The Mechanism Responsible For Mechanically Enhanced Cancer Cell Invasion

Alexander Nicholas Gasparski
Wayne State University, alexgasparski@wayne.edu

Follow this and additional works at: https://digitalcommons.wayne.edu/oa_dissertations

Part of the Biology Commons, and the Cell Biology Commons

Recommended Citation
https://digitalcommons.wayne.edu/oa_dissertations/2098

This Open Access Dissertation is brought to you for free and open access by DigitalCommons@WayneState. It has been accepted for inclusion in Wayne State University Dissertations by an authorized administrator of DigitalCommons@WayneState.
THE MECHANISM RESPONSIBLE FOR MECHANICALLY ENHANCED CANCER CELL INVASION

by

ALEXANDER N. GASPARSKI

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2018

MAJOR: BIOLOGICAL SCIENCES

Approved By:

____________________________________
Advisor                      Date
DEDICATION

I would like to dedicate this dissertation to my parents, Dian and Terry Gasparski, for their unwavering support for my education and career goals. I also dedicate this work to my doctoral advisor, Dr. Karen Beningo, for her amazing support and advice throughout my graduate career at Wayne State University.
ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my doctoral advisor, Dr. Karen Beningo, for her immense trust, support and guidance throughout my graduate career. It is difficult to put into words how much she has helped me both as a scientist and as a person. This work would not have been possible without her scientific guidance and trust in my abilities and skills. Her hard-work and encouragement has made me into a better scientist, writer and overall member of the scientific community. I cannot thank her enough.

I also would like to thank my committee members, Drs. Joy Alcedo, Miriam Greenberg and Raymond Mattingly, for their support, guidance and critiques. I would like to especially thank Dr. Miriam Greenberg for allowing me to do research as an undergraduate in her lab for several years. Moreover, I sincerely thank Dr. Zheni Shen for her guidance and trust while I worked under her in Dr. Greenberg’s lab.

I also thank current and past members of the Beningo laboratory, especially those that came before me that laid the groundwork for my various doctoral projects. I had the privilege of having several hard-working and talented undergraduates work with me, especially Jacob Wilson and Bohdan Matsko.

Thank you to the Lipids@Wayne community for their support and providing numerous opportunities for professional growth.

Finally, thank you to my parents for their support throughout my life and during my educational career. Thank you to my friends for being there for me and listening to me talk about my work throughout the years.
# TABLE OF CONTENTS

Dedication ........................................................................................................................ iii

Acknowledgements ........................................................................................................... iv

List of Figures ..................................................................................................................... vii

CHAPTER 1 – INTRODUCTION ......................................................................................... 1

CHAPTER 2 – TRANSIENT MECHANICAL STRAIN PROMOTES THE MATURATION OF INVADOPODIA AND ENHANCES CANCER CELL INVASION IN VITRO

Abstract ............................................................................................................................ 10

Introduction ....................................................................................................................... 11

Methods .............................................................................................................................. 15

Results ............................................................................................................................... 23

Discussion ......................................................................................................................... 39

CHAPTER 3 – THE ROLE OF PAK1 IN THE MATURATION OF INVADOPODIA WITH TRANSIENT MECHANICAL STIMULATION

Abstract ............................................................................................................................ 46

Introduction ....................................................................................................................... 47

Methods .............................................................................................................................. 50

Results ............................................................................................................................... 54

Discussion ......................................................................................................................... 63

CHAPTER 4 – THE RESPONSE TO IN VITRO DUAL MECHANOSTIMULATION IS AFFECTED BY A CELL’S METASTATIC CAPACITY

Abstract ............................................................................................................................ 66

Introduction ....................................................................................................................... 67

Methods .............................................................................................................................. 68
LIST OF FIGURES

Figure 1.1: The metastatic cascade is a multi-step process ........................................ 2

Figure 1.2: Integrins function as an ECM receptor and can detect changes in the extracellular environment ................................................................. 5

Figure 1.3: Non-integrin mechanoreceptors ................................................................. 6

Figure 1.4: Invadopodia undergo three distinct stages: initiation, assembly and maturation .................................................................................................. 8

Figure 2.1: Design of an in vitro mechano-invasion assay ................................................. 14

Figure 2.2: Differentially expressed genes resulting from mechanical stimulation .... 24

Figure 2.3: Downregulation of integrin β3 expression upon mechanical stimulation enhances cell invasion .................................................................................... 27

Figure 2.4: The activation state of integrin β1 remains unchanged with mechanical stimulation .................................................................................................. 28

Figure 2.5: The decrease in levels of Ser3 phospho-cofilin upon mechanical stimulation is dependent on the downregulation of integrin β3 expression .............. 30

Figure 2.6: Mechanical stimulation produces an increase in the length of invadopodia without affecting the number of invadopodia ........................................ 32

Figure 2.7: Overexpression of integrin β3 and downregulation of cofilin expression both negatively affect the lengthening of invadopodia upon stimulation .... 34

Figure 2.8: MMP-2 protein expression and enzymatic activity is enhanced upon mechanical stimulation and inhibited by integrin β3 overexpression ....... 37

Figure 2.9: Potential pathway for enhanced invasion upon mechanical stimulation .... 43

Figure 3.1: PAK1 expression and activity decreases with mechanical stimulation ...... 55

Figure 3.2: Overexpression of integrin β3 affects PAK1 levels ......................................... 56

Figure 3.3: Enhanced invasion in response to stimulation requires less PAK1 activity ............................................................................................................. 57

Figure 3.4: Invadopodia fail to elongate in response to transient mechanical stimulation with an overactive PAK1 ........................................................................ 59
Figure 3.5: Intracellular MMP-2 expression increases with less PAK1 activity .......... 60
Figure 3.6: A decrease in PAK1 activity promotes more MMP-2 secretion in response to mechanical stimulation ................................................................. 61
Figure 3.7: Lowering PAK1 activity leads to more active MMP-2 degradation levels ........................................................................................................... 62
Figure 4.1: Setup of the dual stimulation assay .......................................................... 72
Figure 4.2: Comparison of bead displacement with transient mechanical stimulation and fibroblast cell contraction ................................................................. 74
Figure 4.3: Normal and non-metastatic cells preferentially respond to a stiffer substrate .................................................................................................................. 76
Figure 4.4: Transient mechanical stimulation overrides sensing of compliance in normal and non-metastatic cells, but not in tumorigenic cells ......................... 78
CHAPTER 1 - INTRODUCTION

Cell migration is a coordinated process utilized by both normal and cancer cells to move throughout their environment. In normal cells, migration is required for development, wound healing and immune system function, among other processes (Ewald et al., 2008; Friedl et al., 2004; Holub et al., 2003; Michaelis, 2014). Proper adhesion to the cell’s substrate is important for migration to occur as a traction force is required to push the cell into motion (Beningo et al., 2001). As a result, the cell’s microenvironment can affect the way that a cell migrates, for example by influencing its directionality and speed. When a cell becomes cancerous, it can upregulate its motility to invade through the extracellular matrix (ECM) to move to another location within its host organism. It can also change the way that it responds to various stimuli within the tumor microenvironment to promote enhanced cell invasion (Clark and Vignjevic, 2015).

Mechanical forces can impact many aspects of cellular physiology, including cell invasion and migration. For example, increased substrate stiffness can increase the rates of cell migration and invasion (Charras and Sahai, 2014; Kourouklis et al., 2016; Schedin and Keely, 2011), while an increase in pressure from fluid flow changes cellular alignment (Baeyens et al., 2016). Additionally, increased tension on cancer cells encourages further progress into the metastatic cascade (Assoian and Klein, 2008; Martino et al., 2018; Paszek and Weaver, 2004; Paszek et al., 2005). These examples highlight a subset of the many different ways that mechanical forces exerted on cells can modify cellular behavior and cancer progression.
Cancer cells advance through a well-defined sequence of events called the metastatic cascade (Figure 1.1) (Gupta and Massague, 2006; Scully et al., 2012). The process starts when cells within a primary tumor gain the necessary migratory phenotype through alterations in the expression of genes related to cell motility and invasion. These cells will begin to leave the primary tumor and invade through the basement membrane into the surrounding ECM. Their movement through the ECM is facilitated by both

![Figure 1.1](image.png)

**Figure 1.1.** The metastatic cascade is a multi-step process. At the primary tumor, cells will invade out from the tumor and move through the ECM until intravasating into a blood or lymph vessel. After travelling to a distant site in the body, the cell will extravasate from the vessel in search of a suitable environment. Once one is located, it will colonize to form a secondary tumor.
mechanical movement and enzymatic processes that degrade the surrounding matrix components, such as collagen, fibronectin and laminin. Cells that are able to locate a blood or lymph vessel will intravasate into it and enter the circulation. From here, cells will locate another site and extravasate from the vessel and move back into the connective tissue at a now distant site from the primary tumor. When these cells find a suitable environment, they will colonize and form a secondary tumor (Scully et al., 2012). Because approximately 90% of cancer-related fatalities are due to secondary tumors, it is important to study this process in an effort to reduce mortality (Chaffer and Weinberg, 2011). Throughout each step of the metastatic cascade, these invasive cells will encounter a wide array of mechanical forces (Kumar and Weaver, 2009; Wirtz et al., 2011). The cells respond to these forces and can utilize them to their advantage during their journey to find another site to colonize.

There are many types of mechanical forces that can affect the initial step of the metastatic cascade. For example, it has been shown that increased stiffness that results from increased collagen deposition around a primary tumor can increase the invasive capacity of cells within the tumor (Paszek and Weaver, 2004; Schedin and Keely, 2011). Additionally, as a tumor grows in size, it exerts a compressive force on the surrounding tissue, which can enhance cell motility (Tse et al., 2012). Furthermore, specific cells within the tumor microenvironment can be highly contractile (e.g., myofibroblasts), enabling them to produce contractile forces as they migrate through and remodel the ECM, which are then transmitted locally to nearby cells (Haage and Schneider, 2014; Wrobel et al., 2002). The nearby cells can detect these forces via their many mechanoreceptors and
induce physiological responses, including increased cell motility and invasion (Haage and Schneider, 2014; Jerrell and Parekh, 2014).

**The Process of Mechanotransduction**

In order for cells to respond to the numerous mechanical forces present within their microenvironment, they must have the ‘machinery’ to detect and then convert the mechanical signal into a physiological response. This process is called mechanotransduction and is facilitated by a multitude of mechanoreceptors located on the exterior of a cell (Gasparski and Beningo, 2015; Ross et al., 2013). One of the most commonly studied groups of mechanoreceptors are the integrins, which are a family of proteins that form heterodimers (comprised of an α and β subunit) that span across the cell membrane (Figure 1.2). Their role is to link the ECM to the internal actin cytoskeleton. The many integrin subunits have very specific ligands within the ECM, and when these subunits are engaged to their extracellular ligand, a conformational change occurs that leads to the formation of nascent adhesions. These structures act as a ‘linker’ between the internal and external environments of the cell and will transduce any external force into an internal force as the integrins are directly connected to the cell’s actin cytoskeleton. Similarly, a force produced internally by the cytoskeleton can be transmitted locally to the ECM in the reverse fashion (Katsumi et al., 2004; Kenny and Connelly, 2015; Ross et al., 2013; Schwartz et al., 2018; Schwarz and Gardel, 2012; Wiesner et al., 2005).

There are also other mechanoreceptors that enable a cell to detect and respond to mechanical forces. Some of these other receptors include mechanically-gated ion
channels, ephrins, the CXCR family of receptors, lipid rafts and the glycocalyx (Figure 1.3). Many of these mechanoreceptors can function in a similar way to the integrins (with a direct cytoskeleton attachment) or can transduce by other means, like a change in phosphorylation to their intracellular residues (Gasparski and Beningo, 2015).

**The Role of Integrins in Cancer**

Because integrins are responsible for the interface between the cell and the ECM, it is not surprising that integrins can affect the metastatic capacity of a cancer cell. As the

**Figure 1.2.** Integrins function as an ECM receptor and can detect changes in the extracellular environment. When the α and β subunits bind to their respective extracellular ligands, a conformational change occurs, which causes signal transduction to occur within the cell to elicit a physiological response.
tumor’s microenvironment is being remodeled by cancer-associated fibroblasts (CAFs), among other cells, the integrins are in a frequent state of activation from the changing external environment (Ross et al., 2013).

Within the large family of integrins, there are numerous subunits that have repeatedly been found to be implicated in cancer and cancer-associated physiologies.

**Figure 1.3.** Non-integrin mechanoreceptors. There are many mechanoreceptors that are not within the integrin family that transduce extracellular mechanical forces into intracellular signaling. Many of these receptors are activated by similar forces, like fluid shear stress and stretch. They have been shown to be involved in a wide variety of normal physiological processes, like cell migration and development, in addition to cancer metastasis. Figure Credit: (Gasparski and Beningo, 2015)
For example, β1 and β3 integrins play a large role in the progression and prognosis of breast cancer (Parvani et al., 2013; Shao et al., 2015). Integrin α3β1 has been found to regulate the production of MMP-2 in breast carcinoma, which modulates the migration and invasion of tumor cells (Giannelli et al., 2002; Giannelli et al., 2001; Sugiura and Berditchevski, 1999). Additionally, there are often conflicting reports when it comes to the function of these various integrins among different types of cancer. For example, the expression of α2β1 integrin has been shown to prevent breast cancer metastasis but also can increase migration in melanoma and prostate cancer (Haidari et al., 2012; Ramirez et al., 2011). These differences are very often found between cell types. Thus, understanding the basis of these differences is complicated by the characteristic large-scale dysregulation of normal cell behavior observed in tumor cells. Therefore, it is important to appreciate the heterogeneity of the microenvironments of various cell types and the receptors they express as this can produce a wide variety of responses to similar stimuli.

**The Invasive Machinery of a Cancer Cell**

In order for a cancer cell to progress through the stages of the metastatic cascade, they must be able to invade through the ECM. Cell invasion is facilitated, in part, through the use of invadopodia, which are actin-containing protrusions that are also enzymatically active. These protrusions extend from the cell membrane and contain an actin-rich core. As these protrusions lengthen, they become more mature and will produce ECM-degrading enzymes. These enzymes, called matrix metalloproteases (MMPs), will degrade the various components of the ECM to allow the cell to progress along its
metastatic journey (Beaty and Condeelis, 2014; Jacob and Prekeris, 2015; Parekh et al., 2011; Yamaguchi et al., 2005).

