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Defining The Mechanism Of Enhanced Cellular Invasion Induced By Mechanical Stimulation

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DEFINING THE MECHANISM OF ENHANCED CELLULAR INVASION INDUCED BY MECHANICAL STIMULATION

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DEDICATION

I dedicate this thesis to

My parents, Sunil and Minal Ozarkar

And my husband, Kaustubh

Who have always believed in me and motivated me to overcome all the hurdles in life.

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CHAPTER 1 - INTRODUCTION

Cancer and Metastasis

According to the World Health Organization (2008), cancer is a leading cause of death worldwide and 90% of these deaths result from the secondary tumors. The secondary tumors are formed by a multistep process called metastasis, which involves migration and invasion of primary tumor cells into different parts of the body (Pantel and Brakenhoff, 2004). The primary tumor mass is surrounded by a basement membrane and stroma. The basement membrane is comprised of a number of extracellular matrix (ECM) proteins such as type IV collagen, laminin, elastin, and fibronectin. The tumor stroma, on the other hand, is composed of many non-tumor cells such as fibroblasts, adipocytes and various immune cells, in addition to the ECM proteins (Bissell and Radisky, 2001). The metastatic cascade begins with the acquisition of invasive properties by the primary tumor cells. By virtue of these invasive or metastatic properties, tumor cells breach the basement membrane of the tissue, invade the stroma and via blood or lymph reach other parts of the body where they can form a secondary tumor (Chambers et al., 2002; Liotta et al., 1986; Poincloux et al., 2009). A great deal of research has been done to understand the causes of origin and spread of cancer. Nevertheless, given the extreme complexity and heterogeneity of the disease etiology, it is still difficult to pinpoint exact cause of cancer development. For decades, genetic and epigenetic factors, predisposing cancer cells to become metastatic, have been the main focus of research groups. This research has resulted in the identification of many biochemical factors such as various growth factors and cytokines that promote the metastatic behavior of cancer cells (Hirakawa et al., 2005; Huang et al., 2002; Oft et al., 1998). However, with increasing knowledge about metastatic progression cancer biologists have begun to appreciate the importance of multiple mechanical factors, such as changes in the structure and

mechanics of the tissue, as well as biophysical changes in the geometry and topology of the ECM in initiating the multi-step metastatic cascade (Kumar and Weaver, 2009).

Effect of Tumor Microenvironment on Metastasis

Along with the development of the tumor mass, structure and composition of the tumor stroma undergoes constant changes, a process known as stromagenesis (Bissell and Radisky, 2001). During this process different non-tumor cells, collectively referred to as tumor associated stromal cells, are also recruited and activated. These non-tumor cells contribute to the progression of cancer through the secretion of several biochemical factors and up-regulated synthesis of ECM proteins. By virtue of its dynamic nature, the tumor stroma provides a unique microenvironment for the tumor mass. This tumor microenvironment possesses the ability to change the neoplastic properties of the tumor. The metastatic cascade begins with changes in the tumor microenvironment which triggers the dissemination of primary tumor cells to the probable sites of secondary tumor formation (Yu et al., 2011). Examination of genetic alterations in the tumor associated stromal cells showed numerous chromosomal rearrangements not found in tumor cells (Moinfar et al., 2000). The sources of mutations in stromal cells are still unknown, but lead to the abnormal cross-signaling between cancer cells and stromal cells (De Wever et al., 2008). Therefore is it extremely important to understand the tumor-stromal interactions.

Biochemical Factors in Invasion

Many biochemical factors from the stroma have been identified as promoters of tumor metastasis. For example, immune cells present in the stroma, such as macrophages, dendritic cells, natural killer cells, and mast cells, produce chemokines, cytokines and soluble cytotoxic mediators. All these biochemical components aid in the process of angiogenesis, cell proliferation, cell motility, and invasion (Tlsty and Coussens, 2006). During tumor development, stromal cells

and cancer cells together form a paracrine loop that regulates cancer progression (De Wever et al., 2008; Pollard, 2004). For example, tumor cells secrete colony stimulating factor-1 (CSF-1) for attracting macrophages and macrophages secrete epithelial growth factor (EGF) causing tumor cell proliferation (Wyckoff et al., 2004). Tumor cells also secrete various chemoattractants such as, transforming growth factor β-1 (TGFβ-1), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and EGF, in response to which stromal fibroblast cells are attracted towards the tumor (Denys et al., 2008; Dong et al., 2004; Lederle et al., 2006; Stuelten et al., 2005). Stromal fibroblasts then undergo differentiation into myofibroblasts and secrete similar growth factors, which cause proliferation of tumor cells (Ao et al., 2007; Cat et al., 2006; Shao et al., 2006), and matrix metalloproteinases, which cause degradation and remodeling of tumor stroma (Stuelten et al., 2005; Taniwaki et al., 2007).

Mechanical Factors in Invasion

In addition to the biochemical factors, physical or mechanical factors in the tumor microenvironment also contribute to cancer progression and metastasis (Desmouliere et al., 2004; Tlsty and Coussens, 2006). The tumor mass is characterized by uncontrolled cell growth and disrupted tissue structure. This leads to the loss of tissue homeostasis and mechanical equilibrium that is maintained in the physiologically normal tissue. The physical state of the tumor also evolves with the evolution of the tumor mass (Yu et al., 2011), due to changes in the cell-cell and cell-ECM interactions, as well as deposition and remodeling of the ECM components surrounding the tumor mass (Borghi et al., 2010; Ingber, 2008; Schwartz and DeSimone, 2008). On account of these changes the tumor mass and its microenvironment are acted upon by different mechanical forces such as hydrostatic pressure, shear stress, compression and tension, and changes in compliance (Butcher et al., 2009).

One of the mechanical forces within the tumor microenvironment results from the expansion of the tumor itself. The uncontrollably growing tumor mass is confined within a limited space in the tissue. These spatial constraints generate high levels of radial stress on the surrounding stroma. At the same time, compression of the stroma generates stress within the tumor itself (Shieh, 2011). Tumor cell spheroids embedded in agarose gels have been shown to generate stresses more than 10 kPa in in-vitro studies (Helmlinger et al., 1997). Interstitial fluid pressure also contributes to compressive forces within the tumor and its microenvironment. It is formed due to highly permeable microvascular networks leading to increased flow of fluids and macromolecules within the tumor and stroma (Boucher and Jain, 1992; Fukumura et al., 2010; Hagendoorn et al., 2006). Nonetheless, most studies of mechanical forces in the tumor and microenvironment have focused on compliance. After all, stiffer stromal tissue has long been a hallmark of different cancer types such as breast, liver and prostate cancer (Boyd et al., 1999; Gang et al., 2009; Hoyt et al., 2008). Mammary tumor tissue is known to have stiffness exceeding 4 kPa, which is much higher than a normal mammary gland that has an elastic modulus of 200 Pa (Paszek et al., 2005). This increase in stiffness or rigidity of the stroma is due to the abnormal production of type I collagen and fibronectin by different stromal cells (Bissell and Radisky, 2001; Tlsty and Coussens, 2006). Recent studies have shown that this rigidity of the tumor stroma positively regulates cancer cell proliferation and dissemination to other sites within the body (Kostic et al., 2009; Levental et al., 2009; Paszek et al., 2005).

Stromal cells associated with tumors also give rise to different mechanical forces, which can promote metastasis. Myofibroblasts are one of the main types of stromal cells that appear early during tumor progression. They were initially discovered to be involved in the process of wound healing (Ryan et al., 1974). In the tumor stroma, myofibroblasts differentiate from normal fibroblasts, fibrocytes, mesenchymal stem cells (MSCs) and other cells of mesenchymal origin

(De Wever et al., 2008; Desmouliere et al., 2004). This differentiation requires various paracrine signals from the cancer cells. TGFβ-1 has been found to play a significant role in this process (Desmouliere et al., 1993). The hallmarks of differentiated myofibroblasts are secretion of ECM proteins like collagen and fibronectin as well as neo-expression of α-smooth muscle actin (α-SMA) (Darby et al., 1990; Serini et al., 1998). This actin isoform bundles with myosin-II and forms contractile stress fibers. A direct correlation between the level of α-SMA expression and myofibroblast contractions has been demonstrated both in vitro (Hinz et al., 2001a) and in vivo (Hinz et al., 2001b). Myofibroblasts are also known to produce large focal adhesions, through which tugging or pulling forces produced by stress fibers are transferred into the surrounding ECM (Castella et al., 2010; Hinz et al., 2003). Using a unique in-vitro invasion assay developed in our lab, it has been shown that mechanical stimuli in the form of pulling forces leads to enhanced invasion by cancer cells (Menon and Beningo, 2011). Thus, it is important to study the mechano-sensitivity of tumor cells to these forces generated by stromal cells and to understand how ECM remodeling, mechanical regulation and stromal cell activities contribute to cancer progression.

In vitro Mechano-Invasion Assay

As mentioned earlier, contractile stromal cells such as myofibroblasts can produce pulling forces in the tumor microenvironment. We have developed an *in vitro* mechano-invasion assay, where cells can be subjected to similar pulling forces in an environment free of any cell secreted factors and the effect of these forces on the invasion response of the cells can be observed. The general set-up of the assay is illustrated in figure 1. In preparation for the invasion assay, substrates made of collagen and fibronectin were prepared. These two proteins are the most abundant ECM proteins in the tumor stroma. Paramagnetic beads, functionalized by carboxylation for protein binding, were also mixed with these collagen/fibronectin substrates. Mechanical stimulation was provided by holding these substrates over a rare earth magnet that is rotated on

