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BIOPHYSICAL AND BIOCHEMICAL FACTORS IN THE CELLULAR MICROENVIRONMENT; EFFECTS ON CELL MIGRATION AND INVASION

by

SHALINI MENON

DISSERTATION

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Approved By:

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Advisor Date

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DEDICATION

I would like to dedicate this thesis to my dissertation advisor, Karen A. Beningo; my parents, Unnikrishna Menon and Sashikala Menon; my sister, Sharika Menon and my husband, Debashish U. Menon. The past six years through graduate school and the final outcome in the form of this thesis dissertation would not have been possible without my advisor's constant encouragement and unparalleled guidance, my parents unconditional love and prayers, my sister's immense ability to make me laugh even during the toughest of times and my husband who has been my best friend and my pillar of support. All of you mean the world to me.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Cell migration is a highly orchestrated cellular process essential for the sustenance of life (Horwitz and Webb, 2003; Lauffenburger and Horwitz, 1996). Aberration in cell motility has been observed in disease states such as tumor metastasis, chronic inflammation, and in developmental malfunctions (Franco and Huttenlocher, 2005; Horwitz and Webb, 2003; Webb et al., 2005). Migration is coordinated temporally and spatially by both chemical and physical factors (Lauffenburger and Horwitz, 1996). It has been long accepted that biochemical signals alter cellular migration, both under normal and disease states, and these biochemical pathways have been studied extensively (Devreotes and Zigmond, 1988; Janetopoulos and Firtel, 2008; Jones, 2000; Keller, 2005; Parent and Devreotes, 1999; Van Haastert and Devreotes, 2004; Wells, 2000). However, although the effects of physical factors on cell migration had been documented as early as 1914 (Harrison, 1914; Weiss, 1934), it is only in the past 20 years that our understanding has advanced considerably. It has now become appreciated that changes in the biophysical properties of the environment, and within cells themselves can significantly modulate cell migration (Curtis and Wilkinson, 1999; Davies, 1995; Duncan and Turner, 1995; Georges and Janmey, 2005; Li et al., 2002; Li et al., 2005; Lo et al., 2000; Palecek et al., 1997).

During migration, cells interact physically with the environment. They generate contractile forces, referred to as traction forces and they can also sense physical signals from the environment, both of which are crucial in propelling their migration. Cells are

able to receive and respond to physical signals from its environment and this enables them to perceive changes in the compliance of the substrate or the spatial arrangement of the ECM (Beningo et al., 2004; Lo et al., 2000; Pelham and Wang, 1997)**.** The ability of a cell to sense mechanical properties and changes that result in varying cellular response can be divided into three major steps – ***mechanosensing, mechanotransduction and mechanoresponse*** (Vogel and Sheetz, 2006). Mechanosensing is a term used to define the ability of a cell to sense the mechanical properties of the environment by means of changes in protein conformation or protein clustering which can lead to biochemical reactions (Bershadsky et al., 2003; Kung, 2005; Martinac, 2004; Shemesh et al., 2005). The downstream result of mechanosensing is termed mechanotransduction and includes the activation of G-protein signaling or kinase activation, and often leads to changes in gene expression (Martinac, 2004; Vogel, 2006). Changes in cell shape, motility and other physiological processes that result from mechanotransduction is referred to as the mechanoresponse (Vogel and Sheetz, 2006). The detailed mechanisms involved in each of these steps is, however, not very well understood and an area of intense study. In the sections below a brief summary of the current literature on migration and the biochemical and biophysical players required for migration (in 2- and 3-dimensions) has been provided.

Migration machinery – the vital components of coordinated cell motility

Cell migration, is a crucial cellular process that must be well regulated to maintain the healthy state of a multi-cellular organism. It requires coordination of a number of events taking place both intracellularly and extracellularly. Migration in response to a

biochemical or biophysical stimulus occurs in 2- or 3-dimensional environments with cells moving on a physical substrate. It is therefore essential that the cell is able to sense both the mechanical and biochemical composition of the environment and respond in an appropriate manner. Given below is a detailed description of the cellular machinery that is crucial to cell migration and invasion.

Focal adhesions:

Focal adhesions serve as the nexus of communication between the inside of the cell and the extracellular environment. Focal adhesions were first observed by electron microscopy in 1971 (Abercrombie et al., 1971), however to date, the complete mechanism of focal adhesion assembly remains ill defined. Focal adhesions are large, heterogeneous, dynamic protein complexes comprised of structural proteins, adaptor proteins, protein tyrosine kinases, serine/threonine kinases, phosphatases, proteases and modulators of small GTPases. Currently more than 150 proteins can be found within a cell substrate adhesion (Zaidel-Bar et al., 2007). Proteins commonly used as focal adhesion markers for immunoflouescence studies include vinculin, paxillin and zyxin.

Adhesions undergo a maturation process during migration. Maturation begins with the formation as nascent focal complexes (a dot-like contact) at the leading edge of the cell (Geiger and Bershadsky, 2001). These complexes form in response to the clustering of integrin receptors (Burridge and Chrzanowska-Wodnicka, 1996; Clark and Brugge, 1995). As the cell continues to move forward, these focal complexes either disappear or mature into the cell interior in a centripetal fashion. Thus, focal adhesions are continuously being assembled and disassembled as the cell migrates. During the

process of maturation these adhesions change from a symmetrical, dot-like structure to an elongated structure (Stricker et al., 2011).

Tyrosine phosphorylation and dephosphorylation of the various focal adhesion proteins is essential for focal adhesion dynamics. The significant kinases are focal adhesion kinase (FAK) and c-Src (Sastry and Burridge, 2000). It has been shown that upon inducing mechanical stress on a cell, the focal adhesion proteins show elevated levels of tyrosine phosphorylation (Schmidt et al., 1998). It has also been shown that the tyrosine phosphorylation levels of proteins in focal adhesions are affected by the mechanical properties of the adhesion substrate (Pelham and Wang, 1997). *In vitro* studies demonstrate that mechanical stress leads to an increase in the phosphorylation level of various focal adhesion proteins (Smith et al., 1998). The extent a single cell spreads on a substrate can be correlated proportionally with an increase in the levels of tyrosine phosphorylation of focal adhesion proteins (Lin et al., 2000). Thus, evidence exists to suggest that tyrosine phosphorylation of focal adhesion proteins regulate traction force and mechanosensing pathways. Further studies will help elucidate the purpose of tyrosine phosphorylation signaling in each of these pathways.

Integrins:

Integrins are transmembrane cell adhesion molecules that mediate cell- ECM interaction, thereby integrating the intracellular and extracellular environments. Integrins are non-covalently associated heterodimeric molecules, composed of an alpha and beta subunit. In vertebrates, there are 18 alpha and 8 beta subunits that associate in various combinations to give rise to 24 different integrin molecules (Arnaout et al., 2007). Each

of these integrins binds a specific ECM protein through its extracellular domain. Ligand binding leads to integrin activation, clustering and focal adhesion protein recruitment. The level and type of integrin expressed has also been correlated to the type of cell and the migratory capacity of a given cell type (Chan et al., 2007). The cytoplasmic domain of integrin recruits and binds focal adhesion proteins. Thus, transmembrane integrin receptors link the cell interior and the physical environment of the cell. The ability of integrins to "integrate" extracellular and intracellular environments helps in "outside-in" signaling: signals transmitted from outside the cell to the inside for bringing about changes in cell motility, proliferation, cell shape etc., and "inside-out" signaling: transmission of forces generated within the cell by the cytoskeletal machinery (Luo et al., 2007).

The cellular cytoskeleton:

The cellular cytoskeleton, an orderly arrangement of protein filaments, provides the framework for the concept that form defines function at the cellular level. The cytoskeleton functions include, defining cell shape, migration, intracellular trafficking of organelles, and chromosome segregation during cell division to name just a few. The three main types of filaments that constitute the cytoskeleton are the intermediate filaments, microtubules and actin filaments. Of these filament types, actin is key to maintaining cell shape and for cell locomotion. Actin and proteins that are involved in actin dynamics form the plasma membrane protrusions that serve as a cell's first line of sensing (Ridley, 2011).

Multiple types of protrusion structures can be found at the leading edge of a single cell. Each of these structures contributes to migration in its own specific manner. Lamellipodia are sheet like structures formed by actin polymerization. Actin in this region is highly branched and devoid of microtubules (Abercrombie et al., 1971). Just behind the lamellipodia is a region termed the lamella. In the lamella the adhesions are coupled to the contractile actin cytoskeleton and associated myosins (Ponti et al., 2004). The Arp2/3 complex, the WASP family of proteins, and formins are the large protein families responsible for actin nucleation in the lamellipodia (Campellone and Welch, 2010; Chesarone and Goode, 2009). Often filopodia are observed protruding from the lamellipodia. Filopodia are finger like projections comprised of parallel bundles of actin thought to function as probes of the extracellular environment (Ridley, 2011). Filopodia are formed as extensions of the lamellipodia by Arp2/3 nucleation aided by fascin which bundles the actin (Gupton and Gertler, 2007). More recently a new structure referred to as blebs, which are formed when the plasma membrane detaches temporarily from the underlying actin cortex, have been found to influence migration and cancer cell invasion (Charras and Paluch, 2008; Fackler and Grosse, 2008). Other structures important for cell invasion and migration include invadopodia and podosomes (Buccione et al., 2009). Like lamellipodia and filopodia, they are also actin rich structures, but have the added feature of releasing proeolytic enzymes that target the ECM (Poincloux et al., 2009). Small and short lived invadopodia are referred to as nascent invadopodia and do not efficiently cleave the ECM. These are usually very motile (Yamaguchi et al., 2005). However, long and mature invadopodia are more stationary and effectively degrade the matrix. Research

has shown that cofilin, a critical protein in actin dynamics is essential for the process of invadopodia maturation. Each of these actin containing protrusions also contain various cell surface and transmembrane proteins that are required for inside-out and outside-in signaling.

The cytoskeleton is also imperative to the production of cellular traction forces. These forces are generated by the coordinated action of the actin and the myosin cytoskeleton and transmitted onto the substrate via the focal adhesion complex through the integrins (Beningo et al., 2001; Fournier et al., 2010). Microtubules also regulate force production (Kaverina et al., 2002; Kaverina et al., 2000; Rape et al., 2011). Properly regulated cellular forces maintain cell shape and migration (Wang and Lin, 2007). However, how mechanical forces are regulated is a topic that is not well understood. Recent studies do suggest that generation of traction force can be controlled by two distinct mechanisms. One is focal adhesion kinase (FAK) dependent, whereas the other is FAK independent and myosin II dependent (Rape et al., 2011). Additionally, we have discovered that a lectin binding protein, when secreted, is essential for regulating the production of mechanical forces by the cell. Thus actin generated forces are controlled by a complex mechanism involving the cellular cytoskeleton, numerous signaling proteins and secreted proteins. Further investigation is required before the pathway is completely deciphered.

Calpain Proteases in the Regulation of Migration

The calpain (Capn) protease family in mammals has sixteen known genes. Fourteen of these genes encode proteins that contain the protease domain, and two genes

encode smaller regulatory proteins. These regulatory proteins associate with the larger catalytic calpains to form heterodimeric holoenzymes. Most calpains are ubiquitously expressed (Franco and Huttenlocher, 2005). The best characterized and most relevant to our study are the two ubiquitous isoforms, Capn1 and Capn2 holoenzymes, referred to as μ-Calpain and M-calpain respectively. The holoenzymes are composed of the large Capn1 and Capn2 subunit respectively, each of which heterodimerizes with the smaller regulatory subunit, Capn4.

Calpain mediated proteolysis plays a major role in numerous cellular processes including, apoptosis, proliferation, endocytosis, and in cell adhesion and migration (Franco and Huttenlocher, 2005; Glading et al., 2002; Sato et al., 1995). Calpains are regulated during cell migration by calcium and phospholipids binding, autolysis, phosphorylation and inhibition by calpastatin (Franco and Huttenlocher, 2005). Calpastatins are endogenous inhibitors of Capn1 and Capn2, and thought to maintain balance in the "calpain system" of the cell. Many proteins found in the adhesion complex are calpain targets, examples of which are talin, paxillin, vinculin, ezrin, cytoplasmic tails of integrins β1, β3, and β4 (Glading et al., 2002). Calpains have also been associated with a variety of pathological conditions such as stroke, ischemia and muscular dystrophy (Franco and Huttenlocher, 2005).

Previously published studies from our lab have implicated Calpain proteases in mechanical aspects of migration (Undyala et al., 2008). In this study, the function of the catalytic and the small regulatory subunit were tested for affects on traction force and mechanosensing. The expression of each of the three calpain subunits, Capn1, Capn2 or

Capn4 was silenced individually by siRNA or by obtaining knockout mouse embryonic fibroblasts. Simultaneous inhibition of Capn1 and Capn2 protease activity was achieved by overexpression of calpastatin. The absence of Capn4 resulted in reduced traction force as compared to wildtype Mouse Embryonic Fibroblast (MEF) cells or cells in which the catalytic subunits were silenced. Additionally, disorganized actin stress fibers formed, fewer focal adhesions were linked to stress fibers, and decreased adhesion strength in Capn4 deficient MEF cells was observed. These defects were not found in the absence of the large subunits or when calpastatin was overexpressed, suggesting that the small non-catalytic subunit Capn4 modulates the production of traction forces independent of the catalytic activity of the protease holoenymes, Calpain 1 and 2.

Our previous studies also demonstrate that the absence of the two large subunits or loss of their proteolytic activity, and also a deficiency in Capn4, resulted in the inability to sense localized tension and a failure to engage dorsal integrins (Undyala et al., 2008). An unpublished result from our laboratory also suggests that fibroblasts are able to sense changes in substrate rigidity (homeostatic tension) without the presence of all three subunits of the two Calpain holoenzymes. These results indicate that the ability to perceive changes in localized tension but not substrate rigidity, require the proteolytic activity of the calpain holoenzymes. These results are summarized in Figure 1.1 and have led to the hypothesis that Capn4 alone and not the proteolytic activity of the calpain holoenzymes, directly or indirectly, modulates traction force production by a mechanism that is separate from the mechanosensing pathway. This hypothesis formed the basis for the studies performed in chapters 3 and 4.

Assay	MEF	*capn4 -/-* MEF	Capn1 KD MEF	Capn2 KD MEF	Calpastatin overexpression
Traction Stress	+	-	+	+	+
Reponse to Localized Tension	+	-	-	-	-
Response to Homeostatic Tension	+	+	+	+	n.a.
Dorsal Integrin Engagement	+	-	-	-	-
Substrate Adhesion	+	-	+	+	n.a.
Adhesion-Stress Fiber	+	-	+	+	n.a.

Figure 1.1: Analysis of the mechanical properties of MEF cells, Capn1, Capn2, Capn4 silenced MEF cells and MEF cells in which Calpastatin (endogenous inhibitor of Capn 1 and Capn 2) has been over expressed.

Different assays were used to study the function of Capn1, Capn2, and Capn4 in (1) regulating traction force, (2) response to localized mechanical tension, (3) dorsal integrin engagement, (4) substrate adhesion strength, (5) focal adhesion and stress fiber formation and (6) migration speed.(+) indicates results observed as seen in MEF cells. However, (-) indicates a lack of response or a reduced response as compared to a wildtype response. (n.a.) indicates that the assay was not performed with the specific cell type.

Migration at the cellular level

Due to technological limitations, studies of cell migration have primarily been performed on two dimensional (2D) planar surfaces. What has emerged from years of these studies, is a model of 2D cellular migration involving three major steps – leading edge attachment onto the surface, cellular contraction, and finally the release of the

trailing edge (rear) of the cell (Lauffenburger and Horwitz, 1996). These steps of migration would apply for most migratory cell types and is referred to as the "mesenchymal" mode of migration (Friedl, 2004). Thus this mode of migration requires a spatio-temporally regulated dynamic interaction between the cell and the substrate on which it moves (Friedl, 2004; Rafelski and Theriot, 2004).

Establishing the leading edge-Actin Protrusions:

Mesenchymal migration begins with the cell assuming a polarized morphology and provides the demarcation of the front and rear of the cell (Lauffenburger and Horwitz, 1996). Polarization is prefaced by the extension of membrane protrusions, lamellipodium and filopodium, in the direction of movement, defining the leading edge of the cell (Condeelis, 1993). The details of how these protrusions are formed is hotly debated, but involves the polymerization of the cytoskeletal protein, actin (Condeelis, 1993; Ridley, 2011; Stossel, 1993). These membrane protrusions are devoid of cytoplasmic organelles (Letourneau, 1983; Small, 1981).

As described earlier, protrusions are primarily composed of actin and actin associated proteins, including actin capping and severing proteins, and those required for actin polymerization and bundling. Polymerized actin forms intricate and dynamic meshworks and bundles of filaments that provide structural support of the protrusion, amongst other functions (Lauffenburger and Horwitz, 1996). Actin polymerization at the leading edge, which has been described as a treadmilling process, provides enough force to push the membrane outward (Bugyi and Carlier, 2010; Le Clainche and Carlier, 2008; Wang, 1985). The Brownian ratchet model suggests the generation of this force results

from actin bundling and branching (Mogilner and Oster, 1996). The outward protrusion of the cell membrane, and the following actin polymerization, also results in a pressure gradient that can then drive the fluid cytosol to the front of the cell (Zhu and Skalak, 1988).

Attachment to substrate and transmission of forces:

Extension of the protrusion is followed by the formation and stabilization of integrin-mediated adhesions at the leading edge. Internal Reflection Microscopy was used to demonstrate that new adhesions form at the leading edge of the cell and grow larger in size as the cell continues to migrate (Izzard and Lochner, 1980; Regen and Horwitz, 1992). These adhesions, as discussed in greater detail above, serve two important roles during cell migration. They serve as a link between the ECM, on which the cell is attached and the acto-myosin cytoskeleton, thereby helping transmit traction forces from the cytoskeleton to the substratum. Adhesions also form loci for the assembly of signaling complexes (Wolfenson et al., 2009). These signaling complexes are vital to cell migration and a number of other cellular processes including cell proliferation and survival.

Traction and contractile forces are generated during the formation and stabilization of the lamellipodia (Lauffenburger and Horwitz, 1996). In a migrating cell the nascent focal adhesions formed at the leading edge transmit larger traction forces as compared to the more mature larger focal adhesions (Beningo et al., 2001). Recent studies also demonstrate that these points of contact are inchoate and dynamic with respect to protein-protein interactions within the adhesions (Hu et al., 2007; Wang, 2007).

These studies have resulted in the formulation of the clutch hypothesis which suggests that when there is no slippage between the actin network and the substrate, the forces transmitted are more effective (Jurado et al., 2005; Lin and Forscher, 1995). Slippage results in retrograde flow of actin. However, a different mechanism referred to as the viscous friction mechanism suggests that velocity of actin flow is directly proportional to the traction forces generated (Theriot and Mitchison, 1992). Gardel et al in 2008 suggested a biphasic relationship observed in epithelial cells which incorporated both these mechanisms and suggested that the switch depends on the actin velocity (Gardel et al., 2008). A similar study performed with neuronal cells suggests that substrate rigidity can also control this switch (Chan and Odde, 2008). The contractility of the actin cytoskeleton for the production of traction forces and its transmission onto the cell exterior are obviously very important, but what regulates traction forces is not well understood. Also, how the switch between the clutch model and the viscous friction mechanism influences traction force and in turn alters cell migration also requires further study.

Detaching the Rear of the Cell:

Finally, efficient migration also requires that the cell releases its adhesions at the rear of the cell so that it can move forward (Chan et al., 2007). Early studies have shown that the release of the rear end determines migration rate, making it the rate limiting step of the entire cycle (Chen, 1981). If the rear is not released properly, as observed in mutant cells, the cell has been known to rip itself apart (Crowley and Horwitz, 1995; Regen and Horwitz, 1992). However, it is not unusual for a normally migrating cell to

leave small pieces of its membrane on the substratum in its wake. A number of membrane receptors can be found in these fragments, including beta 1 integrin molecules (Regen and Horwitz, 1992). The integrins that remain on the cell surface however, are dispersed through the cell body upon detachment or they are endocytosed leading to recycling of the integrins (Palecek et al., 1996; Regen and Horwitz, 1992).

The detailed mechanisms resulting in the release of the rear of the cell are not clearly understood. However, studies have shown that cytoskeleton contractility and signaling mechanisms contained within the focal adhesion complex contribute to rear end detachment (Hendey et al., 1992; Jay et al., 1995; Paterson et al., 1990; Wilson et al., 1991). For instance, Calpain 2, a member of the calpain family of proteases described earlier, has been implicated in mediating rear end detachment by proteolysis of a number of its substrates, most of which are focal adhesion proteins (Cuevas et al., 2003; Franco and Huttenlocher, 2005). To initiate proteolysis, Calpain 2 is activated via the MAP kinase pathway and also requires the adaptor function of FAK (Cuevas et al., 2003).

In summary, efficient migration of cells adopting the mesenchymal mode of migration on a 2-dimensional substrate typically follow the steps outlined above and simply repeat these steps in an orderly fashion during subsequent cycles resulting in what is referred to as a “migratory cycle” (Parsons et al., 2010). Equally important for coordinated migration, although not discussed in detail here, is the temporally and spatially regulated action of the signaling molecules necessary for each event.

Cell migration in the physiological context:

Within the body, cells migrate primarily within three dimensional (3D) environments with only a few exceptions. These exceptions would include migration of epithelial cells during processes, such as wound healing, in which case the cells move on a flat surface (Kirfel and Herzog, 2004). The third dimension presents the cells with physical constraints that are not encountered in 2D migration. The cells are required to traverse through connective tissue composed of a meshwork (sometimes quite dense) of extracellular matrix (ECM) proteins that include rope-like fibers of collagen and fibronectin. Thus, apart from the three major steps observed during 2D migration, namely actin mediated protrusion of the leading edge, attachment onto the substrate, and rear end detachment, there are additional steps that facilitate migration through the ECM protein mesh (Friedl and Wolf, 2009).

Dimensionality imposes changes in the details of the migratory steps described for 2D. To begin, unlike the readily polarized state observed when migrating on a 2-dimensional surface, cells migrating in a 3-dimensional surface are less efficiently polarized, if at all. In 3D a cell extends a psuedopod following chemical or biophysical stimulation, instead of filopodia and lamellipodia (Wolf et al., 2003; Wolf et al., 2007). The cell then attaches to the substrate through focalized adhesion structures that are typically fewer in number in 3D. The forces transmitted through these sites helps realign the extracellular matrix fibers with respect to the cell body (Even-Ram and Yamada, 2005; Miron-Mendoza et al., 2008). The cell front being thin can protrude through small gaps. However, the ECM fibrils are organized such that the nucleus of the cell impedes

its ability to squeeze through small gaps (Friedl and Wolf, 2009). To overcome the physical barriers, focalized proteolysis of the ECM proteins occurs, primarily by the Matrix Metalloproteases (MMPs) family of proteases (Wolf et al., 2007). Multiple perpendicular fibers can be cleaved to generate enough space for the cell to pass unimpeded. The cell can then propel itself forward through these gaps by means of acto-myosin mediated contractility.

Although migration can be divided into general steps in either dimension, a number of factors determine the details. For instance, significantly different modes of migration are observed depending on the cell type. A diversity of cell shapes can be adopted during migration. The kinetics of migration is also largely cell type dependent. Another determinant of cell type dependent migration is the extent of cell-cell and cell-matirx interactions (Friedl and Wolf, 2008; Wolf et al., 2007). During migration not all cell types follow the mesenchymal mode of migration described in the above paragraphs. For example, neutrophils and leukocytes migrate by a gliding mechanism referred to as an "amoeboid" mode of migration (Guck et al., 2010). They exert very weak traction forces onto the substrate on which they migrate. This mode of migration is also mostly integrin independent (Lammermann and Sixt, 2009). Another aspect of amoeboid migration is the ability of the cell to change its shape in response to its environment, such as cell bending to accommodate curvatures along the migration path, elongation of the cell to pass through small pores and also blebbing (Lammermann et al., 2009). Thus, the rate of amoeboid migration depends on the ability of the cell to switch between cell shapes.

With the technological advances in microscopic imaging, multiple modes of three dimensional migration and invasion have emerged (Webb and Horwitz, 2003). Cells migrating in 3D have been observed to migrate either individually or collectively as a group (Wolf et al., 2007). When migrating individually the cells adopt one of two basic morphologies, either that of an elongated mesenchymal cell or the more rounded morphology of an amoeboid cell. These morphologies are interchangeable during 3D migration and cells occasionally alternate between morphologies as they progress towards their final destination (Wolf et al., 2003). The mode of migration is unique to the cell type and the microenvironment in which the cells are present.

The microenvironment and its influence on migration and invasion

The extracellular microenvironment, both at the cellular and the tissue levels, impacts cell adhesion, spreading, migration, invasion and apoptosis (Hynes, 2009; Lu et al., 2012). The mechanical and chemical components of the microenvironment are also known to alter gene expression leading to cellular differentiation and other varied physiological responses (Hynes, 2009). The chemical composition of the cellular microenvironment includes proteins that form the extracellular matrix and biochemical components, such as growth factors released by cells, many of which adhere to the ECM until activated. The mechanical parameters of the microenvironment that influence cellular behavior are diverse. These factors include (but not limited to) substrate rigidity and elasticity, localized tensions generated by contractile forces from cells embedded within the ECM, shear flow, and interstitial fluid pressure.

Extracellular matrix (ECM): Composition, Rigidity and Topography:

The ECM is a highly organized, multimolecular network of glycoproteins, proteoglycans, and polysaccharides all of which are produced and secreted by many of the cells that reside in it (Egeblad et al., 2010). They surround, protect and support cells and tissues, thus forming the physical environment of the cells. The ECM proteins structurally form either the basement membrane or the interstitial stroma (Lu et al., 2012). The basement membrane is formed by the epithelial cells, the endothelial cells or the stromal cells. It is rich in collagen type IV, laminin and fibronectin along with linker proteins entactin and nidogen. The stroma on the other hand is formed solely by stromal cells. It is composed of fibrillar collagen, glycoproteins such as fibronectin and other proteoglycans. The stroma in comparison to the basement membrane is not as compact and is more porous. The stroma is also highly charged and hydrated.

The specific composition and arrangement of the ECM protein are tissue type dependent and their organization and densities contribute to the mechanical stiffness detected by the cell (Discher et al., 2005). Many of the ECM proteins begin as monomers and then form various inter- and intramolecular interactions to make-up large cross-linking polymers of varying rigidity (Vakonakis and Campbell, 2007). These ECM polymers are dynamic and undergo constant remodeling mediated by contractile cells, such as fibroblasts and myofibroblasts, and also by the enzymatic activity of various proteases, including the matrix metalloproteases (MMPs) family (Egeblad et al., 2010; Lu et al., 2011).

Response to substrate stiffness is cell and tissue type dependent (Discher et al., 2005). Fibroblasts have been shown to migrate towards stiffer substrates when plated on softer substrates (Lo et al., 2000). They also tend to spread better on stiff substrates as compared to being rounded on soft substrates (Zemel et al., 2010). However, embryonic mouse neurons extend neuritis on soft substrates but not on hard (Moore and Sheetz, 2011). Stem cell differentiation studies have also shown differentiation into varying cell lineages based on the substrate stiffness on which they are cultured (Engler et al., 2006; Wilda and Adam, 2011). Topography, which includes texture and shape of the substrate, also contributes to proper cellular organization and differentiation (Lu et al., 2012; Petrie et al., 2009; Wilda and Adam, 2011). The ECM, being a charged protein meshwork also serves as a reservoir for a number of signaling molecules, such as fibroblast growth factors, bone morphogenetic proteins, hedgehogs and WNTs (Hynes, 2009). The ECM helps restrict the diffusivity of these proteins and thus regulating its accessibility to its receptor on cells. The ECM can thus help mediate signaling cascades initiated by both biochemical and biophysical cues.

Mechanical cues from the ECM and its protein composition are often times disrupted affecting tensional homeostasis and localized tension. These imbalances can ultimately lead to pathologies such as cancer and cardiovascular diseases (Friedl and Alexander, 2011). Numerous factors are responsible for these alterations. For example, the ECM surrounding a tumor mass is referred to as the “reactive stroma” (Barkan et al., 2010; Hanahan and Coussens, 2012). Cells within this stroma include, not only tumor cells that have begun to leave the primary tumor, but also contain cells such as

fibroblasts, myofibroblasts, macrophages, other cells of the immune system and pericytes that line blood vessels. Many of these cell types are highly contractile in nature and will tug and pull the polymers of the ECM in the stroma. These cells also rampantly remodel the stroma laying out more collagen and fibronectin than non-tumor associated cells. These activities result in a denser ECM surrounding the tumor. Paszek et al have reported a 5-20 times increase in the stiffness of mammary tumor and the surrounding tumor stroma, as compared to the normal mammary gland tissue (Paszek et al., 2005).