The process of invadopodia formation and maturation is a highly regulated process with three phases: initiation, assembly and maturation (Figure 1.4). During initiation, a core structure containing Src, protein kinase C (PKC), N-WASP and Tks5 is formed. At this point, various actin polymerization factors, like coflin, are recruited to the site. Assembly involves the stabilization of these proteins into a complex and actin cleavage by coflin, which produces sites for rapid actin polymerization by the Arp2/3 complex and N-WASP protein. Additionally, the recruitment of cortactin occurs as the invadopodia

**Figure 1.4.** Invadopodia undergo three distinct stages: initiation, assembly and maturation. During the initiation stage, Src, Tks5 and protein kinase C (PKC) are recruited to the membrane and subsequently recruit various actin polymerization factors. During assembly, coflin produces free barbed ends for rapid actin polymerization via N-WASP and the Arp 2/3 complex. The final stage, maturation, is characterized by the presence and/or secretion of various MMP enzymes.
begins to elongate. Once NHE-1, a sodium/hydrogen exchanger, is recruited to invadopodia, the pH-dependent release of cofilin from its inhibitory interaction with cortactin occurs (Beaty and Condeelis, 2014; Magalhaes et al., 2011; Yamaguchi et al., 2005). Cofilin activity can also be regulated by phosphorylation at the Ser3 position by LIM kinase 1 (LIMK1). Only cofilin that is unphosphorylated at this position can promote actin polymerization by generating free barbed ends (Blanchoin et al., 2000). As invadopodia mature, they become proteolytically active, which is characterized by the presence of MMP enzymes, particularly MMP-2, MMP-9 and MT1-MMP (also known as MMP-14). The first two are secreted into the ECM while the third remains anchored to the membrane (Jacob and Prekeris, 2015). It is through these invadopodia structures that a cancer cell can invade through their surrounding microenvironment to progress through the metastatic cascade.

Summary and Rationale

In the following studies in this dissertation, I address two main questions. First, what is the mechanism responsible for enhanced cell invasion in response to a unique transient tugging mechanical force? Second, how do normal and metastatic cancer cells interpret and respond to two simultaneous mechanical stimuli? These questions are important to understanding how both normal and cancer cells interpret the multitude of complex mechanical forces present within their microenvironment. Through these studies, we can better understand the roles of these forces on the prognosis of cancer and its treatment.
CHAPTER 2 - TRANSIENT MECHANICAL STRAIN PROMOTES THE MATURATION OF INVADOPODIA AND ENHANCES CANCER CELL INVASION IN VITRO

This chapter has been published


ABSTRACT

Cell invasion is a process utilized by cancer cells to complete their journey through the metastatic cascade, which results in the formation of deadly secondary tumors. This process is influenced by biomechanical forces that are present within the tumor microenvironment. We have previously shown that transient tugging forces can enhance the invasion of human fibrosarcoma cells in an in vitro mechano-invasion assay. This force is similar to the tugging forces present within the tumor extracellular matrix (ECM) that is produced by local matrix remodeling and nearby cell movements. In a gene expression assay we found the expression of integrin β3 to be down-regulated when this mechanical stimulation is applied. Additionally, the ability for mechanical stimulation to enhance invasion was found to be dependent on the expression of cofilin, a regulator of invadopodia maturation. Invadopodia are actin protrusions, that when enzymatically active, degrade the surrounding ECM to facilitate cell invasion. This present study examines the response of invadopodia to mechanical stimulation. When integrin β3 expression is reduced by stimulation, there is a measurable increase in the average length of invadopodia. This is likely due to increased cofilin-mediated actin polymerization activity via a decrease in Ser-3 phospho-cofilin levels. Correspondingly, as invadopodia
lengthen, they become enzymatically active, thus suggesting that transient tugging forces promote the maturation of invadopodia to enhance the level of cell invasion. Our results are unique in describing a mechanism that affects the invasiveness of cancer cells by means of transient tugging forces present within the tumor ECM, aside from traditionally studied forces such as compression or rigidity.

**INTRODUCTION**

Cell invasion is a process that drives the advancement of cancer through the metastatic cascade. When cells do not have the ability to invade, most tumors could be treated by the removal of the initial tumor and would reduce the need for chemotherapeutics to eliminate invasion-competent cells. Currently, our understanding of how invasive capacity is acquired and how invasion is executed is lacking. Cancer biologists have only recently begun to appreciate the effect of mechanical factors on the metastatic process (Kumar and Weaver, 2009). Some of these biomechanical factors can include tissue changes, such as in the structure and mechanics, as well as changes in both the extracellular matrix’s (ECM) geometry and topology. Many studies have focused on stiffness as the stroma around most tumors tends to become more rigid due to increased levels of collagen (type I) and fibronectin (Miles and Sikes, 2014; Pickup et al., 2014). Because of this increased rigidity, tumor cell proliferation, migration and invasion also increases (Alexander et al., 2008; Charras and Sahai, 2014; Jerrell and Parekh, 2014; Kostic et al., 2009; Parekh and Weaver, 2009; Tilghman et al., 2010; Ulrich et al., 2009; Umesh et al., 2014).

Aside from the matrix stiffness, various cells within the stroma, including the highly contractile myofibroblasts, produce forces within the ECM as the cells move through the
matrix and remodel it (Goffin et al., 2006; Shieh, 2011; Tripathi et al., 2012). A tugging force is produced during this remodeling as the collagen fibers within the ECM are arranged and bundled (Castella et al., 2010; Goffin et al., 2006; Murrell et al., 2015; Oudin et al., 2016). The forces produced by these cells have been known to increase both the invasion and motility of cancer cells (De Wever et al., 2008; Elkabet et al., 2011; Fuyuhiro et al., 2012). Previously, our lab determined that cell invasion rates can be enhanced in highly invasive human fibrosarcoma through the transient tugging on fibers located within a collagen/fibronectin matrix (Menon and Beningo, 2011). Mechanoreceptors located on the cell surface detect these specific mechanical cues present within the ECM (Gasparski and Beningo, 2015). Specifically, the integrin family of mechanoreceptors and their significance in mechanical signaling has been widely studied (Roca-Cusachs et al., 2012; Ross et al., 2013). Various cellular structures, such as lamellipodia, filopodia and invadosomes (invadopodia and podosomes) have been found to be mechanosensitive (Mrkonjic et al., 2016; Schwarz and Gardel, 2012).

Invadopodia are cellular protrusions that contain actin that are present in invasive cancer cells and are responsible for the degradation of the ECM. These structures recruit various matrix metalloproteinases (MMPs) that allow a cancer cell to invade through the basement membrane and to move through the metastatic cascade (Frittoli et al., 2011; Jacob and Prekeris, 2015; Poincloux et al., 2009). Within these structures are many actin-associated proteins, such as cortactin and cofilin (Artym et al., 2006; Clark et al., 2007; Yamaguchi et al., 2005). Invadopodia have three phases of development: initiation, assembly and maturation. The initiation step is characterized by the presence of N-WASP, Tks5, cofilin and cortactin into a nascent, core structure (Artym et al., 2006; Blouw
et al., 2015; Oser et al., 2009). Assembly occurs when this complex becomes stabilized and cofilin activity begins to promote actin polymerization. We have previously shown that cofilin expression is required in cancer cells in order for them to invade (Menon and Beningo, 2011; Nagai et al., 2011; Walter et al., 2009). The activity of cofilin is regulated by phosphorylation at the serine-3 position by LIM kinase (LIMK1). Actin polymerization is promoted when unphosphorylated cofilin binds to F-actin filaments. This means that the free barbed ends necessary for actin polymerization can only be created by unphosphorylated cofilin (Blanchoin et al., 2000).

Invadopodia become enzymatically active when they mature, which is marked by the localization and/or secretion of MMP enzymes. Of the large MMP family, there are three main enzymes that are typically associated with invadopodia: MMP-2, MMP-9 and MT1-MMP (MMP-14) (Jacob and Prekeris, 2015). The fibronectin type II repeats of MMP-2 bind to its collagen substrate (Polette et al., 2004). Once MMP-2 is localized to invadopodia, it is secreted and degrades the surrounding ECM (Clark and Weaver, 2008). When cofilin is overexpressed in invasive cancers, cell invasion and MMP-2 enzymatic activity are both increased (Dang et al., 2006; Yap et al., 2005), however lower cofilin expression inhibits both the maturation of invadopodia and MMP-2 activity (Tahtamouni et al., 2013; Wang et al., 2007).

While much focus has been on the stiffness of the stroma, there exists a wide variety of other mechanical forces within the tumor microenvironment (Kostic et al., 2009; Levental et al., 2009; Paszek et al., 2005). We are using an in vitro mechano-invasion assay (Menon and Beningo, 2011) to determine the level of cell invasion in response to a different type of mechanical force: transient tugging (Figure 2.1). This type of stimulation
is generated by magnetic beads that are randomly attached to anisotropic collagen and fibronectin fibers. The forces alone are not enough to stretch the substrate, nor does it cause the fibers to align in any particular direction. When this type of mechanical force is present, the cell invasion of highly metastatic cells is enhanced above a basal level (Menon and Beningo, 2011). We found that the enhancement of invasion in response to this force could only be achieved in cells that are already highly-invasive since non-

**Figure 2.1.** Design of an in vitro mechano-invasion assay. A well of 1 mm depth is created in a 60 mm cell culture dish by drilling a hole in the bottom of the dish and attaching an activated glass coverslip with vacuum grease. The resulting well is filled with a collagen type I and fibronectin matrix containing 1 μm carboxylated paramagnetic beads, which covalently attach to the fibers upon polymerization. HT1080 fibrosarcoma cells are seeded onto the surface of the matrix and either cultured 1.5 cm above a rotating magnet or outside of the magnetic field (unstimulated). After 24 hours, cells invade into the matrix and are counted to determine the percentage invasion.
invasive cells could not be stimulated to increase their invasion. As a result, it appears that this mechanical force is exploited by metastatic cancer cells. Moreover, both cofilin and fibronectin are required for a cell to respond to the stimulation. However, the mechanism responsible for sensing this type of force is not known. We set out to find candidate genes that are part of a mechanosensitive pathway involved in sensing transient stimulation. Furthermore the role of invadopodia were examined in response to stimulation, as cofilin is necessary for cancer cell invasion (Menon and Beningo, 2011). We hypothesize that the tugging forces produced within the stroma will result in a differential level of expression of genes associated with mechanosensing in cancer cells. In order to determine differentially expressed genes in stimulated invasive cells, real-time PCR analysis was used. The integrin β3-encoding gene showed differential expression in response to mechanical stimulation. Also, the decrease in integrin β3 expression promoted the lengthening (maturation) of invadopodia. As expected, the knockdown of cofilin expression prevented the maturation of invadopodia in response to stimulation. When the invadopodia increased in length, there is an increase in MMP activity from enzymes known to be associated with invadopodia. This is the first study to associate transient tugging to the enhancement of cell invasion levels in metastatic cancer cells via the maturation of invadopodia induced by this unique form of mechanical force.

METHODS

Cell Culture

HT1080 human fibrosarcoma cells were used in this study (ATCC). These cells were maintained in Eagle’s Minimum Essential Medium (EMEM; ATCC) containing 10% fetal bovine serum (FBS; Hyclone) and 1% Penicillin-Streptomycin (100 U/ml penicillin
and 100 µg/ml streptomycin; Life Technologies) at 37°C and 5% CO₂. Cells were trypsinized with 0.25% trypsin (Sigma) until the eighth passage. The cells were authenticated and verified to be clear of mycoplasma in October 2016 by the Karmanos Cancer Institute Biobanking and Correlative Services Core.

**In vitro Mechano-Invasion Assay**

As previously described, the invasion assay was setup and performed (Menon and Beningo, 2011). In summary, 1.5 X 10^4 cells were placed onto a sterile collagen/fibronectin/paramagnetic bead-containing matrix. A rotating rare earth magnet of 12,100 Gauss (25 mm x 5.5 mm) 1.5 cm under the culture on an orbital shaker (Barnstead Thermolyne) at 160 rpm (2.6 Hz) provided the mechanical stimulation. For unstimulated controls, a duplicate plate was placed outside the magnetic field. After a period of 24 hours, the cells located within ten random microscopic fields were counted using a 10X phase objective on an Olympus IX81 microscope. Starting at the matrix surface and at 100 µm/step increments within the z-plane of the matrix, cells were counted. To determine the percent of cells that were invaded, the number of cells located below the matrix surface (invaded into the matrix) was divided by the total number of cells counted. Various controls for this mechano-invasion assay have been previously performed, including confirming that the magnetic beads were not phagocytosed, that cell secretions were not alone causing a response and that the matrices were not being stretched or changed in a way to cause pores to form. Multiple cell lines, both normal and cancerous, have also been tested (Menon and Beningo, 2011).
**Collagen Degradation**

In Hank’s Balanced Salt Solution (Life Technologies), 2 mg/ml solution of collagenase type IV (Worthington Biochemical) was added and warmed to 37˚C. To the matrix, 2 ml of this solution was added after the matrix was physically removed from the culture well using a spatula and added to a sterile tube. The matrix was incubated in a 37˚C water bath with intermittent shaking for 10 min to degrade the collagen. Cells were separated by centrifugation at ~500 x g for 5 min at 37˚C. The resulting pellet contained both whole cells and the paramagnetic beads from the matrix. The pellet was washed with sterile 1X PBS at 37˚C.

**RNA Extraction**

Following degradation of the matrix, RNA was extracted from invaded cells. These matrices used for RNA extraction were seeded with 7 X 10^4 HT1080 cells on larger matrices in wells of 1 mm x 3 cm. Duplicate stimulated and unstimulated matrices were made for each independent experiment. RNA extraction was performed using the Qiagen RNeasy mini kit. The paramagnetic beads were pulled down using a magnet before loading the cell lysate onto the column to prevent clogging. Qiagen on-column DNaseI digestion was used to prevent DNA contamination. The RNA was eluted in 20 µl of DNase/RNase free water. To determine the quality of the obtained RNA, a NanoDrop spectrophotometer (ThermoScientific) was used. Only RNA samples having 260/280 ≥ 2.0, 260/230 ≥ 1.7 and concentration ≥ 40 µg/ml were used.

**PCR Array and qPCR Analysis**

cDNA was synthesized from the RNA of cells with and without mechanical stimulation from the invasion assay to be used for PCR array and qPCR analysis. In each
experiment, 1 µg of RNA from each unstimulated and stimulated sample was converted into cDNA using RT² First Strand Kit (SA Biosciences, for PCR array analysis) or GoScript™ Reverse Transcriptase (Promega, for qPCR). In order to identify genes with differential expression among stimulated and unstimulated conditions we used the following PCR arrays acquired from SA Biosciences: Cell Motility PCR Array, Tumor Metastasis PCR Array and ECM and Adhesion Molecules PCR Array. Each PCR array contained primers against 84 candidate genes related to cell motility, tumor metastasis and ECM and adhesion molecules. PCR array analysis was performed using RT² qPCR SYBR Green/ROX MasterMix-12 (SA Biosciences) on a Stratagene Mx3000P instrument. The raw data were analyzed using the web-based RT² Profiler PCR Array Data Analysis software (SA Biosciences). To normalize gene expression, the following housekeeping genes were used: actin (ACTB), β-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and large ribosomal protein (RPL13A).