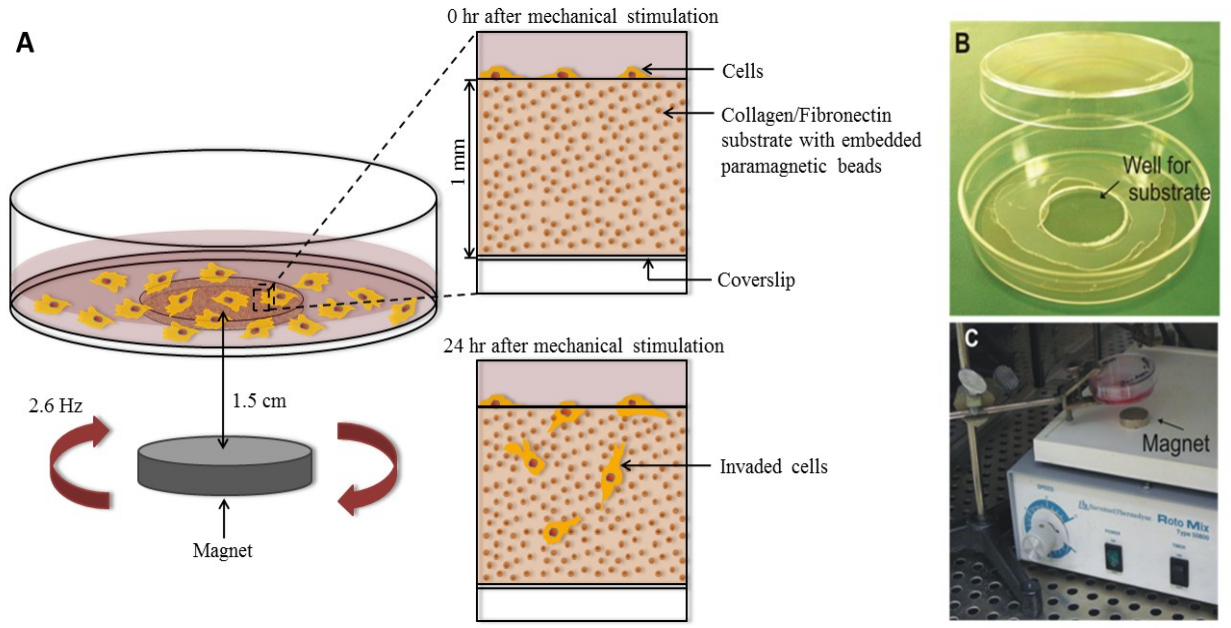


Figure 1. Design of *in vitro* mechano-invasion assay. A) A well is created in a 60mm culture dish and filled with a collagen type-I/fibronectin matrix containing 1 µm carboxylated paramagnetic beads. Cells are seeded onto the surface of the matrix and either cultured outside of a magnetic field or cultured 1.5 cm 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 actual assay within a typical cell culture incubator. (Menon and Beningo, 2012)

an orbital shaker beneath the culture. The human fibrosarcoma cell line, HT1080, was used for this assay. HT1080 cells cultured on collagen/fibronectin matrices incubated without magnetic stimulation were used as controls (Figure 1).

Magnetic stimulation was provided for 24 hours and the number of cells that invaded into the matrix were counted using an inverted phase microscope. HT1080 cells under mechanical stimulation showed enhanced invasion (2-fold) compared to control cells. Similar enhanced invasion was also seen at 36 hours of mechanical stimulation (Figure 2A). This enhanced invasion response by HT1080 cells was stable for frequencies of bead stimulation ranging from 8 to 160

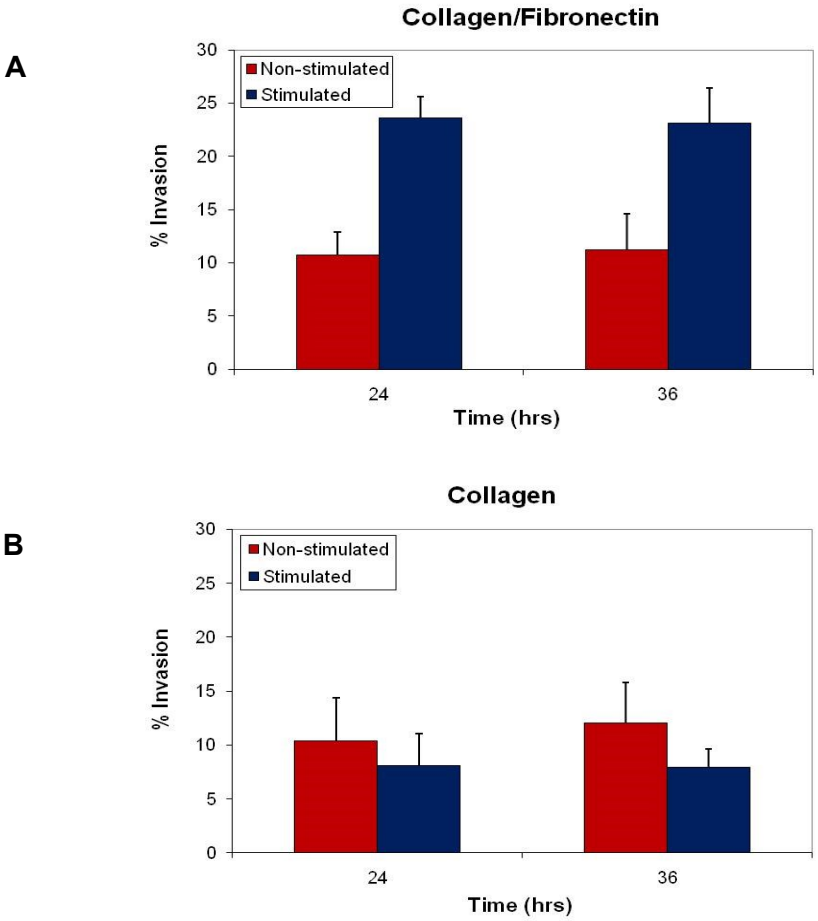


Figure 2. Cancer cells show enhanced invasion in response to mechanical stimulation in the presence of fibronectin. A) HT1080, human fibrosarcoma cells were seeded onto collagen/fibronectin matrices containing paramagnetic beads. 25 fields of cells were counted at multiple depths within the substrate (beginning at the surface of the field and progressing towards the bottom of the plate – farthest depth of invasion was 800 μm) at 24 and 36 hours after seeding. The percent of invading cells was 2-fold higher in stimulated cultures when compared to controls (** P=0.0120). B) The assay was performed as in (A) however only collagen type I was used (2.5mg/ml). (Menon and Beningo, 2011)

rpm. Furthermore, non-invasive mouse embryonic fibroblast (MEF) cells failed to invade in the presence, as well as the absence, of mechanical stimulation, demonstrating that in order to invade upon mechanical stimulation, cells must have a pre-defined ability to invade.

To test the importance of fibronectin in the process of enhanced invasion, the assay was performed in collagen only substrates and compared to collagen/fibronectin substrates. After 24

hours of mechanical stimulation, cells seeded on collagen only substrates failed to show enhanced invasion compared to non-stimulated controls (Figure 2B). This demonstrates the requirement of fibronectin in the extracellular environment for the cancer cell to sense the mechanical stimulation.

Mechanosensing and Mechanotransduction

In order for the cell to respond to mechanical cues in its surrounding, the cells must first be able to sense these stimuli. The process of sensing mechanical stimuli is known as mechanosensing. Secondly, cells should be able to transduce these stimuli within their cytoplasm resulting in changes in gene expression and intracellular biochemistry of the cell, by the process known as mechanotransduction (Wang et al., 2009). The signal assimilated into the cytoplasm is carried forward through a hierarchical mechanochemical signal transduction network that results in the cellular response to the mechanical stimulus. In the case of cancer cells, this response can be in the form of increased proliferation and invasion or secretion of proteases for matrix remodeling (Kumar and Weaver, 2009). Since the mechanical signal spreads once it crosses the cell membrane, this conversion of the mechanical signal into a biochemical signal likely takes place nearing close proximity to the cell membrane. Indeed, many different transmembrane proteins that connect the ECM to the inside of the cell can act as mechanotransductors (Orr et al., 2006). Integrins are one of the most studied families of transmembrane receptors known to act as mechanotransductors. As the name suggests, they integrate the outside of the cell with the inside. However, additional membrane receptors can also act as mechanotransductors. For example, abolishment of G-protein coupled receptors (GPCR) in neutrophils has been shown to inhibit the cellular response to fluid shear stress, implicating GPCRs as mechanosensors (Makino et al., 2006). Unlike all the above mentioned receptors, which bring about mechanotransduction

between ECM and cell, E-cadherin transduces the mechanical signal through cell-cell junctions with the help of other binding partners such as α-catenin and vinculin (Smutny and Yap, 2010).

Integrins are heterodimeric glycoproteins composed of one α-subunit and one β-subunit. There are eighteen α-subunits and eight β-subunits which non-covalently pair with each other to form 24 different integrin molecules (Plow et al., 2000). Both α and β-subunits have larger extracellular domains with which they bind to a specific ligand in the extracellular matrix. The cytoplasmic tail of these subunits is non-catalytic and shorter compared to the extracellular domain. The cytoplasmic tail of the β-subunits bind directly to the actin cytoskeleton of the cell, thus providing a connection between the outside and the inside of the cell (Rathinam and Alahari, 2010; Springer and Wang, 2004). The cellular response to different mechanical forces such as, fluid shear stress, stretch, hydrostatic pressure, has been shown to be dependent on the type integrin that is engaged (Katsumi et al., 2004). Since many signaling cascades are controlled downstream of integrins, these mechanical forces can activate a variety of pathways. The outcome of this phenomenon changes from cell type to cell type and is also dependent on the type of integrin and mechanical forces involved (Schwartz and DeSimone, 2008). Considering their important role in mechanosensing and mechanotransduction, integrins have been implicated in cancer cell proliferation and migration. Even though detailed specifics vary with cancer type, in general the integrin gene expression in cancer cells is found to be in disarray. These defective expression levels of integrins, also lead to abnormal signaling downstream causing the progression of cancer (Guo and Giancotti, 2004). Higher expression levels of certain integrin subunits such as α3, α5, α6, αv, β1, and β3 have been correlated with greater metastatic potential of cancer cells, whereas, some integrins such as α2β1 have been associated with decreased cell proliferation (Wiseman and Werb, 2002).

Integrin β3

Integrin β3 is one of several β-subunits of integrin receptors which non-covalently binds with the αv or αIIb subunits (Hynes, 2002). β3 containing integrins bind to Arg-Gly-Asp (RGD) sequence containing ligands in the extracellular matrix, which include fibronectin, vitronectin, and fibrinogen (Xiong et al., 2002). Expression levels of integrin β3 coupled with integrin αv are shown to be variable in different types of cancers. They are highly up-regulated in cases of melanoma, squamous cell carcinoma and ovarian cancer. Whereas, down-regulated in cases of kidney and colon cancer (Mizejewski, 1999). Integrin β3 has been shown to affect different processes during cancer development and progression such as cell proliferation, apoptosis, angiogenesis and cell invasion (Jin and Varner, 2004). Integrin αvβ3 up-regulation is known induce cell death (Stupack et al., 2001). This agrees with the recent finding that down-regulation of ITGB3 in glioma and hepatocellular carcinoma renders cancer cells resistant to apoptosis and therefore causes aggressive tumor growth (Kim et al., 2011; Wu et al., 2009). Up-regulation of ITGB3 has been seen in melanoma and breast cancer and it is associated with increased tumor invasion and metastasis (Albelda et al., 1990; Gasparini et al., 1998). As integrin β3 is known to aid in the process of tumor progression by increasing cell proliferation and invasion, it has become a potential target for cancer therapy. Different types of blocking peptides and antibodies against integrin β3 are currently in clinical and pre-clinical trials (Eskens et al., 2003; Smith, 2003; Stoeltzing et al., 2003).