Tumor progression is associated with deregulated collagen metabolism (Levental et al., 2009). Collagen expression and deposition levels are elevated; its organization is altered and so is its MMP mediated turnover. Lysyl oxidase activity is also elevated in tumors leading to an increase in the crosslinking of collagen. This promotes tissue stiffness leading to enhanced homeostatic tension. Studies have shown that this increase in collagen stiffness promotes integrin clustering resulting in enhanced PI3K activity leading to enhanced invasion (Miranti and Brugge, 2002). The results were interpreted to mean that tumor metastasis is promoted by increased tissue stiffness. However, studies from our lab have shown that the presence of fibronectin and the following activation of beta 1 integrin provide metastatic cells with the ability to disregard changes in compliance (Indra and Beningo, 2011). This observation provides a possible explanation for how cancer cells are able to migrate and invade through tissues of varying stiffness before they reach the site of secondary metastasis. This is contrary to normal cellular behavior in which changes in stiffness dictates migratory abilities. We have also shown that localized mechanical perturbations can be sensed by tumor cells to promote invasion

(Menon and Beningo, 2011). We explain this as a possible effect of fibronectin dimer opening which exposes cryptic binding sites. These cryptic sites provide access for integrin engagement leading to an undefined pathway resulting in enhanced invasion.

Thus, the ability of both normal and disease cells to perceive and respond to signals from its microenvironment is essential for their survival. There are far reaching medical implications for each of these pathways, but a lot more information needs to be uncovered before potential drug targets or other medical applications are identified.

CHAPTER 2

CANCER CELL INVASION IS ENHANCED BY APPLIED MECHANICAL STIMULATION

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Menon S, Beningo KA (2011) Cancer Cell Invasion Is Enhanced by Applied Mechanical Stimulation. PLoS ONE 6(2): e17277. doi:10.1371/journal.pone.0017277. © 2011 Menon, Beningo. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ABSTRACT

Metastatic cells migrate from the site of the primary tumor, through the stroma, into the blood and lymphatic vessels, finally colonizing various other tissues to form secondary tumors. Numerous studies have been done to identify the stimuli that drive the metastatic cascade. This has led to the identification of multiple biochemical signals that promote metastasis. However, information on the role of mechanical factors in cancer metastasis has been limited to the effect of compliance. Interestingly, the tumor microenvironment is rich in many cell types including highly contractile cells that are responsible for extensive remodeling and production of the dense extracellular matrix surrounding the cancerous tissue. We hypothesize that the mechanical forces produced by remodeling activities of cells in the tumor microenvironment contribute to the invasion efficiency of metastatic cells. We have discovered a significant difference in the extent of invasion in mechanically stimulated versus non-stimulated cell culture environments. Furthermore, this mechanically enhanced invasion is dependent upon substrate protein composition, and influenced by topography. Finally, we have found that the protein

cofilin is needed to sense the mechanical stimuli that enhances invasion. We conclude that other types of mechanical signals in the tumor microenvironment, besides the rigidity, can enhance the invasive abilities of cancer cells *in vitro*. We further propose that *in vivo,* non-cancerous cells located within the tumor micro-environment may be capable of providing the necessary mechanical stimulus during the remodeling of the extracellular matrix surrounding the tumor.

INTRODUCTION

The defining moment in the classification of a tumor as benign or malignant lies in the tumor cells ability to breach the basement membrane. The extension of invasive structures, such as invadopodia, allows the tumor cell to penetrate the basement membrane and interstitial stroma through enzymatic and physical means (Alexander et al., 2008; Busco et al., 2010; Poincloux et al., 2009). However, the tumor cell will not go far without the additional ability to migrate. The tumor cells acquisition of invasive and migratory properties provide the means to enter and exit the lymphatic or the vascular system and establish secondary tumors in foreign tissue, thereby completing the complex sequence of events within the invasion-metastasis cascade (Chambers et al., 2002; Ridley et al., 2003). It is these secondary tumors that account for greater than 90% of cancer deaths, yet our understanding of invasion and metastasis is incomplete. Much of the research has focused on intrinsic genetic and biochemical factors that trigger primary tumor formation and subsequent metastasis. However, more recent studies have identified both physical and biochemical factors within the tumor microenvironment that also contribute to cancer progression (Desmouliere et al., 2004; Tlsty and Coussens, 2006).

The stroma surrounding a tumor is continually changing in composition and structure as the primary tumor cells progress to invasion and metastasis, a process termed stromagenesis (Amatangelo et al., 2005; Bissell and Radisky, 2001). The tumor stroma becomes enriched in extracellular matrix (ECM) proteins and non-tumor cells including fibroblasts, macrophages, adipocytes, and pericytes (Amatangelo et al., 2005; Bissell and Radisky, 2001; Condeelis and Pollard, 2006; Mueller and Fusenig, 2004; Pollard, 2004). Biochemical signaling from the stroma to the tumor cells can promote proliferation and invasiveness. For instance, tumor-associated macrophages establish an EGF-CSF-1 paracrine signaling loop with the tumor cells that promote tumor cell movement (Condeelis and Pollard, 2006). The mechanical properties of the stroma can also enhance tumor progression. For example, the stroma surrounding a tumor is enriched in both type I collagen and fibronectin, creating a denser and mechanically rigid tissue compared to normal tissue (Tlsty and Coussens, 2006). This increased rigidity enhances tumor cell proliferation and dissemination (Kostic et al., 2009; Levental et al., 2009; Paszek et al., 2005). Recent studies also indicate that physically stretching fibronectin can trigger a mechanical response pathway in normal fibroblasts (Friedland et al., 2009; Kostic et al., 2007; Kostic and Sheetz, 2006). Given the increased amount of fibronectin in the stroma, these observations could suggest a potential mechanism for the mechanical response of tumor cells.

There are a number of mechanical forces, aside from the change in compliance, that may impact the progression of cancer. One such force could be derived from stromal cell movements or the matrix remodeling activity of the highly contractile cells of the

stroma, including fibroblasts and myofibroblasts. Myofibroblasts have been shown to differentiate from normal tissue fibroblasts, and their production and remodeling of the ECM enhances proliferation and dissemination of the tumor cells (Bhowmick et al., 2004; Follonier et al., 2008). The accumulation of stromal myofibroblasts are a defining feature of the desmoplasia most commonly associated with invasive cancers of the breast, gastrointestinal tracts, lungs, pancreas, and squamous cell carcinomas to name a few (Amatangelo et al., 2005). In addition to the high level of type I collagen production, myofibroblasts are identified by their expression of alpha-smooth muscle actin (Amatangelo et al., 2005; Desmouliere et al., 1993; Hinz et al., 2001; Tlsty and Coussens, 2006). The alpha-smooth muscle actin associates with non-muscle myosin to form highly contractile microfilamentous units that terminate at the surface of a myofibroblast in a fibronexus (Singer et al., 1984). These are characteristic features of myofibroblasts and form a mechano-transduction system that function in inside-out and outside-in force transmission (Dugina et al., 2001; Singer et al., 1984; Tomasek et al., 2002). In remodeling the ECM within the stroma, the myofibroblasts produce a mechanical stimulus as they tug and pull on the fibers (Goffin et al., 2006). This leads us to the hypothesis we address in this study. We hypothesized that the applied mechanical forces generated by the remodeling of the ECM and pulling on the ECM by stromal cells will contribute to the invasive properties of a tumor cell. We asked if this mechanical stimulus can provide a "come hither" stimulus that encourages the tumor cells to leave the tumor.

Here we report that a mechanical stimulus of pulling and releasing applied to a collagen matrix *in vitro* does indeed enhance the invasion of cancer cells in a fibronectin

dependent manner. This ability appears to be unique to cancer cells that are known to be highly invasive, as poorly invasive and normal cells do not respond in the same way to this stimulus. Finally, using gene silencing we determined that cofilin, a normal component of invadopodia, is required to sense this mechanical signal for enhanced invasion. This study suggests that physical factors, beyond compliance, are involved in promoting existing invasive behavior in cancer cells and that mechanical signals transmitted from the physical activity of cells within the stroma may potentiate cancer progression.

MATERIALS AND METHODS

Cell Culture

HT1080 human fibrosarcoma cells, B16F10 mouse melanoma cells and mouse embryonic fibroblasts (MEF) cells used in this study, were purchased from ATCC and are cultured and maintained in Dulbecco's Modified Eagle's Medium - high glucose (Sigma) and 10% FBS (Hyclone). Cells were passed by trypsinization using 0.25% Trypsin-EDTA, the reaction is terminated with complete media. The passage number of any cell type never exceeds eight passages.

Invasion Substrates

To create a culture well for thick (1mm) substrates, an activated coverslip (Beningo et al., 2002) was attached with vacuum grease to the bottom of a culture dish (Nunclon) into which a 20mm hole had been drilled.

The substrate was composed of 2.5mg/ml (or 4.5mg/ml, Figure S2) type I collagen (PureColl and Nutragen, Advanced Biomatrix), 20μg/ml fibronectin (Sigma) and 4μl of 1-2μm carboxylated paramagnetic beads (Polysciences Inc.). The pH of the mixture was adjusted to 7.4±0.2 with 0.1 N NaOH and 10X PBS. For "Collagen only" substrates, everything except fibronectin is added to the substrate mix. All the components were chilled and mixed at 4°C. 500μl of the substrate solution was added to a chilled culture well, and a 25mm coverslip was dropped onto the gel mixture to obtain a flat surface. For polymerization, the substrate solution was placed at 37°C for 30 minutes. Following polymerization, 3 ml of media was added to the substrates and the top coverslip was removed. The substrates were then sterilized in a culture hood under ultraviolet light for 15 minutes at a distance of 25 inches from the light source.

Invasion Assay

Cells were seeded at $1.5x10^4$ cells/ml onto the sterilized substrates and allowed to adhere for 1 hour at 37°C/5% CO_2. For each experiment, one seeded substrate was incubated at 37°C/5% CO_2 1.5cm above a rare earth magnet of 12,100 Gauss (25mm in diameter and 5.5mm in thickness). A second seeded substrate was incubated outside the magnetic field. The magnet was rotated below the culture at 160 rpm (2.6 Hz) in an orbital field of 2cm on an orbital shaker (Barnstead Thermolyne, Roto Mix-Type 50800). This rotation frequency was maintained the same for all assays described. The invasion assay was also performed with the magnet rotated at lower frequencies (8 and 90 rpm (0.13 and 1.5 Hz)) as indicated. The cellular response was recorded for 25 randomly selected microscope fields at 24 hours using a 10X phase objective on an Olympus IX81

Microscope. Cell counts were recorded at eight increments of 100μm/step within the z-plane of the substrate. Percentage invasion was calculated as the percent of invaded cells in comparison to the total cell count. Statistical analysis was performed using the two-tailed students T-test.

The peptide inhibitor experiments were performed as above; 1.5 x 10^4 cells /ml were seeded onto the substrates followed by 100μg/ml of GRGDS peptide or GRGES (control) peptide (Bachem Americas Inc.) suspended in water. Percent invasion was calculated 24 hours after the start of stimulation.

Upward Invasion Assay

Culture wells without the substrates were prepared as described above. However, cells were first seeded directly onto the glass coverslip coated with a thin layer of type I collagen (200μg/ml) and fibronectin (62.5μg/ml) before overlay of the matrix. The cells were allowed to adhere overnight in media at 37°C and 5% CO_2. The media was removed and cells were then overlaid with the unpolymerized collagen/fibronectin substrate as described above. Media was replaced following polymerization. For each experiment, one seeded overlaid substrate was cultured 1.5cm below a rare earth magnet of 12,100 Gauss (25mm in diameter and 5.5mm in thickness) and a second was maintained outside the magnetic field. The magnet was rotated above the culture held in a stand placed on the orbital shaker (Barnstead Thermolyne, Roto Mix-Type 50800) and rotated at 160 rpm (2.6Hz) in an orbital field of 2cm. Percent invasion and statistical analysis were described above.

Actin Depolymerization

HT1080 cells were seeded onto collagen/fibronectin substrates. After the cells had adhered and spread on the substrates, 2μM of Cytochalasin B (Sigma) resuspended in DMSO or a corresponding volume of DMSO was added to separate plates. These were then directly used for invasion assay.

Cofilin Knockdown

CFL1 siGENOME SMARTpool and non-target siRNA (Dharmacon RNAi Technology, Thermo Scientific) were used to silence the expression of Cofilin and as controls, respectively. RNA's were introduced into cells by nucleofection using an Amaxa Nucleofector II and solutions from Kit T. Control and cofilin siRNA treated HT1080 cells were grown in multiple plates such that they would become 80% confluent in 24, 48 and 72 hours post nucleofection. Proteins were extracted for western analysis from cofilin silenced and control HT1080 cells using a triple detergent lysis buffer (100mM Tris-Cl, 300mM NaCl, 0.5% sodium deoxycholate, 0.2% SDS, 2% Nonidet P 40) containing Protease Inhibitor Cocktail (Sigma) at 24, 48 hours and 72 hours post nucleofection to confirm knockdown. Anti-cofilin monoclonal antibody, ab54532 (Abcam) and anti-mouse HRP-labeled antibody (Amersham) were used to probe the western blots and detected with ECL Plus Western Blotting Detection Reagents (Amersham).

Invasion Assay Using Cofilin siRNA and Cytochalasin B Treated HT1080 Cells

Invasion assay was performed using Control siRNA and Cofilin siRNA treated HT1080 cells. Since cofilin knockdown is efficient 48 hours post nucleofection, the treated cells were seeded onto the substrates at the 48 hour time point. After the cells had adhered, one seeded substrate for each of the conditions was placed above the magnet rotating at 160 rpm (2.6Hz), whereas the other was placed outside the magnetic field. The assay was also performed using Cytochalasin B or DMSO treated cells. In each case, one seeded substrate was provided magnetic stimulation at 160 rpm (2.6Hz) whereas the other substrate was placed outside the magnetic field. The cellular response for each of the four conditions was measured 24 and 48 hours after the start of stimulation. Percentage invasion was calculated and statistical analysis was performed using a two-tailed students T-test.

Western Blot of Fibronectin Secretion by HT1080 Cells

1.5 x 10^4 cells /ml HT1080 cells were grown in serum free DMEM medium and seeded onto collagen-only substrates, prepared as described above, and the standard invasion assay was performed. After 24 hours of stimulation, the cultures were scraped into a microfuge tube containing 2mg/ml of Collagenase Type 4 (Worthington Biochemical Corporation) in Hanks' Balanced Salt Solution (Gibco, Invitrogen). The collagen substrate was solubilized by gently shaking the tube at 37^{o}C and cells were pelleted by centrifugation at 2000 rpm for 5 min, the supernatant was used for analysis. Cell extracts of HT1080 cells and MEF cells cultured on 100mm polystyrene culture dishes to 80% confluency over 48 hours were also prepared. The cell lysis and protein

extraction were performed as described above. SDS-PAGE was performed using 30µg of total protein from MEF and HT1080 cell extracts and 35µl of collagenase suspension. 4-20% Tris-HEPES-SDS precast polyacrylamide gels were used (Pierce, Thermo Scientific). Western blots were prepared and probed with mouse monoclonal [IST-9] to fibronectin (1:300), ab6328 (Abcam) in 5% milk in TBS followed by a HRP Goat Anti-mouse Ig (BD Pharmingen) secondary antibody (1:1000) and detected as above.

RESULTS

Structural Design of the Mechanical Invasion Assay

The goal of this study was to determine if applied mechanical stimulation, such as those simulating the re-modeling of the extracellular matrix, could enhance the process of invasion. To address our hypothesis, we designed a new assay system where mechanical stimulation could be applied in the absence of secreted biochemical factors. Our intention was to create an assay that used commercially available components, required standard equipment, provided control of biochemical and mechanical parameters, all in a framework that was optically compatible with an ordinary fluorescent microscope. We chose to use a type I collagen matrix commonly used for invasion assays, reasoning that the stroma is highly enriched in this extracellular matrix protein. Carboxylated fluorescent paramagnetic micro-beads were embedded within the matrix to provide mechanical stimulation. To produce a transient magnetic pull, without the need for a micron size electro-magnet, we rotated a rare earth magnet on a rotating mixer beneath the culture while the culture was suspended above the magnet (Figure 2.1A). The entire

culture system can be maintained within a standard tissue culture incubator (Figure 2.1B, C).

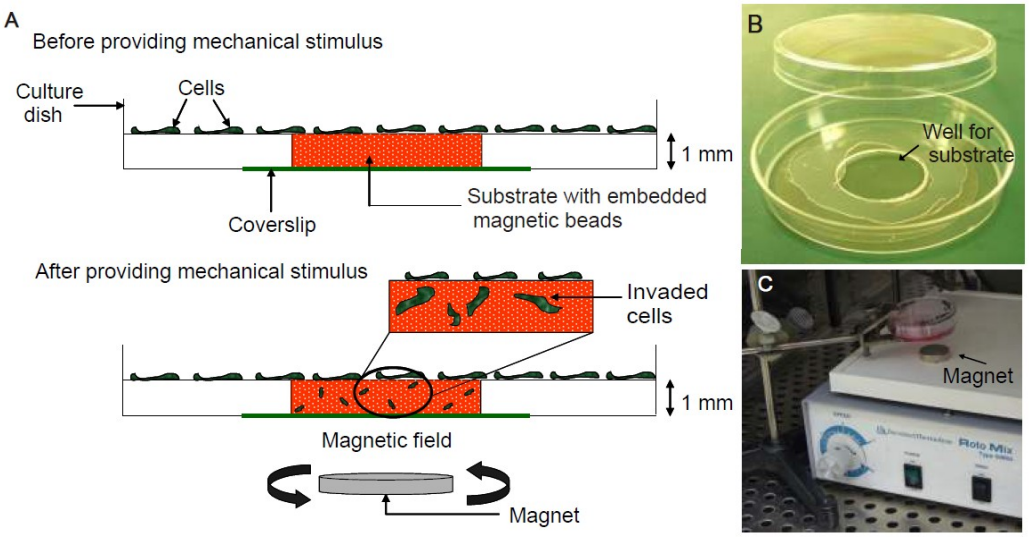

Figure 2.1. The mechanically enhanced invasion assay.

A) A well is created in a 60 mm culture dish and filled with a type I collagen/fibronectin matrix containing 2µm paramagnetic beads. Cells are seeded onto the surface of the matrix and either cultured outside of a magnetic field or cultured 1.5cm above a rotating rare earth magnet. Upon stimulation, cells invade the substrate. **B)** 60mm plate with a 20mm hole drilled into it, with an activated coverslip glued to the bottom, creates a well for the matrix. **C)** The culture is suspended 1.5 cm above a rare earth magnet placed on an orbital shaker within a typical cell culture incubator. See the methods section for details.

To verify that the magnet was capable of producing enough magnetic force and that the embedded beads responded to the force in a transient manner, we used a magnometer to measure the magnetic force at defined experimental distances. We discovered a magnetic bead at a fixed point within the center of the culture could be subjected to a range of 500 to 80 Gauss as the rare earth magnet rotates 1.5cm beneath

the culture dish completing an orbit of 2cm at 160 rpm (2.6Hz) (Figure 2.2A). Simulation at these distances under the microscope resulted in bead displacements of approximately 0.5-5μm (Figure 2.2B, Movie S1, Movie S2). Beads were observed to spring back to their original position in the x-y plane after the magnet was removed, indicative of their attachment to the collagen matrix and maintenance of the integrity of the gel network. To determine the physiological significance of this displacement, we recognized that we could calculate the amount of force that was applied on the bead by the magnet, however a more tangible test would be to observe MEF cells extending and retracting extensions within our controlled culture system. We recorded bead displacements in the x-y plane from cellular extensions of MEF cells that range from 0.08 – 5.1μm (Figure 2.2C, Movie S3). This is a conservative comparison to the types of displacements that could occur in the stroma given that the most contractile cell type found there, the myofibroblasts, produce considerably more force than a MEF (Meshel et al., 2005; Wrobel et al., 2002).

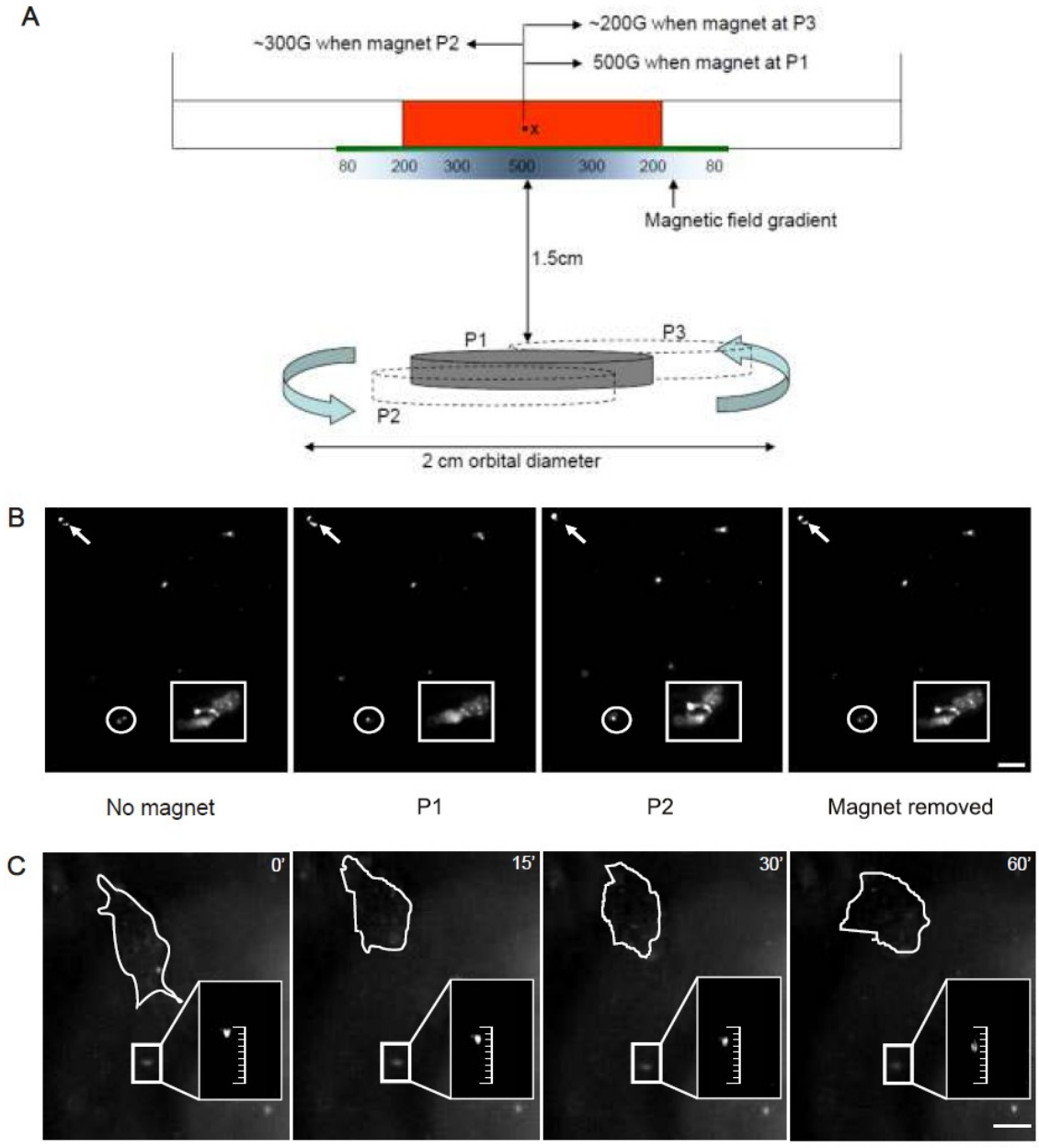

Figure 2.2. Stimulation of paramagnetic beads.

A) A rare earth magnet placed 1.5 cm below a substrate produces a gradient field ranging from 500G to 80G within the substrate as it rotates in a 2cm orbit. A paramagnetic bead at position X would receive a magnetic force of 500G, ~300G and ~200G when the magnet is orbiting at positions P1, P2 and P3 respectively. **B)** Series of four images depicting the displacement of beads by the magnet when held in stationary positions within the orbit. Clusters of beads responding to the mechanical stimulus and

showing a positional shift have been demarcated using a circle, a square and an arrow. From left to right, image one is outside the magnetic field while the second and third images were taken with the magnet held in positions P1 and P2 respectively. The final image demonstrates the beads return to their original position after the magnet is removed. **C)** MEF cellular extensions cause fluorescent bead displacement. Four images (0, 15, 30 and 60 minutes) from a single focal plane were selected from a series of 30 phase images taken every 2 minutes of a MEF cell within a collagen/fibronectin substrate. Cell outlines and corresponding fluorescent bead images are shown. A bead undergoing displacement is outlined using a white rectangular box. The area within the box from all four images has been enlarged and displayed with an inset ruler to show the bead displacement more clearly. The contrast of the magnified images have been altered to better reflect the position of the bead in each case. Mag. Bar = 10 μm

Mechanical Stimulation Enhances the Invasion of Cancer Cells

Invasive structures have previously been described in both inherently normal invasive cells and in those that have acquired their invasive capacity during cancer progression (Gimona et al., 2008). We reasoned that it was unlikely that mechanical stimulation would induce a previously non-invasive cell type to invade and hence we tested cells known to be highly invasive in our assay system. We chose to test the human fibrosarcoma cell line HT1080 and the mouse melanoma cell line B16F10 (K. Beningo, unpublished data), whereas the non-invasive MEF cell line served as control.

These cell types were tested individually for their ability to respond to the mechanical stimulation provided in the assay. In brief, cells were seeded onto prepared matrices, as described in methods, and allowed to adhere for 30 minutes before beginning the stimulation. Cells cultured on substrates of identical composition, but not subjected to magnetic stimulation, served as controls. Cells cultured on substrates lacking magnetic beads, but subjected to magnetic stimulation served as additional controls. Invasion was observed under the microscope beginning at 5μm from the surface to a depth of 800μm

within the substrate (Figure 2.3A). The number of invading and non-invading cells were counted after 24 hours of stimulation and calculated as the percent invasion.

We initially seeded our cells onto matrices comprised only of type I collagen. Upon stimulation we did not observe enhanced invasion (varying between 5 and 10% invasion in stimulated and non-stimulated cultures). However, not only is type I collagen abundant in the stroma but the collagen binding ECM protein fibronectin is also enriched (Ingham et al., 2002; Tlsty and Coussens, 2006). Thus, we compared matrices composed of collagen alone to those of both collagen and fibronectin, with and without stimulation. Under these conditions we observed a significant difference in the number of invading cells in mechanically stimulated verses non-stimulated culture environments for the invasive cell types when collagen/fibronectin matrices were used (Figure 2.3B). A two-fold increase in the percentage of invading cells in the stimulated (23%) as compared to the non-stimulated matrix (10%) was consistently observed in these cultures ($P<0.05$). These results indicated that an applied stimulus was capable of enhancing invasion of cancer cells, but required in the presence of fibronectin for the mechanical response. Furthermore, we found that non-invasive MEF cells failed to invade both in the presence or absence of mechanical stimulation into collagen/fibronectin matrices, suggesting the need for a cell to have a pre-defined ability for invasion.

To confirm the importance of fibronectin for the mechanical response we inhibited cell-fibronectin interactions with RGD inhibitory peptides. Cells were treated with the GRGDS peptide or a control GRGES peptide after seeding onto the collagen/fibronectin substrates. The percent invasion was normal in the presence of the

control GRGES peptide (28% with stimulation and 13% without stimulation) while mechanically stimulated invasion was inhibited by the RGD peptide (9% with stimulation and 11.5% without stimulation, $P>0.05$). These results not only support the fact that fibronectin is necessary for the mechanically stimulated invasion, but suggest the "basal" level of ~10% invasion observed in collagen/fibronectin (non-stimulated) and collagen (stimulated and non-stimulated) cultures is fibronectin independent. In addition, these results confirm that any fibronectin secreted by the HT1080 cells into the matrices (although undetectable by western blot; Figure 2.4) is inconsequential to the mechanical response.

Due to the heterogeneity of cell types and cell numbers within the stroma it is unclear at what frequency the stimulus should be applied. To determine if the frequency of bead stimulation was a factor in enhanced invasion, we adjusted the speed of the rotating magnet, rotating at speeds of 8, 90 and 160 rpm or 0.13, 1.5, and 2.6 Hz, respectively. The percent of invasion did not differ significantly between the cultures stimulated at 8 and 160rpm ($P>0.05$; Figure 2.3C). These results demonstrated that, within a 20-fold range of frequency, enhanced invasion in response to mechanical stimulation is unaffected.

Invasive cells encounter physical barriers within the connective tissue or tumor stroma and are likely to follow the path of least resistance (Friedl and Brocker, 2000). In addition, they are likely to invade along paths in which matrix associated soluble factors have been released (Bhowmick et al., 2004; Pietras et al., 2008; Pietras et al., 2003;

Wipff et al., 2007; Yu et al., 2003). Based on this knowledge, it was important to ensure that neither of these factors contributed to the enhanced invasion observed in our assay.

One way in which our matrix could generate paths of least resistance for cell invasion would be through a permanent remodeling created by the movement of the embedded beads. To test this possibility, we pre-stimulated the matrices over the rotating magnet for 24 hours prior to seeding the cells. After 24 hours of culture on the pre-stimulated substrates, but outside of the magnetic field, we did not observe enhanced invasion (Figure 2.3D, left panel). In addition, the media of the pre-stimulated matrix was not changed prior to seeding the cells. This eliminated the potential that soluble factors in the matrix were being released by the tugging of the beads on the matrix and contributing to the enhanced invasion. However, when these same cell cultures grown on the pre-stimulated matrix were then given magnetic stimulation, enhanced invasion was again observed (Figure 2.3D, right panel). Taken together, these results suggest that any remodeling or release of soluble factors from the matrix due to the movement of the magnetic beads does not contribute to the enhanced invasion we observe upon mechanical stimulation.