In order to confirm the differential expression, qPCR primers were created for genes that had more than 1.35-fold change in expression. To design the necessary qPCR primers, online PrimerQuest software (Integrated DNA Technologies) was used. The following primers were generated: GAPDH (GAPDH-F: 5'-TTCGACAGTCAGCCGCATCTTCTT-3', GAPDH-R: 5'-ACCAAATCCGTTGACTCCGACCTT-3'), ACTN3 (ACTN3-F: 5'-CAATGGCCTCAAACTCATGCTGCT-3', ACTN3-R: 5'-TCTCTTCAGCACCAATGGACACCA-3'), CTTN (CTTN-R: 5'-ACCTGGGTGACATCCTCAAAGGT-3'), MYL9 (MYL9-F: 5'-
5'-GGCCACATCCAATGTTTCGCAAT-3', MYL9-R: 5'-AGAAGTTCATGAGCGAGGCAGTGA-3', PTK2B (PTK2B-F: 5'-AGAAGTT
CATGAGCGAGGCAGTGA-3', PTK2B-R: 5'-ATTCCATGATGATCCAGGTGGGCT-3'),
ITGB3 (ITGB3-F: 5'-TGGACAAGCCTGTGTCACCATACA-3', ITGB3-R: 5'
TTGTAGCCAAAC ATGGGCAAGCAG-3'), MTSS1 (MTSS1-F: 5'-ATCAAGATGGGCTTT
GCCGTTTCC-3', MTSS1-R: 5'-AGCCAAACCAGCTCTGTAGGGTATT-3'). During analysis, the GAPDH housekeeping gene was used for normalization. The qPCR analysis of individual genes was performed using the RT² qPCR SYBR Green/ROX MasterMix-12 (SA Biosciences) on a Stratagene Mx3000P instrument. Each gene had two or more biological replicates in addition to two technical replicates. Stratagene Mx-Pro Mx3000P software was used to obtain and analyze the data. The significance in the difference of gene expression was determined by the Student’s t-test.

**Integrin β3 Overexpression**

Integrin β3 was overexpressed in HT1080 cells by a plasmid encoding the human ITGB3 gene, pcDNA3.1-beta-3, which was provided by Dr. Timothy Springer (Addgene #27289), was used for integrin β3 overexpression in HT1080 cells. Cells were nucleofected using an Amaza Nucleofector 2 (Lonza) with kit T (Lonza) after grown to confluency. Cells were placed onto 100 mm culture dishes for 24 hours and incubated at 37°C with 5% CO₂ after nucleofection. For invasion assays used for extraction of proteins, nucleofected cells were placed directly on the assay matrices.

**Protein Extraction**

Triple detergent lysis buffer (TDLB; 50 mM Tris HCL pH 8.0, 150 mM NaCl, 1%
NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) mixed with Protease Inhibitor Cocktail
(Sigma), and Halt™ Phosphatase Inhibitor Cocktail (ThermoScientific) was used for protein extraction for Western blot analysis. Cells were rinsed with cold 1X PBS and incubated with cold TDLB for 20 min. To remove debris from cells, the lysates were centrifuged at 4°C for 10 min at ~5500 x g.

To extract proteins from cells invaded into the matrices, the matrices were degraded with collagenase and the mixture centrifuged to pellet the cells. Under ice-cold conditions, the cell pellet was incubated with 250 µl TDLB for 20 min. A magnet was used to separate out the paramagnetic beads and then the lysates were centrifuged at 4°C for 5 min at ~7000 x g to remove cell debris. This solution was then mixed with 50 µl of 6X Laemmli buffer (reducing or non-reducing, based on the antibody to be used; Boston BioProducts) and boiled for 10 min.

**Conditioned Media Collection**

To collect secreted MMP-2, the invasion assay was allowed to occur as described above for the first 24 hours. Then, the media were removed, and the matrices were rinsed two times in warm 1X PBS. Serum-free media were then added for an additional 24 hours of stimulation. Conditioned media were then collected from the cultures immediately before lysing the cells. The conditioned media were concentrated using 10K molecular weight cut-off protein concentrators (ThermoFisher). The concentration of proteins was measured using the DC protein assay (Bio-Rad).

**Western Blotting**

Protein samples obtained from stimulated and unstimulated cells after 48 hours were used for Western blotting. Proteins were then transferred onto a PVDF membrane (Bio-Rad) and blocked using 5% milk in 0.1% Tween-80 in PBS (PBS/T; for GAPDH and
cofilin), 5% milk in 0.1% Tween-80 in TBS (TBS/T; for integrin β3, integrin β1 and MMP-2) or 5% BSA in 0.1% TBS/T (for phospho-cofilin). Primary antibody dilutions were made in the same solutions used for blocking (except for integrin β3 for which 1% milk in 0.1% TBS/T was used) and incubated at 4°C overnight (except for cofilin; 4 hours at RT). The membrane was washed in 0.1% PBS/T (GAPDH and cofilin) or 0.1% TBS/T (phospho-cofilin, integrin β3, active integrin β1 and MMP-2). Secondary antibody dilutions were made in the same solution as that of primary and incubated at RT 1 hour. The following antibodies were used: rabbit polyclonal integrin β3 (1:300, Santa Cruz), rat monoclonal active integrin β1 (1:5000, BD Pharmingen), mouse monoclonal cofilin (1:300, Abcam), rabbit monoclonal phospho-cofilin (Ser-3) (1:1000; Cell Signaling Technology), rabbit polyclonal MMP-2 (1:500, Bioss), mouse monoclonal GAPDH (1:15000; Millipore), HRP tagged anti-mouse (Fisher), HRP tagged anti-rat (Abcam), HRP tagged anti-rabbit (Amersham). Following secondary antibody incubation, membranes were washed with 0.1% PBS/T or 0.1% TBS/T and incubated with Amersham ECL Prime Western Blotting Detection Reagent. ImageJ (NIH) software was used to measure and normalize the band intensities.

**Immunofluorescence**

Chemical fixation of the cells invaded within the assay matrix was performed at 37°C with a 2.5% paraformaldehyde (Electron Microscopy Sciences) solution containing 1.33X PBS for 10 min followed by a permeabilization solution of 2.5% paraformaldehyde, 1.33X PBS and 0.2% TritonX-100 for 10 min. The samples were quenched in a 0.5 mg/ml solution of NaBH₄ (Sigma) in 1X PBS for 5 min at RT. Matrices were blocked in a solution containing 5% BSA (Fisher) in 1X PBS overnight at 4°C. A mouse monoclonal α-cortactin
(1:500, Abcam) was prepared in blocking solution for overnight, 4°C. A secondary anti-mouse Alexa 647 (1:450, Life Technologies) and Alexa 546 Phalloidin (1:200, Life Technologies) prepared in blocking solution was incubated at RT for 1 hour and followed by 3 washes in 1X PBS.

**Invadopodia Imaging and Measurement**

Dye-quenched (DQ) Collagen (Type I) (ThermoFisher) was added to the unpolymerized matrix solution at a concentration of 25 µg/ml and mixed thoroughly.

Invasion assay matrices (except cofilin knockdown) were imaged using a Zeiss LSM 550 META NLO confocal microscope with an Achroplan 63x water-immersion objective. The microscope is housed in the Microscopy, Imaging and Cytometry Resources Core located at Wayne State University (Detroit, MI). Matrices with cofilin knockdown cells were imaged using a Zeiss LSM 880 with a Plan-Apochromat 63x oil-immersion objective. Z-stack images were taken at increments of 0.4µm along the z-plane. Three individual trials of at least 5 cells per trial were imaged for each experiment. Zeiss Zen (2012) and ImageJ (NIH) software were used to view the images and merge the fluorescent channels.

To measure invadopodia, individual punctate structures of actin and cortactin co-localization were identified. Measuring was performed by locating the first image within the z-stack where the co-localization first appeared (‘starting’ point) and ending when the co-localization was no longer detectable (‘ending’ point). The distance between these two values was recorded as the length of invadopodia. An average length of invadopodia within each cell was calculated and the Student’s t-test determined statistical significance.
Gelatin Zymography

30μg of concentrated conditioned medium was combined with 6x Laemmli non-reducing loading buffer (Boston BioProducts) and loaded onto an 8% SDS-PAGE gel impregnated with 1 mg/mL gelatin (Sigma). After running for 1.5 hr at 75 V, the gel was incubated in renaturing solution (2.5% Triton X-100) for 30 min at RT. After rinsing the gel twice with water, it was incubated in developing buffer (50mM Tris-HCl, pH 7.8, 0.2M NaCl, 5mM CaCl$_2$ and 0.02% Triton X-100) for 1 hr at RT. The buffer was then replaced with fresh developing buffer for 16 hr at 37°C. The gel was rinsed and stained with 0.05% coomassie blue and destained with methanol/acetic acid until bands were clearly visible. For a negative control, media not used for culture were used. ImageJ (NIH) software was used to quantify band intensities.

RESULTS

Genes Identified by PCR Array Analysis as Having Altered Expression

To identify genes with affected expression under mechanically stimulated conditions, three PCR arrays were used. The RNA obtained from mechanically stimulated and unstimulated cells was used to produce cDNA for PCR array analysis. The genes identified by array analysis were classified into 7 groups based on their function as defined by the manufacturer of the gene array. From the list of 252 total genes examined, 46 had altered expression with mechanical stimulation (using a value of 1.35-fold change as a cut-off): 38 genes had increased expression while 8 genes had down-regulated expression (Figure 2.2A).
Several of the differentially expressed genes were selected because of their connection with invasion in cancer and/or mechanosensing (Calvo et al., 2013; Figure 2.2. Differentially expressed genes resulting from mechanical stimulation. (A) qPCR array analysis found genes having a ±1.35-fold change in expression when subjected to mechanical stimulation. The pie chart illustrates the number of differentially expressed genes within 7 classes of genes and within the table are the total number of genes with either increased or decreased expression within each of the classes. (B) qPCR was used to confirm the down-regulation of select genes from the larger group of genes identified. These differentially expressed genes were selected for further confirmation by qPCR. Three biological replicates were used for qPCR of ITGB3, MTSS1, MYL9 and ACTN3; four biological replicates were used for qPCR of PTK2B. Two technical replicates were performed for each biological replicate; values represent mean±s.e.m. All results were P<0.05 (two-tailed t-test).
Ciobanasu et al., 2013; Prager-Khoutorsky et al., 2011; Shams et al., 2012; Xie et al., 2011) and then confirmed by qPCR. The following genes with down-regulated expression were selected: ITGB3 (integrin β3), MTSS1 (metastatic suppressor 1), PTK2B (protein tyrosine kinase 2B), MYL9 (myosin light chain 9) and ACTN3 (actinin 3). qPCR was performed with the RNA extracted from cells with or without mechanical stimulation for a period of 48 hours. A minimum of two biological replicates were used for each gene and two technical replicates were performed for each biological replicate. To normalize gene expression GAPDH was used. qPCR analysis was used to confirm that mechanical stimulation indeed led to the down-regulation of ITGB3, MTSS1, PTK2B, MYL9 and ACTN3 genes (Figure 2.2B). These data were contributed by Snehal Ozarkar (Gasparski et al., 2017).

**Integrin β3 has Down-regulated Expression with Mechanical Stimulation**

From the confirmed genes with differential expression, ITGB3 was selected for further analysis. The ITGB3 gene encodes for integrin β3, which is one of the β subunits within the integrin family, and is a known mechanosensor (Rathinam and Alahari, 2010). Additionally, integrin β3 pairs with integrin αv or integrin αIIb to form heterodimeric integrin molecules. These integrin β3 containing heterodimers bind to Arginine-Glycine-Aspartic acid (RGD) domains of fibronectin (Xiong et al., 2002). Since fibronectin is required for enhanced cell invasion in our *in vitro* mechano-invasion assay (Menon and Beningo, 2011), we found it interesting that the integrin β3 mechanoreceptor has decreased expression with mechanical stimulation. However, the expression level of integrin β3 can vary among several cancer types and this change in expression can affect the level of cell invasion (Jin and Varner, 2004; Seguin et al., 2015; Sheldrake and Patterson, 2009).
The decrease of integrin β3 expression was first confirmed at the translational level. The lysates from cells either mechanically stimulated or unstimulated for 48 hours were used for Western blot analysis (Figure 2.3A). Mechanically stimulated cells had approximately 0.6 times less integrin β3 level compared to unstimulated cells. This value was similar to gene expression levels, where stimulated samples contained 0.5 times less ITGB3 mRNA compared to unstimulated cells (Figure 2.2B), which confirmed that mechanical stimulation decreased the level of integrin β3 protein.

In order to verify the significance of the down-regulation of integrin β3, we tested the invasive response of HT1080 cells with the gene overexpressed in the mechano-invasion assay. If down-regulation of integrin β3 is necessary for the cells to respond to the mechanical stimulation, then overexpressing the receptor would prevent stimulation from enhancing invasion.

Western blot confirmed that integrin β3 was overexpressed for up to 72 hours in HT1080 cells (Figure 2.3C). These cells overexpressing integrin β3 were placed onto collagen/fibronectin matrices after transfection and incubated with or without 48 hours of mechanical stimulation. Cells with integrin β3 overexpressed did not respond to mechanical stimulation as they had similar levels of invasion compared to unstimulated cells (Figure 2.3D). These results confirmed that the down-regulation of integrin β3 in response to stimulation is necessary for the level of invasion to be enhanced. These data were contributed by Snehal Ozarkar (Gasparski et al., 2017).
Integrin β3 is Unaffected Despite Down-regulated Expression of Integrin β3

Integrins are known to crosstalk among each other, which means that when one integrin binds to its ligand, the expression level or activity of another integrin can be affected. This form of crosstalk has been shown to occur between both integrin β3 and

Figure 2.3. Downregulation of integrin β3 expression upon mechanical stimulation enhances cell invasion. (A) ITGB3 expression with and without mechanical stimulation in cell lysates. (B) The levels of ITGB3 protein levels in cells with and without stimulation was quantified. Results are mean±s.e.m, n=4, *P<0.05 (Student’s t-test). (C) The level of integrin β3 protein levels in cells overexpressing integrin β3 (ITGB3) and mock nucleofected cells (control). (D) The percent cell invasion in mock nucleofected cells (control) and integrin β3 overexpressing (ITGB3) cells. Results are mean±s.e.m, n=4, *P<0.05; n.s., not significant (two-factor ANOVA)
β1, which is another fibronectin-binding family member (Gonzalez et al., 2010). We thought that perhaps the decrease in integrin β3 expression maybe complemented by an increase in integrin β1 expression and/or activity. Our PCR array data found no change in the level of integrin β1 expression with mechanical stimulation (Figure 2.4A). Due to crosstalk between these integrins, expression levels may not change but receptor activation might. To examine this, an antibody exclusive to the active conformation of integrin β1 was used. We found no change in integrin β1 activity with mechanical stimulation (Figure 2.4B & C). This result suggests that there is no apparent crosstalk between the integrin β1 and β3 subunits in this mechanotransduction, therefore

**Figure 2.4.** The activation state of integrin β1 remains unchanged with mechanical stimulation. A) As determined by the PCR array experiment, the level of integrin β1 expression with and without mechanical stimulation. Two-tailed Student’s t-test (P>0.05); values shown as mean±s.e.m. B) Active integrin β1 levels in stimulated and unstimulated cells are shown in a representative Western blot. For a loading control, GAPDH was used. C) The level of active integrin β1 levels in stimulated and unstimulated cells was quantified. Two-tailed Student’s t-test (P>0.05); n=3; values represent mean±s.e.m.
eliminating an obvious divergent pathway. These data were contributed by Snehal Ozarkar (Gasparski et al., 2017).