Integrin Crosstalk

As mentioned earlier, integrin heterodimers bind to specific ligands in the ECM. Integrins in the ligand bound unfolded conformation are said to be “active”, whereas those in unbound folded conformation are said to be “inactive” (Takagi et al., 2002). It has been observed that when

activity of one integrin subunit or the entire heterodimer is inhibited, it sometimes causes perturbation of ligand binding ability of another integrin family member within the same cell. This phenomenon where the activity of one integrin is controlled by other integrin is referred to as "integrin crosstalk" (Diaz-Gonzalez et al., 1996; Gonzalez et al., 2010). Evidence of integrin crosstalk has been reported between integrin β3 and integrin β1. Integrin β1 is also one of the β subunits which can form heterodimers with 12 different α subunits. Integrin α5β1 is a RGD-binding heterodimer, like integrin β3, and it primarily binds to fibronectin (Hynes, 2002). In endothelial cells, reciprocal inhibitory activity of integrin αvβ3 and integrin α5β1 has been observed (Brooks et al., 1994; Eliceiri and Cheresh, 1999). Moreover, in vivo experiments in mice have shown that down-regulation of integrin αvβ3 expression during tumor associated angiogenesis leads to increased activity of integrin β1 (Reynolds et al., 2002). This integrin crosstalk is regulated by different proteins binding to the cytoplasmic tail of the integrins, for example talin. In some instances, it is also regulated by the phosphorylation state of the integrin cytoplasmic tails. There is also evidence that this integrin crosstalk is brought about, not only at the protein level, but also at the level of mRNA. It has been shown that integrin β1 destabilizes the mRNA encoding integrin β3, therefore decreasing the activity of integrin αvβ3 (Retta et al., 2001). Activity of a particular integrin matters because downstream signaling under different integrins is different (Morgan et al., 2009). Thus, the same type of cell having different integrin activity can produce totally different cellular response. For example, it has been demonstrated that the activity of Rho GTPases is regulated differently downstream of integrin αvβ3 and α5β1 (Morgan et al., 2009). Cells with active β3 integrins have low RhoA activity and show persistent migration, conversely, cells with active β1 integrins have high RhoA activity and show random migration (Danen et al., 2005).

Invadopodia: Mechanosensory Protrusions

In 1971 Gabbiani and colleagues first discovered specialized actin-rich protrusions in Rou-

s sarcoma virus (RSV) transformed chicken embryonic fibroblasts making contacts with the ECM and having ECM degradation properties (Gabbiani et al., 1971). These protrusions were named invadopodia and were found to be a characteristic unique to invasive cancer cells and transformed cells. Matrix degradation by invadopodia enhances the process of cancer metastasis *in vivo*, by helping cancer cells breach the basement membrane and navigate through the dense stromal ECM. The process of breaching the basement membrane via invadopodia consists of three stages; 1) formation of invadopodia, 2) maturation of invadopodia by their elongation and recruitment of ECM degrading proteases and finally, 3) degradation of ECM (Schoumacher et al., 2010). The actin core formation is the principle step in invadopodia formation. Hence, different molecules required for actin nucleation and polymerization such as, Arp2/3, N-WASP, cortactin, have been shown to be required for this process. However, the molecule that stands out from these is an actin severing protein cofilin. Cofilin has been shown to be required only for invadopodia maturation and not for the formation stage. When cofilin is knocked down, cells give rise to short-lived invadopodia which are not capable of ECM degradation (Yamaguchi et al., 2005).

Cofilin is an actin binding protein that belongs to a family of “actin depolymerizing factors (ADF)” (Maciver and Hussey, 2002). Cofilin is actively involved in actin tread-milling as it binds to capped ends of the actin filaments and severs them. This function of cofilin is important in two ways; 1) the severing action keeps on refilling the actin monomer pool, 2) it creates more barbed ended actin filaments which can be further elongated (Pollard and Borisy, 2003). The actin depolymerization activity of cofilin is controlled by its phosphorylation at a serine-3 near the N-terminus by LIM-kinase. When phosphorylated, cofilin cannot interact with ADP-actin filaments of monomers and therefore, inactivated (Blanchoin et al., 2000). Rho family GTPases indirectly control the cofilin activity. Active Rho family GTPases activate PAK kinase which in turn activates

LIM-kinase leading to cofilin phosphorylation (Edwards et al., 1999). A phosphatase called slingshot causes dephosphorylation of serine residue and activates cofilin (Niwa et al., 2002). By virtue of its actin depolymerizing ability, cofilin plays an important role in cell motility. Recently it has also been observed that expression profiles of cofilin and its regulators are highly disturbed in invasive cancer cells (Wang et al., 2007). Moreover, in our *in vitro* invasion assay, we found that cofilin knock-down cells fail to show enhanced invasion in response to mechanical stimulus in their microenvironment (Figure 3; Menon and Beningo, 2012). Even though cofilin is closely related to invadopodia maturation and cancer cell invasion, its exact role in the process is still unclear.

In addition to cofilin, many other invadopodia associated proteins have been shown to be required for tumor growth and metastasis (Blouw et al., 2008; Clark et al., 2009). However, the effect of external mechanical forces on the activity of invadopodia is still unknown. Recently it was shown that increased stiffness of ECM leads to enhancement in the activity and number of invadopodia (Alexander et al., 2008). Moreover, localization of active forms of mechanosensing

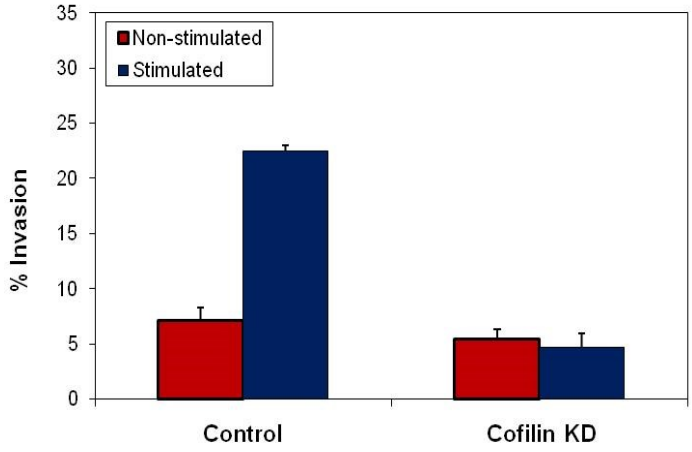


Figure 3. Cofilin is required for enhanced invasion upon mechanical stimulation. HT1080 cells nucleofected with control siRNA or Cofilin siRNA and cultured for 48 hours were seeded onto collagen/fibronectin matrices containing paramagnetic beads. The cells were cultured with or without stimulation for 48hr hours and the percent of invading cells was calculated. Stimulated cells had 3-fold higher invasion as compared to non-stimulated cells (*P*= 0.0065) (Menon and Beningo; 2012).

proteins such as FAK (focal adhesion kinase) and p130Cas suggests that invadopodia are sensitive to mechanical forces in the tumor microenvironment (Parekh and Weaver, 2009). Therefore, the molecular dissection of the process of invadopodia formation and maturation in response to mechanical forces will be interesting.

In conclusion, the tumor stroma serves as a potent carcinogen by being a source of numerous mechanical factors. Recent findings suggest that many of these factors are both necessary and sufficient for the process of cancer progression. Even though influence of mechanical factors is positively linked with enhanced invasion, it is important to integrate this information into our existing knowledge of the molecular and cell biology of cancer. Identification of signaling pathways and molecules related to mechanosensing in cancer can provide new directions to chemotherapeutics, although much remains to be uncovered.

CHAPTER 2 - METHODS

Cell Culture

All the experiments in this study were performed with human fibrosarcoma cells (HT1080) purchased from ATCC. As per ATCC recommendation, these cells were cultured in Eagle's Minimum Essential Medium (EMEM; ATCC) along with 10% fetal bovine serum (FBS; Hyclone) and 1% Penicillin-Streptomycin solution (100 U/ml of penicillin and 100 µg/ml of streptomycin; Gibco by Life Technologies). The cells were maintained in a culture incubator at 37^{o}C with 5% CO_2. Cells were allowed to grow up to 75-80% confluency before passing them into fresh culture dishes. For this purpose, cells were trypsinized with 0.25% trypsin (Sigma) and proteolysis was stopped by addition of fresh complete media. For all experiments, cells up to consecutive sixth passage were used. After the sixth passage all cells were discarded.

Invasion Matrices

Invasion assay matrices were composed of 2.5 µg/ml collagen type I (PureColl, Advanced Biomatrix), 20 µg/ml fibronectin (Sigma). 4 µl of 1 µm carboxylated paramagnetic microspheres (Polysciences Inc.) were added for each 1 ml of matrix solution to provide magnetic stimulation. 0.1N NaOH and 10X PBS were used to adjust the pH to 7.4 +/- 2. All the components were kept cold and mixed together at 4^{o}C. 450 µl of this solution was then polymerized in a culture well, by incubating at 37^{o}C for 30 min. The culture well, 1 mm deep and 2 cm in diameter, was created in 60 mm culture dish (Nunclon) by drilling a hole and attaching an activated coverslip (described in Beningo and Wang, 2002) at the bottom. During polymerization a 25 mm coverslip was dropped on top of the matrix to make the surface flat. For mRNA and protein extraction, 1.1 ml of matrix

mix of the same composition was polymerized in larger wells of 1 mm depth and 3 cm in diameter. The polymerization was carried out at 37^{o}C for 60 min. After polymerization, 4 ml of warm media was added to the plates and coverslips on the top were removed. The matrices were then sterilized under ultraviolet light for 15 min within a laminar flow culture hood. “Collagen only” matrices were prepared in the same manner without adding fibronectin to the matrix mix.

In vitro Mechano-Invasion Assay

For the invasion assay, 1.5 X 10^4 cells were counted by hemocytometer and seeded onto the sterilized matrix. The cells were allowed to adhere and spread for 1 hour in the incubator at 37^{o}C with 5% CO_2. To provide mechanical stimulation, the culture dish with seeded matrix was held over a rare earth magnet 25 mm in diameter and 5.5 mm in thickness with magnetic field of 12,100 Gauss. The distance between matrix and the magnet was kept constant to 1.5 cm for all experiments. An orbital shaker (Barnstead Thermolyne, Roto Mix-Type 50800) was used to rotate the magnet at 160 rpm (2.6 Hz) below the seeded collagen/fibronectin matrix. Another matrix seeded with cells was incubated separately without magnetic stimulation served as a control. At 24 hours (48 hours for integrin β3 overexpression analysis) the invasion response of cells was quantified by selecting 10 random microscopic fields of matrix under 10X phase objective on an Olympus IX81 microscope. Cells on the surface of the matrix were counted and then invaded cells were counted in eight focal planes separated by 100 μm, from 5 μm to 800 μm below the matrix surface. The percent invasion was counted as a percent of number of invaded cells compared to total number of cells counted.