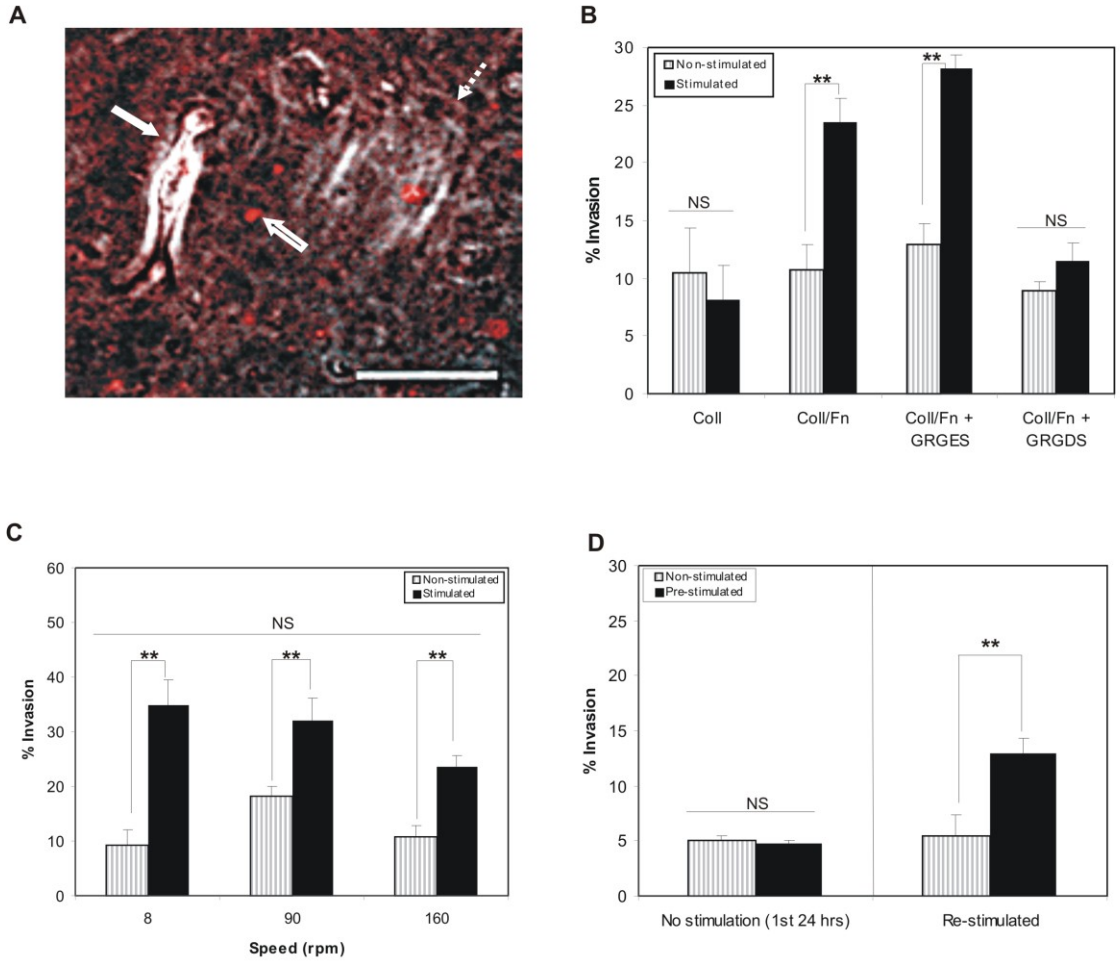

Figure 2.3. Enhanced invasion of mechanically stimulated cultures of cancer cells.

A)HT1080 fibrosarcoma cells were seeded onto type I collagen/fibronectin matrices containing paramagnetic beads and cultured either under magnetic stimulation or without stimulation. A combined phase and fluorescent image of a mechanically stimulated culture were superimposed. The solid arrow points to a cell that has invaded. The dotted arrow indicates a second cell within another focal plane. The empty arrow points to a fluorescent paramagnetic bead. Mag. Bar = 50 μm. **B)** Invasion of HT1080 cells under mechanically stimulated and non-stimulated conditions was performed in matrices containing either type I collagen (2.5mg/ml) or both type I collagen and fibronectin, or collagen/fibronectin in the presence or absence of RGD peptide. 25 fields of cells were counted 24hours after seeding at multiple depths within each substrate beginning 5μm below the surface of the matrix and progressing towards the farthest depth of 800μm. The percent of invading cells was 2-fold higher in stimulated cultures when compared to controls (P<0.05) in substrates containing both ECM proteins.

Similar results were obtained when the control peptide GRGES was added to the media. The percent invasion was approximately the same with or without stimulation when fibronectin was absent. Addition of the GRGDS peptide also resulted in inhibition of enhanced invasion upon mechanical stimulation. **C)** A 20-fold difference in the frequency of stimulation does not influence the percent of cell invasion. The percent of invading cells 24 hours after stimulation at magnetic rotation speeds of 8, 90 and 160 rpm (0.13, 1.5 and 2.6Hz). An insignificant difference was found between cells stimulated at 8 and 160 rpm ($P>0.05$). Data represents three independent experiments, of 25 fields. **D)** Type I collagen/fibronectin matrices containing paramagnetc beads were pre-stimulated for 24 hours. These substrates were then seeded with HT1080 cells and counted 24 hours after seeding, during which period both the pre-stimulated and the control plates were not stimulated (left panel). These cultures were then either continued or placed over the magnet (right panel), data obtained 24 hours after stimulation. Data represents two independent assays of 15 fields of cells at a depth range of 800 μm. Two-tailed analysis using student t-test. For all panels ** denotes $p<0.05$ and NS denotes a non-significant relationship.

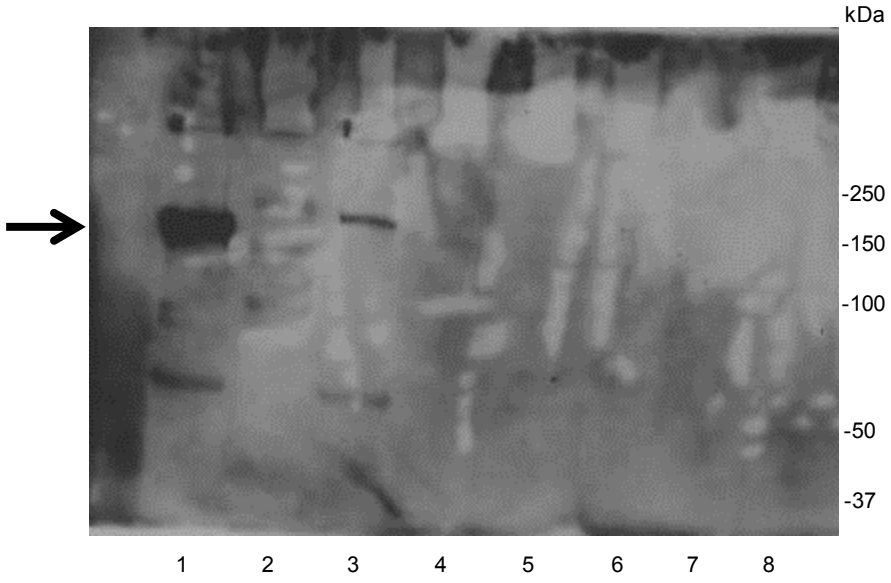

Figure 2.4. Secretion of fibronectin from HT1080 cells is undetectable in collagen-only substrates.

A) Western blot of fibronectin (black solid arrow) from total protein extracts of MEF and HT1080 cells (lanes 1 and 3 respectively), cultured on standard polystyrene dishes, demonstrates reduced amounts of fibronectin from HT1080 cells. Western blot of collagenase treated collagen-only substrates or collagen/fibronectin matrices in which HT1080 cells were cultured and stimulated for 24 hours shows no detectable fibronectin (lanes 5 and 6 respectively). Conditioned media from HT1080 cell cultures grown for 24 hours and for one week also shows no detectable fibronectin (lanes 7 and 8 respectively).

The Invasion Response is Enhanced whether the Stimulus is Delivered from Top or Bottom

The dimensionality of the environment is known to influence cellular behavior. Specifically, HT1080 cells have been shown to change their migration speed and persistence in three dimensions (Fraley et al., 2010). In our initial experiments, the cells are seeded on top of the matrix, invading from the top downward, thus beginning in two-dimensions and moving into three. To address the influence of dimensionality on mechanical invasion we changed the orientation of the stimulus so the cells would invade upwards. To do this, we first seeded the cells onto collagen/fibronectin-coated coverslips before overlaying and polymerizing the collagen/fibronectin/magnetic bead solution over them (Figure 2.5A). The magnetic field was then applied to the top of the culture by rotating the magnet above the stationary culture (Figure 2.5B). After 24 hours of stimulation, we found the cells invaded just as well as they did when they were seeded on top of the substrate prior to stimulation (6% invasion in non-stimulated and 13% in stimulated cultures) (Figure 2.5C). However, we found by 48 hours the difference between non-stimulated invasion and stimulated invasion was even larger such that 12% of the cells invaded in non-stimulated versus 41% invasion in the stimulated cultures. Thus, an even greater enhancement of invasion occurs in the response to applied mechanical stimulation when the cells began in a three-dimensional environment.

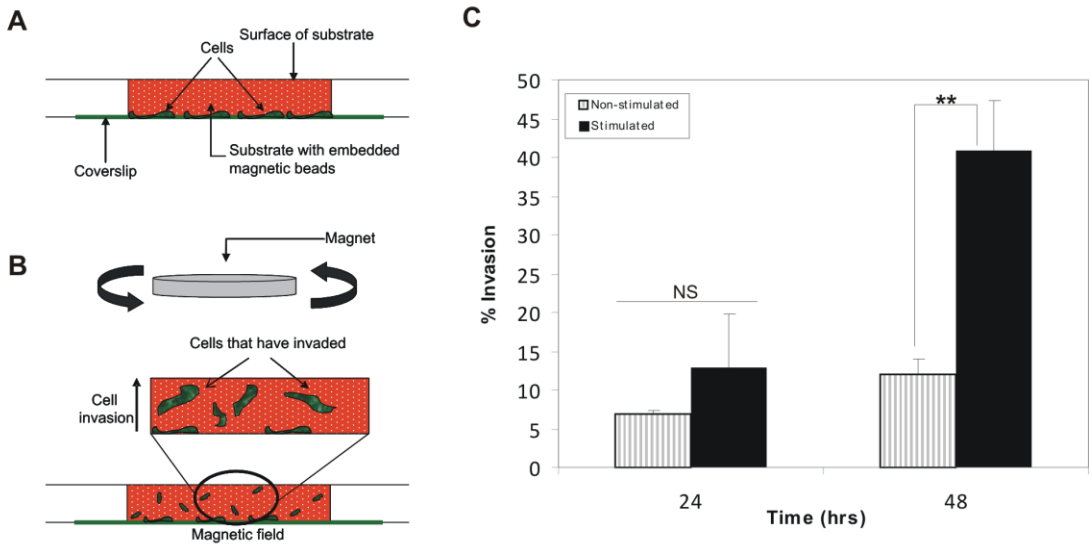

Figure 2.5. Upward Invasion Assay.

A) HT1080 fibrosarcoma cells were seeded onto a collagen/fibronectin coated coverglass at the bottom of the well. After the cells had adhered, a type I collagen/fibronectin solution containing paramagnetic microbeads was overlaid onto the cells and allowed to polymerize. Cultures were either subjected to magnetic stimulation or grown outside the magnetic field. **B)** The magnet is rotated above the culture as cells start to invade up into the substrate. **C)** HT1080 cells seeded on a collagen-fibronectin coated coverslip and overlaid with a collagen/fibronectin substrate were cultured either in the presence or absence of a magnetic field. Percent invasion was calculated 24 and 48 hours following stimulation from three independent trials (15 fields were counted per culture). A difference in invasion (approx. 4–fold higher) between the stimulated cultures as compared to non-stimulated cultures was significant at 48 hours post-stimulation (**$P < 0.005$, NS – non-significant relationship).

Cofilin and Actin are Required for Mechanically Stimulated Invasion

A functioning actin cytoskeleton is required for the invasiveness of a number of tumor cells (Bijman et al., 2008; Bousquet et al., 1990). To confirm the significance of actin dynamics in HT1080 invasion into type I collagen/fibronectin matrix, Cytochalasin B or control DMSO treated cells were tested in the invasion assay. As anticipated, both the mechanically stimulated and the non-stimulated invasion were inhibited. Less than

1% of the cells treated with Cytochalasin B invaded irrespective of whether they were mechanically stimulated (Figure 2.6C). In comparison, 12% of non-stimulated and 29% of stimulated, DMSO treated control cells invaded into the matrix (Figure 2.6C). As expected, invasion into a 3D matrix is dependent on the dynamics of the actin cytoskeleton.

Given that mechanical stimulation enhances an existing ability for invasion, it was important to identify other proteins that might sense the mechanical stimulation, but whose function is not dire to the formation of invasive structures as with actin. We tested the protein cofilin because it is vital for maturation of invadopodia since reduced cofilin expression leads to the formation of less invasive invadopodia, but does not inhibit invasion (Yamaguchi et al., 2005). Cofilin is also important in directional sensing during chemotactic migration and also in 3-dimensional migration (Klemke et al., 2010; Mouneimne et al., 2006). Based on these observations, we silenced cofilin in HT1080 cells using siRNA and tested the cells in our invasion assay. Knockdown was confirmed by western blot and defined 48 hours post-nucleofection as the optimum time point for a 60% knockdown of the cofilin protein (Figure 2.6A).

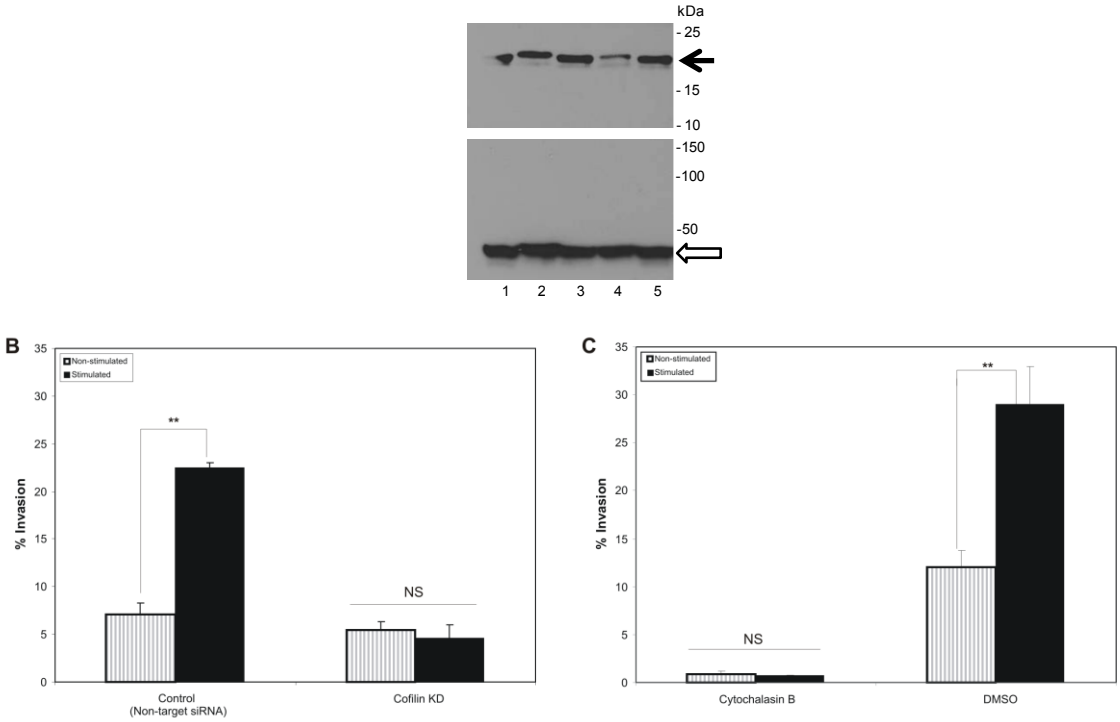

Figure 2.6. Silencing of Cofilin prevents mechanically stimulated invasion.

A) Western blot of cofilin from lysates of wildtype HT1080 cells, HT1080 cells treated with off-target control siRNA (lanes 1 and 2 respectively), and cells cultured for 24, 48 and 72 hours after nucleofection with cofilin siRNA (lanes 3, 4 and 5 respectively). Cofilin expression is reduced 48 hours post-nucleofection (black solid arrow). GAPDH was used as loading control (unfilled arrow). B**)** HT1080 cells nucleofected with control siRNA or Cofilin siRNA and cultured for 48 hours were seeded onto collagen/fibronectin matrices containing paramagnetic beads. The cells were cultured with or without stimulation for 48 hours and the percent of invading cells was calculated. Invasion assays using control siRNA treated cells were repeated twice (15 fields were counted per trial). Stimulated cells had 3-fold higher invasion as compared to non-stimulated cells (**$P< 0.05$). The assay using cofilin silenced cells was repeated four times (15 fields were counted per trial). The percent invasion between stimulated or non-stimulated cultures was insignificant ($P>0.05$, NS – non-significant relationship). **C)** HT1080 cells were seeded onto collagen/fibronectin matrices containing paramagnetic beads. Cells treated with 2 μM Cytochalasin B or DMSO were cultured with or without stimulation for 48 hours and the percent of invading cells was calculated. Data represents three independent assays (** denoted $P<0.05$, NS denotes a non-significant relationship).

We observed that reduced cofilin expression failed to enhance stimulated invasion as compared to silencing HT1080 with off-target siRNA. Approximately 7% of cells treated

with control siRNA invaded without mechanical stimulation, while 22% invaded when given mechanical stimulation, reflective of the enhanced invasion typically observed in untreated cells (Figure 2.6B). In comparison, the cofilin silenced cells showed approximately 5% invasion without mechanical stimulation and showed no significant response to the mechanical stimulation (4% invasion) (Figure 2.6B). Thus, while knockdown of cofilin does not impede basal invasion abilities in our assay, these results establish a role for cofilin in the enhanced invasive response invoked by mechanical stimulation.

DISCUSSION

The progression of cancer, from the formation and growth of the initial tumor through the multi-step metastatic cascade, is sure to be impacted by multiple mechanical factors. Within the tumor mass and in the microenvironment, factors of tissue compliance, shear force and interstitial forces are present (Cheng et al., 2009; Craig and Basson, 2009; Helmlinger et al., 1997; Kumar and Weaver, 2009; Mierke et al., 2008). Indeed it has been known for several years that the compliance of the tumor and its surrounding stroma are more rigid due to an enhanced deposition of ECM (Paszek et al., 2005). Matrix compliance is known to influence cell growth, morphology, differentiation and motility (Engler et al., 2004; Lo et al., 2000; Tilghman et al., 2010; Wozniak et al., 2003; Yeung et al., 2005). Changes in mechanical properties result from the unique repitoire of cells found in the tumor stroma, of most significance are the fibroblasts, myofibroblasts and pericytes (Amatangelo et al., 2005; Bissell and Radisky, 2001; Mueller and Fusenig, 2004). Myofibroblasts are known to extensively remodel the ECM

producing considerable forces on the deposited ECM (Amatangelo et al., 2005; Hinz et al., 2001; Pietras and Ostman, 2010; Tlsty and Coussens, 2006). Pericytes associated with a tumor are different morphologically and physiologically from pericytes of normal blood vessels and forces generated by these tumor associated pericytes have been shown to alter the microvascular niche (Kutcher et al., 2007; Lee and et al., 2010; Morikawa et al., 2002). In our study we have asked whether these mechanical forces generated by remodeling and migrating cells within the stroma could impact cancer cell invasion.

The assay used for this study offers many benefits in its simplicity, yet retains some aspects of physiological relevance. For instance, the study is done in a three-dimensional environment of collagen and fibronectin which are the most abundant ECM proteins found in the stroma of tumors, and are secreted and remodeled by cancer associated fibroblasts (CAF's) and myofibroblasts (Tlsty and Coussens, 2006). We mimic these remodeling forces, without the complication of the secreted biochemical factors that are produced by stromal cells (Kopfstein and Christofori, 2006). The magnetic force generated by the paramagnetic microbeads is tuned to produce displacement forces comparable to normal fibroblasts in this culture environment (Movie S3). Furthermore, we recognized that the stellate shaped fibroblasts within the stroma typically run parallel to the basement membrane of the tumor, hence the forces applied during the remodeling are likely in this orientation, thus we applied the magnetic force in a parallel plane (see Movie S1 and S2). We also considered the range of compliance possible for a tumor and the stroma, with reports ranging from 300-2000Pa (Paszek et al., 2005). We discovered no difference in the invasive response when we tested within a range of 400-1600Pa

(Figure 2.7). The correct combination of these factors resulted in the enhanced invasion we were able to generate upon mechanical stimulation, however there are certain to be other factors that will optimize this method.

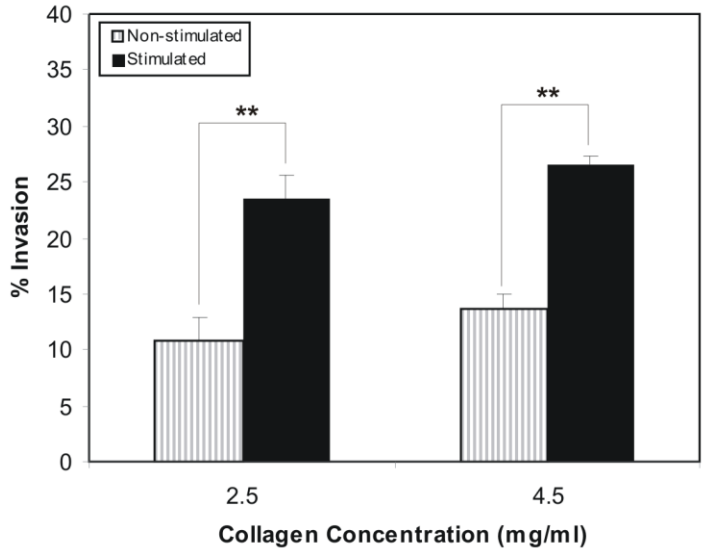

Figure 2.7. Mechanically stimulated invasion is unaffected by collagen concentrations and changes in compliance.

Invasion assays of HT1080 cells in collagen/fibronectin substrates under stimulated and unstimulated conditions. Collagen concentrations of 2.5mg/ml (~400Pa) and 4.5mg/ml (~1600Pa) were used; both produced similar extents of invasion (23.6% and 26.6% respectively. Data represents 3 independent experiments. Statistical analysis was performed using student's t-test (** indicates $P<0.05$).

Given that non-invasive cell types were unable to invade in response to the mechanical stimulation, it is reasonable to presume the necessary molecular machinery for mechanically stimulated invasion is not available. A vital structure used by highly invasive cells is the invadopodia. These structures are enriched in proteases, cytoskeletal proteins, such as actin, and adhesion proteins including α5β1 integrin (Buccione et al., 2009; Poincloux et al., 2009; Weaver, 2006). It is likely that invadopodial structures are important in the mechanical response as they display enhanced activity to changes in

compliance, which also supports our observation that the mechanically stimulated invasion is unaffected when we change the compliance (Figure S2) (Alexander et al., 2008). Cancer cell motility and invasion are actin dependent processes (Bijman et al., 2008; Bousquet et al., 1990; Yamazaki et al., 2005). We also confirmed its requirement for mechanically enhanced invasion. Given that the response to our mechanical stimulus does not induce invasion in non-invasive cells, but enhances the existing processes, suggested a "late comer" to the established machinery (invadopodia) might participate in the mechanical sensing. Based on the fact that cofilin is not involved in the initial formation of invadopodia, but in their maturation, we evaluated it as a potential mechanical responder (Yamaguchi et al., 2005). Our finding that knockdown of cofilin does not affect non-stimulated invasion, but eliminates the enhanced response in our assay, confirms our reasoning. What remains to be determined is if the presumed lack of maturation of the invadopodia is responsible for the loss of our response, or if there is a change in the overall number of invadopdia, or perhaps a change in the proteolytic activity of these structures.

Another intriguing observation is the requirement for fibronectin for the mechanically enhanced invasion. In our study, collagen alone did not provide sufficient signal to the cells to trigger a mechanical response. One obvious explanation is that the sensor, possibly an integrin, possessing the sensing function for enhanced invasion does not bind to collagen, but recognizes only fibronectin as the ligand (Akiyama et al., 1995). The need for fibronectin in the sensing mechanism is also consistent with numerous reports that mechanical load alters the structure of the fibronectin molecule, specifically

the synergy site (Gao et al., 2002; Gee et al., 2008; Krammer et al., 2002; Krammer et al., 1999). Furthermore, more recent studies find that α5β1 integrin switches fibronectin binding states based on mechanical information (Friedland et al., 2009; Garcia et al., 2002; Li et al., 2003). α5β1 integrin is overexpressed in a number of cancers, and is under study as both a therapeutic and diagnostic target (Jin and Varner, 2004; Martinkova et al., 2010; Nam et al., 2010). This integrin is highly expressed at the periphery of invadopodia and is essential for the adhesion process by mediating their formation and extension (Mueller et al., 1999; Stylli et al., 2008). Our data defines significant importance to fibronectin interactions in the mechanical sensing observed in our invasion assay. We speculate the enriched expression of fibronectin receptors at the tip of invadapodia and the enhanced access granted by the pulling of the fibronectin molecules by our magnetic beads are key to this sensing mechanism, though further studies are necessary.

In conclusion, we have discovered that mechanical stimulation applied to a collagen-fibronectin matrix through micro-magnetic beads, can enhance the invasive abilities of invasive cancer cells. This response requires both extracellular and cellular proteins. From our studies we can conclusively state that ECM component fibronectin and the cellular protein cofilin are required for this mechanical response. We further suspect invadopodia in the process of mechanically stimulated invasion. We propose these observations translate to the tumor microenvironment where multiple cell types can be found, including highly contractive cells, and that mechanical forces generated by

these stromal cells could contribute to enhancing the metastatic abilities of invasion competent cells leaving the primary tumor.

ACKNOWLEDGEMENTS

The authors would like to thank Brittany McNeill, Vishnu Undyala, and Wenjia Lou for technical assistance.

CHAPTER 3

GALECTIN-3 SECRETION AND TYROSINE PHOSPHORYLATION IS DEPENDENT ON THE CALPAIN SMALL SUBUNIT, CALPAIN 4

This chapter has been published.

Menon S, Kang C-M, Beningo KA (2011) Galectin-3 secretion and tyrosine phosphorylation is dependent on the calpain small subunit, Calpain 4. Biochemical and Biophysical Research Communications 410: 91-96.

ABSTRACT

Cell adhesion and migration are important events that occur during embryonic development, immune surveillance, wound healing and in tumor metastasis. It is a multi-step process that involves both mechanical and biochemical signaling that results in cell protrusion, adhesion, contraction and retraction. Each of these events generates mechanical forces into the environment measured as traction forces. We have previously found that the calpain small subunit, Calpain 4, is required for normal traction forces, and that this mechanism is independent of the catalytic activities of the holoenzymes that are formed between Calpain 4 and each of the proteolytic heavy chains of Calpain 1 and 2. To define a potential mechanism for the Calpain 4 regulation of traction force, we have evaluated the levels of tyrosine phosphorylation, a hallmark of force dependent signaling within focal adhesions. Using 2D gel electrophoresis we compared tyrosine phosphorylation profiles of Calpain 4 deficient mouse embryonic fibroblasts (MEFs) to the levels in wildtype MEFs and MEF's deficient in the large catalytic subunits, Capn1 and Capn2. Of particular interest, was the identification of Galectin-3, a galactose

binding protein known to interact with integrins. Galectin-3 has previously been shown to regulate cell adhesion and migration in both normal and tumor cells; however its full mechanism remains elusive. We have found that Calpain 4 regulates the tyrosine phosphorylation of galectin-3, and its ultimate secretion from the cell, and speculate that its secretion interferes with the production of traction forces.

INTRODUCTION

Galectins are a family of lectin proteins that bind to β-galactoside carbohydrate structures through their carbohydrate recognition domains (CRDs). Galectin-3 is a unique member of this family because it is chimeric, containing one CRD and a long N-terminal sequence rich in serine phosphorylation sites and glycine repeats, in addition to a tyrosine and proline rich collagen-like sequence (reviewed in (Nakahara and Raz, 2006)). Galectin-3 is ubiquitously expressed in most normal adult tissues and also found in a variety of tumor cell types. In tumor cells, galectin-3 expression varies with cell type and stage of cancer progression (reviewed in (Danguy et al., 2002; Dumic et al., 2006; van den Brule et al., 2004). Within the cell, galectin-3 is localized to the nucleus, cytoplasm and cell surface and is also known to be secreted (reviewed in (Wang et al., 2004)). Cytoplasmic and nuclear localized galectin-3 is involved in apoptosis, cell proliferation, splicing, and Wnt/β-catenin signaling pathway, while cell surface and extracellular galectin-3 modulate cell adhesion (reviewed in (Nakahara and Raz, 2006; van den Brule et al., 2004; Wang et al., 2004)). Extracellular galectin-3 binds to extracellular matrix (ECM) proteins such as fibronectin, laminin, collagen IV, elastin, hensin, tenascin-C and

–R (reviewed in (Dumic et al., 2006)). Interestingly, galectin-3 also binds to a variety of integrin receptors located on the cell surface (reviewed in (Dumic et al., 2006)).