**Mechanical Stimulation Creates More Active Cofilin as a Result of Down-regulated Integrin β3 Expression**

In order to find likely signaling pathways downstream of integrin β3, we examined the interplay between cofilin and the integrins. For invadopodia to mature, cofilin is required to sever actin filaments to promote actin polymerization (Yamaguchi et al., 2005). Cofilin siRNA-treated cells did not have enhanced invasion in response to stimulation, yet retained a basal level of invasion (Menon and Beningo, 2011). Since phosphorylation of cofilin at the Ser-3 position regulates its activity (Pollard and Borisy, 2003), we surmised that there would be lower levels of Ser-3 phospho-cofilin (inactive state) in cells subjected to mechanical stimulation. Furthermore, integrin β3 overexpression should enhance the level of inactive, Ser-3 phospho-cofilin even under mechanical stimulation if the activity of cofilin were dependent on integrin β3 expression.

After 48 hours with or without transient mechanical stimulation, cells within the invasion assay were lysed and total proteins collected to examine the levels of Ser-3 phospho-cofilin. The levels of Ser-3 phospho-cofilin were decreased with mechanical stimulation, which suggests that cofilin is likely more active (Figure 2.5A). A similar decrease in Ser-3 phospho-cofilin levels was detected in mock nucleofected cells with mechanical stimulation. Stimulated cells had 0.65 times lower Ser-3 phospho-cofilin levels compared to cells not given mechanical stimulation in both wild-type cells and mock nucleofected cells (Figure 2.5B). This effect was reversed when integrin β3 was overexpressed, such that similar levels of Ser-3 phospho-cofilin were observed in integrin
β3 overexpressing cells regardless of mechanical stimulation. Moreover, the amount of total cofilin did not change in any of cells regardless of integrin β3 expression level and

**Figure 2.5.** The decrease in levels of Ser-3 phospho-cofilin upon mechanical stimulation is dependent on the downregulation of integrin β3 expression. (A) Wild-type (HT1080), mock nucleofected (Mock) and overexpressing integrin β3 (ITGB3) cells cultured with or without stimulation were used for analysis of Ser-3 phospho-cofilin and total cofilin levels. For a loading control, GAPDH was used. (B) Ser-3 phospho-cofilin levels were quantified in cells with and without stimulation. Results are shown as mean±s.e.m, n=3, *P<0.05; n.s., not significant (two-tailed t-test). (C) Based on the data from (A), the ratio of Ser-3 phospho-cofilin was normalized to the total cofilin level. Results shown as mean±s.e.m, n=3, *P<0.05; n.s., not significant (two-tailed t-test). In both (B) and (C), the data was normalized within each condition.
mechanical stimulation (Figure 2.5B). However, mechanical stimulation does significantly reduce the portion of total cofilin that is Ser-3 phosphorylated in wild-type and mock nucleofected cells. With overexpression of integrin β3, this does not occur (Figure 2.5C). Our data strongly suggest that the amount of active, non-phosphorylated cofilin increases in response to mechanical stimulation and this increase in active cofilin is brought about by the down-regulated expression of the integrin β3 mechanoreceptor.

**Invadopodia Become Longer in Response to In Vitro Mechanical Stimulation**

It is known that when the ECM becomes more rigid, invadopodia also can become more enzymatically active (Jerrell and Parekh, 2016; Parekh and Weaver, 2016). However, it is not known what happens to invadopodia in response to the contractile tugging forces found within the ECM. From our previous results, we know that cell invasion occurs at a basal level, which suggests that invadopodia may be functioning at a basal level without stimulation. When cofilin is made more active by stimulation, it is likely that existing invadopodia would be made more active. We hypothesized that mechanical tugging increases the invasive capacity of these cells by up-regulating maturation of their invadopodia.

To test this, confocal microscopy was utilized to look at both the length and number of invadopodia structures when the cells are stimulated by the force provided by our assay. After the cells were subjected to stimulation (or without stimulation for control cells) for 48 hours, the matrices were chemically fixed. An antibody specific to cortactin, a marker of invadopodia, and phalloidin were used to visually mark invadopodia for immunofluorescence. Punctate signals of actin and cortactin co-localization were
observed in three-dimensional z-stack images to mark individual invadopodia within the cells (Figure 2.6A). The length of these invadopodia were measured and averaged.

It was determined that mechanical stimulation led to an average increase in length of 1.4 µm in stimulated cells compared to unstimulated cells (Figure 2.6B). This result suggests that mechanical stimulation was indeed causing invadopodia to become longer and thus, more mature. Moreover, more mature invadopodia should also be more enzymatically active to degrade the surrounding ECM.

**Figure 2.6.** Mechanical stimulation produces an increase in the length of invadopodia without affecting the number of invadopodia. (A) Images shown are representative of HT1080 cells that are fixed within invasion assay matrix and with and without mechanical stimulation. Red indicates actin and magenta indicates cortactin. Based on the co-localization of cortactin and actin, arrows indicate individual invadopodia. Scale bar: 10 µm. (B) The average length of invadopodia per cell after 48 h with and without stimulation. Results are mean±s.e.m., n=16, *P<0.05 (two-tailed t-test). (C) The average number of invadopodia per cell was calculated with and without stimulation. Results are mean±s.e.m., n=16, n.s., not significant (two-tailed t-test).
When we examined the number of invadopodia per cell with stimulation, we did not find any significant change (P>0.05) compared to unstimulated cells (Figure 2.6C). This was an important finding because an increase in the number of invadopodia could also be responsible for the enhanced invasion, but in response to the force provided by our assay, the invadopodia appear to be only increasing in length, not number.

**The Maturation of Invadopodia is Inhibited with Integrin β3 Overexpression**

Integrin β3 has increased affinity for the ECM when cells are under mechanical stress, including in response to increased stiffness (Jiang et al., 2006; Katsumi et al., 2005). However, in our study, we found that the expression of integrin β3 is actually down-regulated in response to tugging forces, which leads to increased invasion. To determine if the integrin β3 receptor is involved in the maturation of invadopodia in response to stimulation, we overexpressed the receptor in cells and observed invadopodia with confocal microscopy (Figure 2.7A & B). Cells were seeded onto invasion assay plates at 24 hours post-nucleofection as this was the most effective timepoint for maximum protein expression and then subjected to stimulation for 48 hours. Imaging of invadopodia showed that there is no significant change in invadopodia length with transient stimulation when integrin β3 is overexpressed (Figure 2.7C). This suggests that the regulation of a signaling pathway involved in invadopodia maturation with tugging forces is directed by integrin β3.
Figure 2.7. Overexpression of integrin β3 and downregulation of cofilin expression both negatively affect the lengthening of invadopodia upon stimulation. (A) Western blot confirming overexpression of integrin β3 in protein lysates from control vector (WT) and integrin β3 (ITGB3)-overexpressing cells after 48 h. (B) A representative confocal fluorescent image of a fixed cell with integrin β3 overexpression residing within the
Cofilin Knockdown Prevents the Elongation of Invadopodia in Response to Mechanical Stimulation

We have previously shown that cancer cells require expression of cofilin in order for them to be invasive, including in response to transient tugging (Menon and Beninag, 2011; Nagai et al., 2011). Moreover, actin polymerization within invadopodia is facilitated by cofilin activity, so if cofilin expression is decreased, the maturation of invadopodia in response to mechanical stimulation should be inhibited. To test this, siRNA was used to knockdown cofilin expression and off-target siRNA was used as a negative control. (Figure 2.7D). After mechanical stimulation, cells with normal and reduced cofilin levels were immunolabelled to examine invadopodia (Figure 2.7E). When cells have reduced cofilin expression, they have significantly shorter invadopodia, even when mechanically stimulated, compared to control siRNA nucleofected cells (Figure 2.7F). From this result,
we suggest that the maturation of invadopodia stimulated by our assay is promoted by coflin activity.

**MMP-2 Exhibits Increased Expression and Activity with Mechanical Stimulation**

The degradation of a cell’s ECM is performed by MMP enzymes that are expressed and secreted from invadopodia. MMP-2 is one such invadopodia-associated enzyme that can recognize both collagen and fibronectin as a substrate. There are two isoforms of MMP-2 that are detectable: an inactive pro-form (72 kDa) and a proteolytically active form (66 kDa). The expression and secretion of MMP-2 was measured with and without stimulation. To do this, conditioned media was collected from cells containing either the control vector or the integrin β3 overexpression plasmid.

We found that MMP-2 expression is increased in response to mechanical stimulation, which supports our hypothesis that the stimulation is promoting increased invadopodia maturation. With mechanical stimulation, control vector cells exhibit approximately 50% and 30% more expression of the inactive and active isoforms of MMP-2, respectively (Figure 2.8A & B). Additionally, there was no observed increase in expression of either MMP-2 isoform when integrin β3 was overexpressed (Figure 2.8A & B). This result suggests that the maturation of invadopodia is inhibited when integrin β3 is overexpressed since MMP-2 expression is a marker of mature invadopodia.
(Figure legend on next page)
To examine MMP-2 activity, gelatin zymography was performed with collected conditioned media from stimulated and unstimulated cells. Our results show that control vector cells had a typical increase in MMP-2 enzymatic activity while media from integrin β3 overexpressing cells had no increase in MMP-2 activity with stimulation (Figure 2.8C & D). This definitely confirms that these longer invadopodia promoted by transient tugging in HT1080 cells are indeed more active and more mature as both MMP-2 expression and secretion are increased. When the integrin β3 receptor is up-regulated, the maturation of invadopodia is prevented and less MMP-2 is expressed, secreted and active.

**Figure 2.8.** MMP-2 protein expression and enzymatic activity is enhanced upon mechanical stimulation and inhibited by integrin β3 overexpression. (A) After 48 hours, a Western blot analysis of proteins obtained from control vector (WT) and integrin β3 (ITGB3) overexpressing cells. Two isoforms of MMP-2 are able to be detected: the inactive form (72 kDa) and the active form (66 kDa). For a loading control, GAPDH was used. (B) The change in expression of MMP-2 inactive and active isoforms upon stimulation in WT and ITGB3 cells. Results are shown as mean±s.e.m., n=3. (C) Gelatin zymography of conditioned medium collected from control vector (WT) and ITGB3 cells with and without mechanical stimulation. For a negative control, unused culture media was used. Unstained bands (white) indicate enzymatic proteolysis. (D) Quantification of zymogram band intensities in WT and ITGB3 cells. Results are mean±s.e.m. from three biological replicates. (E) Matrices containing DQ collagen from cells with and without stimulation. Punctate structures with actin, cortactin and DQ collagen colocalization are invadopodia. Select invadopodia are indicated by the arrows. *P<0.01, n.s., not significant (two-tailed t-test). Scale bar: 10 μm.
In order to visually confirm that more collagen degradation was occurring \textit{in vitro}, DQ collagen type I was mixed with the matrices prior to polymerization. This type of collagen allows for the microscopic analysis of proteolysis as the excessive amount of fluorescent dyes attached to it have a quenching effect. Once it has been enzymatically degraded, the local area becomes fluorescent because the quenching has been abolished (Jedezsko et al., 2008). As a result, collagenase activity, such as from MMP-2, can be surmised to have occurred at areas of DQ collagen fluorescence. We found areas of DQ collagen fluorescence at invadopodia that were said to be mature. The intensity of fluorescence was higher at these invadopodia locations in cells that had been mechanically stimulated (Figure 2.8E). This suggests that mechanical stimulation is producing more ECM-proteolysis.

**DISCUSSION**

Metastasis is a complex process affected by numerous biochemical and mechanical factors within the tumor cell’s microenvironment. We have mimicked the tugging forces that are generated by cellular movements within the ECM by our \textit{in vitro} mechano-invasion assay. These forces produced by our assay enhance cell invasion above a basal level (Menon and Beningo, 2011). Using this assay, we have identified a potential mechanism that is responsible for producing enhanced invasion with mechanical stimulation that is similar to that found \textit{in vivo}.

Our study has found several genes that have altered expression between stimulated and unstimulated conditions in HT1080 human fibrosarcoma cells. Many of these genes are known to be involved in cell invasion and migration. For example, genes that are involved in membrane protrusion were found to be up-regulated (Albiges-Rizo et
al., 2009; Alexander et al., 2008). Since these genes had increased expression with mechanical stimulation, it suggests that invadopodia in these cells could be more active. Furthermore, we found that more cell adhesion-related genes had down-regulated expression with stimulation rather than up-regulated, which supports the finding that stimulation increases a cell’s invasive capacity. Within these genes, the integrin β3 receptor (ITGB3) had down-regulated expression with stimulation. When integrin β3 was overexpressed, stimulation no longer enhanced invasion, which confirms that the down-regulation of integrin β3 is necessary for HT1080 cells to respond to transient tugging.