Collagenase Degradation

A 2 mg/ml solution of collagenase was prepared by dissolving collagenase type 4 (Worthington Biochemical Corporation) in Hank’s Balanced Salt Solution (HBSS, Gibco by Life

Technologies) warmed to 37°C. The media on top of the collagen/fibronectin matrix was aspirated and the matrix was physically removed from the culture well with a spatula. Each matrix (3 cm in diameter) was dropped into a tube containing 2 ml of collagenase solution and kept in water bath at 37°C. With intermittent shaking for approximately 10 min, the collagen matrix was degraded. Cells were separated by centrifugation at ~500 x g for 5 min in eppendorf centrifuge 5810 R at 37°C. The resulting pellet contained both whole cells and the paramagnetic beads from the collagen/fibronectin matrix. The pellet was washed with sterile 1X PBS at 37°C.

RNA Extraction

The RNA was extracted from invaded cells after the matrix had been degraded as mentioned above. The original matrices had been seeded with 7 X 10^4 HT1080 cells. For each independent experiment, control and stimulated matrices were made in duplicates. During collagenase degradation cells from duplicate cultures were pooled together. Qiagen RNeasy Mini kit was used for RNA extraction from pelleted cells. 700 µl of lysis buffer from the kit was added to the pellet. To prevent plugging the column, paramagnetic beads from the pellet were pulled down with a magnet, before loading the lysate on to the column. Genomic DNA contamination was removed by Qiagen on-column DNaseI digestion. RNA was eluted in 20 µl of DNase/RNase free water. The quality of the acquired RNA was tested spectrophotometrically using a NanoDrop spectrophotometer (Thermo Scientific). Only RNA samples having 260/280 ≥ 2.0, 260/230 ≥ 1.7 and concentration ≥ 40 µg/ml were used for further studies.

PCR Array and qPCR Analysis

RNA obtained from both control and mechanically stimulated cells from the invasion assay were used to make cDNA that was used for PCR array as well as qPCR analysis. For each experiment, 1 µg of RNA from each control and stimulated sample was converted into cDNA using

RT² First Strand Kit (SA Biosciences, for PCR array analysis) or GoScript™ Reverse Transcriptase (Promega, for qPCR). To identify differentially expressed genes between non-stimiulated and stimulated conditions from the invasion assay, the following PCR arrays were purchased from SA Biosciences; Cell Motility PCR Array, Tumor Metastasis PCR Array and ECM and Adhesion Molecules PCR Array. PCR array analysis was performed using RT² qPCR SYBR Green/ROX MasterMix-12 (SA Biosciences) on a Stratagene Mx3000P instrument. The raw data obtained were analyzed using the web-based RT^2 Profiler PCR Array Data Analysis software (SA Biosciences). For confirming the differential expression, qPCR primers were designed for the genes showing more than 1.35 fold differential expression in PCR array analysis. For designing qPCR primers online PrimerQuest software (Integrated DNA technologies) was used. The following primers were synthesized; GAPDH (GAPDH-F: 5'-TTCGACAGTCAGCCGCATCTTCTT- 3', GAPDH-R: 5'- ACCAAATCCGTTGACTCCGACCTT-3'), ACT3 (ACT3-F: 5'- CAATGGCCTCAAACTCATGCTGCT- 3', ACT3-R: 5'- TCTCTTCAGCA CCAATGGACACCA- 3'), SRC (SRC-F: 5'- ATCCTACTGTGTGCTGGAAAGCGA- 3', SRC-R: 5'-GGTGCAGATGTTCACAAACAGCCA-3'), MMP14 (MMP14-F: 5'- TGATGGATGGATACCCAA TGCCCA- 3', MMP14-R: 5'- CGCCTCATCAAACACCCAATGCTT- 3'), CTTN (CTTN-F: 5'-TCCA AAGGATTCGGCGGGAAGTAT-3', CTTN-R: 5'-ACCTGGGTGACATCCTCAAAGGTT-3'), DIAPH1 (DIAPH1-F: 5'-TGAAGGCTTGGCCTCCTTATTGGA-3', DIAPH1-R: 5'-TCTCATGCTT GTTCCGGCTATCGT-3'), MYL9 (MYL9-F: 5'-GGCCACATCCAATGTCTTCGCAAT-3', MYL9-R: 5'-AGCCATCACGGTTCTGGTCAATCA-3'), PTK2B (PTK2B-F: 5'-AGAAGTTCATGAGCGAGG CAGTGA-3', PTK2B-R:5'- ATTCCATGATGATCCAGGTGGGCT-3'), ITGB3 (ITGB3-F: 5'-TGGACAAGCCTGTGTCACCATACA-3', ITGB3-R: 5'-TTGTAGCCAAAC ATGGGCAAGCAG-3'), IGF1 (IGF1-F: 5'-TGAAGATGCACACCATGTCCTCCT-3', IGF1-R: 5'-AACTGAAGAGCATCCA CCAGCTCA-3'), MTSS1 (MTSS1-F: 5'-ATCAAGATGGGCTTTGCCGTTTCC-3', MTSS1-R: 5'-

AGCCAAACCGCTCTGTAGGGTATT-3'), TSHR (TSHR-F: 5'-ACCCGTGTGAAGACATAATGGG CT-3', TSHR-R: 5'-AGGACAAAGACATTGCCCAGGAGA-3'). The GAPDH gene was used as the normalizing gene in the analysis. The qPCR analysis of individual genes was performed using RT² qPCR SYBR Green/ROX MasterMix-12 (SA Biosciences) on Stratagene Mx3000P instrument. For every gene, at least two biological replicates were performed and for every biological replicate two technical replicates were performed. The raw data obtained were analyzed using Stratagene Mx-Pro Mx3000P software. Student's t-test was performed to determine the statistical significance of difference in gene expression.

Integrin β3 Overexpression

A plasmid with human ITGB3 gene, pcDNA3.1-beta-3, provided by Dr. Timothy Springer (Addgene plasmid #27289), was used for integrin β3 overexpression in HT1080 cells. HT1080 cells were grown to approximately 85% confluency and nucleofected using the Amaxa Nucleofector 2 with the Amaxa nucleofector kit T (Lonza). 4 µg of pcDNA3.1-beta-3 plasmid were used for each nucleofection. The cells were also cotransfected with pmaxGFP® supplied with the kit, to determine the efficiency of nucleofection. For mock nucleofection, HT1080 cells were also transfected with pmaxGFP or with Amaxa nucleofector kit T (Lonza) alone. After nucleofection, cells were seeded into 100 mm culture dish for 24 hours and incubated at 37°C with 5% CO_2. In case of invasion assays performed for protein extractions, nucleofected cells were directly seeded on to collagen/fibronectin matrices prior to incubation.

Protein Extraction

Triple detergent lysis buffer (TDLB; 50 mM Tris HCL pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) mixed with Protease Inhibitor Cocktail (Sigma), and Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific) was used for protein extraction for

western blot analysis. In studies checking for integrin β3, proteins were extracted from HT1080 cells, mock nucleofected HT1080 cells and HT1080 cells nucleofected with integrin β3 plasmid at 24, 48 and 72 hours post nucleofection. All cells were rinsed with 1X PBS and incubated with TDLB for 20 min under ice-cold conditions. Lysates were centrifuged at 4^{o}C for 10 min at ~5500 x g to remove cell debris. Protein estimation was done using the DC assay (Bio-Rad).

For protein extraction from invaded cells, collagen/fibronectin matrices were prepared as mentioned above and seeded with HT1080 cells, mock nucleofected HT1080 cells or HT1080 cells nucleofected with integrin β3 plasmid. For each experiment, control and stimulated matrices were made in duplicates. After 48 hours of mechanical stimulation, matrices were degraded with collagenase and cells were pelleted down by centrifugation as described above. The cell pellet was incubated with 250 µl TDLB for 20 min under ice-cold conditions. The paramagnetic beads were separated from lysates with a magnet. Lysates were centrifuged at 4^{o}C for 5 min at ~7000g to remove cell debris. The supernatant was again mixed with separated paramagnetic beads, in order to avoid loss of protein bound to paramagnetic beads. This solution was then mixed with 50 µl of 6X lamelli buffer (reducing or non-reducing, based on the antibody to be used) and boiled for 10 min. Immediately following boiling, paramagnetic beads were separated from the solution using a magnet, without letting the solution cool down. Protein estimation was done by the RC-DC assay (Bio-Rad).

Western Blots

Western blots were performed with protein samples collected from HT1080 cells, mock transfected HT1080 cells, and integrin β3 transfected HT1080 cells. Invasion assays were performed with all the above cell conditions and western blots were performed with protein samples collected from each condition following 48 hours of mechanical stimulation. 15 µg – 25

μg of protein was run on a 4-20% Precise™ Protein Gel (Thermo Scientific) for one hour at 100 V. Proteins were then transferred onto a PVDF membrane (Bio-Rad) using a Transblot® SD Semi-Dry Transfer Cell (Bio-Rad) at 20 V for 30 min. Following transfer, the PVDF membrane was blocked for 1 hour using 5% milk in 0.1% PBS/T (for GAPDH and cofilin), 5% milk in 0.1% TBS/T (for integrin β3 and integrin β1) or 5% BSA in 0.1% TBS/T (for phospho-cofilin (ser3)). Primary antibody dilutions were made in the same solutions used for blocking (except for integrin β3 for which 1% milk in 0.1% TBS/T was used) and incubated at 4°C overnight (except for cofilin; 4 hours at RT). The following day, the membrane was washed three times for 10 minutes each either with 0.1% PBS/T (GAPDH and cofilin) or 0.1% TBS/T (phospho-cofilin (ser3), integrin β3 and active integrin β1). Secondary antibody dilutions were made in the same solution as that of primary antibody and incubation was carried out at RT for 1 hour. The following antibodies were used for these studies; rabbit polyclonal integrin β3 antibody (1:300, Santa Cruz Biotechnology), rat monoclonal active integrin β1 antibody (1:5000, BD Pharmingen™), mouse monoclonal cofilin antibody (1:300, Abcam), rabbit monoclonal phospho-cofilin (ser3) antibody (1:1000; Cell Signaling Technology), mouse monoclonal GAPDH antibody (1:15000; Millipore), HRP tagged anti-mouse antibody (Fisher), HRP tagged anti-rat (Abcam), HRP tagged anti-rabbit (Amersham GE Healthcare). GAPDH served as a loading control in all experiments. Following secondary antibody incubation, membranes were washed again with 0.1% PBS/T or 0.1% TBS/T three times for 10 minutes each and for detection incubated with Amersham ECL Prime Western Blotting Detection Reagent. After development, band intensity readings were taken using ImageJ software.