Despite knowing that galectin-3 has multiple interactions in the extracellular environment, its function in this niche is not clear, and the exact mechanism by which galectin-3 is secreted is not understood. It is known that galectin-3 is secreted by a non-classical secretion pathway which bypasses the ER/Golgi, it also lacks a conventional secretion signal sequence (Lindstedt et al., 1993; Sato et al., 1993). However, there are indications that the N-terminal 11-amino acids introduce structural changes in the protein that may mediate secretion (Gong et al., 1999; Mehul and Hughes, 1997; Menon and Hughes, 1999). There is some evidence of vesicle mediated secretion of galectin-3 and it has been found associated with exosomes (Menon and Hughes, 1999; Thery et al., 2001). Nonetheless, how extracellular galectin-3 effects cell adhesion is ambiguous. Previous studies have described either an enhancement or abrogation of cellular adhesion and spreading, dependent on cell type and galectin-3 concentration (reviewed in (Dumic et al., 2006)). For example, extracellular galectin-3 may form a lattice along with Mgat5 to induce clustering and activation of β1 integrin. On the other hand, it has also been shown that extracellular galectin-3 is needed for internalization of the β1 integrin receptor (Furtak et al., 2001; Goetz et al., 2008; Lagana et al., 2006). A better understanding of how galectin-3 is secreted and what purpose it serves in the extracellular environment is needed. In this paper we have found a previously unknown link between secretion of galectin-3 and the calpain small subunit, calpain 4.

Calpains are a family of intracellular calcium-dependent proteases involved in a plethora of physiological processes including cell migration, apoptosis, and cell proliferation to name a few (reviewed in (Suzuki et al., 2004). The calpain system includes the Calpain 1 and 2 holoenzymes, and their endogenous inhibitor calpastatin. Calpain 1 and 2 holoenzymes are comprised of the large catalytic subunits calpain 1 and 2 and a common small regulatory subunit, calpain 4, often referred to as Css-1 (reviewed in (Goll et al., 2003)). This specific system impacts cell adhesion and migration, likely by controlling the turnover of focal adhesions (reviewed in (Franco and Huttenlocher, 2005; Glading et al., 2002; Goll et al., 2003; Huttenlocher, 2005). We have previously found that the small subunit not only acts as a regulatory protein, but also functions independent of the catalytic activity of the large subunits to produce mechanical forces on the ECM, known as traction forces (Undyala et al., 2008). Calpain 4 deficient mouse embryonic fibroblasts (MEF) produce less traction force in comparison to wildtype MEF, MEF deficient in the large catalytic subunits or MEF overexpressing the endogenous inhibitor calpastatin.

Exactly how traction forces are produced is currently an area of intense research in which mechanisms involving tyrosine phosphorylation have become a focus. Early studies established that enhanced tyrosine phosphorylation occurs at focal adhesions upon the application of mechanical stress (Chrzanowska-Wodnicka and Burridge, 1996; Pelham and Wang, 1997). Subsequent studies have substantiated these original observations (as reviewed in (Giannone and Sheetz, 2006)). To further elucidate the force generation pathway, we have compared the tyrosine phosphorylation patterns of

intracellular proteins from calpain 4 deficient MEFs to wildtype MEFs. We have identified galectin-3 as differentially tyrosine phosphorylated in these cells. Most significantly, we have made the unique observation that calpain 4 alone, and not the catalytic subunits of the Calpain 1 and 2 holoenzymes, as essential for galectin-3 secretion.

MATERIALS AND METHODS

Cell Culture and Plasmids

Mouse embryonic fibroblasts (MEF) cells (immortalized by SV40 large T-antigen transfection), Capn4 -/- MEFs, Capn1 knockdown MEFs, Capn2 knockdown MEFs were used in this study (Arthur et al., 2000; Dourdin et al., 2001; Franco et al., 2004). All cell lines were cultured and maintained in Dulbecco's Modified Eagle's Medium - high glucose (Sigma), supplemented with 10% fetal bovine serum (Hyclone) and 1% Penicillin/Streptomycin /Glutamine (Gibco) and incubated at 37^{o}C under 5% humidified CO_2. 0.1 % Trypsin-EDTA was used for cell passages, never exceeding eight passages for a given cell line. Calpain 4 deletion was rescued by nucleofection of *capn4-/-* MEFs with the plasmid pSBC-r28kDa encoding the full-length rat calpain small subunit as described by Dourdin et al., 2001 (Dourdin et al., 2001). Calpastatin was over-expression in wildtype MEF cells from the plasmid hrEGFP-calpastatin (Bhatt et al., 2002).

Protein Extraction and Collection of Conditioned Media

Proteins were extracted from each cell line with triple detergent lysis buffer (100mM Tris-Cl, 300mM NaCl, 0.5% sodium deoxycholate, 0.2% SDS, 2% NP 40)

containing Protease Inhibitor Cocktail (Sigma) and also Halt™ Phosphatase Inhibitor Cocktail. Protein from cells grown to 80% confluency on three 100mm cell culture dishes were extracted, concentrated and further prepared for two-dimensional polyacrylamide gel electrophoresis as described below.

To test for the secretion of galectin-3, conditioned media was collected from two 80% confluent 60mm culture dishes containing 2.5ml of culture media. Equal volume of conditioned media from each cell type was loaded onto standard 4-20% Tris-HEPES-SDS polyacrylamide gels and used for western blot or for Coomassie Brilliant Blue staining to ensure equal loading.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)

The protocol used for 2-D PAGE has been described in detail with minor modification to the sample preparation (Kang et al., 2005). Briefly, the protein extracts from each cell line was concentrated using Amicon Ultra-4 5K filter units of 5000 Da Nominal Molecular Weight Limit (NMWL). The concentrated proteins were then solubilized in sample buffer (8M Urea, 50mM DTT, 4% CHAPS, 0.2% Carrier ampholytes, 0.0002% Bromophenol Blue). 200 micrograms of protein for each sample was rehydrated into isoelectric focusing strips with a pH range of 3-10 (Bio-Rad). Isoelectric focusing was then performed at 35,000 V-h in a PROTEAN IEF Cell (Bio-Rad). Following this, second-dimension SDS PAGE was performed using 4-12% Bis-Tris precast polyacrylamide gels (Bio-Rad).

Western Blot

Protein samples resolved either by the 2-D PAGE method or by standard SDS-PAGE (4-20% gradient Tris-HEPES-SDS precast polyacrylamide gel system, Pierce) were transferred (semi-dry) onto PVDF membranes (Bio-Rad). Buffers used for the transfer have been previously described (Canelle et al., 2005). The blots were probed with one of the following antibodies; anti-phosphotyrosine antibody clone PY20 (Millipore), monoclonal rat anti-Galectin-3 antibody and polyclonal rabbit anti-Galectin-3 antibody (gifts from Dr. A. Raz, Karmanos Cancer Institute, MI). Commercially available HRP conjugated secondary antibodies were detected with ECL Plus Western Blotting Detection Reagents (Amersham).

Identification of Differentially Tyrosine Phosphorylated Proteins

Protein samples from MEF cells and *capn4-/-* MEF cells were resolved by 2-D PAGE. The proteins were partially electro transferred onto PVDF membranes. As mentioned above, the blots were probed using anti-phosphotyrosine antibody clone PY20 (Millipore). The gel containing residual proteins were stained using SYPRO-Ruby Protein Gel Stain (Bio-Rad). Images from the immunoblot and stained gels were superimposed to select protein spots that were differentially phosphorylated in the two cell types (Described previously by Kang et al., 2005 (Kang et al., 2005)). These spots were then excised and identified by mass spectrometric analysis by the Protein Core Facility, Columbia University.

Immunofluorescence and Microscopy

Cultured cells were fixed for immunofluorescence using 4% paraformaldehyde and 0.1% Triton X-100 for 10 min, blocked for one hour with 5% BSA in PBS, followed by Anti-Galectin-3 antibodies and the species appropriate secondary Alexa Fluor 546 antibody. All images were acquired using an Olympus IX81 ZDC inverted microscope. Images were captured using a Diagnostic Instruments Boost EM-CCD-BT2000 back-thinned camera driven by IPLab software.

RESULTS

Identification of Galectin-3 as a Non-Tyrosine Phosphorylated Protein in Calpain 4 Deficient Cells

Protein lysates were prepared from four different cell lines; MEF cells, MEF cells deficient in either the large Calpain 1 or 2 subunits, and MEF's deficient in the small subunit Calpain 4. These lysates were resolved by two-dimensional gel electrophoresis as described in the materials and methods section. Western blotting with an anti-tyrosine antibody revealed numerous protein spots that differed in their levels of tyrosine phosphorylation between the four cell lines (Figure 3.1). A total of six protein spots were selected based on their differential presence on the four gels and analyzed by mass spectrometry. An isolated protein spot within the 30kDa range which was present in wildtype cells and Capn1 and 2 deficient cells, but absent in *capn4-/-* lysates was identified as galectin-3. Using an anti-Galectin-3 antibody, we confirmed that the protein was expressed in *capn4-/-* cells and at levels equivalent to its expression in wildtype MEF cells (Figure 3.2). Expression levels of galectin-3 were also unchanged when the two

large catalytic subunits, Capn1 and Capn2, were silenced (data not shown). These results strongly indicate that calpain 4 is essential for the tyrosine phosphorylation of galectin-3.

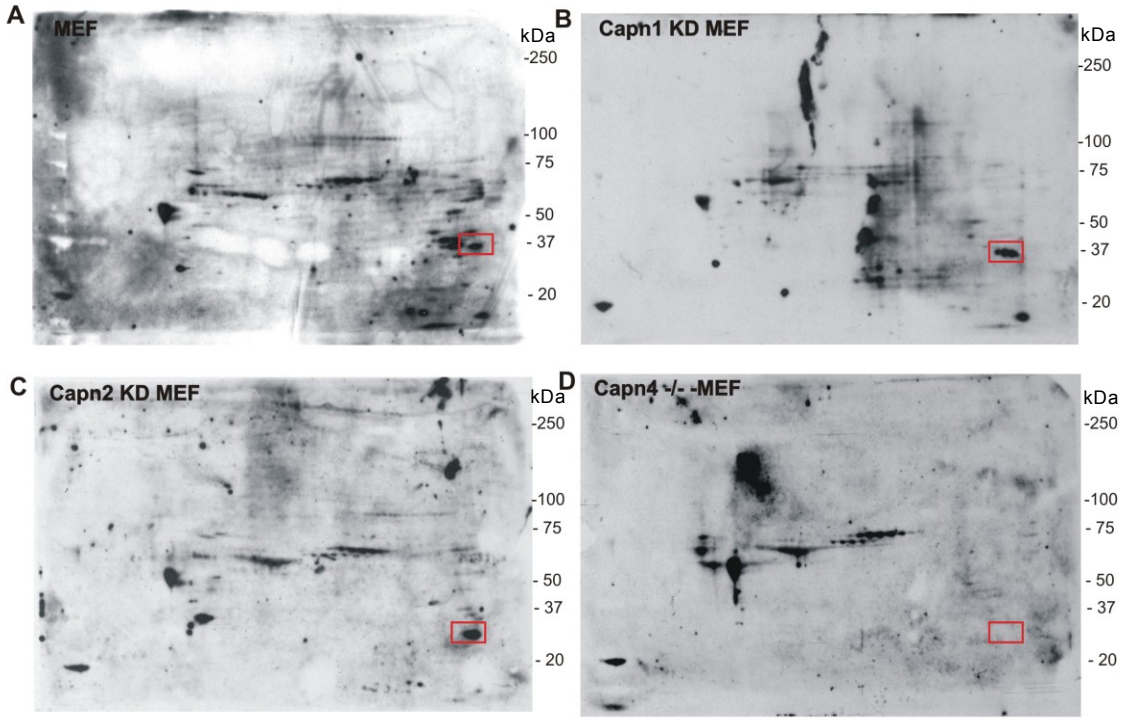

Figure 3.1: Galectin-3 is not tyrosine phosphorylated in the absence of the calpain small subunit, Capn4.

Total cellular protein from MEF cells (A), Capn1 silenced MEF cells (B), Capn2 silenced MEF cells (C) and *capn4-/-* MEF cells (D) were resolved by two-dimensional gel electrophoresis, transferred onto a PVDF membrane and probed for tyrosine phosphorylated proteins. Numerous protein spots are differentially phosphorylated in the four different cellular backgrounds. However, one prominent protein spot (red rectangular box) was phosphorylated in all cell types except in Capn4 silenced MEF cells. The protein spot was identified as the 30kDa protein Galectin-3 by mass spectrometric analysis.

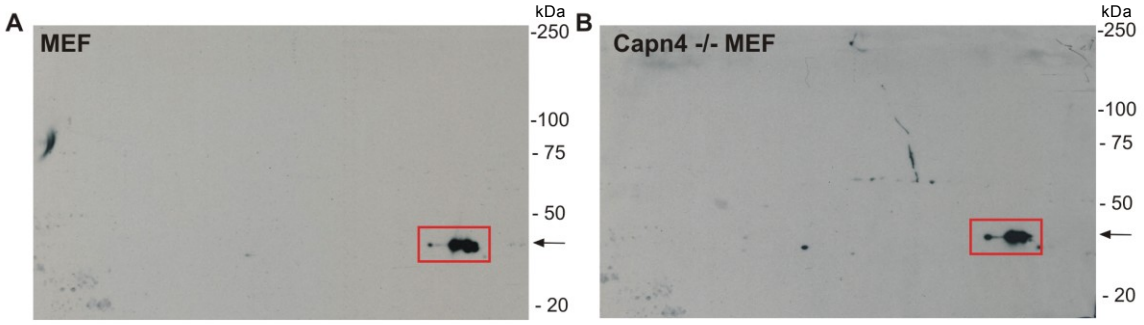

Figure 3.2: Galectin-3 expression is normal in the absence of Capn4.

Total cellular protein from MEF cells (A) and c*apn4* -/- MEF cells (B) were resolved by two-dimensional gel electrophoresis, transferred onto a PVDF membrane and probed using an anti-Galectin-3 antibody. A distinct Galectin-3 spot (red rectangular box and black arrow) was obtained in both cell types, indicating that Galectin-3 expression is normal in the absence of Capn4.

Galectin-3 Fails to Localize to the Cell Periphery in the Absence of Calpain 4

Post-translational modifications of proteins are known to affect numerous facets of a protein including its function, interacting partners, localization and stability. We tested if the intracellular localization of the non-tryosine phosphorylated form of galectin-3 differed in the Calpain 4 deficient cells. In our immunofluorescence studies we used four different antibodies each recognizing a distinct region of the protein. We observed that the non-tyrosine phosphorylated form of galectin-3 does not localize to the periphery of the *capn4-/-* cell (Figure 3.3). However, when tyrosine phosphorylated, as is the case in MEF cells and cells deficient in the catalytic heavy chains, galectin-3 is ubiquitously expressed in the cell and is not excluded from the cell periphery. This prompted us to ask if secretion of galectin-3 was disrupted in the Calpain 4 deficient cells.

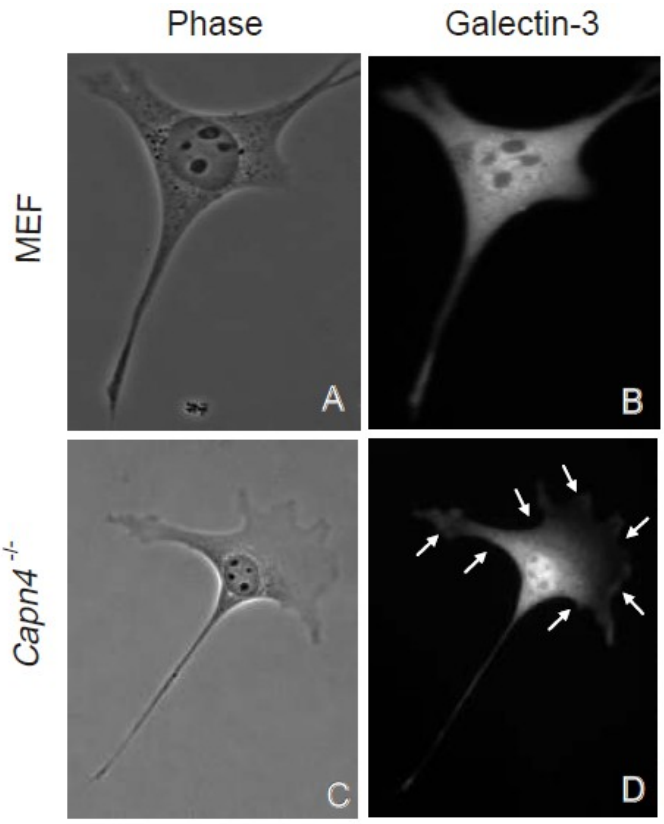

Figure 3.3: Absence of calpain 4 potentially affects galectin-3 localization.
MEF cells and Capn4-/- MEF cells were fixed and immunofluorescence was performed using a monoclonal anti-Galectin-3. Galectin-3 is present uniformly throughout the wildtype cell. However, in a Capn4 -/- MEF cell, galectin-3 was localized within the nucleus and in the peri-nuclear region, but absent from the cell periphery as indicated by white arrows around the cell periphery.

Calpain 4 Regulates Secretion of Galectin-3

Galectin-3 can be found on the surface of the cell and is also known to be secreted, although the mechanism is currently unknown. Galectin-3's extracellular activities are known to impact cell adhesion and migration in both normal and tumor cells. Since we were seeing an absence in the localization of galectin-3 to the cell periphery in Calpain 4 deficient cells, we tested for its secretion into the media of these cells. Conditioned media was collected on day 2 from cultures of all four cell lines; MEF,

Capn1 and 2 deficient cultures and Calpain 4 deficient cell cultures. Consistent with our immunofluorescence results, we found that galectin-3 was not being secreted from the *capn4-/-* cells, cells in which galectin-3 was not tyrosine phosphorylated (Figure 3.4A). In addition to the four cell lines, we also tested the conditioned media of cultured cells in which an endogenous inhibitor (calpastatin) of the calpain holoenzymes was overexpressed, thereby ensuring that the catalytic activity of both the large subunits would be abrogated. As expected, conditioned media from these cell cultures also showed an abundance of secreted Galectin-3 (Figure 3.4B). To further support our observation, we rescued *capn4-/-* cells using a recombinant rat Capn4 cDNA plasmid. Conditioned media from these cells showed that galectin-3 was now being secreted by these cells indicating that the calpain small subunit (calpain 4), but not the catalytic activities of calpain 1 and calpain 2, is essential for galectin-3 secretion (Figure 3.4C).

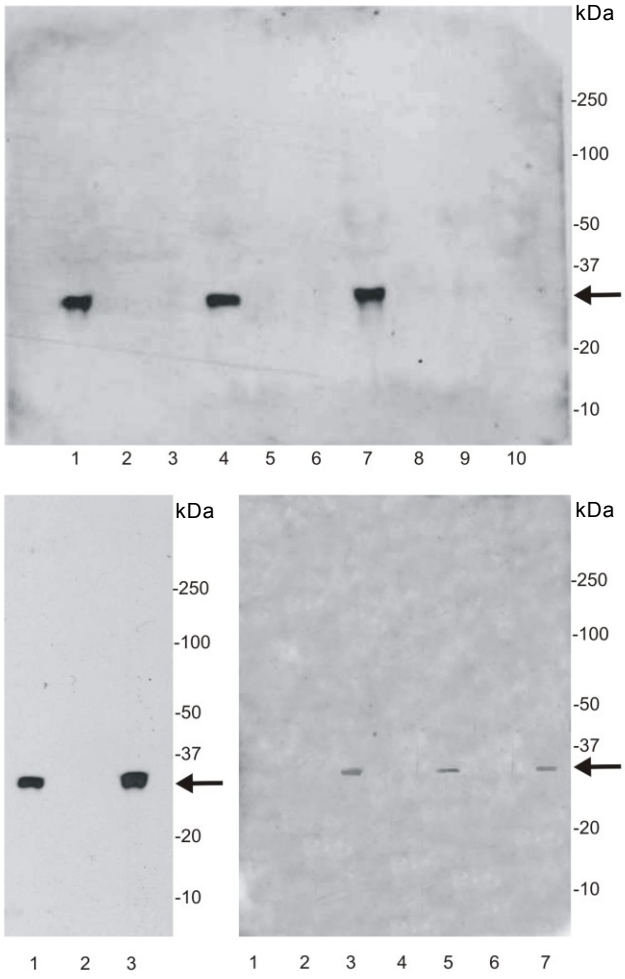

Figure 3.4: Capn4 regulates secretion of galectin-3.

A) Western blot of galectin-3 (black solid arrow) from media of MEF (lane 1), Capn1 silenced MEF (lane 4), Capn2 silenced MEF (lane 7) and *capn* -/- MEF cell (lane 10). B) Calpastatin overexpressing MEF cells secrete Galectin-3 at levels similar to mock-nucleofected MEF cells (lanes 3 and 1 respectively)). C) *capn4-/-* MEF cells rescued by exogenous expression of Calpain 4 secrete galectin-3 (lanes 3, 5 and 7) as compared to *capn4-/-* cells (lane 1). Lanes 3, 5 and 7 shows results from conditioned media collected from Calpain 4 rescued cells 24, 48 and 72 hours post nucleofection. The amount of secreted galectin-3 decreases as the proportion of Calpain 4 rescued cells is overgrown by *capn4-/-* cells.

DISCUSSION

Cell migration is a complex process and both biochemical and mechanical components of the environment impact how a cell migrates. Environmental information is transmitted into the cell through transmembrane receptors, such as GPCRs, hormone receptors, and integrins to name a few. Activation of a complex system of overlapping signaling cascades ultimately lead to altered cytoskeletal and focal adhesion dynamics necessary for cell spreading or migration.

As a cell migrates it generates its strongest traction forces at the leading edge of a migrating cell resulting in the maturation of focal complexes into focal adhesions (Beningo et al., 2001). Much of how traction forces are generated in a migrating cell remains to be elucidated though previous studies have identified proteolytic and phosphorylation activities as significant events. As part of identifying the role of calcium-dependent proteases in mechanical signaling, our group has previously established that the calpain small subunit (calpain 4) regulates traction forces and strengthening of adhesions, independent of the catalytic activity of the large subunits (Undyala et al., 2008). Furthermore, various studies have identified Src family kinases, focal adhesion kinase, the SH2 domain-containing phosphates and receptor-like protein tyrosine phophatases as important components of the force-dependent signal transduction pathways (Giannone and Sheetz, 2006; Pelham and Wang, 1997). Based on this evidence we compared the tyrosine phosphorylation levels of proteins extracted from MEF cells and MEF cells in which the expression of each of the three subunits of the Calpain 1 and

2 holoenzymes were silenced independently. From this screen galectin-3 was found to be differentially phosphorylated.

Until recently, phosphorylation of galectin-3 was believed to occur only on Serine residues 6 and 12 at the amino terminus of galectin-3. A single study suggested that galectin-3 is was phosphorylated on tyrosine residues at the N-terminal PGAY or PXXY motifs (Menon and Hughes, 1999). It was recently confirmed that galectin-3 is also phosphorylated on tyrosine residues 79, 107 and 118 and suggested that c-Abl kinase is the responsible kinase (Balan et al., 2010; Li et al., 2010). However, the functional significance of tyrosine phosphorylation of galectin-3 residues has not yet been established until now.

Many studies have confirmed the importance of this protein in the extracellular environment of cells and its impact on cell adhesion and migration under both normal and disease conditions. It has been shown *in-vivo* that circulating galectin-3 promotes tumor progression (Iurisci et al., 2000). However the literature is conflicting on how extracellular galectin-3 is influencing migration. For example, one study finds that *in-vitro* galectin-3, along with caveolin-1, bind to N-glycans that have been modified by Mgat5 and recruit conformationally active α5β1 integrin to adhesions, resulting in the activation of FAK and PI3K, hence enhancing the formation of adhesions (Goetz et al., 2008; Lagana et al., 2006). Conversely, a second study found that galectin-3 is involved in internalization of β1 integrin, thereby working against the formation of adhesions (Furtak et al., 2001). Despite these different roles attributed to extracellular galectin-3 in

cell adhesion and migration, a potential mechanism for the secretion of galectin-3 has not been identified.

Galectin-3 is not secreted by the classical secretion pathway and adopts a non-classical mechanism (Lindstedt et al., 1993; Sato et al., 1993). It has been reported, that the first 11 amino acids of galectin-3 act to regulate of the localization of galectin-3, as truncation of this region eliminates secretion and nuclear localization (Gong et al., 1999). However little else is known about how this protein is secreted. In this study we have found a previously unknown link between calpain-4 and the secretion of galectin-3. More specifically, we have made the novel observation that the tyrosine phosphorylation status of galectin-3, indirectly modulated by calpain-4, influences its secretion. We speculate that phosphorylation of tyrosine residues 79, 107 and 118 (Balan et al., 2010) alters the quaternary structure of galectin-3, making the N-terminal 11 amino acids unavailable for mediating galectin-3 secretion. Nonetheless, the lack of galectin-3 secretion serves to explain the defects observed in traction forces and migration found in calpain 4 deficient cells and further experimentation is currently underway to establish this fact.

ACKNOWLEDGEMENTS

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CHAPTER 4

THE REGULATION OF TRACTION FORCES AND FOCAL ADHESION DYNAMICS THROUGH CALPAIN 4 MEDIATED SECRETION OF GALECTIN-3

ABSTRACT

Traction forces generated by the acto-myosin cytoskeleton of a cell are vital to cellular migration. These forces are transmitted via focal adhesions and integrins onto the substrate on which the cells are adhered. However, the signaling mechanism that leads to the generation and regulation of these forces is not fully understood. We have previously found that calpain 4 (Capn4), the small non-catalytic subunit of the Calpain 1 and 2 proteases, is involved in the production of traction force independent of the proteolytic activity of the larger subunits. We further showed that Capn4 mediates tyrosine phosphorylation of galectin-3 followed by its secretion. Since *capn4-/-* Mouse Embryonic Fibroblasts (MEF) cells are defective in traction force production, focal adhesion maturation and adhesion strength we asked if addition of recombinant galectin-3 externally to the *capn4-/-* MEF cell culture would help rescue the defects observed with these cells. As hypothesized, traction force microscopy indicates that extracellular galectin-3 was able to enhance traction forces generated by *capn4-/-* MEF cells. Our current studies indicate that recombinant galectin-3 added externally to the media rescues focal adhesion turnover and maturation defects seen in *capn4-/-* MEF cells. Similarly, addition of recombinant galectin-3 to the media also enhances the adhesion strength of weakly adhered *capn4-/-* MEF cells. However, extracellular galectin-3 does not influence mechanosensing, both homeostatic and localized tension. Our experiments also suggest

that galectin-3 mediated regulation of traction force production is potentially independent of β1 integrin activation or signaling pathways triggered by FAK Y397 autophosphorylation. Further experiments need to be performed to establish the signaling mechanism downstream of galectin-3 secretion.

INTRODUCTION

The orchestration of adhesion events during the migration of a cell requires coordinated cues from both intracellular and extracellular factors (Gardel et al., 2010; Huttenlocher et al., 1995; Li et al., 2005). The extracellular matrix (ECM) provides numerous cues, these cues being both chemical and physical in nature. These signals coming from regions extraneous to the cell would constitute part of what is referred to as outside-in signaling (Kim et al., 2011; Luo et al., 2007). These extracellular cues need to be transmitted into the cell and is carried out mainly by transmembrane receptors called integrins (Hu and Luo, 2012; Luo et al., 2007). Integrins bind specifically to various ECM proteins such as fibronectin, collagen, laminin, etc. Apart from integrins numerous other membrane receptors and proteins in the extracellular environment also play a role in transmitting biochemical and biophysical cues into the cell. An example would include growth factor signaling (Beattie et al., 2010; Kim et al., 2011; Streuli and Akhtar, 2009). These signals, whether on 2-dimensional or in 3-dimensional environments, are then transmitted to the cell nucleus via various signaling cascades and can then either promote cell migration or can halt the process (Ingber, 1991; Martins et al., 2012; Wang et al., 2009). This response from the cell is referred to as inside-out signaling (Faull and

Ginsberg, 1996). It could involve changing cell polarity, cell spreading, change in the rate and direction of migration and many more (Ginsberg et al., 1992; Huveneers and Danen, 2009).

Some of the extracellular physical cues include substrate compliance, topography, local stimulus such as the contractile forces generated by neighboring cells and shear flow (Freund et al., 2012; Guilak et al., 2009). Perception of these stimuli by a cell is referred to as mechanosensing that is translated into a mechanoresponse (De et al., 2010; Vogel and Sheetz, 2006). Evidence suggests that the sensing and force production mechanisms are linked (Fouchard et al., 2011; Prager-Khoutorsky et al., 2011; Weng and Fu, 2011). For instance, substrate stiffness, in which the cell is exposed to homeostatic tension, can affect the traction forces generated by the acto-myosin cytoskeleton of the cell (Califano and Reinhart-King, 2010; Chan and Odde, 2008; Fouchard et al., 2011; Trichet et al., 2012; Wang, 2009). The process by which traction forces and sensing are processed is poorly understood. Early speculations proposed a feedback loop between mechanosensing and traction force generation, although new data is emerging that suggests a more complicated relationship. The mechanistic details of how traction forces are produced are complex on their own.