The exact mechanism responsible for decreasing integrin β3 expression in response to stimulation is not known. As fibronectin is necessary for cells to respond to stimulation (Menon and Beningo, 2011), it is possible that a feedback mechanism initiated by mechanosensing is occurring through integrin β3 to down-regulate itself. However, another integrin subunit, such as integrin β1, could crosstalk to integrin β3 to down-regulate its expression. It was surprising that a mechanoreceptor would have decreased expression in response to mechanical stimulation. There are other studies, however, that have found the expression of integrin β3 to vary in many cancers with other forms of mechanical stimulation (Felding-Habermann et al., 2001; Page et al., 2015). For example, a recent study found that HT1080 cells can exhibit a “nuclear piston” invasion method that is independent of MMPs but does requires integrin β3 activity (Petrie et al., 2017). It is possible that cells use integrin β3 expression to ‘select’ between these two modes of invasion, meaning that down-regulation of integrin β3 promotes MMP-dependent invasion while normal levels of expression favor the “nuclear piston” MMP-independent invasion.
Integrin β1 and β3 bind to the RGD motif (Branch et al., 2012) and are involved in regulating the maturation of invadopodia (Beaty and Condeelis, 2014; Beaty et al., 2013; Knowles et al., 2013). Since integrin β3 expression is reduced with transient tugging, we examined if there was an accompanying increase in integrin β1 activity since this is a known receptor to localize to invadopodia (Mueller et al., 1999). We found that the activity level of integrin β1 did not change significantly with mechanical stimulation. It could be possible, however, that the decrease in integrin β3 expression may affect the localization of integrin β1 within a cell. For example, integrin α5β1 may become enriched at invadopodia and have increased engagement with fibronectin within the ECM to increase a cell’s mechanosensory ability. It is known that integrin β1 can localize to invadopodia and has been shown to increase invadopodia maturation and MMP secretion when it does (Antelmi et al., 2013; Beaty et al., 2013).

In order to confirm that transient tugging was causing invadopodia to become more mature, it was necessary to measure invadopodia-associated enzymatic activity as this is a hallmark of maturation (Jacob and Prekeris, 2015). Many MMPs are located to and secreted from mature invadopodia, including MMP-2, which had up-regulated expression as a result of the mechanical stimulation provided by our assay. When we overexpressed integrin β3, MMP-2 expression remained constant between stimulated and unstimulated cells, which suggests that the maturation of invadopodia requires the decreased expression of integrin β3. Performing confocal microscopy of the matrices containing DQ collagen showed significant degradation of collagen at and around invadopodia with mechanical stimulation. Together these results confirm that the maturation of invadopodia is enhanced with transient tugging and that the observable increase in the length of
invadopodia coincides with increased MMP activity. How these invadopodia go about becoming more mature is currently unknown. Transient tugging may speed up the rate of maturation or prevent more nascent invadopodia from disassembling compared to unstimulated cells. However, since the number of invadopodia within a cell was not significantly affected by stimulation, it is likely that the speed of maturation is promoted rather than a disruption in disassembly.

Cofilin activity is likely regulated by the reduced expression of the integrin β3 receptor in a signaling pathway downstream of the mechanoreceptor. We know that cofilin expression is critical for enhanced invasion in response to stimulation provided by our mechano-invasion assay (Menon and Beningo, 2011). Cofilin’s actin severing activity is regulated by phosphorylation by LIM kinase 1 (LIMK1) (Pollard and Borisy, 2003; Yamaguchi et al., 2005). In metastatic T-lymphoma and carcinoma cells derived from highly metastatic cell lines the levels of Ser-3 phospho-cofilin are low (Nebl et al., 1996) and the invasiveness of cancer cells is correlated with overall activity of the cofilin pathway (Wang et al., 2006; Zebda et al., 2000). Both the ligand binding ability of αvβ3 and MMP-2 expression has been shown to be linked to Ser-3 phosphorylation of cofilin (Dang et al., 2006). We show that cofilin is more active (less Ser-3 phosphorylation) with mechanical stimulation and that this increased activity can be reduced by integrin β3 overexpression. This suggests that a signaling pathway between integrin β3 and cofilin that enhances the maturation of invadopodia is modulated by mechanical stimulation.

We have developed a model signaling pathway linking integrin β3 and cofilin activity to the maturation of invadopodia based on both our observations and prior literature (Figure 2.9). We hypothesize that Rac1, a Rho GTPase, is activated by integrin
engagement, which has been shown to be regulated by integrin β3 in focal adhesions (Morgan et al., 2009). When mechanical stimulation is applied to cells, Rac1 has lower activation due to the down-regulated expression of integrin β3. As a result, the inactive...
Rac1 does not activate its down-stream effector, p21 activated kinase-1 (PAK1) (del Pozo et al., 2000). Because of the decrease in PAK1 activity, there is consequently less activation of LIMK1, which leads to higher levels of active cofilin that is unphosphorylated at the Ser-3 position. Hence, when cells are unstimulated, the cellular pool of cofilin is less active but it is still active enough to cause a basal level of invasion and invadopodia maturation. However, as more cofilin is active with stimulation, higher actin polymerization activity occurs within nascent invadopodia leading to increased maturation.

Furthermore, this study supports the idea that various types of forces utilize different signaling pathways, with the force acting as a different ligand. In our previous study we found that increasing the stiffness of the invasion assay matrix (4.5mg/ml) does not affect the enhancement of invasion from tugging forces compared to a less stiff matrix (2.5mg/ml). The level of invasion was not affected by only an increase in stiffness without transient tugging (Menon and Beningo, 2011). Importantly, it is worth noting that the matrix that we are using in this assay has a comparable stiffness to the stroma surrounding a tumor (Paszek et al., 2005), yet only with the transient tugging do invadopodia mature and enhance invasion. This means that it is not the stiffness of the matrix responsible for the maturation of invadopodia but the transient tugging force. Several other studies performed using two-dimensional cultures have found that increasing matrix stiffness results in an increase in the quantity of invadopodia (Alexander et al., 2008; Artym et al., 2015; Das et al., 2013). Our study found that the length, not number, of invadopodia is affected by stimulation. These collective results and studies suggest that highly invasive cancer cells have the ability to differentiate a change in matrix
stiffness from transient tugging. However, additional study is required to examine this idea of selective cellular mechanical communication.

In conclusion, we have discovered a mechanosensitive signaling pathway that enhances invasion via the maturation of invadopodia in response to transient tugging forces. This type of mechanical force acts as a unique biomechanical cue to promote cell invasion and adds another force to the many types of forces known to affect cancer progression and metastasis. This mechanical cue could occur in any extracellular microenvironment and can be used by a highly invasive metastatic cell to further enhance its metastatic capacity. Non-invasive cells, on the other hand, do not appear to have the necessary capacity to utilize this mechanical signal. Untangling the multitude of biomechanical cues encountered by a cancer cell will take us closer to understanding how mechanical cues are used by the cancer cell for the progression of the disease.

ACKNOWLEDGEMENTS

The authors wish to thank Imjoo Jang for his careful reading of the manuscript and the Microscopy, Imaging and Cytometry Resources Core at Wayne State University for their technical support. The Microscopy, Imaging and Cytometry Resources Core is supported, in part, by a National Institutes of Health center grant (P30 CA022453) to the Karmanos Cancer Institute at Wayne State University, and the Perinatology Research Branch of the National Institutes of Child Health and Development at Wayne State University.
CHAPTER 3 - THE ROLE OF PAK1 IN THE MATURATION OF INVADOPODIA WITH TRANSIENT MECHANICAL STIMULATION

ABSTRACT

Cancer cells are affected by a wide range of mechanical forces within their extracellular environment. It has been widely shown that these forces can lead to increased metastatic capacity of these cells. One such force is a transient tugging-like force that is similar to contractile forces present within the tumor microenvironment. When this force is simulated in vitro with a mechano-invasion assay, human fibrosarcoma cells exhibit enhanced cell invasion in a 3D collagen-fibronectin matrix by downregulating the expression of integrin β3. Recently, it was found that the tugging force produces this increase in cell invasion by enhancing the maturation of invadopodia in an integrin β3-dependent manner. The increase in invadopodia maturation is also accompanied by an increase in cofilin activity and MMP-2 secretion. The present study aims to elucidate part of the signaling pathway that is affected by the decrease in integrin β3 signaling in response to mechanical stimulation. It was found that p21-activated kinase 1 (PAK1) has decreased activity, as detected by a decrease in Ser144 phosphorylation, with mechanical stimulation. However, this loss in activity can be reversed if integrin β3 is overexpressed. Furthermore, PAK1 mutants show a correlated response in MMP-2 enzyme expression and activity in addition to the lengthening of invadopodia in response to stimulation. This suggests that a novel mechano-sensitive response in human fibrosarcoma utilizes PAK1 as a signaling player located downstream of integrin β3.
INTRODUCTION

Cancer cells within a tumor are subjected to a wide array of biomechanical forces that can affect their metastatic capacity. Although the biochemical factors that influence metastasis have been well-studied, the mechanisms by which mechanical forces modulate the invasive capacity of cancer cells have received considerably less attention. Cells receive, interpret and respond to these forces via a process known as mechanotransduction, which is when an external mechanical signal is converted into an intracellular biochemical signal (Martino et al., 2018). Examples of biomechanical forces can include the stiffness of the extracellular matrix (ECM), interstitial flow and transient tugging forces (Joyce and Pollard, 2009; Kostic et al., 2009; Kourouklis et al., 2016; Menon and Beningo, 2011). Highly contractile cells that are located near a tumor, such as myofibroblasts, can produce a transient tugging force that is transmitted locally to nearby cells as they remodel the ECM (Wrobel et al., 2002). This type of transient force has been shown to increase the cell invasion of human fibrosarcoma cells within a 3D in vitro mechano-invasion assay. Increased cell invasion is dependent on the presence of fibronectin, an abundant ECM protein, and the expression of cofilin, an actin polymerization factor (Menon and Beningo, 2011). The transient tugging force causes decreased expression of the integrin β3 mechanoreceptor, which in turn causes protrusive structures called invadopodia to lengthen and produce more ECM-degrading matrix metalloprotease 2 (MMP-2) (Gasparski et al., 2017). Due to the increase in MMP-2 secretion and activity, fibrosarcoma cells increase their invasive capacity as they progress through the metastatic cascade. At present, the exact signaling mechanism that
links mechanical force to integrin β3 receptor downregulation and subsequently to increased cofilin activity is uncertain.

There is already an existing signaling pathway that links integrin β3 to the regulation of cofilin activity, but its role in this process is unknown. Integrin β3 signals to Rac1 leading to the activation of p21-activated kinase 1 (PAK1) at the membrane by PAK1 autophosphorylation (Morgan et al., 2009). PAK1 then phosphorylates LIM kinase 1 (LIMK1) at the Tyr507 position, which reduces cofilin activity by phosphorylating cofilin at the Ser3 residue (del Pozo et al., 2000; Pollard and Borisy, 2003; Yamaguchi et al., 2005). However, it is not known if this pathway is relevant to the finding that transient mechanical stimulation produces an increase in cofilin activity, and as a result, increased invasion via the maturation of invadopodia. It is likely that this pathway could be downregulated, as this would produce more active, unphosphorylated cofilin leading to the maturation of invadopodia.

PAK1 is part of the six-member PAK serine-threonine-protein kinase family. It contains three main domains: a kinase domain in the C-terminal region, an auto-inhibitory domain and a p21-binding domain (Kumar et al., 2017; Rane and Minden, 2018). The auto-inhibitory domain within a single PAK inhibits the catalytic activity of its own kinase domain. A single PAK1 molecule is inactive but becomes active when its auto-inhibitory domain binds to another molecule’s kinase domain (Kumar et al., 2017). PAK1 is known to regulate the remodeling of the cytoskeleton, cell motility and invasion, metastasis and angiogenesis (Hammer and Diakonova, 2015; Hammer et al., 2013; Kumar and Li, 2016). The PAK family is an important link between the Rho family of GTPases and various cytoskeletal processes. For example, Rac1 is known to affect PAK1 signaling in both cell
motility and invasion (Meyer Zum Buschenfelde et al., 2018). There is also significant
evidence that PAK1 is involved in many types of cancer, especially in the regulation of
metastatic capacity of invasive cells (Hammer and Diakonova, 2015; Hammer et al.,
2013; Kumar and Li, 2016; Yang et al., 2015). However, to our knowledge, the present
study is the first to examine the role of PAK1 in the response to this type of transient
tugging force.

Through the use of an in vitro mechano-invasion assay that has been previously
developed, we examined the role of PAK1 in the upregulation of invasion in response to
transient mechanical stimulation. We found that PAK1 has both decreased expression
and activity (as shown by phospho-Ser144 levels) when transient stimulation is applied
to human fibrosarcoma cells. When integrin β3 is overexpressed, phospho-PAK1 (p-
Ser144-PAK1) levels are higher in stimulated cells, suggesting that PAK1 is more active.
When mutants of PAK1 were expressed in these cells, the ‘kinase dead’ mutants
exhibited increased cell invasion, invadopodia maturation and corresponding MMP-2
secretion. Conversely, constitutively active PAK1 mutants showed less invasion, shorter
invadopodia and less MMP-2 activity. These results suggest that a decrease in PAK1
activity is necessary for fibrosarcoma cells to enhance their invasiveness in response to
mechanical stimulation. Elucidating the signaling pathway that is affected by mechanical
stimulation within the tumor microenvironment can lead to a greater understanding of how
cells can gain the invasive capacity and progress through the metastatic cascade.
METHODS

Cell Culture

Human HT1080 fibrosarcoma cells obtained from ATCC were cultured in Eagle’s Minimum Essential Media (EMEM; ATCC) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin solution (100 U/ml penicillin and 100 μg/ml streptomycin; Life Technologies) at 37°C and 5% CO₂ in a standard cell culture incubator. Cells were passaged via Trypsinization with 0.25% trypsin (Sigma) and maintained up to the eighth consecutive passage. Cells were authenticated and tested negative for mycoplasma by the Biobanking and Correlative Services Core at the Karmanos Cancer Institute (Detroit, Michigan) in October 2016.

Integrin β3 Overexpression and PAK1 Mutants

For experiments requiring the overexpression of integrin β3, a plasmid containing the human ITGB3 gene (pcDNA3.1-beta-3) from Timothy Spranger (Addgene #272289) was used in HT1080 cells. For PAK1 mutant experiments, three plasmids were used: empty vector, PAK1-K299R (kinase inactive) and PAK1-T423E (constitutively active). These plasmids were a generous gift from Dr. Raymond Mattingly from the Department of Pharmacology at the Wayne State University School of Medicine (Detroit, MI).

For all experiments using the above-mentioned plasmids, cells were allowed to grow to ~80% confluency and nucleofected using the Amaxa Nucleofector 2 device (Lonza) with kit T (Lonza).

Mechano-invasion Assay

The in vitro mechano-invasion assay was setup as described previously (Menon and Beningo, 2011). Briefly, a sterilized matrix containing collagen, fibronectin and
paramagnetic beads was polymerized into a culture dish containing a well. Approximately 1.5 x 10^4 HT1080 cells were seeded on top of the matrix. The entire assay was placed above a rotating rare earth magnet inside of a cell culture incubator for a period of 24 hours. For unstimulated controls, the plate was kept outside the magnetic field.