CHAPTER 3 - RESULTS

Many types of mechanical forces are produced in the tumor stroma. With the growing awareness about these mechanical forces, a new door for research has opened with respect to their effect on the process of metastasis. Despite the different known mechanical forces produced in the tumor microenvironment, efforts have focused heavily on only one kind of mechanical force, increased rigidity of the stroma. Recently it has been shown that the rigidity of tumor stroma is responsible for an increase in number and activity of invadopodia as well as cell proliferation and dissemination (Kostic et al., 2009; Levental et al., 2009; Paszek et al., 2005). We have developed an in vitro mechano-invasion assay, where we tested the impact of mechanical stimuli in the form of pulling forces on the cells ability to invade the stroma. We have found a significant increase in the invasion efficiency of cells in this mechanically stimulated culture environment compared to that in a non-stimulated culture environment (Menon and Beningo, 2011). However, the mechanistic details about this process of mechanosensing still remain elusive. Our objective is to uncover candidate genes involved in this mechanosensing pathway, which ultimately leads to cancer metastasis. Based on our preliminary data, we hypothesized that in response to mechanical forces in the stroma, cancer cells will show an altered expression of genes involved in mechanosensing. To test our hypothesis, we used real-time PCR analysis to identify differentially expressed genes in mechanically stimulated cancer cells compared to non-stimulated cancer cells.

RNA Extraction Standardization

In order to obtain an RNA yield sufficient for real-time PCR experiments, the previously described *in vitro* invasion assay was scaled up. For scaling up, HT1080 cells were seeded on larger collagen/fibronectin substrates (3 cm in diameter) and mechanical stimulation was provided

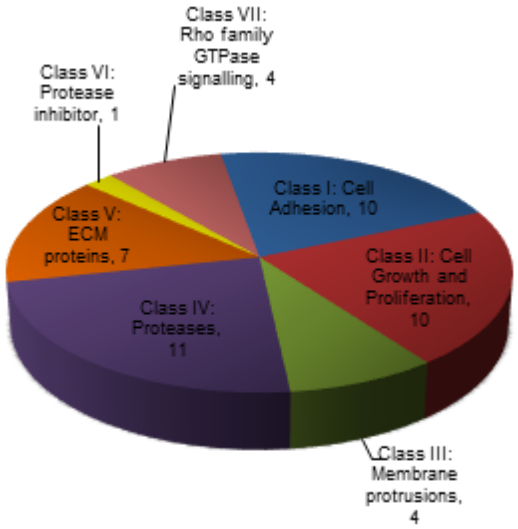
for 48 hours. For real-time PCR it is recommended to use RNA with 260/280 ≥ 2.0 and 260/230 ≥ 1.7, in order to have RNA free of protein and chemical contamination. As the cells were invaded into the collagen/fibronectin matrix, it was necessary to degrade the matrix to isolate the cells for RNA extraction. For standardization of matrix degradation and RNA extraction two different methods were used. In the first method, the collagen/fibronectin matrix was degraded with TRIzol, which degrades the matrix and lyses the cells simultaneously. Therefore, the matrix degradation will be directly followed by RNA extraction with chloroform as per the TRIzol RNA extraction protocol. Despite numerous attempts, this method consistently produced values of 260/230 ratio significantly less than 1.7 indicating phenol contamination in the purified RNA. As an alternative approach, the degradative enzyme collagenase in Hanks's balanced salt solution at 37^{o}C was used to degrade the matrix. However, as collagenase is known to have a low tryptic and protease activity, the concentration of collagenase solution to be used for matrix degradation, without damaging the cells, required standardization. The concentration of collagenase was standardized to 2 mg/ml which degraded the matrix within 10 min. The cells were separated by centrifugation and RNA was extracted with the Qiagen RNeasy mini kit. Any genomic DNA contamination was removed by Qiagen on-column DNaseI digestion. An RNA with sufficient concentration and of high quality was consistently obtained using this method and therefore it was used for RNA extraction.

Identification of Differentially Expressed Genes by PCR Array Analysis

For identification of differentially expressed genes under stimulated and non-stimulated conditions, three sets of PCR arrays were purchased from SA Biosciences. Each PCR array contained primers against 84 candidate genes related to cell motility, tumor metastasis and ECM and adhesion molecules. The RNA was obtained from mechanically stimulated and non-stimulated cells as described above and was then used to prepare cDNA. This cDNA was used

for PCR array analysis. The gene expression was normalized with five housekeeping genes, actin (ACTB), β-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and large ribosomal protein (RPL13A). The differentially expressed genes identified by PCR array analysis were classified into 7 groups based on their function (Figure 4). With cut-off value of 1.35, 46 genes were found to be differentially expressed, out of which 39 genes were up-regulated (Table 1) and 8 genes were down-regulated (Table 2).

From the differentially expressed genes listed in table 1 and 2, select genes were chosen for further confirmation of gene expression by qPCR. The factors for selection were based on literature survey, genes with known association with cancer cell invasion and genes associated with mechanosensing. The genes chosen were, CTTN (cortactin), DIAPH1 (diaphanous homologue 1), SRC (V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog), TSHR (thyroid stimulating hormone receptor), ITGB3 (integrin β3), MTSS1 (metastatic suppressor 1),



Gene Class	No. of Up-regulated Genes	No. of Down-regulated Genes
Class I: Cell Adhesion	4	6
Class II: Cell Growth and Proliferation	10	0
Class III: Membrane protrusions	4	0
Class IV: Proteases	10	1
Class V: ECM proteins	7	0
Class VI: Protease inhibitor	1	0
Class VII: Rho family GTPase signaling	2	1

Figure 4. Differentially expressed genes identified by PCR array. Genes found to be more than equal to +/- 1.35-fold differentially expressed upon mechanical stimulation were classified into seven groups. The pie chart illustrates the number of differentially expressed genes in each class. The table demonstrates the number of up-regulated and down-regulated genes within each class.

Up-regulated genes (> 1.35 fold)					
Gene name	**Fold regulation**	**Gene name**	**Fold regulation**	**Gene name**	**Fold regulation**
Cell adhesion genes		**Membrane projections**		**ECM proteins**	
BCAR1	1.40	CTTN†	1.47	COL14A1	2.35
LAMA2	1.62	DIAPH1†	1.49	COL7A1	1.75
PNN	1.42	RDX	1.39	VCAN	1.72
ROCK1	1.37	SRC†	1.79	HAS1	1.61
				SPP1	1.48
Cell growth and proliferation					
		Proteases		THBS3	1.64
CCL7	3.22	ADAMTS13	1.57	VTN	1.64
CXCR4	1.43	ADAMTS8	1.69		
				Protease inhibitor	
DENR	1.43	MMP10*	2.59 +/- 0.8		
GNRH1	1.86	MMP12	2.20	KAL1	1.47
KISS1R	1.70	MMP13	1.73		
				Rho family GTPase	
HTATIP2	2.31	MMP15	2.27		
IL8RB	1.59	MMP16	1.51	ARHGDIA	1.37
SET	1.39	MMP2	1.55	RAC2	1.39
TSHR†	5.46	MMP7	1.40		
VEGFA	1.45	SPG7	1.84		

Table 1: Up-regulated genes in mechanically stimulated cells obtained by PCR array analysis. (*genes tested in more than one array; †inconsistent results)

Down-regulated genes (> -1.35 fold)			
Gene name	**Fold regulation**	**Gene name**	**Fold regulation**
Cell adhesion genes		**Integrin mediated signaling**	
			-1.73 +/-
ACTN3**	-3.89	ITGB3*	0.2
CLEC3B	-2.17	**Proteases**	
MYL9**	-1.57	MMP8	-2.13
MTSS1**	-1.37	**Rho family GTPase signaling**	
PTK2B**	-1.67	RHO	-1.58

Table 2: Down-regulated genes in mechanically stimulated cells obtained by PCR array analysis. (*genes tested in more than one array; **genes tested individually by qPCR)

PTK2B (protein tyrosine kinase 2B), MYL9 (myosin light chain 9), ACTN3 (actinin 3), MMP14 (matrix metalloproteinase 14). Similar to PCR array analysis, qPCR was performed with RNA extracted from cells incubated under stimulated and non-stimulated conditions for 48 hours. For every gene, at least two biological replicates were used and for every biological replicate two technical replicates were performed. The housekeeping gene GAPDH was used for normalization of gene expression. After repeating the qPCR, CTTN, DIAPH1, SRC and TSHR did not show a consistent change in gene expression ($P > 0.05$). However, differential expression of ITGB3, MTSS1, PTK2B, MYL9, ACTN3 and MMP14 were confirmed by qPCR. Out of these genes, ITGB3, MTSS1, PTK2B, MYL9 and ACTN3 were confirmed to be down-regulated in response to

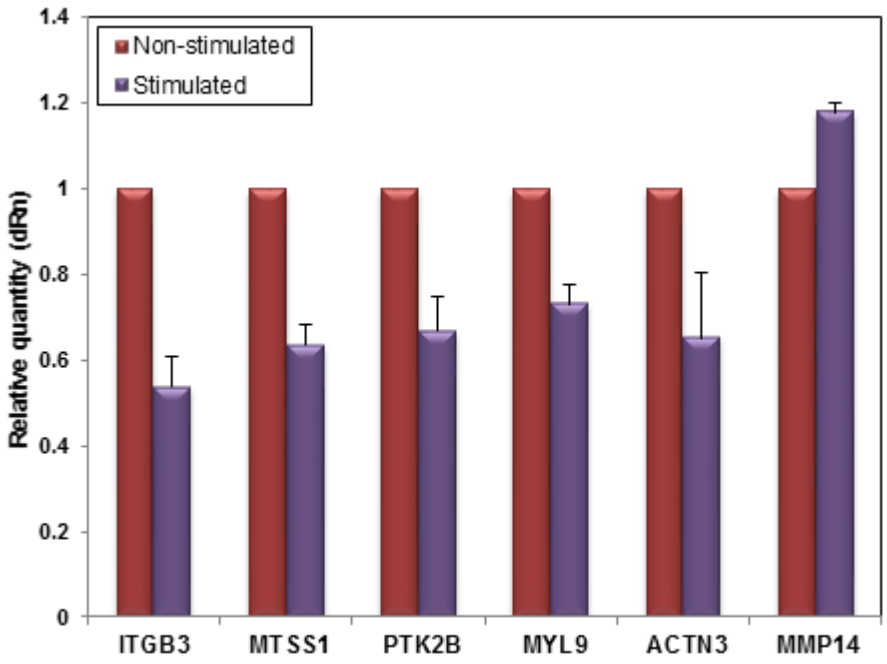


Figure 5. Genes confirmed to be differentially expressed upon mechanical stimulation. ITGB3 – β3 integrin; MTSS1 – Metastasis suppressor 1; PTK2B – Protein kinase 2 β; MYL9 – Myosin light chain 9; ACTN3 – Alpha 3 actinin; MMP14 – Matrix metalloproteinase 14. Above differentially expressed genes were selected for further confirmation by qPCR. Two biological replicates were used for qPCR of MMP14; three biological replicates were used for qPCR of MTSS1, ITGB3, MYL9 and ACTN3; four biological replicates were used for qPCR of PTK2B. For every biological replicate two technical replicates were also performed. Statistical significance was tested by performing Student's t-test ($P<0.05$).

mechanical stimulation, whereas, MMP14 was up-regulated after mechanical stimulation (Figure 5).