A recent study from by Rape et al., has suggested a two way signaling mechanism that controls force generation upon microtubule depolymerization (Rape et al., 2011). One is a myosin-II dependent pathway and the other is Focal Adhesion Kinase (FAK) dependent and both these pathways are independent of each other. Our group has previously looked into the role of the proteolytic enzymes Calpains 1 and 2 and

successfully established that the large catalytic subunits of each of these holoenzymes do not play a role in the generation of traction forces (Undyala et al., 2008). However, calpain small subunit 1 (calpain 4), the common regulatory subunit, plays a role independent of the large subunits in force generation. This interesting result suggested that the small subunit has a role other than its preconceived role as just the regulatory subunit of the calpains.

To further understand the specific role calpain 4 plays in the traction force pathway, we adopted a method whereby we looked at differential tyrosine phosphorylation levels of cellular proteins from wildtype mouse embryonic fibroblasts (MEFs) and MEF cells in which one of the calpain subunits have been silenced. We determined that one such protein that showed reduced tyrosine phosphorylation level in the absence of calpain 4 was galectin-3 (Menon et al., 2011). Galectin-3 is a lectin binding protein and is an atypical member of the galectin family of proteins (Krzeslak and Lipinska, 2004; Nakahara and Raz, 2006). Three of its tyrosine residues, 79, 107 and 118, were recently identified as the residues that are phosphorylated, potentially by Abl-kinase (Balan et al., 2010; Li et al., 2010). The protein has numerous roles both within and outside the cell indicating that its localization is important for each of its roles (Dumic et al., 2006; Haudek et al., 2010; Liu et al., 2002; Nakahara and Raz, 2006). Extracellularly, galectin-3 has been shown to play a role in cell adhesion and migration (Ochieng et al., 2004). Goetz et al., have shown that the extracellular galectin-3 lattice results in integrin clustering and focal adhesion turnover (Goetz et al., 2008). However, other than knowing galectin-3 is secreted by the non-classical mode, the mechanistic

details for its secretion are unknown (Gong et al., 1999; Lindstedt et al., 1993; Sato et al., 1993; Zhu and Ochieng, 2001). We have found that the absence of calpain 4, which resulted in a reduction in its tyrosine phosphorylation levels, also altered its secretion pattern (Menon et al., 2011).

Having identified a connection between calpain 4 and the secretion of galectin-3, we hypothesized that extracellular galectin-3 would positively regulate the production of traction force. To address this hypothesis, we measured traction stress, adhesion strength, focal adhesion turnover rate, both in the presence and absence of recombinant galectin-3 added to calpain deficient cells. We also studied the effect of this secreted protein in the ability of the cell to sense both environmental and transient mechanical cues.

MATERIALS AND METHODS

Cell culture and reagents

All cell lines used in this study including Mouse Embryonic Fibroblasts (MEFs) (immortalized by SV40 large T-antigen transfection), calpain 1 and calpain 2 silenced MEFs, *capn4-/-* MEFs have been previously described. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, high glucose) (Sigma) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin/glutamine (Gibco) and grown at 37˚C under 5% CO_2 in a humidified cell culture incubator. Cells were passaged using 0.1% Trypsin–EDTA (Invitrogen) and was not allowed to exceed eight passages for a given cell line. Lyophilised recombinant galectin-3 (rGal3) was purchased from R&D Systems and reconstituted at 250 μg/ml following the manufacturer's protocol. For all

experiments, rGal3 was added at 2 μg/ml concentration. For calpain 4 and galectin-3 gene knockdowns siGENOME SMARTpool siRNA from Dharmacon RNAi Technology (Thermo Scientific) were used. Non-target siRNA, also from Dharmacon RNAi Technology, was used as controls whenever applicable. Appropriate RNA was introduced into cells by a method called nucleofection performed using an Amaxa Nucleofector II device and MEF compatible nucleofection reagents (MEF 2 nucleofector solution, Lonza). Primary antibodies used for immunofluorescence and western blot included mouse anti-vinculin monoclonal antibody (V4505, Sigma), rabbit anti-FAK [pY^{397}] polyclonal antibody (44-624G, Invitrogen) and mouse anti-GAPDH monoclonal antibody (MAB374, Millipore) as the loading control. The secondary antibody used for immunofluorescence was Alexa Fluor® 488 goat anti-mouse IgG (H+L) (A11001, Invitrogen). ECL™ anti-mouse and anti-rabbit IgG, HRP linked whole antibodies (NA931 and NA934 respectively, GE Healthcare) were used as secondary antibodies for western blot experiments. For immunofluorescence experiments, actin was stained using Alexa Fluor® 546 phalloidin (A22283, Invitrogen).

Traction force microscopy and analysis

Cells were seeded on flexible 5% acrylamide and 0.08% N,N-methylene-bis-acrylamide polyacrylamide substrates prepared as described previously. These substrates were coated with fibronectin at a concentration of 5 μg/cm^2. The previously estimated Young's modulus of polyacrylamide substrates made up of the above mentioned concentration is $2.4x10^4$ N/m^2. After the cells were allowed to adhere to the substrates by incubating them overnight under regular cell culture conditions, traction force

microscopy was perfomed as described earlier. Briefly, three sets of images were taken per field – a bright field image of the cell followed by two fluorescent images of the embedded beads, with and without the cell on the substrate. Bead displacement maps and the cell and nuclear boundaries were then used to calculate and render traction stress values using a custom made algorithm provided to us by Dr. Micah Dembo (Boston University). The algorithm has been previously described.

Mechanosensing experiments

To study the effect of substrate stiffness on cellular morphology (spread versus round), cells were seeded on polyacrylamide substrates of two varying stiffness achieved by varying the N,N-methylene-bis-acrylamide concentration (0.1% and 0.04% for hard and soft substrates respectively) keeping acrylamide concentration constant at 5%. The substrates were coated with fibronectin. Cells were seeded and incubated overnight and then images were taken at 10X magnification. The number of spread and round cells as observed visually by their area were then counted from six random fields for each cell line seeded on either of the two substrates. The average cell count was compared.

The effect of a locally applied mechanical stimulus on cell migration was also studied by seeding cells on 5%/0.1% acrylamide/N,N-methylene-bis-acrylamide polyacrylamide substrates coated with fibronectin. The experiment was performed as described previously. Briefly, a blunted microneedle was used to gently push the substrate in front of a migrating cell. This leads to a decrease in the tension within the substrate. Cells respond morphologically to this difference in tension by rounding up or altering their migratory trajectory away from the needle. A “no response” is recorded

when it remains on its trajectory towards the needle without gross morphological change. The response is observed by taking images every 3 min for approximately one hour.

Cell adhesion assay

A centrifugation assay was used to evaluate adhesion strength and has been described previously (Guo et al., 2006; Undyala et al., 2008). Briefly, cells were seeded onto 5%/0.08% acrylamide/bis-acrylamide substrates coated with fibronectin. The cells were allowed to adhere at 37˚C for 30 minutes. Adhered cells from 10 random fields were counted before and after centrifugation. Percentage adhesion for each cell line was calculated and compared.

Cell migration assay

Cells were seeded onto fibronectin coated coverglass and incubated overnight at 37˚C. The migration pattern of a cell was then observed at 40X magnification, images were collected at 2 minute intervals for 2 hours. Linear speed (microns/min) and persistence (min) of each cell was then calculated using the custom built dynamic image analysis system software (DIM, Y-L. Wang) based on the x,y coordinates of cell centroids.

Immunofluorescence

Cells were seeded on fibronectin coated coverglass and incubated overnight under regular cell culture conditions. The cells were then fixed and permeabilized using a teo-step protocol – first with paraformaldehyde, followed by a second step with both paraformaldehyde and 0.1% Triton X-100. This was followed by blocking with 5% BSA in PBS for 1 hour at room temperature. Following this, anti-vinculin antibody (Sigma,

V4505) was added at a 1:200 dilution and incubated at room temperature for 3 hours. Alexa Fluor® 488 anti-mouse secondary antibody (Invitrogen) was then added at a 1:500 dilution and incubated for 1 hour at room temperature followed by Alexa Fluor® 546 phalloidin (Invitrogen, a22283) staining at a 1:500 dilution also for 1 hour at room temperature. Each step was followed by PBS washes (3 x 15 min each). Images were then acquired using appropriate filters. The number and size of vinculin containing plaques were measured using the NIH Image J software.

Microscopy

Images for all experiments described above were acquired using an Olympus IX81 ZDC inverted microscope fitted with a custom built stage incubator to maintain cells at 37˚C under 5% CO2 for live cell imaging and a Diagnostic Instruments Boost EM-CCD-BT2000 back-thinned camera. The camera was driven by the IPLab software (BD Biosciences).

Polyacrylamide gel electrophoresis and western blotting

Each cell line was cultured to 80% confluency in 2 x 60mm culture dishes coated with fibronectin for protein extraction. Cells were lysed with triple detergent lysis buffer (100 mM Tris–Cl, 300 mM NaCl, 0.5% sodium deoxycholate, 0.2% SDS, 2% NP 40) containing Protease Inhibitor Cocktail (Sigma) and also HaltTM Phosphatase Inhibitor Cocktail (Thermo Scientific). Protein concentrations were estimated using the Bio-Rad *DC* protein assay kit. Equal protein concentration for all cell lines were then loaded onto 4-20% gradient Tris–HEPES–SDS precast polyacrylamide gel system (Pierce) and resolved at 100V. The proteins were then transferred using the Bio-Rad semi-dry transfer

apparatus onto PVDF membranes (Bio-Rad). Buffers used for transfer have been previously described. After transfer, the blots were blocked and then probed with appropriate antibodies. Rabbit anti-FAK [pY^{397}] polyclonal antibody (Invitrogen, 44-624G) was used at a 1:1000 dilution in 1% BSA in Tris Buffered Saline – 0.1% Tween (TBS-0.1% Tween). The antibody incubation was done overnight at 4˚C. Anti- active β1 integrin antibody (clone 9EG7, BD Pharmingen) was used at a 1:500 dilution in 5% non-fat blotting grade milk in phosphate buffered saline – 0.1% Tween (PBS-0.1% Tween). Levels of GAPDH, the loading control, was detected using anti-GAPDH monoclonal antibody (Millipore, MAB374) diluted to 1:7000 in 5% non-fat blotting grade dry milk in PBS-0.1% Tween. Commercially available HRP conjugated secondary antibodies (Amersham) were used were detected with ECL Plus Western Blotting Detection Reagents (Amersham). Washes before and after the secondary antibody treatment in each case was done using TBS-0.1% Tween or PBS-0.1% Tween for FAK [pY^{397}] and GAPDH respectively.

RESULTS

Galectin-3 Positively Regulates the Generation of Cellular Traction Force

In previous studies we discovered that cells deficient in calpain 4 are impaired in their ability to produce traction forces (Undyala et al., 2008). Furthermore, this function is unique to the small subunit, as knockdown or suppression of the catalytic activity of the heavy chains, Calpain 1 and Calpain 2, did not affect traction forces. In subsequent experiments, we discovered that calpain 4 indirectly alters the phosphorylation status of

multiple proteins, including the lectin binding protein galectin-3, and does so independent of the catalytic subunits (Menon et al., 2011). Although the intracellular expression level of galectin-3 remains unaffected in the absence of calpain 4, its level of tyrosine phosphorylation is reduced. Furthermore, the absence of calpain 4 and a potential defect in phosphorylation prevents the secretion of galectin-3 from the cell. Extracellular galectin-3 is known to regulate cell adhesion and migration, although the specifics are lacking (Goetz et al., 2008; Ochieng et al., 2004).

To solidify the functional connection between calpain4 and galectin-3 we have asked if extracellular galectin-3 influences the magnitude of traction forces produced by migrating fibroblasts. We used traction force microscopy (TFM), performed on polyacrylamide gels of moderate stiffness (Y=2.4E10+5), to measure the magnitude of stress. Measurements from MEF cells, under conditions of calpain4 and galectin-3 deficiencies, were compared to wild-type and non-target siRNA control cells. As for previous experiments, the *capn4-/-* MEF cells (avg. 1.49 kPa) produced three-fold less traction than the control cells (avg. 4.34 kPa) (Figure 4.1A,B). Likewise, the silencing of galectin-3 resulted in MEF cells with impaired traction (avg. 2.15 kPa), thus mirroring the *capn4-/-* traction phenotype. To address whether the secretion of galectin-3 contributed to the shared traction phenotype, we added exogenous recombinant galectin-3 to the medium of *capn4-/-* cells. The addition of galectin-3 not only rescued the defect in traction observed in the *capn4-/-* MEF cells, it actually enhanced the magnitude beyond that of the control cells (avg. 5.83kPa) (Figure 4.1A,B). These results suggest that the

defects in traction force observed in calpain 4 deficient cells results from a lack of secreted galectin-3 and is likely indirectly mediated by calpain 4 itself.

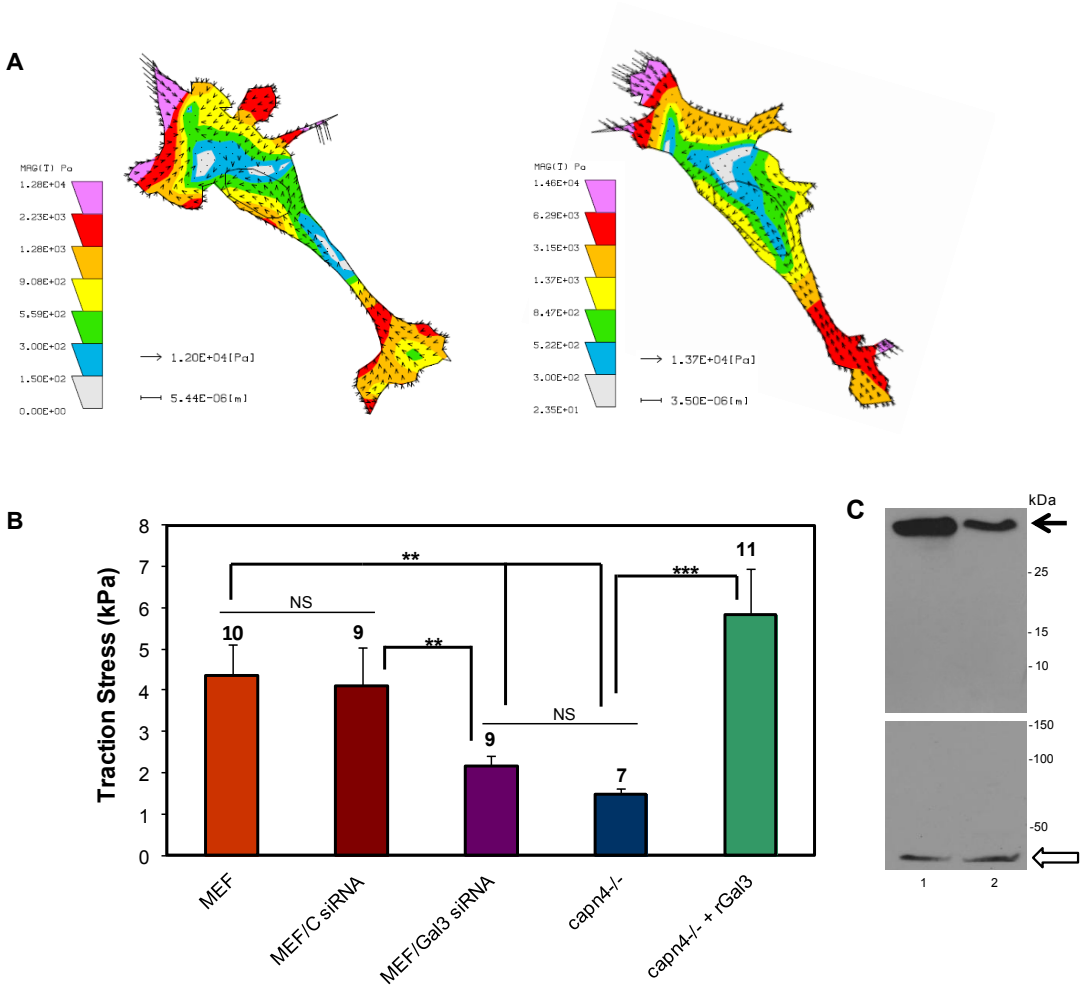


Figure 4.1: Extracellular galectin-3 rescues the defect in traction force in *capn4-/-* MEF cells.
A) Vector plots show the magnitude and direction of traction stress exerted by a *capn4-/-* cell (left) and a *capn4-/-* cell with recombinant galectin-3 (2µg/ml) added externally. The vectors indicate the direction and magnitude of traction stress. The color map illustrates magnitude. B) Average traction stress exerted by MEF cells, MEF cells treated with either control siRNA and Gal3 siRNA, *capn4-/-* MEF cells and *capn4-/-* cells with recombinant galectin-3 added to the media as a bar graph. Number of cells chosen for each cell type is denoted above the respective bar. Statistical analysis was performed by student's t-test (** indicates $p<0.05$, *** indicates $p<0.005$, NS indicates a non-significant relationship). C) siRNA mediated knockdown of galectin-3 (solid black

arrow) in MEF cells is effective 48 hours post-nucleofection as seen in lane 2. MEF cells nucleofected with control siRNA is loaded in lane 1.

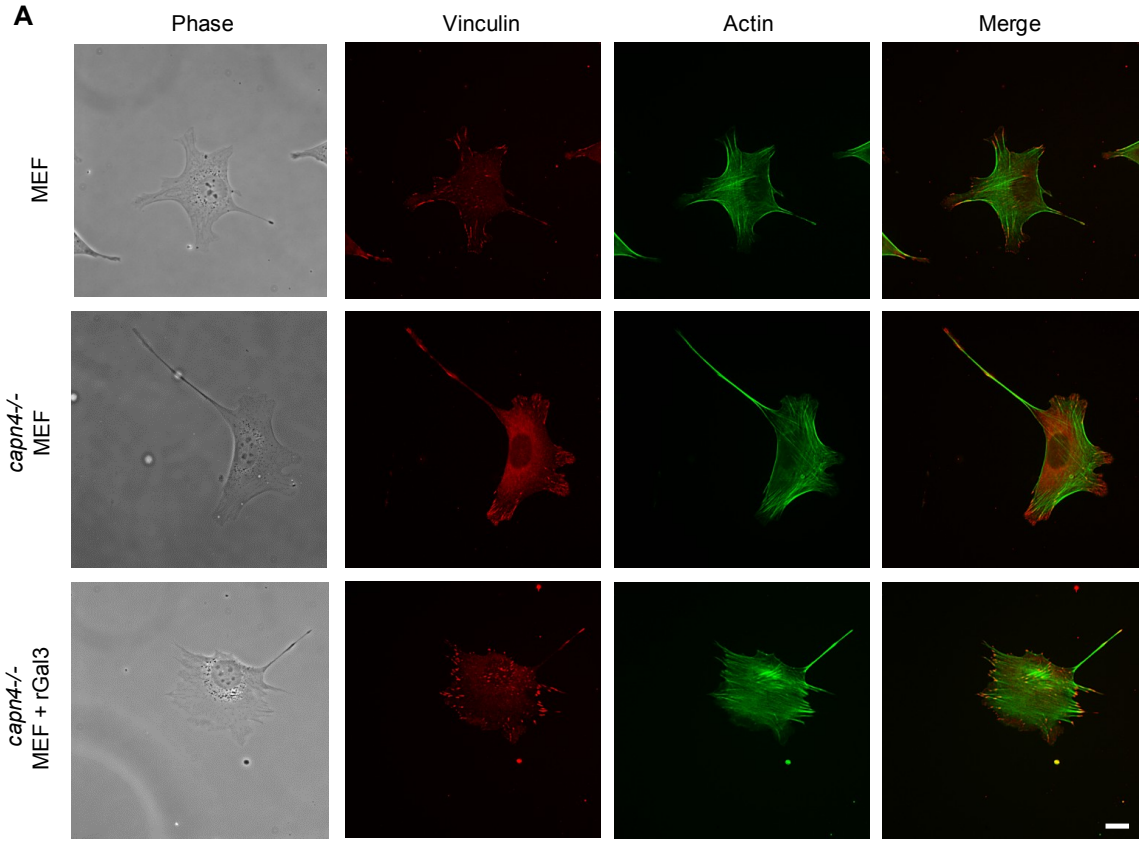
Extracellular Galectin-3 Enhances the Maturation and Strength of Focal Adhesions

Capn4 deficient MEF cells are defective in the maturation of focal adhesions (Undyala et al., 2008). MEF cells typically display adhesions of varying sizes ranging from small complexes that form at the edge of the cell and the more mature adhesions increasing in size as they grow and move towards the center of the cell (Papusheva and Heisenberg, 2010; Wolfenson et al., 2009). However, in *capn4-/-* cells this maturation processes is perturbed and adhesions of fairly uniform sizes can be found at the periphery of the cell, with few found within the cell body. This abnormality in focal adhesion maturation was also accompanied by a decrease in adhesion strength of the *capn4-/-* MEF cells (Undyala et al., 2008). These defects in adhesion dynamics and strength likely explain the reduction in traction force that we see in the absence of calpain 4. Since we were able to rescue the traction force defect of *capn4-/-* MEF cells by the external addition of rGal3 to the media, we tested the effect of extracellular galectin-3 on adhesion maturation and strength. To identify size defects the focal adhesion protein vinculin was immune-stained using anti-vinculin and fluorescently labeled secondary antibodies in formaldehyde fixed wildtype MEF cells, *capn4-/-* MEF cells and *capn4-/-* cells with rGal3 added to the media. Actin was also visualized using rhodamine-phalloidin. As expected, mature adhesions (as determined by their size and location) were observed in the cell body of wildtype MEF cells as compared to *capn4-/-* cells, where the adhesions primarily localized to the periphery of the cell (Figure 4.2A). In contrast, when rGal3 was added to the media of the *capn4-/-* cells, the number of focal adhesions within the cell

body increased greatly (Figure 4.2A). Quantification of the size and number of focal adhesions in each of these cell lines showed a significant increase (p=0.02) in the number of adhesions ranging from 0.5 to 1.5 microns in *capn4-/-* cells treated with rGal3 (Figure 4.2B). The numbers of adhesions in *capn4-/-* cells with rGal3 added externally showed no significant difference from those in wildtype MEF cells (p=0.13). As seen in figure 4.2B, *capn4-/-* MEF cells had more than 50% of focal adhesions that were smaller than 0.5 microns (focal complexes) and were localized to the periphery of the cell. However, the number of adhesions greater than 1.5 microns was not significantly different in any of these treated or non-treated cell lines. These results could suggest two things; first, that secreted galectin-3 potentially mediates proper focal adhesion maturation and second that focal adhesions grow in size in calpain 4 deficient cells but do not mature into the cell body. Further studies need to be performed to identify the galectin-3 mediated mechanism of focal adhesion maturation and turnover.

To test for the strength of adhesiveness to the substrates, we used a previously described centrifugation assay (Guo et al., 2006; Undyala et al., 2008). Using the same set of cells described above, we measured the adhesion strength, in addition we tested MEF in which capn4 was silenced by siRNA. We found that in the absence of calpain 4 approximately 50% of the cells remained adhered to the polyacrylamide substrate on which they were seeded (Figure 4.2C). In comparison, approximately 80-85% of MEF cells and MEF cells treated with control siRNA, remained adhered after centrifugation (Figure 4.2C). Addition of rGal3 to the media enhanced the adhesive strength of *capn4-/-* cells and we found that more than 95% of cells stayed adhered to the substrate (Figure

4.2C). Consistent with these observations, silencing of galectin-3 through siRNA in MEF cells also reduced the strength considerably to approximately 67% (Figure 4.2C). Our results indicate that galectin-3 in the extracellular environment also contributes to the adhesive strength of focal adhesions.



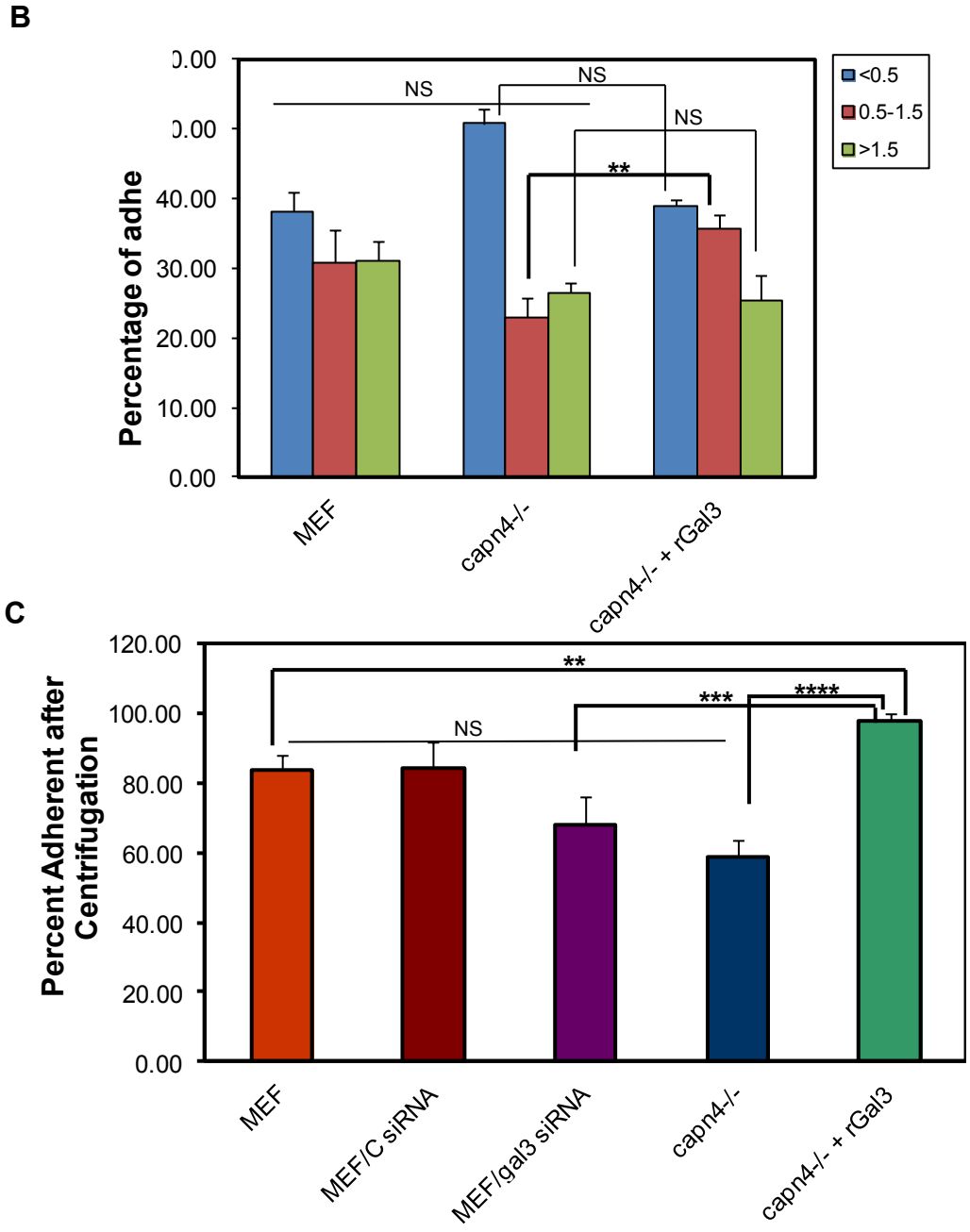


Figure 4.2: **Galectin-3 mediates focal adhesion maturation and enhances adhesion strength.**

A) Immunofluorescence of focal adhesions with anti-vinculin antibody illustrates adhesions maturing into the cell body in MEF cells (top row) and in *capn4-/-* MEF cells treated with recombinant galectin-3 (bottom row). Focal adhesions fail to mature in *capn4-/-* MEF cells (middle row). Similarly, stress fibers visualized by actin staining shows well formed stress fibers in MEF and *capn4-/-* MEF cells treated with recombinant galectin-3, unlike in *capn4-/-* MEF cells (Mag. bar = 10μm). B) Bar graph represents the

average number of adhesions of varying sizes in each of the three cell lines. The focal adhesion counts were obtained from 3-5 cells in two different trials. Nascent adhesions (0.5 – 1.5 sq.μm) are significantly greater when recombinant galectin-3 is added to *capn4-/-* MEF cells in comparison to untreated cells as analyzed by student's t-test (p=0.02). NS represents a non-significant relationship. C) Adhesion strength, expressed as a percentage of the number of cells that remain adhered after centrifugation, is also rescued when galectin-3 is added to *capn4-/-* MEF cells. Almost 97% of treated *capn4-/-* MEF cells remain adhered after centrifugation as compared to 57% that remained adhered when calpain 4 is deficient (Student's t-test analysis **** $p<0.00005$)). Galectin-3 knockdown also results in a reduction in the adhesion strength of MEF cells bringing down the value from approx. 84% in MEF and control siRNA treated MEF cells to 64% upon knockdown of galectin-3. Data represents three independent trials each performed in duplicates. Analysis performed by student's t-test (** indicates $p<0.05$, *** indicates $p<0.005$, NS indicates a non-significant relationship).