**Collagen Gel Degradation**

The invasion assay matrix was degraded as previously described to collect the cells embedded on top of or invaded within the substrate (Menon and Beningo, 2011). Briefly, a 2 mg/ml solution of collagenase type IV (Worthington Biochemical) was prepared in Hank’s Balanced Salt Solution (Life Technologies) and warmed to 37°C. The matrix was physically removed from the culture dish and placed into the collagenase solution. With intermittent shaking, the matrix was allowed to degrade over a period of 10 minutes within a 37°C water bath. Cells were separated from the dissolved collagen by centrifugation and the pellet was washed once with sterile, warmed 1x phosphate buffered saline (PBS).

**Protein Extraction**

Proteins were extracted from the collected cells as previously described (Menon and Beningo, 2011). Briefly, triple-detergent lysis buffer (TDLB; 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) was mixed with Protease Inhibitor Cocktail (Sigma) and Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific). The cell pellet was incubated with the lysis buffer solution for 20 minutes under ice-cold conditions. The solution was then centrifuged at 4°C for 10 minutes to isolate protein from cell debris. Protein concentration was determined by the DC protein assay (Bio-Rad).
Conditioned Medium Collection

To collect secreted MMP-2 enzyme, the HT1080 cells were allowed to attach as normal to the invasion assay as described above. After the cells were attached, the medium was removed, and the matrix was rinsed twice with warmed 1x PBS. The medium was then replaced with serum-free medium for the duration of mechanical stimulation. The medium was collected by pipetting immediately before the matrix was degraded as described above. The collected medium was concentrated using 10 kDa molecular weight cut off protein concentrators (ThermoFisher). Protein concentration was determined by the DC protein assay (Bio-Rad).

SDS-PAGE and Western Blotting

Equal amounts of protein were mixed with 6x Laemmli buffer (Boston BioProducts) and boiled for 10 minutes (except for conditioned media for zymography, which was not boiled to retain enzyme activity). Samples were loaded onto an SDS-PAGE gel and allowed to separate. Proteins were transferred onto a PVDF membrane (ThermoFisher) and blocked for 1 hr at room temperature using 5% milk in 0.1% Tween-80 in PBS (PBS/T; for GAPDH), 5% milk in 0.1% Tween-80 in TBS (TBS/T; for integrin β3, MMP-2 and PAK1) or 5% bovine serum albumin (BSA; Santa Cruz) in 0.1% Tween-80 (BSA-TBS/T; for p-PAK1). Primary antibody dilutions were made in the same solution used for blocking and incubated overnight at 4°C. The following antibodies were used: rabbit polyclonal total PAK1 (1:1000; Bethyl Laboratories), rabbit monoclonal Ser-144 p-PAK1 (1:1000; Cell Signaling Technologies), rabbit polyclonal integrin β3 (1:300; Santa Cruz), mouse monoclonal GAPDH (1:7000; Millipore), HRP tagged anti-mouse (1:8000; Fisher) and HRP tagged anti-rabbit (1:10000; Amersham). The membranes were washed 3x for 10
mins each in the same solutions used for blocking, but without milk or BSA added. Secondary antibody dilutions were made in the same solution used for the primary antibody and were incubated for 1 hour at room temperature. After washing the membranes three times, the membranes were incubated with FemtoGlow WesternPLUS ECL reagent (Michigan Diagnostics). Band intensity readings were measured and normalized using ImageJ (NIH).

**Gelatin Zymography**

Zymography was performed as described previously (Gasparski et al., 2017). Briefly, concentrated conditioned medium was mixed with 6x Laemmli non-reducing buffer (Boston BioProducts) and loaded onto an 8% SDS-PAGE gel containing 1mg/ml gelatin (Sigma). After separation, the gel was incubated for 30 mins at room temperature in renaturing solution (2.5% Triton X-100). The gel was then rinsed twice in water and incubated for 1 hr at room temperature in developing buffer (50 mM Tris-HCl, pH 7.8, 0.2 M NaCl, 5 mM CaCl$_2$ and 0.2% Triton X-100). The buffer was replaced with fresh developing buffer and incubated overnight at 37°C. The gel was rinsed with water and stained using 0.05% Coomassie blue and destained with methanol and acetic acid solution. Band intensities were measured using ImageJ (NIH).

**Immunofluorescence**

Cells attached to the top and invaded within the invasion assay matrices were chemically fixed in a solution of 2.5% paraformaldehyde (Electron Microscopy Sciences) in 37°C 1.33x PBS for 10 mins followed by a warmed solution of 2.5% paraformaldehyde, 1.33x PBS and 0.02% Triton X-100 for 10 mins. Quenching was performed with a 0.5 mg/ml solution of NaBH$_4$ (Sigma) in 1x PBS for 5 mins at room temperature. Blocking
was performed using 5% BSA (Santa Cruz) in 1x PBS overnight at 4°C. A mouse monoclonal anti-cortactin (1:500, cat# ab33333, Abcam) antibody was prepared in blocking solution overnight at 4°C. Samples were then incubated with secondary anti-mouse-IgG conjugated to Alexa Fluor 647 (1:450, Life Technologies) and Alexa Fluor 546 phalloidin (1:200, Life Technologies) prepared in blocking solution at room temperature for 1 h and followed by 3 washes in 1x PBS.

Matrices were imaged using a Leica SP8 confocal microscope with a 63x oil-immersion objective maintained by the Microscopy, Imaging and Cytometry Resources Core at Wayne State University (Detroit, MI). Images were taken as z-stacks at 0.3 μm along the z-axis. Image stacks were analyzed and individual invadopodia measured using LAS X software (Leica) and as previously described (Gasparski et al., 2017).

RESULTS

PAK1 Expression and Phosphorylation Levels Decrease with Mechanical Stimulation

In order to determine if PAK1 is involved in the response to mechanical stimulation in human fibrosarcoma cells, it was necessary to examine the total expression level of PAK1 in addition to its activity. PAK1’s activity is regulated by phosphorylation at the serine 144 position, so we also examined Ser144 p-PAK1 with and without stimulation. When mechanical stimulation is provided by the mechano-invasion assay, the expression of total PAK1 is reduced by approximately 50% compared to unstimulated cells (Figure 3.1). Similarly, the level of Ser144 p-PAK1 is also reduced by ~60% with transient tugging mechanical force. These results suggest that mechanical stimulation reduces both the
expression of PAK1 and the level of active PAK1. It is possible that the reduction of p-PAK1 is due to the dramatic decrease in total PAK1 expression, but nonetheless, reduced PAK1 activity is the net result of the stimulation provided by this assay.

**Overexpression of Integrin β3 Increases PAK1 Phosphorylation Levels**

Since we had previously shown that the integrin β3 mechanoreceptor has downregulated expression when transient mechanical stimulation is applied to HT1080 cells (Gasparski et al., 2017), it seemed prudent to examine if affecting integrin β3 levels would change PAK1 activity. Confirmation of this result would suggest that PAK1 is
involved in the intracellular signaling cascade that is affected by the loss of integrin β3 expression. To do this, we overexpressed the human integrin β3 gene (ITGB3) in HT1080 cells and examined Ser144 PAK1 phosphorylation levels with and without stimulation. We found that when integrin β3 is overexpressed, the level of p-PAK1 increases with stimulation, which suggests PAK1 is more active when being stimulated by integrin β3 and its downstream effectors in the signaling axis (Figure 3.2). This is compared to the empty vector control where mechanical stimulation continued to produce a decrease in p-PAK1 levels. These results confirm that the regulation of PAK1 activity is downstream of integrin β3 signaling in response to stimulation.

Enhanced Cell Invasion in Response to Stimulation Depends on Decreased PAK1 Activity

We have previously shown that transient mechanical stimulation leads to enhanced cell invasion (Menon and Beningo, 2011), but the signaling pathway that is
involved in this response has not been elucidated. To determine if PAK1 is a member of the signaling pathway that produces enhanced invasion in response to stimulation, we modified the activity of PAK1 in cells through the use of two PAK1 mutants. To decrease PAK1 activity, we expressed a plasmid containing the PAK1 gene with a K299R substitution (PAK1-K299R) in HT1080 cells and tested their invasion in our mechano-invasion assay. After stimulation, cells with decreased PAK1 activity exhibited increased invasion in response to stimulation, similar to the cells expressing the negative control vector (Figure 3.3). To examine when PAK1 activity is constitutively active, a plasmid containing a T423E substitution in the PAK1 gene (PAK1-T423E) was nucleofected into the cells and mechanical stimulation was applied via our assay. Upon doing so, the

![Bar graph showing invasion levels](image)

**Figure 3.3.** Enhanced invasion in response to stimulation requires less PAK1 activity. In cells with PAK1 activity reduced (K299R), mechanical stimulation results in an enhanced level of cell invasion. When PAK1 activity is increased (T423E), stimulation no longer leads to more cell invasion. *P<0.05, **P<0.01 by Student’s t-test, data from at least three independent experiments. Error bars represent SEM ± mean.
enhancement of invasion was lost, which suggests that a decrease in PAK1 activity is necessary to promote enhanced invasion in response to transient mechanical stimulation.

**Invadopodia Become Shorter in Length with More PAK1 Activity**

In a previous study, we found that transient mechanical stimulation promotes the elongation of invadopodia through the downregulation of the integrin β3 receptor (Gasparski et al., 2017). However, the exact signaling mechanism for this is not clear. To determine if PAK1 is involved in this pathway, we increased and decreased its activity level and measured invadopodia with and without stimulation. To modify PAK1 activity, we expressed the PAK1 mutant vectors in HT1080 cells and plated them on our mechano-invasion assay. The cells in the collagen-fibronectin matrix were then fixed within the matrix, stained with fluorescent antibodies for cortactin and actin (invadopodia markers) and imaged using z-stack confocal microscopy. When PAK1 activity is reduced in cells subjected to mechanical stimulation (PAK1-K229R), invadopodia elongate in length similar to stimulated cells containing the control empty vector (Figure 3.4). Furthermore, when PAK1 activity was made constitutively active via the PAK1-T423E plasmid, invadopodia did not exhibit any change in length between unstimulated and stimulated cells. This suggests that having less PAK1 activity is necessary for mechanical stimulation to promote the lengthening of invadopodia in fibrosarcoma.

**Lowering the Activity of PAK1 Increases MMP-2 Expression and Secretion**

In order to further confirm that PAK1 activity is affecting the maturation of invadopodia, we used the same three PAK1 mutant plasmids to examine the expression and secretion of MMP-2, an invadopodia-associated enzyme. MMP-2 is a matrix
metalloprotease that has been shown to be secreted by fully mature invadopodia (Jacob and Prekeris, 2015). Its function is to degrade the surrounding ECM components, primarily collagen, to facilitate easier cell invasion. We have previously shown that transient mechanical stimulation increases both MMP-2 expression and secretion (Gasparski et al., 2017). As a result, we should expect a corresponding change in MMP-2 activity when PAK1 is either more or less active.

When PAK1 is made inactive via expression of the PAK1-K299R plasmid, we found that there is an increase in intracellular MMP-2 expression with stimulation. Conversely, when PAK1-T423E is expressed in cells, mechanical stimulation significantly decreases (~60%) the expression of MMP-2 (Figure 3.5). This result suggests that more MMP-2 is being intracellularly expressed when PAK1 is less active.
Because MMP-2 is a secreted protease, it is important to measure the level of it within the culture medium. To do this, conditioned medium was collected from the invasion assay plates just prior to collagen degradation and protein extraction. The medium was concentrated using a protein concentration column to remove proteins that are vastly different sizes than MMP-2. We found that kinase inactive (PAK1-K299R) cells had increased levels of both inactive and active isoforms of MMP-2 within the media (Figure 3.6). Furthermore, cells expressing the constitutively active PAK1 (PAK1-T423E) had no significant change in MMP-2 secretion with mechanical stimulation. These results suggest that having less active PAK1 causes more MMP-2 to be secreted from more mature invadopodia.

These results further confirm that changing PAK1 activity in fibrosarcoma cells affects invadopodia maturation in response to mechanical stimulation. Because MMP-2
expression is a hallmark of mature invadopodia, there should be an increase in both expression and secretion of MMP-2 if invadopodia are indeed becoming more mature in response to mechanical stimulation.

**Figure 3.6.** A decrease in PAK1 activity promotes more MMP-2 secretion in response to mechanical stimulation. (A) A representative western blot of PAK1 mutants (K299R: kinase inactive; T423E: constitutively active) and inactive/active MMP-2 isoforms. (B) When PAK1 is made inactive, there is more inactive MMP-2 secretion with stimulation compared to overactive PAK1. (C) With stimulation, a less active PAK1 causes a significant increase in MMP-2 section of the active isoform. If PAK1 is constitutively active, no change in MMP-2 secretion occurs with stimulation. *P<0.05, **P<0.01 by Student’s t-test; n=3. Error bars represent SEM ± mean.
Less Active PAK1 Activity Promotes More MMP-2 Degradative Activity

As MMP-2 is an enzyme, it is important to measure the activity of the secreted enzyme, because only active MMP-2 can lead to the increase in cell invasion through degradation of the surrounding ECM. To do this, gelatin zymography was used to determine the activity level of the MMP-2 within the collected conditioned medium. We found that with mechanical stimulation, kinase inactive PAK1 (PAK1-K299R) had significantly greater (~2.5 fold) gelatin degradation activity compared to unstimulated cells (Figure 3.7). When PAK1 is constitutively active (PAK1-T423E), no change is observed in the extent of gelatin degradation between mechanically stimulated and unstimulated cells. These data indicate that the elevated MMP-2 secreted from the kinase inactive PAK1 cells is also more enzymatically active. Since there is an increase in longer

**Figure 3.7.** Lowering PAK1 activity leads to more active MMP-2-mediated degradation. (A) When PAK1 has low activity level (K299R), there is a significantly greater gelatin degradation with mechanical stimulation. However, when PAK1 is highly active (T423E), mechanical stimulation does not affect MMP-2’s enzymatic activity. *P<0.05 by Student’s t-test, n=3. Error bars represent SEM ± mean. (B) A representative image of a gelatin zymography using conditioned medium from empty vector and PAK1 mutant cells.
invadopodia in these cells, a corresponding increase in MMP-2 expression and activity suggests that these invadopodia are also more mature (thus, have become enzymatically active).

**DISCUSSION**

Metastasis is a multi-step process that requires a cell to invade and migrate through several different varying environments. As an invading tumor cell moves through these different environments, it will encounter a wide array of mechanical forces. How the cell reacts and responds to these forces can affect its ability to progress through the metastatic cascade. Therefore, it is important to study these forces and the cellular mechanisms involved in responding to them. One such force being explored in this study is a transient tugging force that is produced by nearby cells migrating through and remodeling the ECM near a tumor. Using a previously developed *in vitro* mechano-invasion assay (Menon and Beningo, 2011), we mimicked these transient tugging forces on a collagen-fibronectin matrix and examined the cell’s invasive physiology. This force is one that has been shown to enhance the invasion of highly invasive cancer cells through increasing the maturation of invadopodia (Gasparski et al., 2017). However, the signaling mechanism that is involved in this process is not yet clear.