Down-regulation of the Integrin β3 Protein upon Mechanical Stimulation

From the above confirmed differentially expressed genes, ITGB3 was chosen for further analysis for a number of reasons. ITGB3 codes for integrin β3, one of the β subunits of membrane receptors called integrins. Integrins bind to extracellular membrane proteins and are known to relay signals from outside of the cell to inside and vice-versa and they are considered to be potential mechanosensors (Rathinam and Alahari, 2010). Integrin β3 has been found to be differentially expressed in different types of cancer and this differential expression is associated with increased cell proliferation and invasion (Jin and Varner, 2004). More importantly, integrin β3 pairs with integrin αv or integrin αIIb to form heterodimeric integrin molecules. These integrin β3 containing heterodimers are known to bind Arginine-Glycine-Aspartic acid (RGD) domains of

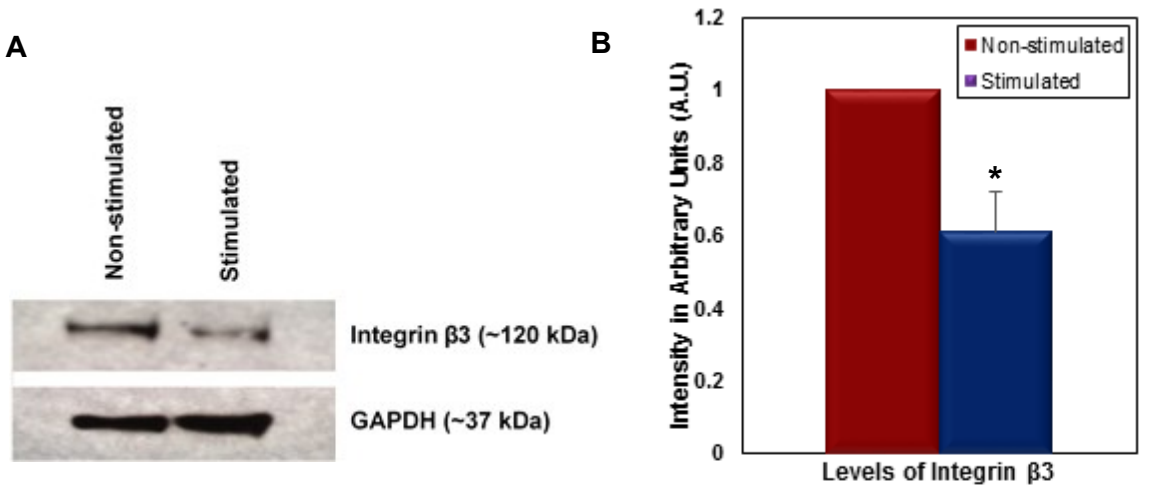


Figure 6. Transcriptional down-regulation of integrin β3 is also reflected at protein level. A) HT1080 cells were seeded on collagen/fibronectin matrix and incubated with or without mechanical stimulation for 48 hours. Protein was extracted from cells separated out of the matrix by collagenase treatment. Western blot confirmed the down-regulation of integrin β3 at protein level under stimulated condition compared to non-stimulated condition. B) Graph illustrating approximately 0.6 times down-regulation of protein expression of integrin β3 after stimulation. Significance was tested using Student's t test (P = 0.0059).

fibronectin (Xiong et al., 2002). As fibronectin has been shown to be required for enhanced cellular response in our invasion assay (Menon and Beningo, 2011), the down-regulation of integrin β3 upon mechanical stimulation was intriguing.

Given that we were sure β3 levels were down-regulated at the transcriptional level, we confirmed the down-regulation of integrin β3 at translational level. To test for β3 protein levels, HT1080 cells were seeded on larger collagen/fibronectin substrates and incubated with and without mechanical stimulation for 48 hours. After 48 hours, cells were removed from within the matrix by collagenase treatment and protein was extracted from them using triple detergent lysis buffer. Western blot analysis performed using these cell lysates showed down-regulation of integrin β3 protein levels when mechanically stimulated in the invasion assay (Figure 6A). Cell lysates from mechanically stimulated cells had approximately 0.6 times integrin β3 compared to that from non-stimulated cells. This value was comparable to gene expression levels, where stimulated samples contained 0.5 times ITGB3 mRNA compared to non-stimulated samples (Figure 5 and 6B).

Enhanced Invasion is Inhibited by Over-expression of Integrin β3

Once the down-regulation of integrin β3 at transcriptional and translational levels was confirmed, we addressed the functional significance of this down-regulation. Naturally we speculated that its down-regulation is directly involved in sensing the mechanical stimulus. However, it is entirely possible that down-regulation of integrin β3 is just an outcome of enhanced invasion and not required for sensing the mechanical stimulus. To test the functional significance of the down-regulation of integrin β3 we over-expressed integrin β3 in HT1080 cells and tested the invasion response of these cells in the mechano-invasion assay. If the down-regulation of integrin β3 is required for cells to sense the mechanical stimulus and show enhanced invasion re-

sponse, overexpression of integrin β3 should inhibit the enhanced invasion observed in these cells.

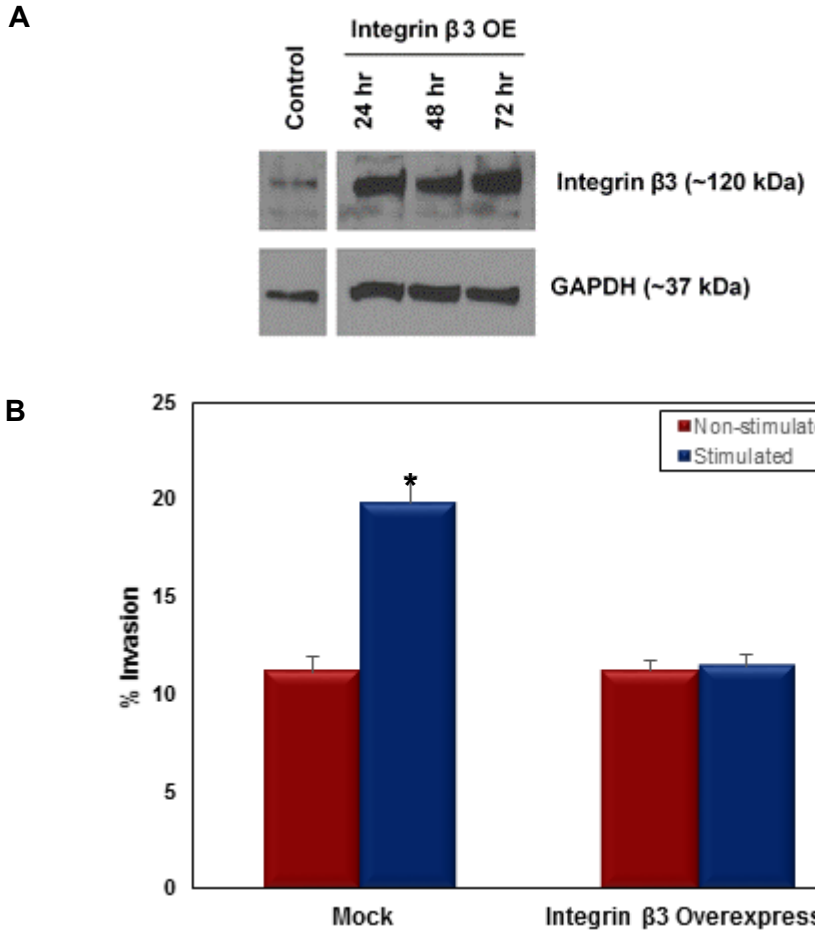


Figure 7. Down-regulation of integrin β3 is required for enhanced invasion. A) HT1080 cells nucleofected with pCDNA3.1-beta3 plasmid overexpressed integrin β3 by approximately two-fold for up to 72 hours compared to mock nucleofected HT1080 cells (control). B) Integrin β3 overexpressing cells and control cells cultured for 24 hours were seeded on collagen/fibronectin substrate. The substrates were incubated with or without mechanical stimulation for 48 hours. Number of cells invaded into the substrate were counted in 10 different fields and percent invasion was calculated in comparison with cells on surface of the substrate. Mock nucleofected cells showed 2-fold enhanced invasion in response to mechanical stimulation over non-stimulated condition. Whereas, cells over-expressing integrin β3 failed to show enhanced invasion upon mechanical stimulation and showed basal level of invasion as non-stimulated culture. The significance was tested using two factor ANOVA ($P<0.05$).

Integrin β3 was overexpressed in HT1080 cells by nucleofecting them with pcDNA3.1-beta-3 plasmid. The cells were also co-nucleofected with GFP plasmid to estimate the nucleofection efficiency. Fluorescence microscopy, indicated a 75-80% nucleofection efficiency was obtained for every experiment. HT1080 cells nucleofected with only GFP plasmid were used as control for these experiments. Western blot confirmed that ITGB3 gene was stably overexpressed up to 72 hours and approximately two-fold over-expression was obtained (Figure 7A). After nucleofection cells were incubated at 37^{o}C for 24 hours to let them recover and allow stable over-expression of integrin β3. After 24 hours these cells were seeded on collagen/fibronectin substrates and incubated with or without mechanical stimulation for 48 hours. After 48 hours, the number of cells invaded into the collagen/fibronectin matrix, were counted. It was observed that cells overexpressing integrin β3 failed to respond to mechanical stimulation and did not display enhanced invasion. However, control cells nucleofected with GFP plasmid showed the typical 2-fold enhanced invasion with mechanical stimulation compared to those under non-stimulated conditions (Figure 7B). This confirmed that down-regulation of integrin β3 is required for the process of enhanced invasion and if the integrin β3 down-regulation is inhibited, cells fail to show enhanced invasion in response to mechanical stimulation.

Down-regulation of Integrin β3 is Not Accompanied by Increased Activity of Integrin β1

Numerous instances of integrin crosstalk have been reported, where binding of one integrin to its ligand causes perturbation in the expression levels or activity of other integrins. Such crosstalk is known to occur between integrin β3 and integrin β1 (Gonzalez et al., 2010). Integrin β1 is another fibronectin binding β subunit of integrin. To address the potential of integrin cross-talk between these two integrins we tested if down-regulation of integrin β3 is accompanied by an increase in the activation of integrin β1. A re-evaluation of the PCR array analysis indicated there was no difference in the expression levels of integrin β1 under stimulated and non-

stimulated conditions (Figure 8A). However, as a result of integrin crosstalk between integrin β1 and β3 the regulation may not occur at the level of expression but in the degree of receptor activation of β1. To address this possibility, we tested if levels of active integrin β1 are increased upon mechanical stimulation compared to non-stimulated cultures.