Galectin-3 Does Not Rescue the Mechanosensing Defect of Capn4 Deficient Cells

Cells are able to sense substrate stiffness, topography, and localized mechanical forces generated by neighboring cells, through a phenomenon referred to as mechanosensing. There is evidence that different forms of external stimulation produce different responses (Freund et al., 2012; Guilak et al., 2009; Menon and Beningo, 2011; Vogel and Sheetz, 2006). Cells prefer to spread and migrate on substrates whose stiffness closely matches its endogenous substrate (Discher et al., 2005; Discher et al., 2009; Engler et al., 2006; Flanagan et al., 2002; Pelham and Wang, 1997; Pittenger et al., 1999; Saha et al., 2008). For example, MEF cells spread and migrate better on stiffer substrates as compared to soft polyacrylamide substrates coated equally with the ECM protein fibronectin (Pelham and Wang, 1997). When we tested the calpain deficient cells on hard and soft substrates we have found that MEF cells deficient in Calpains 1, 2 or 4, are also capable of sensing a difference in stiffness and behaved similar to MEF cells on these same substrates. In contrast, Calpain 1, 2, or 4 deficient cells are unable to sense locally

applied stimuli provided by pushing on the substrate with a blunted needle, immediately in front of a migrating cell (Undyala et al., 2008). A MEF cell responds to the local stimulus by rounding up or changing migratory trajectory to avoid the stimulus. Given that secreted galectin-3 is essential for rescuing defects in traction force and adhesions, a process linked to sensing of external mechanical stimuli, we asked if exogenous galectin-3 could rescue the sensing defects of *capn4-/-* MEF cells.

Using fibronectin coated hard and soft polyacrylamide substrates we tested the ability of cells to spread normally. As expected, all cell lines seeded on hard substrates responded similarly and 90-95% percent of the cells were spread as wild-type. However, when plated on soft substrates, almost half the number of MEF cells and MEF cells treated with control non-target siRNA or galectin-3 siRNA remained rounded (Figure 4.3A,B). However, 85% of *capn4-/-* MEF cells failed to sense the soft substrate and spread normally. The addition of exogenous rGal3 failed to rescue this defect and 80% of *capn4-/-* MEF cells with rGal3 added externally to the culture medium spread normally (Figure 4.3A,B). These results suggest that galectin-3 is not involved in the mechanosensing pathway adopted by cells to sense the static stiffness of its environment.

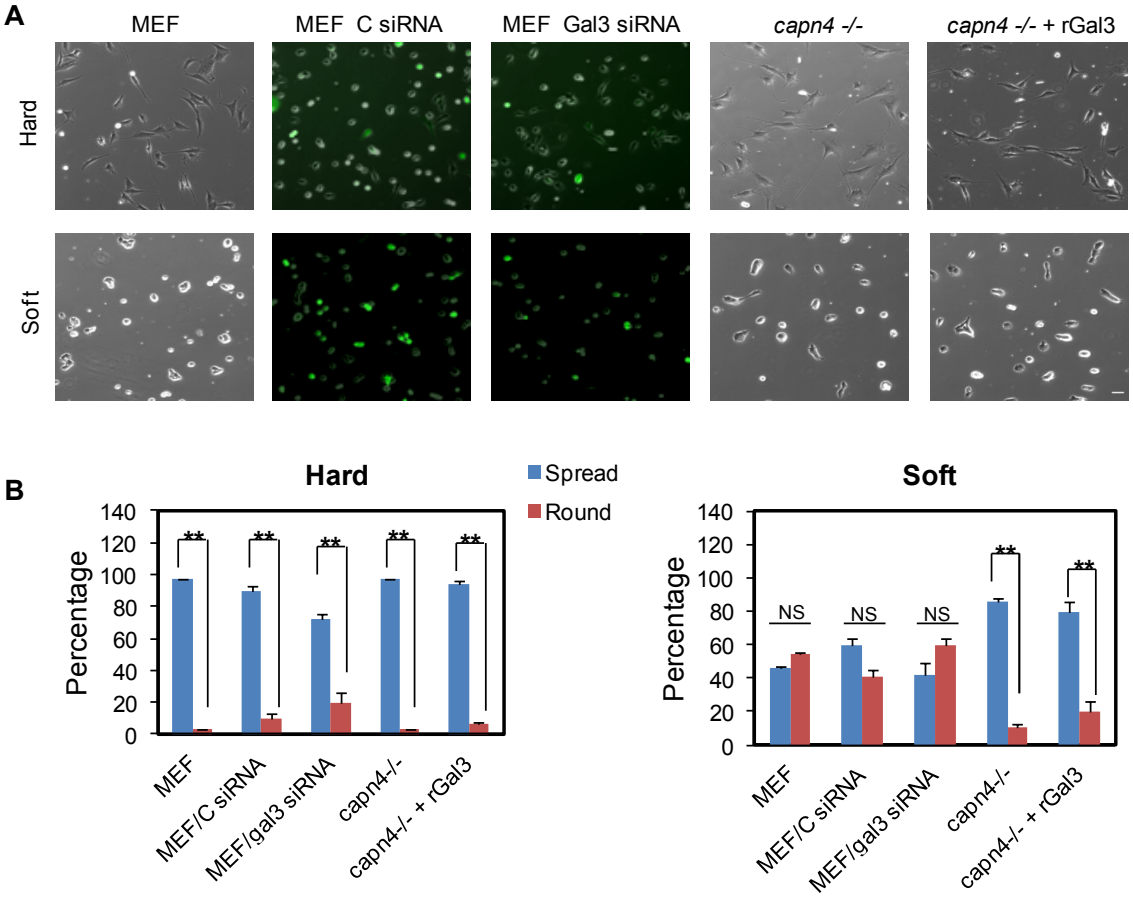
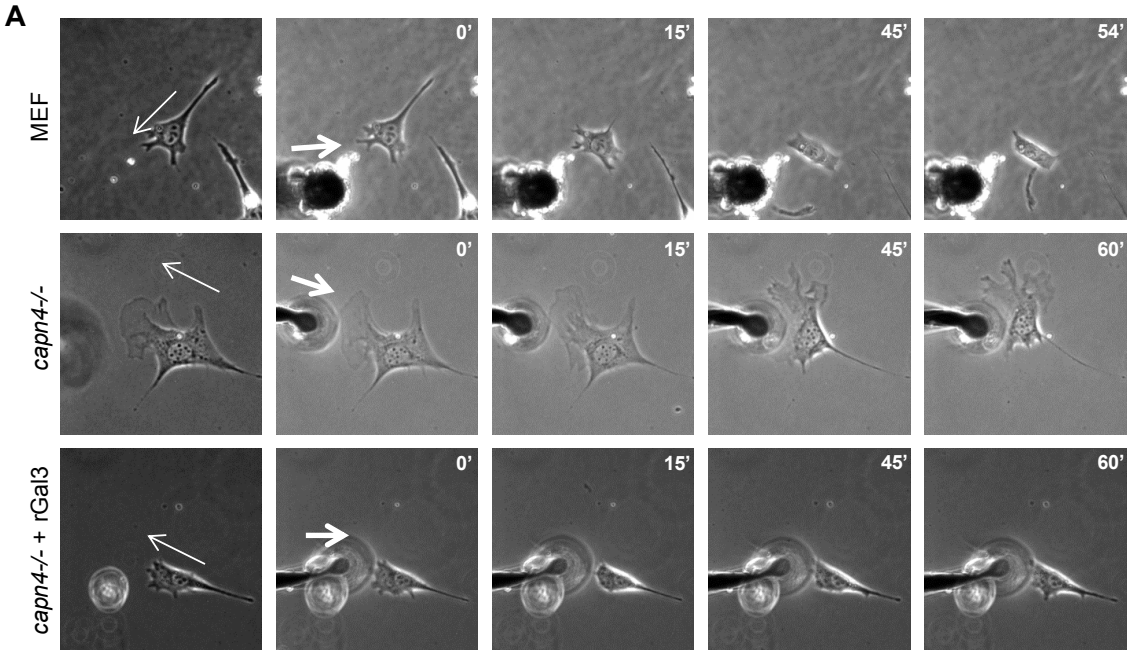


Figure 4.3: Extracellular galectin-3 is not required for sensing the homeostatic tension of the underlying substrate.
A) Representative images (10X) of cells seeded on hard and soft polyacrylamide substrates shows that majority of MEF cells and MEF cells treated with control and galectin-3 siRNA display spread morphology on hard substrates and round up on soft substrates. siRNA treated cells have been co-nucleofected with a GFP plasmid to ensure that only nucleofected cells are considered during cell counting. Recombinant galectin-3 added to the media is not able to rescue the sensing defect seen in *capn4-/-* MEF cells. Most cells remain spread even when seeded on soft substrates (Mag bar=50µm). B) Bar graphs represent the average number of spread and round cells when seeded on hard or soft polyacrylamide substrates. The values are averages obtained from two trials. Statistical analysis perfomed by student's t-test (** indicates $p<0.05$, NS indicates a non-significant relationship).

To address the ability of *capn4-/-* MEF cells treated with rGal3 to sense a locally applied stimulus, we subjected these cells to the needle pushing assay described earlier.

None of the calpain 4 deficient cells treated with rGal3 observed during the course of the experiment responded to the stimulus (n=6) (Figure 4.4A). Similarly, 12 out of 16 *capn4-/-* cells did not respond to the externally applied local stimulus (Figure 4.4B). However, most of the MEF cells (n=9), control siRNA (n=5 of 6) or galectin-3 siRNA treated MEF (n=5 of 6) cells responded to the applied stimulus (Figure 4.4B). These results suggest that galectin-3 is not important for cellular mechanotransduction in response to homeostatic tension or to a locally applied stimulus, although galectin-3 clearly effects the production of traction forces.



Cell type	MEF	MEF/ C siRNA	MEF/ gal3 siRNA	*capn4-/-*	*capn4-/-* + rGal3
Response	+	+	+	-	-
n =	9 of 9	5 of 6	5 of 6	12 of 16	6 of 6

Figure 4.4: Galectin-3 in not involved in sensing a locally applied mechanical stimulus.
A) Representative time lapse images display cellular responses of a MEF cell (top row), *capn4-/-* MEF cell (middle row) and *capn4-/-* MEF cell with recombinant galectin-3 added to the media in response to an externally applied local mechanical stimulus. The migration trajectory is indicated (thin arrow). The thick white arrow in the second column denotes the orientation in which the blunted needle is pushed. B) The table summarizes the response observed for each cell type. (+) indicates a positive response (rounding up of the cell or migrating away from the stimulus) whereas (-) is used when the cells fail to respond and continue moving towards the stimulus. Number of cells that showed a (+) or (-) response has also been listed.

Extracelluar Galectin-3 Impacts Linear Speed and Persistence of Migration

Cells are known to migrate individually and collectively (Ilina and Friedl, 2009; Lauffenburger and Horwitz, 1996). Biochemical and biophysical signals, both extracellular and intracellular, can alter the directionality (persistence) and speed of migration (Petrie et al., 2009). Parameters such as adhesiveness and strength of traction stress can modulate speed and persistence (Munevar et al., 2001). We measured persistence and the linear speed of wildtype MEF cells and *capn4-/-* MEF cells when the cells were seeded on 5 μg/cm^2 fibronectin coated glass coverslips or polyacrylamide substrates. We found that on fibronectin coated glass coverslips both persistence and linear speed of *capn4-/-* MEF cells were greater than those measured for MEF cells (Figure 4.5). We added rGal3 to capn4-/- MEF cells cultured on fibronectin coated coverslips to see if this would rescue the abnormal migration trend of the *capn4-/-* knockout cells. Surprisingly, both linear speed and persistence were reduced upon rGal3 addition, returning the values to those obtained for wild-type (p=4.65E-05 and p=0.0009 respectively) (Figure 4.5). However, siRNA mediated silencing of galectin-3 did not affect these properties, suggesting that even small amounts of secreted galectin-3 may suffice to support normal migration speeds and persistence.

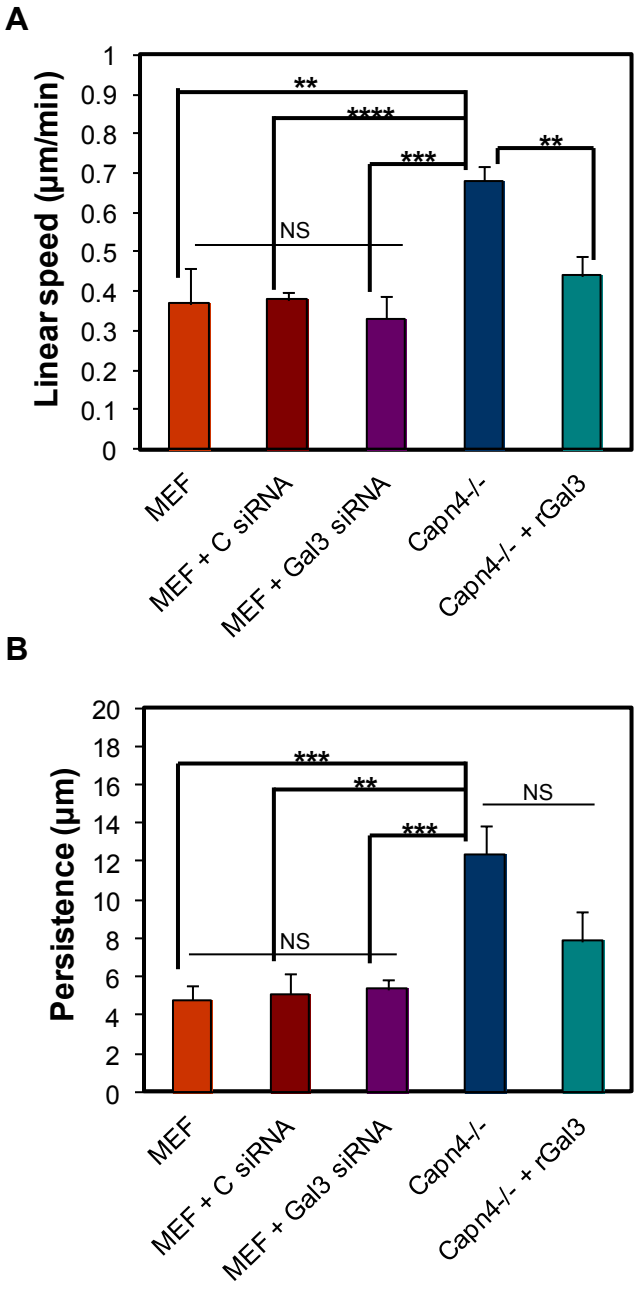


Figure 4.5: Extracellular galectin-3 influences migration speed and persistence. A) *capn4-/-* MEF cells when seeded on fibronectin coated glass coverslips migrates almost two times faster than MEF cells. Addition of galectin-3 externally reduces the linear speed of *capn4-/-* MEF cell migration to levels comparable to MEF cells or MEF cells treated with control or galectin-3 siRNA. B) Similar results were obtained when persistence of migration was measured. Addition of recombinant galectin-3 to the media reduced the directional persistence observed during *capn4-/-* MEF cell migration. An

average of 10 cells was observed for each cell type. Statistical analysis was performed by student's t-test (** denotes $p<0.05$, *** denotes $p<0.005$, NS denotes a non-significant relationship).

Extracellular galectin-3 may not influence β1 integrin activation and FAK auto-phosphorylation

Previous studies have implicated galectin-3 in the clustering of integrin receptors (Goetz et al., 2008). Given that all of the experiments used in this study have involved surface coating of fibronectin we reasoned that galectin-3 could be working through a fibronectin receptor to modulated traction force, adhesion maturation and strengthening. Integrins that serve as the receptors for fibronectin include $\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_{II}\beta_3$ and $\alpha_V\beta_3$ (Plow et al., 2000). Specifically, $\alpha_5\beta_1$ clustering is required for the formation of strong fibronectin bound adhesions (Roca-Cusachs et al., 2009). Therefore, we asked if the galectin-3 mediated increase in adhesion strength of calpain 4 deficient cells could be due to the activation of β_1 integrin. We measured, through western analysis, the levels of active β_1 integrin in cellular protein extracts from MEF cells, MEF cells treated with control and galectin-3 siRNA, *capn4-/-* cells and *capn4-/-* cells exogenously treated with rGal3, all grown on fibronectin coated surfaces. Surprisingly we found that total active β_1 integrin levels were not different in any of these cell types at a given time point (Figure 4.6A,B). This led us to conclude that extracellular galectin-3 mediated increase in adhesion strength and focal adhesion turnover is probably not mediated through β_1 integrin activation, although further studies confirming this conclusion would be necessary.

Tyrosine phosphorylation of FAK residue Y397 occurs through autophosphorylation (Mitra et al., 2005). It is the initial step in the activation of FAK leading to the phosphorylation of numerous other FAK tyrosine residues. Tyrosine phosphorylation of FAK is associated with cell migration including mechanosensing and traction force (Michael et al., 2009; Pirone et al., 2006; Schober et al., 2007; Wang et al., 2001). To determine if galectin-3 mediated regulation of traction force, focal adhesion turnover and adhesion strength is mediated through the autophosphorylation of FAK, we checked the levels of Y397 phosphorylated FAK present in MEF cells and *capn4-/-* MEF cells. Levels of Y397 in *capn4-/-* MEF cells or MEFs in which the expression of calpain 4 has been silenced are approximately two fold higher than levels in MEF cells (Figure 4.6C,D). Addition of rGal3 to *capn4-/-* MEF cells further elevated the FAK Y397 phosphorylation as compared to levels in untreated *capn4-/-* MEF cells (Figure 4.6C,D). This elevation in the tyrosine phosphorylation level is consistent with previously published observations of elevated FAK Y397 phosphorylation levels upon externally addition of galectin-3. However, this does not explain our results. The silencing of galectin-3 in MEF cells did not alter levels of Y397 FAK phosphorylation when proteins were extracted from cells grown to 80% confluency (Figure 4.6C,D). This result suggests that galectin-3 mediated regulation of cell migration may not be modulated through the FAK pathway and is possibly playing a role in the myosin II mediated traction force pathway by which the forces produced are stronger.

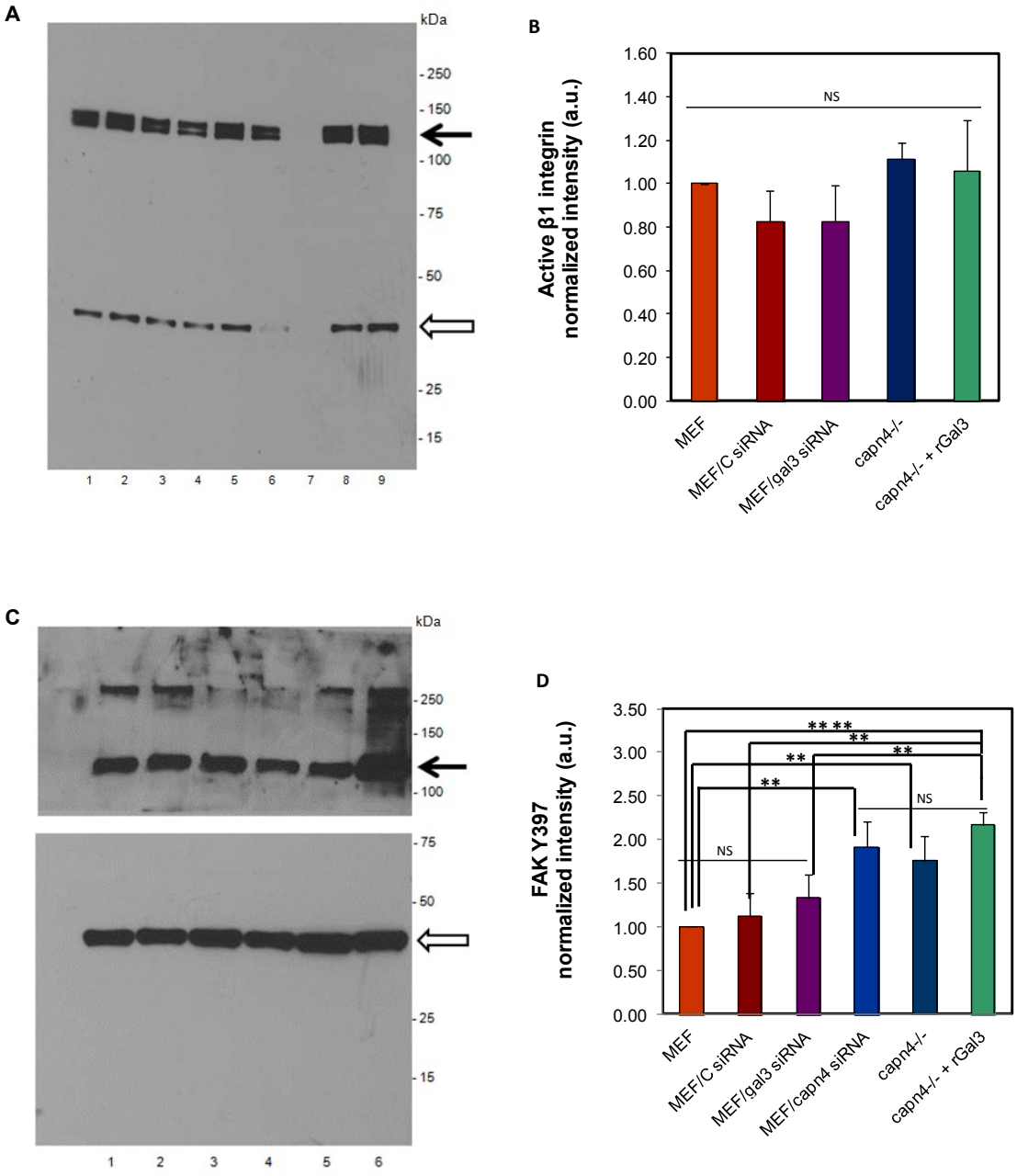


Figure 4.6: Galectin-3 mediated regulation of traction stress and focal adhesion maturation does not likely involve β1 integrin or autophosphorylation of FAK at Y397. A,B) Active β1 integrin levels are not significantly different when each of the cell lines, treated or untreated, are grown on fibronectin coated culture dishes (Lanes 1 through 6: MEF, MEF-mock nucleofected, MEF- nucleofected with non-target control siRNA, MEF – Gal3 siRNA nucleofected, MEF treated with recombinant galectin-3, MEF – nucleofected with Capn4 siRNA. Lanes 8 and 9 – *capn4*-/- and *capn4*-/- treated with recombinant galectin-3. Active β1 integrin bands are indicated by the black solid

arrow and GAPDH used as the loading control is marked by the unfilled arrow. C,D) Autophosphorylation of FAK at Y397 is elevated in *capn4-/-* MEF cells when compared to MEF, control and galectin-3 siRNA treated MEF cells. However, addition of recombinant galectin-3 to *capn4-/-* MEF cells does not lower the levels of FAK Y397 autophosphorylation (Lanes 1 through 6: MEF, *capn4-/-*, MEF- nucleofected with non-target control siRNA, MEF – Gal3 siRNA nucleofected lysates 48 hrs p.n., MEF – Gal3 siRNA nucleofected lysates obtained >48 hrs p.n., *capn4-/-* treated with recombinant galectin-3, molecular weight marker). Normalized intensity as expressed in arbitrary units in the bar graphs is an average of three separate experiments. (p.n. – post nucleofection) Statistical analysis performed using student's t-test (** denotes $p<0.05$, **** denotes $p<0.005$, NS denotes a non-significant relationship).

DISCUSSION

Cell migration is a process that is influenced by a myriad of factors, both intracellular and extracellular, that may be biochemical or biophysical in function. It is carefully coordinated by multiple signal transduction pathways, many of which are not fully understood. The cell takes up information from its immediate environment ("outside-in" signaling) and responds in an appropriate fashion ("inside-out" signaling). The role of the chemical environment and the resulting cellular responses with respect to migration has been under investigation for a long time (Devreotes and Zigmond, 1988; Janetopoulos and Firtel, 2008; Jones, 2000; Keller, 2005; Parent and Devreotes, 1999; Van Haastert and Devreotes, 2004; Wells, 2000). However, the role of the bio-physical environment and the biophysical traits associated with cell growth, spreading and migration are also being appreciated (Curtis and Wilkinson, 1999; Davies, 1995; Duncan and Turner, 1995; Georges and Janmey, 2005; Li et al., 2002; Li et al., 2005; Lo et al., 2000; Palecek et al., 1996). These physical parameters play a major role in various diseases such as cancer (Indra and Beningo, 2011; Menon and Beningo, 2011; Mierke, 2011; Schedin and Keely, 2011; Yu et al., 2011). It is also important for development and

tissue bio-engineering studies (Butler et al., 2009; Huang and Li, 2011; Mammoto and Ingber, 2010). In line with this, our group had previously shown the importance of the calcium dependent family of proteases, namely calpains, in the regulation of two bio-physical parameters, traction force and mechanosensing (Undyala et al., 2008). In addition, it was found that focal adhesion dynamics and strengthening were also altered by the calpain family. We concluded that calpain 4 plays a role independent of the proteolytic activity of the large catalytic subunits in the process of traction force. The large subunits, calpains 1 and 2, along with the small subunit are involved in sensing global stiffness changes and also locally applied mechanical stimulations. Thus the calpains have provided a means to separate spatially and temporally, traction force and mechanosensing.

How traction forces are generated and how they can be measured is being investigated by a number of groups (Kraning-Rush et al., 2012; Wang and Lin, 2007). These studies have established the importance of tyrosine phosphorylation and dephosphorylation of a number of cellular proteins mediated by kinases such as FAK, Src family kinases and phosphatases such as the SH2 domain containing phosphatases, receptor-like tyrosine phosphatases in this process. Therefore, to further understand the role of calpains in the force generation pathway and mechanosensing we decided to look at differential tyrosine phosphorylation levels in the absence of each of the calpain subunits. We found that the protein galectin-3 was not phosphorylated in the absence of calpain 4. We also discovered that the absence of calpain 4 and a corresponding reduction

in tyrosine phosphorylation prevented the protein from being secreted (Menon et al., 2011).

Galectin-3 has been known to play a role in cell migration. Most studies suggest that it modulates migration from the outside of the cell, both under normal conditions and in cancer cells (Goetz et al., 2008; Ochieng et al., 2004). Thus, the fact that galectin-3 secretion was likely mediated by tyrosine phopshorylation which is indirectly regulated by calpain 4 prompted us to look at its role in producing traction force when added externally to calpain 4 deficient cells. Our results obtained upon the addition of recombinant galectin-3 to the culture medium concur with studies done by other groups suggesting the role of the protein in the extracellular environment. We were able to rescue traction force defects observed in a calpain 4 deficient background cell. Addition of recombinant galectin-3 also mediated maturation of adhesions (point contacts to mature adhesions) and also helped strengthen the adhesions. It has previously been shown that forces are greatest at the leading edge of migrating cells and that nascent adhesions generate greater forces (Beningo et al., 2001). Addition of recombinant galectin-3 resulted in fewer point contacts (adhesions that were less than 0.5 μm) as compared to calpain 4 deficient fibroblasts that had significantly greater number of point contacts. The number of nascent focal adhesions increased upon the addition of recombinant galectin-3. This increase correlated with an increase in traction stress and supporting the fact that nascent adhesions generate greater forces. The number of adhesions that were larger than 2 μm was almost the same with and without the addition of galectin-3. The number of adhesions and their size could also explain the increase in

adhesion strength seen in the presence of extracellular galectin-3. Together these results suggest that galectin-3 in the extracellular environment forms a lattice, which then helps cluster and activate integrins (Goetz et al., 2008). Once integrins are activated it can activate numerous intracellular signal transduction pathways that can ultimately lead to increased adhesion maturation, improved strength and greater forces. The primary fibronectin receptor $\alpha_5\beta_1$ provides a reasonable target as its function in adhesion strengthening and migration is well documented (Roca-Cusachs et al., 2009). However, contrary to previous studies we find that galectin-3 mediated changes in cell adhesion and migration is probably not via β_1 integrin activation. Moving further downstream from beta-1 integrin activation is the FAK autophosphorylation at tyrosine 397 residue. Upon galectin-3 addition, we however, do not see a dramatic change in the already elevated levels of Y397 phosphorylation observed in calpain 4 deficient cells. The simplest explanation for our data is that extracellular galectin-3 activates pathways that do not require $\alpha_5\beta_1$ integrin followed by FAK Y397 phosphorylation. Instead, as proposed in other literature, it may transduce through β_3 integrin clustering and activation leading to Src kinase activation independent of FAK autophosphorylation (Arias-Salgado et al., 2003). This would identify a previously unknown mechanotransduction pathway that signals only for the production of traction forces and not mechanosensing.

Unlike previous studies that have shown slower migration rates for *capn4-/-* cells on fibronectin coated surfaces as compared to wildtype cells, we see that the rate of migration of capn4-/- cells is higher than fibroblasts (Dourdin et al., 2001). However, the concentration of fibronectin (5 $\mu g/cm^2$) used for our studies is higher than concentrations

used previously for migration studies with calpain 4 deficient cells. Previous research has also shown that high concentrations of fibronectin reduces rate of migration by modulating Rho GTPases through integrins (Cox et al., 2001). This may explain the reduction in linear speed observed when recombinant galectin-3 is added externally to calpain deficient fibroblast cultures, as galectin-3 has been shown to cluster and activate integrins. Galectin-3 is also proposed to form a lattice which promotes fibrillogenesis providing another potential route to modulate rate and direction of migration (Lagana et al., 2006).