We already found that integrin β3 is important in this process as its downregulation is necessary to enhance invasion in response to transient stimulation (Gasparski et al., 2017). Based on our previous studies and current literature, we believe integrin β3 to be connected to the regulation of cofilin activity via a PAK1 signaling axis. We wanted to investigate if this signaling pathway involving PAK1 is utilized by cells to upregulate their invasion in response to mechanical stimulation. Using our mechano-invasion assay we
tested PAK1 expression and activity with mechanical stimulation, in addition to measuring invadopodia and MMP-2 levels.

We found that transient mechanical stimulation caused a decrease in PAK1 expression and activity level, as measured by Ser144 phosphorylation. Also, when integrin β3 is overexpressed, the decrease in PAK1 expression and activity is abolished, which suggests that PAK1 is downstream of integrin β3. To determine if invadopodia maturation is also regulated by PAK1 activity, we expressed PAK1 mutants (kinase inactive and constitutively active) in cells and measured invadopodia. It was found that when PAK1 has less activity, invadopodia still become longer in response to mechanical stimulation. Furthermore, when PAK1 activity is increased, invadopodia do not lengthen with stimulation, which suggests that a decrease in PAK1 activity is required for the cell to produce longer invadopodia to upregulate their invasion in response to tugging forces.

Because increased matrix metalloprotease activity is a hallmark of mature invadopodia, we tested the levels of MMP-2, an invadopodia-associated protease, in PAK1 mutant cells (Jacob and Prekeris, 2015). When PAK1 activity is decreased, more MMP-2 is secreted and enzymatically active compared to when PAK1 activity is increased. This again confirms that a decrease in PAK1 activity is important in producing mature, enzymatically active invadopodia.

There are other components of the signaling pathway that need to be tested, as PAK1 is not known to directly interact with cofilin. The kinase that is responsible for phosphorylating cofilin at the Ser3 position to regulate its activity is LIM kinase 1 (LIMK1) (Pollard and Borisy, 2003; Yamaguchi et al., 2005). PAK1 is a known regulator of LIMK1 (Park and Kim, 2017), so it is reasonable to hypothesize that a decrease in PAK1 activity
produces less active LIMK1, which would mean less Ser3-phosphorylated, inactive cofilin. With cofilin being more active, it would be able to promote further actin polymerization at nascent invadopodia to cause them to elongate and become more mature in response to this type of mechanical stimulation. Further experiments need to be performed to confirm that LIMK1 is part of this integrin β3-Rac1-PAK1 signaling axis. It is also possible that PAK1 might be affecting another aspect of invadopodia maturation, such as modifying the activity of another molecule involved in promoting their maturation. Additionally, this process should be explored in other cancer types beyond fibrosarcoma, as there are often cell type-dependent differences.

Overall, this study demonstrates the importance of examining the impact of mechanical forces within the tumor microenvironment. It is becoming increasingly evident that these mechanical forces can cause cancer cells to become more invasive and thus, more metastatic. Understanding the mechanotransduction signaling pathways affected by these forces can lead to therapeutic targets for future treatments to reduce cancer cell metastasis.

ACKNOWLEDGEMENTS

The authors wish to thank Bohdan Matsko for his help in performing some of the experiments in this study. We also thank the Wayne State University Microscopy, Imaging and Cytometry Resource Core for the use of the confocal microscopy facility and technical support. The Microscopy, Imaging and Cytometry Resources Core is supported, in part, by a National Institutes of Health center grant (P30 CA022453) to the Karmanos Cancer Institute at Wayne State University, and the Perinatology Research Branch of the National Institutes of Child Health and Development at Wayne State University.
CHAPTER 4 - THE RESPONSE TO IN VITRO DUAL MECHANOSTIMULATION IS AFFECTED BY A CELL’S METASTATIC CAPACITY

This chapter has been submitted for Publication (in re-review) and portions appear in the Dissertation of Indrajyoti Indra, WSU 2012

Indra, I., Gasparski, A.N., and K.A. Beningo

ABSTRACT

Within the in vivo environment, cells are acted upon by many different forms of biomechanical forces. For example, the surrounding extracellular matrix (ECM) can have increased stiffness, fluid shear stress and transient tugging forces, all of which will simultaneously act upon a cell. Much study has been done to understand the mechanotransduction processes that occur, but the nature of how a cell responds to multiple mechanical forces at a time is not well-studied. In this study, a series of normal and increasingly metastatic cell lines are subjected to two mechanical forces simultaneously (substrate compliance and transient mechanical tugging). We have previously shown that the metastatic capacity of a cell correlates with their response to substrate stiffness. Moreover, transient mechanical stimulation will increase cell motility in highly invasive cells. We found that when provided with competing, dual mechanical stimuli, a cell will limit their response to only one at a time. In both the normal and non-metastatic cells, the transient stimulation dominates over compliance while the metastatic cells were unresponsive to either cue. This suggests that as cells gain more metastatic capacity, there is a significant change in mechanotransduction signaling that can affect their response to complex in vivo mechanical cues.
INTRODUCTION

Mechanotransduction is an important process that affects a wide array of cellular behaviors, from wound healing to development to cancer cell invasion. Not surprisingly there are a multitude of mechanoreceptors that a cell expresses, such as integrins, cadherins, stretch-activated ion channels and ephrins (Gasparski and Beningo, 2015; Ross et al., 2013). When these receptors are stimulated by their respective mechanical signals, a change in cell behavior occurs, such as an increase in actin polymerization, phosphorylation or cell migration among other behaviors (Ciobanasu et al., 2013; Gasparski and Beningo, 2015; Mrkonjic et al., 2016; Ross et al., 2013; Zebda et al., 2012). However, most of the studies regarding mechanotransduction have been limited to the application of one mechanical stimulus at a time, which is not physiologically relevant to in vivo conditions where multiple, simultaneous mechanical stimuli exist. Therefore, it is important to study what happens when a cell is confronted with more than one mechanical cue at a time, especially in the process of cancer cell invasion, which is already known to be affected by various forms of mechanical stimulation.

The role that mechanical cues play in vivo is important in many physiological processes, such as wound healing and tissue development (Hamada, 2015; Kenny and Connelly, 2015). It has been shown that cells use these mechanical inputs to aid in various processes, for example wound healing being dependent on the stiffening of the local tissue (Tomasek et al., 2002). Also, the formation of the ductal tree during mammary gland development requires increased deposition of ECM and recruitment of contractile fibroblasts (Schedin and Keely, 2011). As such, these mechanical cues present in the cell's microenvironment are important in guiding tissue development and maintenance,
so studying the cellular response to simultaneous mechanical cues is important in both normal and disease situations.

In this present study, we developed a novel in vitro assay to provide dual, simultaneous mechanical inputs to cells to study their response. The two stimuli we have chosen are substrate compliance and transient tugging, as these have been shown to affect both cell migration and invasion, making them relevant to normal and disease contexts (Gasparski et al., 2017; Indra and Beningo, 2011; Menon and Beningo, 2011). We tested the response of a panel of normal, non-metastatic and metastatic murine breast epithelial cells in this assay. We found that the normal and non-metastatic cells preferentially respond to the transient tugging over compliance when simultaneously stimulated. However, metastatic cancer cells did not respond to either cue, which suggests that increased metastatic capacity might be associated with the down-regulation of mechanotransduction signaling. These results suggest that a cell’s metastatic capacity may correlate with a change in mechanotransduction signaling and the ability to respond to certain in vivo biomechanical signals.

**METHODS**

**Cell Culture**

Four sub-populations of murine breast cancer epithelial cell lines derived from the same primary tumor but having variable metastatic capacities (a gift from Dr. Fred Miller, Karmanos Cancer Institute, Detroit, MI), and a normal murine mammary gland cell line (NmuMg) purchased from ATCC (CRL-1636) were used for this study. All cells are adherent and are able to form spheroids using the method described below. Mouse embryonic fibroblasts (MEFs) were purchased from ATCC. Cultures were maintained in
Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (Hyclone), and supplemented with 100 U/mL penicillin, 2 mM L-glutamine, and 100 μg/mL streptomycin (Gibco). Cells were grown in a standard cell culture incubator at 37°C with 5% CO₂.

3D Spheroid Preparation

Multicellular 3D spheroids were prepared by culturing cells on agar-coated 96-well plates. Briefly, 96-well plates were coated with 50 µL of sterile 2% agar and UV sterilized for 30 minutes. Trypsinized cells were resuspended in cell culture medium and 1 X 10⁴ cells/mL were pipetted into each well. For spheroid development, the plate was placed on a platform rotating at 1.83 Hertz inside the cell culture incubator until rounded spheroids formed. The spheroids were kept in culture for approximately 24 hours until ready to use to allow them to proliferate to a suitable compactness and size.

Substrate Preparation

Polyacrylamide gels were prepared with a few modifications as described previously (Beningo et al., 2002). The flexibility of the substrate was setup by keeping the total acrylamide concentration constant at 5% while varying the bis-acrylamide concentration between 0.04% (Young’s modulus: 330 Pa, referred to as “soft”) and 0.1% (Young’s modulus: 1980 Pa, referred to as “hard”). The substrates were embedded with 50 µL of fluorescently labeled beads (0.2 µm carboxylated microspheres). A 20 mm hole was drilled with 1 mm thickness through the bottom of a 60 mm culture dish (Nunclon) and a chemically-treated coverslip was attached with vacuum grease to the bottom of the culture dish. Approximately 250 µL of hard substrate solution treated with ammonium per sulfate (APS) and tetramethylmethylenediamine
(TEMED) was pipetted into the well filling half of the well volume. A silanized square coverslip (25 x 25 µm) was placed on top of the solution leaving a gap on the opposite side of the well while the hard substrate polymerized for several minutes. Next, 250 µL of APS- and TEMED-treated soft substrate was pipetted into the opposite half of the well. Then, a paramagnetic bead of 800 µm diameter (Cospheric) was quickly placed into the unpolymerized soft substrate using fine-tipped tweezers and positioned approximately 0.5-1 mm away from the border of the two substrates. The coverslip was moved over the top of the two substrates during polymerization. After the substrates had polymerized, the coverslip was carefully removed. To facilitate cell adhesion, bovine plasma fibronectin (Sigma) at a concentration of 5 µg/cm² was conjugated to the surface of the polyacrylamide substrates as described previously (Beningo et al., 2002). After overnight incubation with fibronectin at 4°C, the substrates were rinsed with 1X PBS twice and UV sterilized for 30 minutes.

**Spheroid Placement**

After UV sterilization, 500 uL of culture medium was pipetted onto the substrate and placed into a cell culture incubator for 10 minutes to allow for temperature equilibration. A uniformly circular spheroid was selected and gently removed from the 96-well plate using a cut, sterile pipette tip. The spheroid was allowed to settle to the bottom of the cut tip via gravity and the tip was then gently transferred to the border between the hard and soft substrates. The spheroid was positioned on the border using a fine micropipette tip and allowed to attach for a period of 1-5 hours in a minimal amount of culture medium inside the humidified incubator to prevent drying. Once attached, 4 mL of culture medium was gently added to the plate.
Application of the Mechanical Stimulus

Mechanical stimulation was applied as described previously with slight modification. Briefly, the assay plate was positioned 0.5 cm above a rare-earth magnet of 12,000 Gauss (25 mm in diameter, 5.5 mm in thickness). The magnet was rotated below the culture plate at 160 rpm (2.6 Hz) in an orbital field of 2 cm on an orbital rotator (Barnstead, Roto Mix Type 50800) for 36 hours. The distance of the assay plate and the rotational speed of the magnet were adjusted based on the data obtained from bead displacements previously observed from cultured fibroblasts (as described in the results section).

Measurement of Cell Dissemination

An Olympus IX81 ZDC inverted microscope was used to acquire phase contrast images of the spheroid both before and 36 hours after mechanical stimulation was applied. Live spheroids were imaged at 37°C with 5% CO₂ within a custom stage incubator. Images were captured using a 10x/0.25NA CP-Achromat lens and SPOT Boost EM-CCD-BT2000 camera (Diagnostic Instruments) driven by IPLab software (BD Biosciences). The distance that cells disseminated from the spheroid was measured by drawing a line from the spheroid edge to the furthest cell disseminated using ImageJ (NIH)

RESULTS

An Assay for the Simultaneous Application of Dual Mechanical Stimuli

The aim of this study was to examine the changes that occur in the sensing of substrate compliance and transient mechanical stimulation and to test if these responses correlate with metastatic progression. In order to do this, we designed an in vitro dual
stimulation assay whereby two mechanical cues are provided simultaneously to normal, non-metastatic and metastatic cells (Figure 4.1A-B). Two polyacrylamide gels of varying rigidities were cast side-by-side and the entire gel surface was conjugated with fibronectin, an abundant ECM protein, to produce a uniform surface for optimal cell attachment. Fibronectin was chosen because it is necessary in order for compliance to be sensed by this series of cells according to our previous study (Indra and Beningo, 2011). In order to apply transient mechanical stimulation to the softer polyacrylamide gel, a paramagnetic bead of 800 μm diameter was embedded within the soft half of the substrate. It was carefully positioned ~0.5-1 mm away from the border between the substrates. To produce the necessary transient tugging force on the softer side of the gel, a paramagnetic bead of 800 μm diameter was embedded within the soft half of the substrate. It was carefully positioned ~0.5-1 mm away from the border between the substrates. To produce the necessary transient tugging force on the softer side of the substrate.

![Figure 4.1. Setup of the dual stimulation assay.](image)

**Figure 4.1.** Setup of the dual stimulation assay. (A) A schematic drawing of the dual stimulation assay. Two polyacrylamide gels of differing rigidity (hard and soft) are polymerized adjacent to each other. A paramagnetic bead is placed just within the order of the soft substrate. A 3D spheroid of cells is allowed to attach at the border of the two gels. The entire assay is placed above a rotating rare earth magnet. (B) A photograph of the assay.
substrate, the entire assay plate was positioned above a rotating rare earth magnet within a standard tissue culture incubator. Three-dimensional spheroids containing one of the cell lines were then placed at the border of the two gels and allowed to attach. Once attached, the transient mechanical stimulation was applied for a period of 36 hours. An unstimulated plate served as the negative control for all experiments.

