modulator, mucin-4 (muc4), was eliminated from the pancreatic cancer cell genome, it resulted in a change in pancreatic stellate cell migration characteristics when co-cultured, further indicating the importance muc4 in the cancer-stellate communication. Muc4 has been shown to contain three EGF-like domains which are used to regulate growth, motility, and differentiation. Because both eliminating the function of either muc4 or MMP-14 actually caused co-cultured pancreatic cancer and stellate cells to disorganize aligned collagen fibers, they possibly represent good therapeutic approaches for reprogramming the ECM in the tumor microenvironment from cancerous (aligned collagen fibers) to normal (disorganized collagen fibers). Given that we observed pancreatic cancer stellate cells dramatically alter alignment and migration in collagen gels, we were interested in how other additional important tumor microenvironmental components such as hyaluronan may affect migration.

Hyaluronan, a scaffold component found to be overproduced in pancreatic cancer, appears to be important in modulating invasion and limiting apoptosis, but its role in directed migration is unknown. The presence of hyaluronan physically limits the drugs from spreading throughout the tumor by forming large tangles within the TME and absorbing water. Its role in
limiting cell apoptosis has also been connected with drug resistance through increased drug export through ATP-dependent efflux pumps which makes it an interesting protein to study. Consequently, hyaluronidases are being paired with therapeutics which result in better transport of chemotherapeutics into the pancreatic tumor. Due to the overproduction of HA within the pancreatic ECM and its effect on density and viscoelastic properties of the ECM, it was hypothesized that this negatively charged polysaccharide may be important in contact guidance. While we did not observe directed migration by pancreatic cancer cells in aligned collagen fiber networks, we did observe significant directed migration in aligned hyaluronan:collagen composite matrices. While not as significant as the pancreatic stellate cells, the directional migration suggests that ECM guided pancreatic cancer cell migration may be present in pancreatic tumors. This explains the somewhat baffling result that pancreatic cancer cells don’t engage in contact guidance, yet aligned fibers are a prominent feature of invasive pancreatic cancer. Perhaps hyaluronan is a necessary ECM component that turns on directional migration and merits investigation going forward. To begin to determine the mechanism of hyaluronan’s effect on directed migration we analyzed the extensions made by cells embedded in both collagen and hyaluronan:collagen composite matrices. Extension analysis of the pancreatic cancer and pancreatic stellate cells resulted in cell specific differences, however there were few differences resulting from the addition of hyaluronan to the collagen. This demonstrates that hyaluronan is not altering the protrusion of the cell, but may be impacting other steps in the migration sequence, including adhesion or contraction or matrix degradation.

Overall, a reliable and simple collagen fiber alignment method was presented to study contact guidance. Collagen fiber alignment was confirmed by analyzing both the collagen structure directly as well as the directed cell response to that structure. This rotational alignment
technique allows us to study not only directional migration, but also the organizing and disorganizing of the ECM in a way never seen before. The aligned collagen networks that we create can be used to model how stromal therapies may affect the cells ability to disorganize an aligned fiber network, transforming it from a pro-cancer ECM to a normal ECM. Pancreatic cancer cells were subjected to this cue but did not migrate directionality on its own, however it was determined that making the matrix more similar to the TME by co-culturing them with pancreatic stellate cells resulted in a change in some migration characteristics for both cell types. Further increasing the similarity between our artificial TME and the in vivo TME through the addition of hyaluronan to the aligned collagen gels resulted in directional migration by the pancreatic cell, which is believed to occur in vivo.
CHAPTER 7: FUTURE DIRECTIONS AND IMPACT ON ONCOLOGY

This project has yielded an effective tool to study contact guidance and help understand the importance of cell-cell and cell-ECM communication. I will discuss the impact that this work has on oncology. However, there is still considerable room for the implementation of this technique in understanding cancer in general or pancreatic cancer, specifically. Here I present two separate research avenues with the overall goal of furthering our understanding of how contact guidance affects metastasis. They outline the impact in fundamental cancer research as well as in applied cancer research.

7.1 Impact of my Work on the Cancer Field

I have developed an easy and inexpensive way to induce collagen fiber alignment to mimic what seen within the body. This will allow for the continued study of contact guidance in a 3D environment. I have shown that this rotational alignment method can allow for the study of mechanisms that regulate collagen fiber alignment as well as collagen fiber disorganization, which is not currently unavailable. This technique, coupled with fiber orientation analysis such as confocal reflectance microscopy (CRM) or second harmonic generation (SHG) can be used to study how effective a therapeutic is in limiting enhancement of alignment or disrupting existing alignment. The latter is important in leading to regression of the tumor. As we learn more about contact guidance and its role on cancer invasion and metastasis, easy to use and inexpensive techniques will be more important and in this, I have provided a viable option.

Another important characteristic of this technique, which I did not mention in my motivation, is the fact that this is the only method which can be used in vivo. The other
techniques such as magnetic beads, flow and cell-based are exclusively confined for use in vitro, but this acupuncture technique can be used to probe the role of collagen alignment in promoting invasion of subcutaneous tumors. Creating alignment within ECM subcutaneously will allow for in vivo testing of metastatic potential as a function of alignment to better understand to global effects of contact guidance on metastasis. Furthermore, alignment disruption techniques, such as sonic pulses or over twisting of acupuncture needles, can be evaluated for their ability to limit local contact guidance and global metastatic potential. We know that alignment is a poor prognosis in certain cancers, however there is a lack of evidence which shows that the alignment in vivo results in contact guidance. Using an acupuncture needle to alter or disrupt local collagen alignment for tumors sets it apart from other alignment techniques.

7.2 Impact of my Work on Therapeutics

Cancer therapeutics is a wide ranging field and new therapeutic targets are being identified every year. Recently, there has been a push to target the tumors mechanobiology. Inhibitors that block integrin binding, contractility, and ECM degradation are currently in the clinical trial phase as of 2018. Due to the aligned fibers and nature of cell migration, my research has helped build a case for other therapeutic targets, as well as indicate a lack of potential for others. Increasing stiffness in breast cancer cells gave varying results in aligned gels with mesenchymal cells decreasing in speed while amoeboid cells showed an increase in speed. Contractility inhibitors proved to be more effective in limiting cellular migration. Inhibiting myosin II resulted in slower cellular migration, but did not affect directionality. Rho kinase inhibition, however, resulted in both decreased migration speed and decreased directionality indicating that this may be a good therapeutic target. There are other therapeutic targets which
focus on cell-cell and cell-ECM interactions. These include mucin-4 and MMP-14. Mucin-4 has been shown to drive metastatic migration in pancreatic cancer. Our results indicate that removing the gene, disrupts the alignment, possibly by resulting in higher F-actin fiber formation. This further supports mucin-4 as a potential therapeutic target. MMPs are proteinases which the cell uses to degrade the matrix and are upregulated in cancer. Inhibition of MMP-14 resulted in lower cell speed and disruption of alignment in pancreatic cancer, indicating its potential as a therapeutic target. Finally, hyaluronan was also shown as an important therapeutic target. Already proteinases which degrade hyaluronan are being paired with therapeutics, increasing transport to the tumor. My research also indicated that it is crucial for pancreatic cancer contact guidance, indicating that its receptors, CD44 and RHAMM, may be effective in limiting contact guidance.