A recent study proposes that microtubule depolymerization induced traction force regulation can be mediated by two distinct pathways – a myosin-II dependent, FAK independent pathway and a FAK-regulated, myosin-II independent pathway (Rape et al., 2011). Since upon addition of extracellular galectin-3 we see an increase in traction force generated by *capn4-/-* cells without a correspondingly significant increase in the levels of FAK Y397 phosphorylation levels, it is plausible that galectin-3/calpain 4 mediated regulation of force occurs via the myosin-II dependent pathway. Furthermore, we find that the FAK Y397 phosphorylation levels are higher in *capn4-/-* cells as compared to wildtype MEF cells, but the levels of tractions force produced is inversely correlated. An alternative mechanism pieced together from the literature, is that calpain 4 could possibly bind to a phosphatase interacting protein, such as PSTPIP1 through its SH3 domain. Previous studies have shown that calpain 4 binds to proteins through SH3 domains present in the interacting partner (Rosenberger et al., 2005). Similarly PSTPIP1 also binds to its partners through SH3 domains present in its structure (Baum et al., 2005; Wu

et al., 1998). PSTPIP1 has been shown to direct PEST type protein tyrosine phosphatase to Abl kinase (Cong et al., 2000). However,,if it interacts with calpain 4 it will probably prevent PSTIP1-PEST PTP interaction, thus preventing the delivery of PEST-type protein tyrosine phosphatase to Abl kinase. Thus, Abl kinase remains active, phosphorylating its substrate galectin-3. Phosphorylated galectin-3 is then secreted by a mechanism yet to be understood. Once secreted, galectin-3 brings about fibrillogenesis followed by integrin clustering and activation and mechanotransduction (Lagana et al., 2006). This is then translated into force generation via a myosin-II mediated, FAK independent pathway (Rape et al., 2011). Since, addition of galectin-3 is not able to rescue calpain4-/- defects in global and applied mechanosensing, we have been able to strengthen our previous observation that calpain 4 plays a role independent of the large subunits in the production of traction forces. Furthermore, we are able to mechanistically separate, through the galectin-3 connection, a pathway independent of the mechanosensing pathway.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Mechanical forces are an integral part of migration both in two-dimensional and three-dimensional environments. Traction force, force generated by the acto-myosin cytoskeleton and transmitted onto the extracellular matrix via focal adhesions and integrins, is essential for cell adhesion, spreading and migration. However, the signaling mechanism involved in generating and regulating this force is not understood. Cells use these traction forces to both propel themselves forward and to remodel the extracellular matrix surrounding them. Both of these adhesive forces create transient tension, a tug and pull, on the extracellular matrix fibers that is dynamic and heterogeneous, depending on the process and the cell type. A fine example of such highly localized and heterodynamic tension can be seen in the tumor stroma. With a vast number of non-tumor cells residing in the tumor stroma, both migrating through it and remodeling it, the localized tension is dynamic. Whether this locally generated tension influences tumor cell migration is another question in the field of mechanobiology that remains unanswered. In this dissertation I have addressed two different aspects of cell generated forces: 1) Is there a consequence to cell generated forces if they are sensed by neighboring tumor cells? 2) How is traction force generation regulated?

In Chapter 2 I have successfully demonstrated that naturally invasive cells can sense locally generated heterodynamic tension. They respond by invading to a greater extent than non-stimulated cells. For this study, I standardized a novel *in-vitro* invasion assay by incorporating paramagnetic beads into a collagen typeI/fibronectin matrix which

were held over a rotating magnet. The design was intended to mimic the physical forces that would be observed in the tumor stroma, without the interference of biochemical signaling. HT1080 cells showed a two-three fold enhancement in percentage invasion. This enhanced invasion is dependent on substrate composition, but independent of the orientation in which the stimulus is provided. This enhanced mechano-invasion was dependent on fibronectin. Additionally, the proteins actin and cofilin are essential to the process, suggesting that invadopodia maturation probably helps tumor cells invade towards the local stimulus. Further studies are underway to identify other key proteins involved in this process and also to understand the role of invadopodia in enhancing invasion in response to a transient mechanical stimulus.

In Chapters 3 and 4, I investigated the role of the calpain small subunit, calpain 4, in regulating the production of traction force. Exploiting the fact that upon application of stress on a cell, tyrosine phosphorylation increases, I identified that calpain 4 indirectly regulates galectin-3 tyrosine phosphorylation. I was able to show that galectin-3 tyrosine phosphorylation influences its localization to the cell periphery. Moreover, I also discovered that calpain 4 influences galectin-3 secretion from the cell, thus identifying a component of the yet unidentified galectin-3 secretion pathway. Furthermore, I have established a role for extracellular galectin-3 in positively regulating traction force production. Extracellular galectin-3 also promotes focal adhesion maturation and enhances adhesion strength. I have shown that beta-1 integrin activation and tyrosine phosphorylation of FAK Y397 are possibly not required for these processes, but the precise signaling mechanism needs to be identified.

In conclusion, I have shown that extracellular factors, both physical and biochemical, present in the microenvironment of cells can influence cellular behavior. Cell generated mechanical forces altering the local substrate tension can influence cancer cell invasion. Extracellular galectin-3, a protein that is either downregulated or overexpressed in a number of cancer cells, positively regulates mechanical forces generated by the cell. Further investigation of the mechanisms involved in traction force generation and invasion in response to cell generated mechanical forces can eventually help identify potential drug targets for cancer treatment.

APPENDIX A

DEFINITIONS

1. Homeostatic tension: An environment where the mechanical tension is spatially equal and temporally non-dynamic.
2. Mechanosensing: The ability of a cell to sense physical parameters such as rigidity, topography and local mechanical stimuli in its immediate environment by means of changes in protein conformation or protein clustering.
3. Mechanotransduction: Conformation dependent biochemical reactions occurring as a result of mechanosensing. This leads to the activation of downstream signaling cascades such as activation of G-protein signaling or kinase activation, and often leads to changes in gene expression.
4. Mechanoresponse: Spatio-temporal integration of signal transduction resulting in changes in various physiological processes such as cell division and cell migration.
5. Traction force: Contractile forces generated by the acto-myosin cytoskeleton of the cell and transmitted via focal adhesions and detected on the substratum.

REFERENCES

Abercrombie, M., J.E. Heaysman, and S.M. Pegrum. 1971. The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. *Exp Cell Res*. 67:359-367.

Akiyama, S.K., K. Olden, and K.M. Yamada. 1995. Fibronectin and integrins in invasion and metastasis. *Cancer Metastasis Rev*. 14:173-189.

Alexander, N.R., K.M. Branch, A. Parekh, E.S. Clark, I.C. Iwueke, S.A. Guelcher, and A.M. Weaver. 2008. Extracellular matrix rigidity promotes invadopodia activity. *Curr Biol*. 18:1295-1299.

Amatangelo, M.D., D.E. Bassi, A.J. Klein-Szanto, and E. Cukierman. 2005. Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts. *Am J Pathol*. 167:475-488.

Arias-Salgado, E.G., S. Lizano, S. Sarkar, J.S. Brugge, M.H. Ginsberg, and S.J. Shattil. 2003. Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci U S A*. 100:13298-13302.

Arnaout, M.A., S.L. Goodman, and J.P. Xiong. 2007. Structure and mechanics of integrin-based cell adhesion. *Curr Opin Cell Biol*. 19:495-507.

Arthur, J.S., J.S. Elce, C. Hegadorn, K. Williams, and P.A. Greer. 2000. Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. *Mol Cell Biol*. 20:4474-4481.

Balan, V., P. Nangia-Makker, Y.S. Jung, Y. Wang, and A. Raz. 2010. Galectin-3: A novel substrate for c-Abl kinase. *Biochim Biophys Acta*. 1803:1198-1205.

Barkan, D., J.E. Green, and A.F. Chambers. 2010. Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. *Eur J Cancer*. 46:1181-1188.

Baum, W., V. Kirkin, S.B. Fernandez, R. Pick, M. Lettau, O. Janssen, and M. Zornig. 2005. Binding of the intracellular Fas ligand (FasL) domain to the adaptor protein PSTPIP results in a cytoplasmic localization of FasL. *J Biol Chem*. 280:40012-40024.

Beattie, J., L. McIntosh, and C.F. van der Walle. 2010. Cross-talk between the insulin-like growth factor (IGF) axis and membrane integrins to regulate cell physiology. *J Cell Physiol*. 224:605-611.

Beningo, K.A., M. Dembo, I. Kaverina, J.V. Small, and Y.L. Wang. 2001. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J Cell Biol*. 153:881-888.

Beningo, K.A., M. Dembo, and Y.L. Wang. 2004. Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. *Proc Natl Acad Sci U S A*. 101:18024-18029.

Beningo, K.A., C.M. Lo, and Y.L. Wang. 2002. Flexible polyacrylamide substrata for the analysis of mechanical interactions at cell-substratum adhesions. *Methods Cell Biol*. 69:325-339.

Bershadsky, A.D., N.Q. Balaban, and B. Geiger. 2003. Adhesion-dependent cell mechanosensitivity. *Annu Rev Cell Dev Biol*. 19:677-695.

Bhatt, A., I. Kaverina, C. Otey, and A. Huttenlocher. 2002. Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain. *J Cell Sci*. 115:3415-3425.

Bhowmick, N.A., E.G. Neilson, and H.L. Moses. 2004. Stromal fibroblasts in cancer initiation and progression. *Nature*. 432:332-337.

Bijman, M.N., M.P. van Berkel, G.P. van Nieuw Amerongen, and E. Boven. 2008. Interference with actin dynamics is superior to disturbance of microtubule function in the inhibition of human ovarian cancer cell motility. *Biochem Pharmacol*. 76:707-716.

Bissell, M.J., and D. Radisky. 2001. Putting tumours in context. *Nat Rev Cancer*. 1:46-54.

Bousquet, P.F., L.A. Paulsen, C. Fondy, K.M. Lipski, K.J. Loucy, and T.P. Fondy. 1990. Effects of cytochalasin B in culture and in vivo on murine Madison 109 lung carcinoma and on B16 melanoma. *Cancer Res*. 50:1431-1439.

Buccione, R., G. Caldieri, and I. Ayala. 2009. Invadopodia: specialized tumor cell structures for the focal degradation of the extracellular matrix. *Cancer Metastasis Rev*. 28:137-149.

Bugyi, B., and M.F. Carlier. 2010. Control of actin filament treadmilling in cell motility. *Annu Rev Biophys*. 39:449-470.

Burridge, K., and M. Chrzanowska-Wodnicka. 1996. Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol*. 12:463-518.

Busco, G., R.A. Cardone, M.R. Greco, A. Bellizzi, M. Colella, E. Antelmi, M.T. Mancini, M.E. Dell'aquila, V. Casavola, A. Paradiso, and S.J. Reshkin. 2010. NHE1 promotes invadopodial ECM proteolysis through acidification of the peri-invadopodial space. *FASEB J*.

Butler, D.L., S.A. Goldstein, R.E. Guldberg, X.E. Guo, R. Kamm, C.T. Laurencin, L.V. McIntire, V.C. Mow, R.M. Nerem, R.L. Sah, L.J. Soslowsky, R.L. Spilker, and R.T. Tranquillo. 2009. The impact of biomechanics in tissue engineering and regenerative medicine. *Tissue Eng Part B Rev*. 15:477-484.

Califano, J.P., and C.A. Reinhart-King. 2010. Substrate Stiffness and Cell Area Predict Cellular Traction Stresses in Single Cells and Cells in Contact. *Cell Mol Bioeng*. 3:68-75.

Campellone, K.G., and M.D. Welch. 2010. A nucleator arms race: cellular control of actin assembly. *Nat Rev Mol Cell Biol*. 11:237-251.

Canelle, L., J. Bousquet, C. Pionneau, L. Deneux, N. Imam-Sghiouar, M. Caron, and R. Joubert-Caron. 2005. An efficient proteomics-based approach for the screening of autoantibodies. *J Immunol Methods*. 299:77-89.

Chambers, A.F., A.C. Groom, and I.C. MacDonald. 2002. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer*. 2:563-572.

Chan, C.E., and D.J. Odde. 2008. Traction dynamics of filopodia on compliant substrates. *Science*. 322:1687-1691.

Chan, K.T., C.L. Cortesio, and A. Huttenlocher. 2007. Integrins in cell migration. *Methods Enzymol*. 426:47-67.

Charras, G., and E. Paluch. 2008. Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol*. 9:730-736.

Chen, W.T. 1981. Mechanism of retraction of the trailing edge during fibroblast movement. *J Cell Biol*. 90:187-200.

Cheng, G., J. Tse, R.K. Jain, and L.L. Munn. 2009. Micro-environmental mechanical stress controls tumor spheroid size and morphology by suppressing proliferation and inducing apoptosis in cancer cells. *PLoS One*. 4:e4632.

Chesarone, M.A., and B.L. Goode. 2009. Actin nucleation and elongation factors: mechanisms and interplay. *Curr Opin Cell Biol*. 21:28-37.

Chrzanowska-Wodnicka, M., and K. Burridge. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol*. 133:1403-1415.

Clark, E.A., and J.S. Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science*. 268:233-239.

Condeelis, J. 1993. Life at the leading edge: the formation of cell protrusions. *Annu Rev Cell Biol*. 9:411-444.

Condeelis, J., and J.W. Pollard. 2006. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell*. 124:263-266.

Cong, F., S. Spencer, J.F. Cote, Y. Wu, M.L. Tremblay, L.A. Lasky, and S.P. Goff. 2000. Cytoskeletal protein PSTPIP1 directs the PEST-type protein tyrosine phosphatase to the c-Abl kinase to mediate Abl dephosphorylation. *Molecular cell*. 6:1413-1423.

Cox, E.A., S.K. Sastry, and A. Huttenlocher. 2001. Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases. *Mol Biol Cell*. 12:265-277.

Craig, D.H., and M.D. Basson. 2009. Biological impact of mechanical stimuli on tumor metastasis. *Cell Cycle*. 8:828-831.

Crowley, E., and A.F. Horwitz. 1995. Tyrosine phosphorylation and cytoskeletal tension regulate the release of fibroblast adhesions. *J Cell Biol*. 131:525-537.

Cuevas, B.D., A.N. Abell, J.A. Witowsky, T. Yujiri, N.L. Johnson, K. Kesavan, M. Ware, P.L. Jones, S.A. Weed, R.L. DeBiasi, Y. Oka, K.L. Tyler, and G.L. Johnson. 2003. MEKK1 regulates calpain-dependent proteolysis of focal adhesion proteins for rear-end detachment of migrating fibroblasts. *EMBO J*. 22:3346-3355.

Curtis, A., and C. Wilkinson. 1999. New depths in cell behaviour: reactions of cells to nanotopography. *Biochem Soc Symp*. 65:15-26.

Danguy, A., I. Camby, and R. Kiss. 2002. Galectins and cancer. *Biochim Biophys Acta*. 1572:285-293.

Davies, P.F. 1995. Flow-mediated endothelial mechanotransduction. *Physiol Rev*. 75:519-560.

De, R., A. Zemel, and S.A. Safran. 2010. Theoretical concepts and models of cellular mechanosensing. *Methods Cell Biol*. 98:143-175.

Desmouliere, A., A. Geinoz, F. Gabbiani, and G. Gabbiani. 1993. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue

myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol*. 122:103-111.

Desmouliere, A., C. Guyot, and G. Gabbiani. 2004. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. *Int J Dev Biol*. 48:509-517.

Devreotes, P.N., and S.H. Zigmond. 1988. Chemotaxis in eukaryotic cells: a focus on leukocytes and Dictyostelium. *Annu Rev Cell Biol*. 4:649-686.

Discher, D.E., P. Janmey, and Y.L. Wang. 2005. Tissue cells feel and respond to the stiffness of their substrate. *Science*. 310:1139-1143.

Discher, D.E., D.J. Mooney, and P.W. Zandstra. 2009. Growth factors, matrices, and forces combine and control stem cells. *Science*. 324:1673-1677.

Dourdin, N., A.K. Bhatt, P. Dutt, P.A. Greer, J.S. Arthur, J.S. Elce, and A. Huttenlocher. 2001. Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J Biol Chem*. 276:48382-48388.

Dugina, V., L. Fontao, C. Chaponnier, J. Vasiliev, and G. Gabbiani. 2001. Focal adhesion features during myofibroblastic differentiation are controlled by intracellular and extracellular factors. *J Cell Sci*. 114:3285-3296.

Dumic, J., S. Dabelic, and M. Flogel. 2006. Galectin-3: an open-ended story. *Biochim Biophys Acta*. 1760:616-635.

Duncan, R.L., and C.H. Turner. 1995. Mechanotransduction and the functional response of bone to mechanical strain. *Calcif Tissue Int*. 57:344-358.

Egeblad, M., M.G. Rasch, and V.M. Weaver. 2010. Dynamic interplay between the collagen scaffold and tumor evolution. *Curr Opin Cell Biol*. 22:697-706.

Engler, A.J., M.A. Griffin, S. Sen, C.G. Bonnemann, H.L. Sweeney, and D.E. Discher. 2004. Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. *J Cell Biol*. 166:877-887.

Engler, A.J., S. Sen, H.L. Sweeney, and D.E. Discher. 2006. Matrix elasticity directs stem cell lineage specification. *Cell*. 126:677-689.

Even-Ram, S., and K.M. Yamada. 2005. Cell migration in 3D matrix. *Curr Opin Cell Biol*. 17:524-532.

Fackler, O.T., and R. Grosse. 2008. Cell motility through plasma membrane blebbing. *J Cell Biol*. 181:879-884.

Faull, R.J., and M.H. Ginsberg. 1996. Inside-out signaling through integrins. *J Am Soc Nephrol*. 7:1091-1097.

Flanagan, L.A., Y.E. Ju, B. Marg, M. Osterfield, and P.A. Janmey. 2002. Neurite branching on deformable substrates. *Neuroreport*. 13:2411-2415.

Follonier, L., S. Schaub, J.J. Meister, and B. Hinz. 2008. Myofibroblast communication is controlled by intercellular mechanical coupling. *J Cell Sci*. 121:3305-3316.

Fouchard, J., D. Mitrossilis, and A. Asnacios. 2011. Acto-myosin based response to stiffness and rigidity sensing. *Cell Adh Migr*. 5:16-19.

Fournier, M.F., R. Sauser, D. Ambrosi, J.J. Meister, and A.B. Verkhovsky. 2010. Force transmission in migrating cells. *J Cell Biol*. 188:287-297.

Fraley, S.I., Y. Feng, R. Krishnamurthy, D.H. Kim, A. Celedon, G.D. Longmore, and D. Wirtz. 2010. A distinctive role for focal adhesion proteins in three-dimensional cell motility. *Nat Cell Biol*. 12:598-604.

Franco, S., B. Perrin, and A. Huttenlocher. 2004. Isoform specific function of calpain 2 in regulating membrane protrusion. *Exp Cell Res*. 299:179-187.

Franco, S.J., and A. Huttenlocher. 2005. Regulating cell migration: calpains make the cut. *J Cell Sci*. 118:3829-3838.

Freund, J.B., J.G. Goetz, K.L. Hill, and J. Vermot. 2012. Fluid flows and forces in development: functions, features and biophysical principles. *Development*. 139:1229-1245.

Friedl, P. 2004. Prespecification and plasticity: shifting mechanisms of cell migration. *Curr Opin Cell Biol*. 16:14-23.

Friedl, P., and S. Alexander. 2011. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell*. 147:992-1009.

Friedl, P., and E.B. Brocker. 2000. The biology of cell locomotion within three-dimensional extracellular matrix. *Cell Mol Life Sci*. 57:41-64.

Friedl, P., and K. Wolf. 2008. Tube travel: the role of proteases in individual and collective cancer cell invasion. *Cancer Res*. 68:7247-7249.

Friedl, P., and K. Wolf. 2009. Proteolytic interstitial cell migration: a five-step process. *Cancer Metastasis Rev*. 28:129-135.

Friedland, J.C., M.H. Lee, and D. Boettiger. 2009. Mechanically activated integrin switch controls alpha5beta1 function. *Science*. 323:642-644.

Furtak, V., F. Hatcher, and J. Ochieng. 2001. Galectin-3 mediates the endocytosis of beta-1 integrins by breast carcinoma cells. *Biochem Biophys Res Commun*. 289:845-850.

Gao, M., D. Craig, V. Vogel, and K. Schulten. 2002. Identifying unfolding intermediates of FN-III(10) by steered molecular dynamics. *J Mol Biol*. 323:939-950.

Garcia, A.J., J.E. Schwarzbauer, and D. Boettiger. 2002. Distinct activation states of alpha5beta1 integrin show differential binding to RGD and synergy domains of fibronectin. *Biochemistry*. 41:9063-9069.

Gardel, M.L., B. Sabass, L. Ji, G. Danuser, U.S. Schwarz, and C.M. Waterman. 2008. Traction stress in focal adhesions correlates biphasically with actin retrograde flow speed. *J Cell Biol*. 183:999-1005.

Gardel, M.L., I.C. Schneider, Y. Aratyn-Schaus, and C.M. Waterman. 2010. Mechanical integration of actin and adhesion dynamics in cell migration. *Annu Rev Cell Dev Biol*. 26:315-333.

Gee, E.P., D.E. Ingber, and C.M. Stultz. 2008. Fibronectin unfolding revisited: modeling cell traction-mediated unfolding of the tenth type-III repeat. *PLoS One*. 3:e2373.

Geiger, B., and A. Bershadsky. 2001. Assembly and mechanosensory function of focal contacts. *Curr Opin Cell Biol*. 13:584-592.

Georges, P.C., and P.A. Janmey. 2005. Cell type-specific response to growth on soft materials. *J Appl Physiol*. 98:1547-1553.

Giannone, G., and M.P. Sheetz. 2006. Substrate rigidity and force define form through tyrosine phosphatase and kinase pathways. *Trends Cell Biol*. 16:213-223.

Gimona, M., R. Buccione, S.A. Courtneidge, and S. Linder. 2008. Assembly and biological role of podosomes and invadopodia. *Curr Opin Cell Biol*. 20:235-241.

Ginsberg, M.H., X. Du, and E.F. Plow. 1992. Inside-out integrin signalling. *Curr Opin Cell Biol*. 4:766-771.

Glading, A., D.A. Lauffenburger, and A. Wells. 2002. Cutting to the chase: calpain proteases in cell motility. *Trends Cell Biol*. 12:46-54.

Goetz, J.G., B. Joshi, P. Lajoie, S.S. Strugnell, T. Scudamore, L.D. Kojic, and I.R. Nabi. 2008. Concerted regulation of focal adhesion dynamics by galectin-3 and tyrosine-phosphorylated caveolin-1. *J Cell Biol*. 180:1261-1275.

Goffin, J.M., P. Pittet, G. Csucs, J.W. Lussi, J.J. Meister, and B. Hinz. 2006. Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *J Cell Biol*. 172:259-268.

Goll, D.E., V.F. Thompson, H. Li, W. Wei, and J. Cong. 2003. The calpain system. *Physiol Rev*. 83:731-801.

Gong, H.C., Y. Honjo, P. Nangia-Makker, V. Hogan, N. Mazurak, R.S. Bresalier, and A. Raz. 1999. The NH2 terminus of galectin-3 governs cellular compartmentalization and functions in cancer cells. *Cancer Res*. 59:6239-6245.

Guck, J., F. Lautenschlager, S. Paschke, and M. Beil. 2010. Critical review: cellular mechanobiology and amoeboid migration. *Integr Biol (Camb)*. 2:575-583.

Guilak, F., D.M. Cohen, B.T. Estes, J.M. Gimble, W. Liedtke, and C.S. Chen. 2009. Control of stem cell fate by physical interactions with the extracellular matrix. *Cell Stem Cell*. 5:17-26.

Guo, W.H., M.T. Frey, N.A. Burnham, and Y.L. Wang. 2006. Substrate rigidity regulates the formation and maintenance of tissues. *Biophys J*. 90:2213-2220.

Gupton, S.L., and F.B. Gertler. 2007. Filopodia: the fingers that do the walking. *Sci STKE*. 2007:re5.

Hanahan, D., and L.M. Coussens. 2012. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 21:309-322.

Harrison, R.G. 1914. The reaction of embryonic cells to solid structures. *Journal of Experimental Zoology*. 17:521-544.

Haudek, K.C., K.J. Spronk, P.G. Voss, R.J. Patterson, J.L. Wang, and E.J. Arnoys. 2010. Dynamics of galectin-3 in the nucleus and cytoplasm. *Biochim Biophys Acta*. 1800:181-189.

Helmlinger, G., P.A. Netti, H.C. Lichtenbeld, R.J. Melder, and R.K. Jain. 1997. Solid stress inhibits the growth of multicellular tumor spheroids. *Nat Biotechnol*. 15:778-783.

Hendey, B., C.B. Klee, and F.R. Maxfield. 1992. Inhibition of neutrophil chemokinesis on vitronectin by inhibitors of calcineurin. *Science*. 258:296-299.

Hinz, B., G. Celetta, J.J. Tomasek, G. Gabbiani, and C. Chaponnier. 2001. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell*. 12:2730-2741.

Horwitz, R., and D. Webb. 2003. Cell migration. *Curr Biol*. 13:R756-759.

Hu, K., L. Ji, K.T. Applegate, G. Danuser, and C.M. Waterman-Storer. 2007. Differential transmission of actin motion within focal adhesions. *Science*. 315:111-115.

Hu, P., and B.H. Luo. 2012. Integrin bidirectional signaling across the plasma membrane. *J Cell Physiol*.

Huang, N.F., and S. Li. 2011. Regulation of the matrix microenvironment for stem cell engineering and regenerative medicine. *Ann Biomed Eng*. 39:1201-1214.

Huttenlocher, A. 2005. Cell polarization mechanisms during directed cell migration. *Nat Cell Biol*. 7:336-337.

Huttenlocher, A., R.R. Sandborg, and A.F. Horwitz. 1995. Adhesion in cell migration. *Curr Opin Cell Biol*. 7:697-706.

Huveneers, S., and E.H. Danen. 2009. Adhesion signaling - crosstalk between integrins, Src and Rho. *J Cell Sci*. 122:1059-1069.

Hynes, R.O. 2009. The extracellular matrix: not just pretty fibrils. *Science*. 326:1216-1219.

Ilina, O., and P. Friedl. 2009. Mechanisms of collective cell migration at a glance. *J Cell Sci*. 122:3203-3208.

Indra, I., and K.A. Beningo. 2011. An in vitro correlation of metastatic capacity, substrate rigidity, and ECM composition. *J Cell Biochem*. 112:3151-3158.

Ingber, D. 1991. Integrins as mechanochemical transducers. *Curr Opin Cell Biol*. 3:841-848.

Ingham, K.C., S.A. Brew, and M. Migliorini. 2002. Type I collagen contains at least 14 cryptic fibronectin binding sites of similar affinity. *Arch Biochem Biophys*. 407:217-223.

Iurisci, I., N. Tinari, C. Natoli, D. Angelucci, E. Cianchetti, and S. Iacobelli. 2000. Concentrations of galectin-3 in the sera of normal controls and cancer patients. *Clin Cancer Res*. 6:1389-1393.

Izzard, C.S., and L.R. Lochner. 1980. Formation of cell-to-substrate contacts during fibroblast motility: an interference-reflexion study. *J Cell Sci*. 42:81-116.

Janetopoulos, C., and R.A. Firtel. 2008. Directional sensing during chemotaxis. *FEBS Lett*. 582:2075-2085.

Jay, P.Y., P.A. Pham, S.A. Wong, and E.L. Elson. 1995. A mechanical function of myosin II in cell motility. *J Cell Sci*. 108 (Pt 1):387-393.

Jin, H., and J. Varner. 2004. Integrins: roles in cancer development and as treatment targets. *Br J Cancer*. 90:561-565.

Jones, G.E. 2000. Cellular signaling in macrophage migration and chemotaxis. *J Leukoc Biol*. 68:593-602.

Jurado, C., J.R. Haserick, and J. Lee. 2005. Slipping or gripping? Fluorescent speckle microscopy in fish keratocytes reveals two different mechanisms for generating a retrograde flow of actin. *Mol Biol Cell*. 16:507-518.

Kang, C.M., D.W. Abbott, S.T. Park, C.C. Dascher, L.C. Cantley, and R.N. Husson. 2005. The Mycobacterium tuberculosis serine/threonine kinases PknA and PknB: substrate identification and regulation of cell shape. *Genes Dev*. 19:1692-1704.

Kaverina, I., O. Krylyshkina, K. Beningo, K. Anderson, Y.L. Wang, and J.V. Small. 2002. Tensile stress stimulates microtubule outgrowth in living cells. *J Cell Sci*. 115:2283-2291.

Kaverina, I., O. Krylyshkina, M. Gimona, K. Beningo, Y.L. Wang, and J.V. Small. 2000. Enforced polarisation and locomotion of fibroblasts lacking microtubules. *Curr Biol*. 10:739-742.

Keller, R. 2005. Cell migration during gastrulation. *Curr Opin Cell Biol*. 17:533-541.

Kim, S.H., J. Turnbull, and S. Guimond. 2011. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J Endocrinol*. 209:139-151.

Kirfel, G., and V. Herzog. 2004. Migration of epidermal keratinocytes: mechanisms, regulation, and biological significance. *Protoplasma*. 223:67-78.

Klemke, M., E. Kramer, M.H. Konstandin, G.H. Wabnitz, and Y. Samstag. 2010. An MEK-cofilin signalling module controls migration of human T cells in 3D but not 2D environments. *EMBO J*. 29:2915-2929.

Kopfstein, L., and G. Christofori. 2006. Metastasis: cell-autonomous mechanisms versus contributions by the tumor microenvironment. *Cell Mol Life Sci*. 63:449-468.