**Physiological Relevance of the Transient Mechanical Stimulation**

Because we wanted to examine the response of these murine mammary epithelial cells to the contractile forces that are produced by nearby cells, we needed to ensure that the transient mechanical forces applied by our assay are physiologically relevant to fibroblasts. First, we determined that a 12,000 Gauss rare earth magnet of 25 mm in diameter and 5.5 mm thickness rotating under the assay at 2.33 Hertz and a 2 cm orbit can produce approximately 800 Gauss of magnetic force. This was reproduced under the microscope and we measured the displacement of fluorescent microbeads embedded within the gel from the transient mechanical force. The fluorescent beads were displaced 0.15-0.25 μm and 0.17-0.28 μm in the x and y axis, respectively, due to the tugging force produced by the paramagnetic bead under the applied magnetic force (Figure 4.2A). We compared this displacement to a monolayer of mouse embryonic fibroblasts (MEF) cells cultured on the same substrate. When we measured the displacement of the fluorescent beads directly adjacent to the MEF monolayer, we observed a displacement of 0.19-0.89 μm and 0.09-0.21 μm in the x and y direction, respectively (Figure 4.2B). This indicates that the forces produced by the cells themselves are greater relative to the transient mechanical stimulation applied by our *in vitro* assay. Therefore, we assume that the data obtained by this assay is more
conservative than what occurs *in vivo*, as the myofibroblasts that are located within the mammary gland stroma are more contractile than the MEF cells used for this comparison. These data were contributed by Indrajoyti Indra (2012).

**Figure 4.2.** Comparison of bead displacement with transient mechanical stimulation and fibroblast cell contraction. (A) The displacement of fluorescent microbeads embedded within the hydrogel was measured with magnetic stimulation. The X and Y coordinates of the bead positions are shown in four different timepoints (M1-M4) around the rotational axis of the magnet. (B) The X and Y displacement of fluorescent
microbeads nearby a monolayer of MEF cells are shown at 15-minute intervals. (Indrajoyti Indra, 2012)

Normal Mammary Epithelial Cells Preferentially Respond to Substrate Compliance Over Transient Mechanical Stimulation

In normal tissue, mammary epithelial cells encounter variances in both the compliance of the ECM and transient mechanical tugging. Because of this, we started by testing the response of normal murine mammary gland cells (NmuMg) in our dual stimulation assay. A multicellular three-dimensional spheroid made up of NmuMg cells was placed at the border of the two fibronectin-coated polyacrylamide gels. Once the spheroid became attached, it was subjected to 36 hours of magnetic stimulation to generate the transient tugging force. As a negative control, a duplicate plate was kept out of the magnetic field. Images of the spheroids were taken at 0 hours and after 36 hours. To determine the cell dissemination distance from the spheroid, a line was drawn from the spheroid's edge to the cell farthest disseminated from the spheroid and measured using ImageJ.

We found that the dissemination of normal NmuMg cells depended on the compliance of the substrate, which agreed with our previous findings. When provided with stimulation by the substrate stiffness only, NmuMg cells disseminated approximately 100 μm on the soft substrate and approximately 221 μm on the hard substrate (Figure 4.3).
This suggests that normal cells preferentially respond to a stiffer substrate. However, when transient mechanical stimulation is applied in conjunction with a choice of stiffness,
Figure 4.3. Normal and non-metastatic cells preferentially respond to a stiffer substrate. (A) The dissemination of cells from spheroids given only compliance stimulation at 0 hours (left column), and 36 hours on soft (middle column) and hard (right column) substrates. Scale bar: 10 μm. (B) Average distance of dissemination for all cell lines on the soft and hard substrates are shown in the bar graph. *P<0.05 by Student's t-test from at least three separate experiments. Error bars represent SEM ± mean.

the normal epithelial cells respond preferentially to the tugging force, as they disseminated approximately 415 μm on the soft substrate (toward the stimulation) and only 211 μm on the hard substrate (away from the stimulation). This result suggests that transient mechanical stimulation overrides the stiffness cue in normal epithelial mammary cells.

Cells Lose the Ability to Sense Compliance and Transient Mechanical Stimulation with Increased Metastatic Capacity

It is known that these mammary epithelial cells have a decreased ability to sense a change in substrate compliance as they gain metastatic capacity (Indra and Beningo, 2011). However, it is not known if these cell’s ability to sense and respond to transient mechanical stimulation is affected by a change in metastatic capacity. To test this, we used a panel of murine breast cancer cell lines of increasing metastatic capacity within our dual mechanical stimulation assay.

When stimulated only by substrate stiffness, 67NR and 168FARN cells preferentially disseminated to the stiffer of the two substrates at a distance of 106 μm and 83 μm, respectively (Figure 4.4). The most metastatic of the cell lines, 66cl4, did not
**Figure 4.4.** Transient mechanical stimulation overrides sensing of compliance in normal and non-metastatic cells, but not in tumorigenic cells. (A) Cell dissemination
from spheroids with applied transient mechanical stimulation at 0 hours (left column) and 36 hours on the soft (middle column) and hard (right column) substrates. Scale bar: 10 μm. (B) Average cell dissemination distances for all cell lines are shown in the bar graph. *P<0.05 by the Student’s t-test from at least three independent experiments. Error bars represent SEM ± mean.

appear to prefer either substrate over the other, as the difference between its dissemination was not significant (P>0.05). However, when transient mechanical stimulation is applied in conjunction with substrate stiffness, 67NR and 168FARN cells exhibited robust dissemination toward the stimulation onto the soft substrate at a distance of 415 μm and 403 μm, respectively. This result is in contrast to the most metastatic cell line, 66cl4, having no preference to either substrate compliance or transient mechanical stimulation. These results suggest that the breast cancer cells within this panel lose their ability to sense both compliance and applied transient tugging forces as they become more metastatic.

DISCUSSION

It is widely accepted that mechanical forces play an important role in regulating various aspects of cellular behavior. Until now, these studies have limited the application of mechanical stimuli to a single stimulus at a time, which is not physiologically relevant to in vivo conditions where a cell is subjected to many forces simultaneously. In this study, we have provided dual, competing mechanical cues (compliance and transient tugging) in a simultaneous manner to ask how cells respond to these forces. Their response was also studied in conjunction with their metastatic capacity.
The two forces provided in our dual mechanical stimulation assay are similar to two forces present in both normal and cancerous tissues. It is known that normal and cancer tissue can have varying levels of stiffness, with cancer tissue sometimes being stiffer. The stiffnesses used in our assay reflect that of breast cancer tumor formation and progression. The transient tugging also applied by our assay is similar to the contractile forces generated by native cells in the tumor microenvironment, such as fibroblasts. Furthermore, we used three-dimensional spheroids instead of a cell monolayer to more accurately represent a tissue.

In a previous study, we showed that a cell’s ability to sense a change in substrate stiffness decreases as cells become more metastatic. This study shows that when transient mechanical tugging is applied in concert with compliance, the normal (NmuMg) and non-metastatic cancer cells (67NR and 168FARN) responded only to tugging and ignored stiffness. The most metastatic cell line, 66cl4, did not show any change in dissemination with transiently applied tugging stimulation. This implies that with increasing metastatic capacity, the ability to sense these two stimuli is turned off, or greatly inhibited. Moreover, we suggest that the transient tugging force dominates over the substrate compliance cue in both normal and non-tumorigenic breast cancer cells. This also means that cells may only respond to one mechanical cue at a time, at least when presented with the two forms of mechanical forces produced by our assay. This response is also likely cell type dependent as different mechanoreceptors and their associated signaling pathways vary among different cell types, especially among various cancers. Furthermore, there may be some interplay between biochemical cues and these competing mechanical stimuli that may affect a cell’s behavior.
From the conclusions in this study, it is necessary to study the response to various other competing mechanical cues, such as shear flow and osmotic stress, in conjunction with substrate stiffness and transient tugging. In a cell’s complex in vivo environment, it is subjected to many forces simultaneously, so it would not be surprising if introducing additional combinations of forces may elicit different behaviors. Also, this response is likely to be cell type dependent, even among cancers, so examining other types of cells is crucial to understanding this process. The various mechanoreceptors expressed by the subset of murine breast cancer cells used in this study are also likely to be responsible for their observed responses to these forms of stimulation. For example, a change in expression of an integrin subunit may significantly alter the response to these forms of stimulation. We have previously shown that the down-regulated expression of the integrin β3 mechanoreceptor is necessary for fibrosarcoma cells to enhance their cell invasion in response to transient tugging forces (Gasparski et al., 2017). Because that is the same as the force used as in this present study, it is possible that integrin β3 may also be involved in this ‘decision’ process that a cell faces when presented with simultaneous stimuli. Additional studies should be performed to examine the expression and activation levels of mechanoreceptors in response to compliance and transient tugging, as there could be interplay between the receptors that are typically affected by these two forces.

It is important to study the responses to the various mechanical forces that exist within both normal and disease tissues. This is especially important in cancer because many studies have shown that cancer cell invasion can be affected by many different types of mechanical forces. Knowing the effects of simultaneous mechanical stimuli on
cells can help to understand how the \textit{in vivo} cellular microenvironment can affect both normal and disease processes.
CHAPTER 5 - SUMMARY AND CONCLUSIONS

In this dissertation, I have investigated the effects of various mechanical forces on the invasion and migration of cancer cells. In Chapter 2, I demonstrated that invadopodia become increasingly mature in highly invasive fibrosarcoma cells in response to transient tugging mechanical stimulation, which allows them to enhance their cell invasion. Using an \textit{in vitro} mechano-invasion assay and confocal microscopy, I found that invadopodia structures become longer with stimulation. Moreover, there is an increase in invadopodia-associated MMP enzyme expression and activity with stimulation. This response also occurs due to a down-regulation of the integrin β3 receptor and decreased coflin Ser3 phosphorylation. Therefore, I can conclude that mechanical stimulation causes a decrease in integrin β3 signaling, which leads to more active coflin for the elongation invadopodia allowing for enzyme-facilitated cell invasion in individual cancer cells of mesenchymal origin. In Chapter 3, I investigated the signaling pathway that is affected by the down-regulated expression of the integrin β3 receptor in response to mechanical stimulation. I found that invadopodia are affected in this process, but the signaling pathway involved in this response is not yet clear. The data demonstrated that PAK1 has decreased expression and activity with mechanical stimulation and by using PAK1 expression mutants, decreased PAK1 activity produces longer invadopodia and increased MMP-2 activity. Conversely, expression of a constitutively active PAK1 reduces the maturation of invadopodia in response to stimulation. Therefore, I conclude that PAK1 is downstream of integrin β3 in the signaling pathway that is involved in the up-regulation of cell invasion in response to transient mechanical stimulation. In Chapter 4, I examined the response when a series of normal and progressively metastatic breast cancer cell
lines were given two simultaneous mechanical cues (substrate compliance and transient tugging). Cells are presented with multiple mechanical stimuli in their *in vivo* environments, so it is important to understand how normal and metastatic cells might differentially respond to these multiple cues. Using a panel of murine mammary breast epithelial cells, I showed that normal and non-metastatic cancer cells will only respond to one of the two cues at a time. The cells preferentially responded to the transient tugging cue. However, the most highly metastatic cell line ignored both cues and didn’t show any preference. This suggests that as a carcinoma cell gains metastatic capacity, its response to complex mechanical cues within its microenvironment are altered. This may arise via the altered expression or activity of various mechanoreceptors expressed on the cell’s surface or a change in activity of intracellular signaling molecules.

From the data that I have presented in this dissertation, I can conclude that highly invasive individual fibrosarcoma cells show enhanced invadopodia maturation in response to transient tugging mechanical stimulation in order to increase their cell invasion. Moreover, when cells of epithelial origin are presented with multiple mechanical cues simultaneously, only the highly metastatic cells will ignore both cues while the normal/non-metastatic cells preferentially respond to one cue over the other.
REFERENCES


ABSTRACT - THE MECHANISM RESPONSIBLE FOR MECHANICALLY ENHANCED CANCER CELL INVASION

by

ALEXANDER N. GASPARSKI

December 2018

Advisor: Dr. Karen A. Beningo

Major: Biological Sciences

Degree: Doctor of Philosophy

Cell invasion is an important process utilized by cancer cells to progress through the metastatic cascade to form deadly secondary tumors. This process can be influenced by the wide array of biomechanical forces that cancer cells within and around a tumor face in their microenvironment. It is not completely clear how these forces, either alone or simultaneously combined with other forces, can impact the metastatic capacity of cancer cells. To address this, we have utilized an in vitro mechano-invasion assay to mimic a transient tugging force that exists within the tumor microenvironment caused by the remodeling of the extracellular matrix by highly contractile cells, such as myofibroblasts. Furthermore, we have used a novel dual-stimulation assay to compare the response between normal, non-metastatic and metastatic cells to two simultaneous mechanical stimuli. Our results show that transient mechanical stimulation leads to increased invadopodia maturation in highly invasive fibrosarcoma cells, as shown by confocal microscopy and the activity of invadopodia-associated matrix-degrading enzymes. This increase in invadopodia maturation is caused by the down-regulation of the integrin β3 mechano-receptor, a decrease in p21-activated kinase 1 (PAK1) activity.
and an increase in cofilin activity. Additionally, when normal and non-metastatic cells are simultaneously stimulated with a change in substrate compliance and mechanical tugging, they preferentially responded to the tugging force over compliance. The metastatic cells did not preferentially respond to either mechanical cue. Together, these data indicate that highly invasive cells can upregulate their cell invasion in response to transient mechanical stimulation through increasing the maturation of invadopodia. Also, when metastatic cells are simultaneously given conflicting mechanical cues, they can preferentially ignore them, whereas non-metastatic cells do not. This suggests that there is a major interplay between the mechanical forces that exist near a tumor, the physiological nature of the cancer cells themselves and the level of metastatic capacity of these cells.
AUTOBIOGRAPHICAL STATEMENT

ALEXANDER N. GASPARKSI

Education

2013-2018: Doctor of Philosophy, Biological Science, Wayne State University, Detroit, Michigan

2008-2012: Bachelor of Science, Biological Science, Wayne State University, Detroit, Michigan

Publications


Selected Oral Presentations

Toledo CellulART, Department of Biological Sciences, The University of Toledo, September 2017 – Transient Mechanical Strain Affects PAK1 Signaling to Promote the Maturation of Invadopodia

Department of Biological Sciences Annual Retreat, Wayne State University, March 2017 – Mechanical Stimulation Enhances Cancer Cell Invasion and Invadopodia Maturation

Lipids@Wayne Seminar, Wayne State University, April 2015 – Mechanical Stimulation Enhances Cancer Cell Invasion and Invadopodia Maturation

Selected Poster Presentations

ASCB Annual Meeting, Dec 2017 – Transient Mechanical Strain Affects PAK1 Signaling to Promote the Maturation of Invadopodia

ASCB Annual Meeting, Dec 2016 – Transient Mechanical Strain Promotes the Maturation of Invadopodia and Enhances Cancer Cell Invasion in Vitro

Selected Awards & Honors

Thomas C. Rumble University Graduate Fellowship – The Graduate School, Wayne State University, Aug 2017 - May 2018

Exceptional Research Award – Dept. of Biological Sciences, Wayne State University, Aug 2017