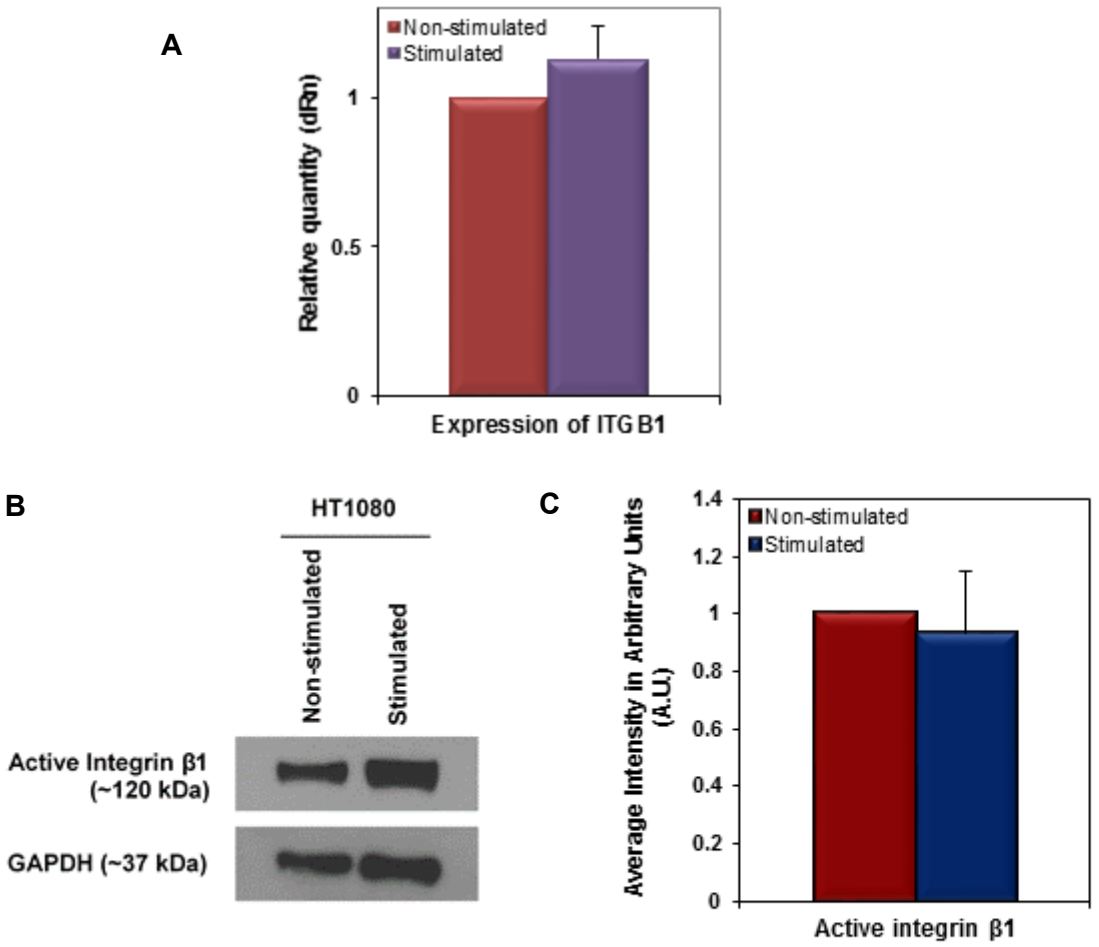


Figure 8. No change in activation of integrin β1 upon mechanical stimulation. A) Expression levels of integrin β1 were found to be similar under stimulated and non-stimulated conditions by PCR array analysis. Statistical analysis was performed by Student's t-test ($P>0.05$) B) HT1080 cells cultured on collagen/fibronectin substrates under mechanically stimulated or non-stimulated conditions for 48 hours. Cell lysates collected from these cells were used to perform western blot for active integrin β1. C) Graph showing average levels of active integrin β1 in four biological samples under mechanically stimulated and non-stimulated conditions. No significant difference between levels of active integrin β1 was observed between stimulated and non-stimulated conditions. Significance was tested using Student's t-test ($P=0.28$).

We used an antibody that specifically recognizes the activated conformation of integrin β1 and probed western blots prepared from HT1080 cell lysates. HT1080 cells incubated with or without mechanical stimulation for 48 hours, were separated from collagen/fibronectin matrix and lysed to collect total protein. Western blot performed with these protein extracts determined that levels of activated integrin β1 upon mechanical stimulation were not significantly different from those in non-stimulated cells (Figure 8B and 8C). Our results suggest that no crosstalk occurs between integrin β1 and integrin B3 in the invasive response observed upon mechanical stimulation.

Down-regulation of Integrin β3 causes Decrease in Levels of Ser3 Phospho-cofilin upon Mechanical Stimulation

To identify potential down-stream pathways of integrin β3, we focused on the relationship between integrins and cofilin. As mentioned previously, cofilin is an actin binding protein that severs actin filaments and have been shown to be required for invadopodia maturation (Yamaguchi et al., 2005). Moreover, in our *in vitro* mechano-invasion assay, cofilin siRNA treated cells failed to sense the mechanical stimulation and did not show enhanced invasion, yet retained basal levels of invasion (Menon and Beningo, 2011). Cofilin activity within the cell is controlled by its phosphorylation at serine-3 by LIM kinase. When phosphorylated at serine-3, cofilin loses its ability to bind and sever actin filaments and is considered to be inactive. The phosphorylation status of cofilin is under indirect control of Rho GTPases (Pollard and Borisy, 2003). As Rho GTPases such as Rho, Rac and Cdc42 are differentially regulated under different integrins, differences in integrin signaling might also affect down-stream cofilin activity. We hypothesized that under mechanically stimulated conditions levels of Ser3 phospho-cofilin (inactive) were lower compared to those in non-stimulated conditions, leading to enhanced invasion. In addition, if the

cofilin activity is dependent on integrin β3 expression, overexpression of integrin β3 should increase the levels of phospho-cofilin upon mechanical stimulation.

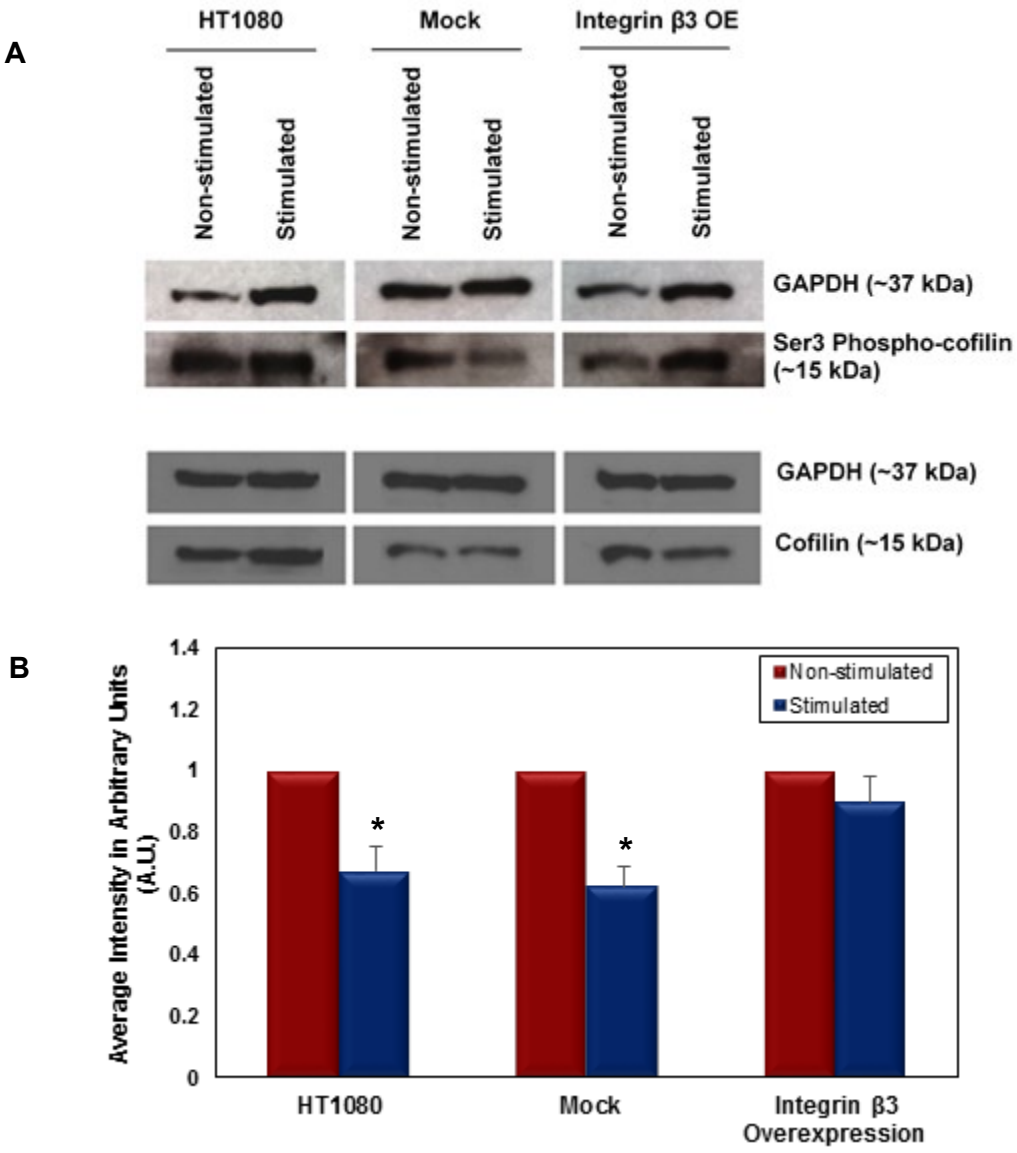


Figure 9. Decrease in levels of Ser3 phospho-cofilin upon mechanical stimulation is dependent on down-regulation of integrin β3. A) Cell lysates from HT1080s, HT1080s overexpressing integrin β3 and mock nucleofected HT1080 cultured on collagen/fibronectin substrate with or without stimulation for 48 hours, were used for western blot for Ser3 phospho-cofilin and total cofilin. B) Quantitation of band intensities showed significant decrease in phospho-cofilin in HT1080 and control cells upon stimulation, but not in integrin β3 overexpressing cells. Significance was tested using two-factor ANOVA ($P<0.05$).