Kostic, A., C.D. Lynch, and M.P. Sheetz. 2009. Differential matrix rigidity response in breast cancer cell lines correlates with the tissue tropism. *PLoS One*. 4:e6361.

Kostic, A., J. Sap, and M.P. Sheetz. 2007. RPTPalpha is required for rigidity-dependent inhibition of extension and differentiation of hippocampal neurons. *J Cell Sci*. 120:3895-3904.

Kostic, A., and M.P. Sheetz. 2006. Fibronectin rigidity response through Fyn and p130Cas recruitment to the leading edge. *Mol Biol Cell*. 17:2684-2695.

Krammer, A., D. Craig, W.E. Thomas, K. Schulten, and V. Vogel. 2002. A structural model for force regulated integrin binding to fibronectin's RGD-synergy site. *Matrix Biol*. 21:139-147.

Krammer, A., H. Lu, B. Isralewitz, K. Schulten, and V. Vogel. 1999. Forced unfolding of the fibronectin type III module reveals a tensile molecular recognition switch. *Proc Natl Acad Sci U S A*. 96:1351-1356.

Kraning-Rush, C.M., S.P. Carey, J.P. Califano, and C.A. Reinhart-King. 2012. Quantifying traction stresses in adherent cells. *Methods Cell Biol*. 110:139-178.

Krzeslak, A., and A. Lipinska. 2004. Galectin-3 as a multifunctional protein. *Cell Mol Biol Lett*. 9:305-328.

Kumar, S., and V.M. Weaver. 2009. Mechanics, malignancy, and metastasis: the force journey of a tumor cell. *Cancer Metastasis Rev*. 28:113-127.

Kung, C. 2005. A possible unifying principle for mechanosensation. *Nature*. 436:647-654.

Kutcher, M.E., A.Y. Kolyada, H.K. Surks, and I.M. Herman. 2007. Pericyte Rho GTPase mediates both pericyte contractile phenotype and capillary endothelial growth state. *Am J Pathol*. 171:693-701.

Lagana, A., J.G. Goetz, P. Cheung, A. Raz, J.W. Dennis, and I.R. Nabi. 2006. Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells. *Mol Cell Biol*. 26:3181-3193.

Lammermann, T., J. Renkawitz, X. Wu, K. Hirsch, C. Brakebusch, and M. Sixt. 2009. Cdc42-dependent leading edge coordination is essential for interstitial dendritic cell migration. *Blood*. 113:5703-5710.

Lammermann, T., and M. Sixt. 2009. Mechanical modes of 'amoeboid' cell migration. *Curr Opin Cell Biol*. 21:636-644.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell*. 84:359-369.

Le Clainche, C., and M.F. Carlier. 2008. Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiol Rev*. 88:489-513.

Lee, S., and et al. 2010. Pericyte actomyosin-mediated contraction at the cell–material interface can modulate the microvascular niche. *Journal of Physics: Condensed Matter*. 22:194115.

Letourneau, P.C. 1983. Differences in the organization of actin in the growth cones compared with the neurites of cultured neurons from chick embryos. *J Cell Biol*. 97:963-973.

Levental, K.R., H. Yu, L. Kass, J.N. Lakins, M. Egeblad, J.T. Erler, S.F. Fong, K. Csiszar, A. Giaccia, W. Weninger, M. Yamauchi, D.L. Gasser, and V.M. Weaver. 2009. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*. 139:891-906.

Li, F., S.D. Redick, H.P. Erickson, and V.T. Moy. 2003. Force measurements of the alpha5beta1 integrin-fibronectin interaction. *Biophys J*. 84:1252-1262.

Li, S., P. Butler, Y. Wang, Y. Hu, D.C. Han, S. Usami, J.L. Guan, and S. Chien. 2002. The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells. *Proc Natl Acad Sci U S A*. 99:3546-3551.

Li, S., J.L. Guan, and S. Chien. 2005. Biochemistry and biomechanics of cell motility. *Annu Rev Biomed Eng*. 7:105-150.

Li, X., Q. Ma, J. Wang, X. Liu, Y. Yang, H. Zhao, Y. Wang, Y. Jin, J. Zeng, J. Li, L. Song, P. Li, X. Qian, and C. Cao. 2010. c-Abl and Arg tyrosine kinases regulate lysosomal degradation of the oncoprotein Galectin-3. *Cell Death Differ*. 17:1277-1287.

Lin, C.H., and P. Forscher. 1995. Growth cone advance is inversely proportional to retrograde F-actin flow. *Neuron*. 14:763-771.

Lin, V.C., E.H. Ng, S.E. Aw, M.G. Tan, E.H. Ng, and B.H. Bay. 2000. Progesterone induces focal adhesion in breast cancer cells MDA-MB-231 transfected with progesterone receptor complementary DNA. *Molecular endocrinology (Baltimore, Md*. 14:348-358.

Lindstedt, R., G. Apodaca, S.H. Barondes, K.E. Mostov, and H. Leffler. 1993. Apical secretion of a cytosolic protein by Madin-Darby canine kidney cells. Evidence for polarized release of an endogenous lectin by a nonclassical secretory pathway. *J Biol Chem*. 268:11750-11757.

Liu, F.T., R.J. Patterson, and J.L. Wang. 2002. Intracellular functions of galectins. *Biochim Biophys Acta*. 1572:263-273.

Lo, C.M., H.B. Wang, M. Dembo, and Y.L. Wang. 2000. Cell movement is guided by the rigidity of the substrate. *Biophys J*. 79:144-152.

Lu, P., K. Takai, V.M. Weaver, and Z. Werb. 2011. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb Perspect Biol*. 3.

Lu, P., V.M. Weaver, and Z. Werb. 2012. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol*. 196:395-406.

Luo, B.H., C.V. Carman, and T.A. Springer. 2007. Structural basis of integrin regulation and signaling. *Annu Rev Immunol*. 25:619-647.

Mammoto, T., and D.E. Ingber. 2010. Mechanical control of tissue and organ development. *Development*. 137:1407-1420.

Martinac, B. 2004. Mechanosensitive ion channels: molecules of mechanotransduction. *J Cell Sci*. 117:2449-2460.

Martinkova, E., A. Maglott, D.Y. Leger, D. Bonnet, M. Stiborova, K. Takeda, S. Martin, and M. Dontenwill. 2010. alpha5beta1 integrin antagonists reduce chemotherapy-induced premature senescence and facilitate apoptosis in human glioblastoma cells. *Int J Cancer*. 127:1240-1248.

Martins, R.P., J.D. Finan, F. Guilak, and D.A. Lee. 2012. Mechanical Regulation of Nuclear Structure and Function. *Annu Rev Biomed Eng*.

Mehul, B., and R.C. Hughes. 1997. Plasma membrane targetting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion. *J Cell Sci*. 110 (Pt 10):1169-1178.

Menon, R.P., and R.C. Hughes. 1999. Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-Golgi complex. *Eur J Biochem*. 264:569-576.

Menon, S., and K.A. Beningo. 2011. Cancer cell invasion is enhanced by applied mechanical stimulation. *PLoS One*. 6:e17277.

Menon, S., C.M. Kang, and K.A. Beningo. 2011. Galectin-3 secretion and tyrosine phosphorylation is dependent on the calpain small subunit, Calpain 4. *Biochem Biophys Res Commun*. 410:91-96.

Meshel, A.S., Q. Wei, R.S. Adelstein, and M.P. Sheetz. 2005. Basic mechanism of three-dimensional collagen fibre transport by fibroblasts. *Nat Cell Biol*. 7:157-164.

Michael, K.E., D.W. Dumbauld, K.L. Burns, S.K. Hanks, and A.J. Garcia. 2009. Focal adhesion kinase modulates cell adhesion strengthening via integrin activation. *Mol Biol Cell*. 20:2508-2519.

Mierke, C.T. 2011. The biomechanical properties of 3d extracellular matrices and embedded cells regulate the invasiveness of cancer cells. *Cell Biochem Biophys*. 61:217-236.

Mierke, C.T., D. Rosel, B. Fabry, and J. Brabek. 2008. Contractile forces in tumor cell migration. *Eur J Cell Biol*. 87:669-676.

Miranti, C.K., and J.S. Brugge. 2002. Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol*. 4:E83-90.

Miron-Mendoza, M., J. Seemann, and F. Grinnell. 2008. Collagen fibril flow and tissue translocation coupled to fibroblast migration in 3D collagen matrices. *Mol Biol Cell*. 19:2051-2058.

Mitra, S.K., D.A. Hanson, and D.D. Schlaepfer. 2005. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol*. 6:56-68.

Mogilner, A., and G. Oster. 1996. Cell motility driven by actin polymerization. *Biophys J*. 71:3030-3045.

Moore, S.W., and M.P. Sheetz. 2011. Biophysics of substrate interaction: influence on neural motility, differentiation, and repair. *Dev Neurobiol*. 71:1090-1101.

Morikawa, S., P. Baluk, T. Kaidoh, A. Haskell, R.K. Jain, and D.M. McDonald. 2002. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol*. 160:985-1000.

Mouneimne, G., V. DesMarais, M. Sidani, E. Scemes, W. Wang, X. Song, R. Eddy, and J. Condeelis. 2006. Spatial and temporal control of cofilin activity is required for directional sensing during chemotaxis. *Curr Biol*. 16:2193-2205.

Mueller, M.M., and N.E. Fusenig. 2004. Friends or foes - bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer*. 4:839-849.

Mueller, S.C., G. Ghersi, S.K. Akiyama, Q.X. Sang, L. Howard, M. Pineiro-Sanchez, H. Nakahara, Y. Yeh, and W.T. Chen. 1999. A novel protease-docking function of integrin at invadopodia. *J Biol Chem*. 274:24947-24952.

Munevar, S., Y.L. Wang, and M. Dembo. 2001. Distinct roles of frontal and rear cell-substrate adhesions in fibroblast migration. *Mol Biol Cell*. 12:3947-3954.

Nakahara, S., and A. Raz. 2006. On the role of galectins in signal transduction. *Methods Enzymol*. 417:273-289.

Nam, J.M., Y. Onodera, M.J. Bissell, and C.C. Park. 2010. Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin. *Cancer Res*. 70:5238-5248.

Ochieng, J., V. Furtak, and P. Lukyanov. 2004. Extracellular functions of galectin-3. *Glycoconj J*. 19:527-535.

Palecek, S.P., J.C. Loftus, M.H. Ginsberg, D.A. Lauffenburger, and A.F. Horwitz. 1997. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature*. 385:537-540.

Palecek, S.P., C.E. Schmidt, D.A. Lauffenburger, and A.F. Horwitz. 1996. Integrin dynamics on the tail region of migrating fibroblasts. *J Cell Sci*. 109 (Pt 5):941-952.

Papusheva, E., and C.P. Heisenberg. 2010. Spatial organization of adhesion: force-dependent regulation and function in tissue morphogenesis. *EMBO J*. 29:2753-2768.

Parent, C.A., and P.N. Devreotes. 1999. A cell's sense of direction. *Science*. 284:765-770.

Parsons, J.T., A.R. Horwitz, and M.A. Schwartz. 2010. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat Rev Mol Cell Biol*. 11:633-643.

Paszek, M.J., N. Zahir, K.R. Johnson, J.N. Lakins, G.I. Rozenberg, A. Gefen, C.A. Reinhart-King, S.S. Margulies, M. Dembo, D. Boettiger, D.A. Hammer, and V.M.

Weaver. 2005. Tensional homeostasis and the malignant phenotype. *Cancer Cell*. 8:241-254.

Paterson, H.F., A.J. Self, M.D. Garrett, I. Just, K. Aktories, and A. Hall. 1990. Microinjection of recombinant p21rho induces rapid changes in cell morphology. *J Cell Biol*. 111:1001-1007.

Pelham, R.J., Jr., and Y. Wang. 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A*. 94:13661-13665.

Petrie, R.J., A.D. Doyle, and K.M. Yamada. 2009. Random versus directionally persistent cell migration. *Nat Rev Mol Cell Biol*. 10:538-549.

Pietras, K., and A. Ostman. 2010. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res*. 316:1324-1331.

Pietras, K., J. Pahler, G. Bergers, and D. Hanahan. 2008. Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. *PLoS Med*. 5:e19.

Pietras, K., T. Sjoblom, K. Rubin, C.H. Heldin, and A. Ostman. 2003. PDGF receptors as cancer drug targets. *Cancer Cell*. 3:439-443.

Pirone, D.M., W.F. Liu, S.A. Ruiz, L. Gao, S. Raghavan, C.A. Lemmon, L.H. Romer, and C.S. Chen. 2006. An inhibitory role for FAK in regulating proliferation: a link between limited adhesion and RhoA-ROCK signaling. *J Cell Biol*. 174:277-288.

Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science*. 284:143-147.

Plow, E.F., T.A. Haas, L. Zhang, J. Loftus, and J.W. Smith. 2000. Ligand binding to integrins. *J Biol Chem*. 275:21785-21788.

Poincloux, R., F. Lizarraga, and P. Chavrier. 2009. Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia. *J Cell Sci*. 122:3015-3024.

Pollard, J.W. 2004. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*. 4:71-78.

Ponti, A., M. Machacek, S.L. Gupton, C.M. Waterman-Storer, and G. Danuser. 2004. Two distinct actin networks drive the protrusion of migrating cells. *Science*. 305:1782-1786.

Prager-Khoutorsky, M., A. Lichtenstein, R. Krishnan, K. Rajendran, A. Mayo, Z. Kam, B. Geiger, and A.D. Bershadsky. 2011. Fibroblast polarization is a matrix-rigidity-dependent process controlled by focal adhesion mechanosensing. *Nat Cell Biol*. 13:1457-1465.

Rafelski, S.M., and J.A. Theriot. 2004. Crawling toward a unified model of cell mobility: spatial and temporal regulation of actin dynamics. *Annu Rev Biochem*. 73:209-239.

Rape, A., W.H. Guo, and Y.L. Wang. 2011. Microtubule depolymerization induces traction force increase through two distinct pathways. *J Cell Sci*. 124:4233-4240.

Regen, C.M., and A.F. Horwitz. 1992. Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. *J Cell Biol*. 119:1347-1359.

Ridley, A.J. 2011. Life at the leading edge. *Cell*. 145:1012-1022.

Ridley, A.J., M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, and A.R. Horwitz. 2003. Cell migration: integrating signals from front to back. *Science*. 302:1704-1709.

Roca-Cusachs, P., N.C. Gauthier, A. Del Rio, and M.P. Sheetz. 2009. Clustering of alpha(5)beta(1) integrins determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. *Proc Natl Acad Sci U S A*. 106:16245-16250.

Rosenberger, G., A. Gal, and K. Kutsche. 2005. AlphaPIX associates with calpain 4, the small subunit of calpain, and has a dual role in integrin-mediated cell spreading. *J Biol Chem*. 280:6879-6889.

Saha, K., A.J. Keung, E.F. Irwin, Y. Li, L. Little, D.V. Schaffer, and K.E. Healy. 2008. Substrate modulus directs neural stem cell behavior. *Biophys J*. 95:4426-4438.

Sastry, S.K., and K. Burridge. 2000. Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. *Exp Cell Res*. 261:25-36.

Sato, K., Y. Saito, and S. Kawashima. 1995. Identification and characterization of membrane-bound calpains in clathrin-coated vesicles from bovine brain. *Eur J Biochem*. 230:25-31.

Sato, S., I. Burdett, and R.C. Hughes. 1993. Secretion of the baby hamster kidney 30-kDa galactose-binding lectin from polarized and nonpolarized cells: a pathway

independent of the endoplasmic reticulum-Golgi complex. *Exp Cell Res*. 207:8-18.

Schedin, P., and P.J. Keely. 2011. Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression. *Cold Spring Harb Perspect Biol*. 3:a003228.

Schmidt, C., H. Pommerenke, F. Durr, B. Nebe, and J. Rychly. 1998. Mechanical stressing of integrin receptors induces enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. *J Biol Chem*. 273:5081-5085.

Schober, M., S. Raghavan, M. Nikolova, L. Polak, H.A. Pasolli, H.E. Beggs, L.F. Reichardt, and E. Fuchs. 2007. Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. *J Cell Biol*. 176:667-680.

Shemesh, T., B. Geiger, A.D. Bershadsky, and M.M. Kozlov. 2005. Focal adhesions as mechanosensors: a physical mechanism. *Proc Natl Acad Sci U S A*. 102:12383-12388.

Singer, II, D.W. Kawka, D.M. Kazazis, and R.A. Clark. 1984. In vivo co-distribution of fibronectin and actin fibers in granulation tissue: immunofluorescence and electron microscope studies of the fibronexus at the myofibroblast surface. *J Cell Biol*. 98:2091-2106.

Small, J.V. 1981. Organization of actin in the leading edge of cultured cells: influence of osmium tetroxide and dehydration on the ultrastructure of actin meshworks. *J Cell Biol*. 91:695-705.

Smith, P.G., R. Garcia, and L. Kogerman. 1998. Mechanical strain increases protein tyrosine phosphorylation in airway smooth muscle cells. *Exp Cell Res*. 239:353-360.

Stossel, T.P. 1993. On the crawling of animal cells. *Science*. 260:1086-1094.

Streuli, C.H., and N. Akhtar. 2009. Signal co-operation between integrins and other receptor systems. *Biochem J*. 418:491-506.

Stricker, J., Y. Aratyn-Schaus, P.W. Oakes, and M.L. Gardel. 2011. Spatiotemporal constraints on the force-dependent growth of focal adhesions. *Biophys J*. 100:2883-2893.

Stylli, S.S., A.H. Kaye, and P. Lock. 2008. Invadopodia: at the cutting edge of tumour invasion. *J Clin Neurosci*. 15:725-737.

Suzuki, K., S. Hata, Y. Kawabata, and H. Sorimachi. 2004. Structure, activation, and biology of calpain. *Diabetes*. 53 Suppl 1:S12-18.

Theriot, J.A., and T.J. Mitchison. 1992. Comparison of actin and cell surface dynamics in motile fibroblasts. *J Cell Biol*. 119:367-377.

Thery, C., M. Boussac, P. Veron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin, and S. Amigorena. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol*. 166:7309-7318.

Tilghman, R.W., C.R. Cowan, J.D. Mih, Y. Koryakina, D. Gioeli, J.K. Slack-Davis, B.R. Blackman, D.J. Tschumperlin, and J.T. Parsons. 2010. Matrix rigidity regulates cancer cell growth and cellular phenotype. *PLoS One*. 5:e12905.

Tlsty, T.D., and L.M. Coussens. 2006. Tumor stroma and regulation of cancer development. *Annu Rev Pathol*. 1:119-150.

Tomasek, J.J., G. Gabbiani, B. Hinz, C. Chaponnier, and R.A. Brown. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol*. 3:349-363.

Trichet, L., J. Le Digabel, R.J. Hawkins, S.R. Vedula, M. Gupta, C. Ribrault, P. Hersen, R. Voituriez, and B. Ladoux. 2012. Evidence of a large-scale mechanosensing mechanism for cellular adaptation to substrate stiffness. *Proc Natl Acad Sci U S A*. 109:6933-6938.

Undyala, V.V., M. Dembo, K. Cembrola, B.J. Perrin, A. Huttenlocher, J.S. Elce, P.A. Greer, Y.L. Wang, and K.A. Beningo. 2008. The calpain small subunit regulates cell-substrate mechanical interactions during fibroblast migration. *J Cell Sci*. 121:3581-3588.

Vakonakis, I., and I.D. Campbell. 2007. Extracellular matrix: from atomic resolution to ultrastructure. *Curr Opin Cell Biol*. 19:578-583.

van den Brule, F., S. Califice, and V. Castronovo. 2004. Expression of galectins in cancer: a critical review. *Glycoconj J*. 19:537-542.

Van Haastert, P.J., and P.N. Devreotes. 2004. Chemotaxis: signalling the way forward. *Nat Rev Mol Cell Biol*. 5:626-634.

Vogel, V. 2006. Mechanotransduction involving multimodular proteins: converting force into biochemical signals. *Annu Rev Biophys Biomol Struct*. 35:459-488.

Vogel, V., and M. Sheetz. 2006. Local force and geometry sensing regulate cell functions. *Nat Rev Mol Cell Biol*. 7:265-275.

Wang, H.B., M. Dembo, S.K. Hanks, and Y. Wang. 2001. Focal adhesion kinase is involved in mechanosensing during fibroblast migration. *Proc Natl Acad Sci U S A*. 98:11295-11300.

Wang, J.H., and J.S. Lin. 2007. Cell traction force and measurement methods. *Biomech Model Mechanobiol*. 6:361-371.

Wang, J.L., R.M. Gray, K.C. Haudek, and R.J. Patterson. 2004. Nucleocytoplasmic lectins. *Biochim Biophys Acta*. 1673:75-93.

Wang, N., J.D. Tytell, and D.E. Ingber. 2009. Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. *Nat Rev Mol Cell Biol*. 10:75-82.

Wang, Y.L. 1985. Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. *J Cell Biol*. 101:597-602.

Wang, Y.L. 2007. Flux at focal adhesions: slippage clutch, mechanical gauge, or signal depot. *Sci STKE*. 2007:pe10.

Wang, Y.L. 2009. Traction forces and rigidity sensing of adherent cells. *Conf Proc IEEE Eng Med Biol Soc*. 2009:3339-3340.

Weaver, A.M. 2006. Invadopodia: specialized cell structures for cancer invasion. *Clin Exp Metastasis*. 23:97-105.

Webb, D.J., and A.F. Horwitz. 2003. New dimensions in cell migration. *Nat Cell Biol*. 5:690-692.

Webb, D.J., H. Zhang, and A.F. Horwitz. 2005. Cell migration: an overview. *Methods Mol Biol*. 294:3-11.

Weiss, P. 1934. In vitro experiments on the factors determining the course of the outgrowing nerve fiber. *Journal of Experimental Zoology*. 68:393-448.

Wells, A. 2000. Tumor invasion: role of growth factor-induced cell motility. *Adv Cancer Res*. 78:31-101.

Weng, S., and J. Fu. 2011. Synergistic regulation of cell function by matrix rigidity and adhesive pattern. *Biomaterials*. 32:9584-9593.

Wilda, H., and E. Adam. 2011. Mechanobiology in the Stem Cell Niche. *In* Mechanobiology Handbook. CRC Press. 439-455.

Wilson, A.K., G. Gorgas, W.D. Claypool, and P. de Lanerolle. 1991. An increase or a decrease in myosin II phosphorylation inhibits macrophage motility. *J Cell Biol*. 114:277-283.

Wipff, P.J., D.B. Rifkin, J.J. Meister, and B. Hinz. 2007. Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol*. 179:1311-1323.

Wolf, K., I. Mazo, H. Leung, K. Engelke, U.H. von Andrian, E.I. Deryugina, A.Y. Strongin, E.B. Brocker, and P. Friedl. 2003. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol*. 160:267-277.

Wolf, K., Y.I. Wu, Y. Liu, J. Geiger, E. Tam, C. Overall, M.S. Stack, and P. Friedl. 2007. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nat Cell Biol*. 9:893-904.

Wolfenson, H., Y.I. Henis, B. Geiger, and A.D. Bershadsky. 2009. The heel and toe of the cell's foot: a multifaceted approach for understanding the structure and dynamics of focal adhesions. *Cell Motil Cytoskeleton*. 66:1017-1029.

Wozniak, M.A., R. Desai, P.A. Solski, C.J. Der, and P.J. Keely. 2003. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *J Cell Biol*. 163:583-595.

Wrobel, L.K., T.R. Fray, J.E. Molloy, J.J. Adams, M.P. Armitage, and J.C. Sparrow. 2002. Contractility of single human dermal myofibroblasts and fibroblasts. *Cell Motil Cytoskeleton*. 52:82-90.

Wu, Y., S.D. Spencer, and L.A. Lasky. 1998. Tyrosine phosphorylation regulates the SH3-mediated binding of the Wiskott-Aldrich syndrome protein to PSTPIP, a cytoskeletal-associated protein. *J Biol Chem*. 273:5765-5770.

Yamaguchi, H., M. Lorenz, S. Kempiak, C. Sarmiento, S. Coniglio, M. Symons, J. Segall, R. Eddy, H. Miki, T. Takenawa, and J. Condeelis. 2005. Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin. *J Cell Biol*. 168:441-452.

Yamazaki, D., S. Kurisu, and T. Takenawa. 2005. Regulation of cancer cell motility through actin reorganization. *Cancer Sci*. 96:379-386.

Yeung, T., P.C. Georges, L.A. Flanagan, B. Marg, M. Ortiz, M. Funaki, N. Zahir, W. Ming, V. Weaver, and P.A. Janmey. 2005. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton*. 60:24-34.

Yu, H., J.K. Mouw, and V.M. Weaver. 2011. Forcing form and function: biomechanical regulation of tumor evolution. *Trends Cell Biol*. 21:47-56.

Yu, J., C. Ustach, and H.R. Kim. 2003. Platelet-derived growth factor signaling and human cancer. *J Biochem Mol Biol*. 36:49-59.

Zaidel-Bar, R., S. Itzkovitz, A. Ma'ayan, R. Iyengar, and B. Geiger. 2007. Functional atlas of the integrin adhesome. *Nat Cell Biol*. 9:858-867.

Zemel, A., F. Rehfeldt, A.E. Brown, D.E. Discher, and S.A. Safran. 2010. Cell shape, spreading symmetry and the polarization of stress-fibers in cells. *J Phys Condens Matter*. 22:194110.

Zhu, C., and R. Skalak. 1988. A continuum model of protrusion of pseudopod in leukocytes. *Biophys J*. 54:1115-1137.

Zhu, W.Q., and J. Ochieng. 2001. Rapid release of intracellular galectin-3 from breast carcinoma cells by fetuin. *Cancer Res*. 61:1869-1873.

ABSTRACT

BIOPHYSICAL AND BIOCHEMICAL FACTORS IN THE CELLULAR MICROENVIRONMENT; EFFECTS ON CELL MIGRATION AND INVASION

by

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Cellular migration is a vital process central to many physiological events including development, immune surveillance and wound healing. However, migration and invasion are not unique to normal physiology, they are also key determinants in the progression of disease states such as cancer. Given the significance of migration it is important that we understand how the process is regulated intracellularly and the various stimuli that can promote it. Even though the role of biochemical factors in mediating migration has been studied extensively, the role of biophysical factors in modulating migration and invasion is less appreciation. The biochemical and biophysical components of cell and tissue microenvironments influence cellular behavior. This is true for both normal and disease conditions. For example, the role of substrate stiffness and extracellular matrix (ECM) composition in cell proliferation, spreading, preferential migration and even stem cell differentiation has been observed. However, a number of questions remain unanswered, such as the ability of cells to sense locally applied mechanical stimuli and how this mechanosensing is regulated. Would the regulation be

different if cancer cells were to sense the applied stimulus? Studies have shown that as cells migrate they produce contractile forces called traction forces that are generated by the cellular cytoskeleton and transmitted onto the substrate. Yet the signaling mechanism that promotes this force production or how is it regulated is not well characterized. In attempt at address these questions, we have identified the importance of locally applied mechanical stimuli in cancer cell invasion and we have also identified a major link in the traction force production pathway. Our study on the influence of local mechanical stimuli on cancer cell invasion suggests that the stimuli produced as a result of ECM remodeling by and migration of non-cancerous cells present in the tumor microenvironment could enhance tumor cell invasion. This enhanced invasion is dependent on actin and cofilin, and the ECM protein, fibronectin.

In gaining understanding of the mechanisms and interplay between traction force and mechanosensing we have focused on the Calpain protease. We previously identified that the calpain small subunit, calpain 4 (Capn4), influences force production independent of the proteolytic activity of the catalytic subunits calpain 1 and 2, yet their mechanosensing mechanism overlaps. To further explore the relationship, we asked how Capn4 could regulate force production. We have found that Capn4 indirectly mediates tyrosine phosphorylation of a lectin binding protein, galectin-3. This phosphorylation potentially helps in galectin-3 secretion into the ECM from where it is able to modulate traction force and associated events involving focal adhesion maturation and adhesion strength. It however, does not influence mechanosensing. Together these results further emphasize the point that cell migration and invasion is significantly influenced by the

biochemical and biophysical components and properties on the microenvironment. Further studies will elucidate these pathways and provide greater insight for bioengineering and medical advances.

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1. Menon, S., Kang C-M and Beningo, K.A. (2011). Galectin-3 secretion and tyrosine phosphorylation is dependent on the calpain small subunit, Calpain 4. ***Biochem Biophys Res Commun.*** 410(1):91-6.

2. Menon, S. and Beningo, K.A. (2011). Cancer cell invasion is enhanced by applied mechanical stimulation. ***PLoS One*** 6: e17277.

3. Mishra GR, Suresh M, Kumaran K, Kannabiran N, Suresh S, Bala P, Shivakumar K, Anuradha N, Reddy R, Raghavan TM, **Menon S,** Hanumanthu G, Gupta M, Upendran S, Gupta S, Mahesh M, Jacob B, Mathew P, Chatterjee P, Arun KS, Sharma S, Chandrika KN, Deshpande N, Palvankar K, Raghavnath R, Krishnakanth R, Karathia H, Rekha B, Nayak R, Vishnupriya G, Kumar HG, Nagini M, Kumar GS, Jose R, Deepthi P, Mohan SS, Gandhi TK, Harsha HC, Deshpande KS, Sarker M, Prasad TS, ,Pandey A (2006) Human protein reference database-2006 update. ***Nucleic Acids Res***. (Database issue): D411-4