To test our hypothesis, cell lysates were collected from HT1080 cells cultured on collagen/fibronectin substrates and incubated with or without mechanical stimulation for 48 hours. The western blot performed with these cell lysates determined that levels of Ser3 phospho-cofilin were indeed decreased under mechanically stimulated conditions compared to non-stimulated conditions indicating that more cofilin was likely to be active (Figure 9A). Cell lysates collected from mock nucleofected cells also showed similar decrease in Ser3 phospho-cofilin upon mechanical stimulation. Quantitation of band intensities found that Ser3 phospho-cofilin upon mechanical stimulation were approximately 0.65 times lower when compared to the non-stimulated samples in both HT1080 cells and mock nucleofected HT1080 cells (Figure 9B). However, when integrin β3 was over-expressed within the cells, the effect of mechanical stimulation on Ser3 phospho-cofilin level was reversed, such that similar levels of Ser3 phospho-cofilin were observed in integrin β3 overexpressing HT1080 cells with or without mechanical stimulation (Figure 9A and 9B). In addition, the amount of total cofilin was unchanged in HT1080 cells, integrin β3 overexpressing HT1080 cells and mock nucleofected HT1080 cells, between stimulated and non-stimulated conditions (Figure 9A). Our data strongly suggests that the amount of active cofilin increases in response to mechanical stimulation and this increase in active cofilin is brought about by down-regulation of integrin β3.

CHAPTER 4 - DISCUSSION

Metastasis is a multistep process controlled by various biochemical and mechanical factors in the tumor microenvironment. Secondary tumors formed by metastatic cancer cells are the leading cause of death in cancer patients. In order to recognize potential therapeutic targets against metastasis, it is important to understand the effects of factors that instigate the process in the first place. In this study, I have focused on the effects of mechanical factors in the tumor microenvironment that can enhance cancer cell invasion. In our *in vitro* mechano-invasion assay we used magnetic beads embedded within the collagen/fibronectin matrix to mimic the pulling forces produced by cellular movements within the extracellular matrix (Menon and Beningo, 2011). Under the effect of these pulling forces, a greater number of cancer cells will invade into the ECM (Figure 2). In this study I wanted to identify molecular players in the process of mechanosensing that eventually leads to the enhanced invasion of cancer cells in our assay. My approach was to identify genes that are differentially expressed between mechanically stimulated and non-stimulated conditions by qPCR. As mRNA is the least stable biomolecule within the cell, it was challenging to standardize a method for RNA extraction from invaded cells. With the help of collagenase, invaded cells were taken out of the matrix within 15 min and RNA was successfully extracted from them.

Using PCR arrays I was able to screen over 200 genes that could be differentially regulated between mechanically stimulated and non-stimulated conditions. Candidate genes were identified and categorized based on their function. These genes belonged to the following categories; cell adhesion, cell growth and proliferation, membrane protrusions, proteases, ECM proteins, protease inhibitors, Rho family GTPase signaling (Figure 4). The pattern of overall gene regulation in some of the above categories was interesting. For instance, in the case of cell adhesion genes, a greater number of genes were down-regulated than up-regulated. This

observation is consistent with the idea that in both 3D invasion and 2D migration, optimal motility of cells occurs when cells possess an intermediate degree of adhesiveness (Palecek et al., 1997; Zaman et al., 2006). Therefore, down-regulation of cell adhesion genes is suggestive of an increase in invasive ability of the cells. Also, in my screen, many genes involved in membrane protrusions were found to be up-regulated. As these genes are known to be required for invadopodia formation in tumor cells, their up-regulation could indicate formation of more invadopodia under mechanical stimulation (Albiges-Rizo et al., 2009; Alexander et al., 2008). In addition, I also found that many genes coding for different proteases were up-regulated under mechanical stimulation. During invasion, cells need to breach the physical barriers formed by the basement membrane (BM) and stroma, and they secrete proteases to degrade the ECM proteins that form these barriers (Poincloux et al., 2009). Hence, this increase in proteases also suggests increased invasive activity by cells. In addition to these, differential regulation of Rho GTPase signaling molecules, which are the key regulators of actin dynamics, also point to modulation of cell motility upon stimulation (Jaffe and Hall, 2005).

From the above candidate genes I was able to confirm the differential expression of six genes between mechanically stimulated and non-stimulated conditions of our mechano-invasion assay. Out of these six genes, ITGB3 that codes for integrin β3 was selected for further studies because it was the only integrin subunit found to be differentially regulated upon mechanical stimulation. Other genes confirmed to be differentially expressed were as follows. 1) MTSS1, also known as ‘Missing-in-metastasis (MIM)’, is expressed in low levels in cancer cells (Lee et al., 2002). It codes for an actin binding scaffold protein that regulates actin dynamics through its interactions with cortactin and protein tyrosine phosphatase δ (PTPδ) (Gonzalez-Quevedo et al., 2005; Lin et al., 2005; Quinones et al., 2010; Woodings et al., 2003). 2) MYL9 codes for myosin light chain (MLC) which is a regulatory component whose phosphorylation state dictates myosin

motor binding to actin filaments. The actomyosin contractility is required by cells to apply traction force on its substrate as well as for retracting the trailing edge of the cell as it moves forward hence it is mechanistically important in cell motility. (Jaffe and Hall, 2005; Ridley et al., 2003). 3) ACTN3 codes for a homologue of α-actinin. It is an actin crosslinking protein generally found at different cell-cell and cell-matrix adhesions. It connects transmembrane proteins such as tails of β integrins and intracellular adhesion molecule-1 (ICAM-1) to the actin cytoskeleton (Carpen et al., 1992; Otey et al., 1990). It also acts as a scaffold to bring together different signaling molecules and plays an important role in cell migration (Otey and Carpen, 2004). 4) PTK2B codes for a non-receptor protein tyrosine kinase that binds to cytoplasmic tails of integrins and is activated in response to cues from fibronectin in many developmental processes (Ma et al 1997, Schaller and Sasaki, 1997). Down-regulation of PTK2B is required for the differential cell response to rigidity and compliance in human foreskin fibroblasts (Prager-Khoutorsky et al., 2011). The kinase activity of PTK2B has been correlated with increased malignancy of cancer cells and the catalytic domain of PTK2B has become a therapeutic target (Lipinski and Loftus, 2010). 5) MMP14 encodes a zinc-dependent endopeptidase called matrix metalloproteinase 14 that causes collagenolysis (Li et al., 2008; Sabeh et al., 2004). In tumor cells, MMP14 is enriched at invadopodia. This provides invadopodial structures with an ECM degrading property, thereby increasing the rate of invasion of tumor cells through the ECM (Clark and Weaver, 2008; Linder, 2007).

As described earlier, being transmembrane proteins, β subunits of integrin are able to bind extracellular ligands outside the cell and actin cytoskeleton and a variety of signaling proteins inside the cell (Rathinam and Alahari, 2010; Springer and Wang, 2004). By virtue of this property, β subunits serve as potential mechanosensors and mechanotransductors. Using qPCR and western blot analysis I confirmed that integrin β3 is down-regulated upon mechanical stimulation

at both the transcriptional and translational levels. As it is a candidate mechanosensing molecule, one would expect to see up-regulation of integrin β3 expression upon mechanical stimulation, hence this decrease in integrin β3 expression was rather surprising. To confirm that the down-regulation of integrin β3 upon mechanical stimulation is required for enhanced invasion of cells, I overexpressed integrin β3 and inhibited the enhanced invasion normally seen upon mechanical stimulation. This indicated that integrin β3 must be down-regulated upon mechanical stimulation in order for the cells to respond to the stimulus. The upstream mechanism of how the mechanical stimulation causes the down-regulation of integrin β3 is unclear. Since fibronectin in the ECM is required for sensing this mechanical stimulus, it is entirely possible that down-regulation of integrin β3 is caused by a feedback mechanism that is initiated by mechanosensing through integrin β3 itself. Alternatively, a second integrin could be responsible for the down-regulation I have observed.

Different signaling pathways are controlled down-stream of different integrins and can result in very different cellular responses. To control the display and engagement of the correct integrins at the proper time, cells use a mechanism of integrin crosstalk, where one type of integrin regulates expression and activity of another type of integrin (Gonzalez et al., 2010). Such integrin crosstalk has been observed between integrin β3 and another fibronectin binding integrin, integrin β1. As integrin β3 is down-regulated upon mechanical stimulation, I checked if it was accompanied by an increase in activity of integrin β1. Unfortunately, we did not observe any significant changes in the levels of activation of integrin β1 upon mechanical stimulation. However, another possibility is that down-regulation of integrin β3 might change localization of integrin β1 within the cell. For example, down-regulation of integrin β3 might enrich integrin α5β1 in invadopodia and thus increasing their interaction with fibronectin resulting in enhanced mechanosensing.

The downstream pathway resulting from the down-regulation of integrin β3, and eventual increase in cellular invasion upon mechanical stimulation, may not be as obscure as its upstream pathway. To begin, our previous studies have shown the requirement of cofilin for sensing the mechanical stimulation in our mechano-invasion assay (Menon and Beningo, 2011). As mentioned previously cofilin is an actin binding protein, also required for maturation of invadopodia and its activity is dependent on phosphorylation by LIM kinase (Pollard and Borisy, 2003; Yamaguchi et al., 2005). (Blanchoin et al., 2000). My results suggest cofilin is in its active state (low phosphorylation at Ser-9) upon mechanical stimulation and that this activation can be inhibited upon overexpression of integrin β3. This evidence provides a clue to a known pathway leading from integrin beta 3 to cofilin and potentially to the maturation of invadopodia.

As cofilin is a key regulator of the actin cytoskeleton, the balance between its active and inactive forms is very important with respect to cell motility and invasion. The levels of Ser3 phosphorylated cofilin are low in highly metastatic cell lines derived from T-lymphoma and carcinoma (Nebl et al., 1996). In *in vitro* as well as *in vivo* experiments, invasiveness of cancer cells has been correlated with overall output of the cofilin pathway (Wang et al., 2006; Zebda et al., 2000). The mammary tumors formed by LIMK1 over-expressing cancer cells have been shown to have low metastatic potential. The tumors with dominant negative LIMK1 expressing cells, on the other hand, show increased invasive abilities (Wang et al., 2006). The phosphorylation of cofilin is linked to the ligand binding ability of integrin αvβ3. Murine melanoma cells expressing integrin αvβ3 have 10-fold higher amounts of Ser3 phospho-cofilin than integrin αvβ3 negative cells. Moreover, expression of pseudophosphorylated and non-phosphorylatable mutant cofilin in these cells showed that cofilin activity is required for expression of MMP2 and MMP14 (Dang et al., 2006).

Based on our observations and current literature we have developed a model that could explain the pathway connecting integrin β3 and cofilin. It is known that integrin engagement and activation leads to activation Rho GTPases. Activity of Rho GTPase Rac1 can be regulated by integrin β3 in focal complexes (Morgan et al., 2009). We hypothesize that in conditions without mechanical stimulation, signaling down-stream of integrin β3 leads to activation of Rac1. The active GTP bound Rac1 phosphorylates and activates its down-stream effector p21 activated kinase-1 (PAK1) (del Pozo et al., 2000). Active PAK1 activates LIM kinase-1 (LIMK1), which in turn phosphorylates its substrate cofilin at Ser-3 thereby inactivating it. Therefore, under non-

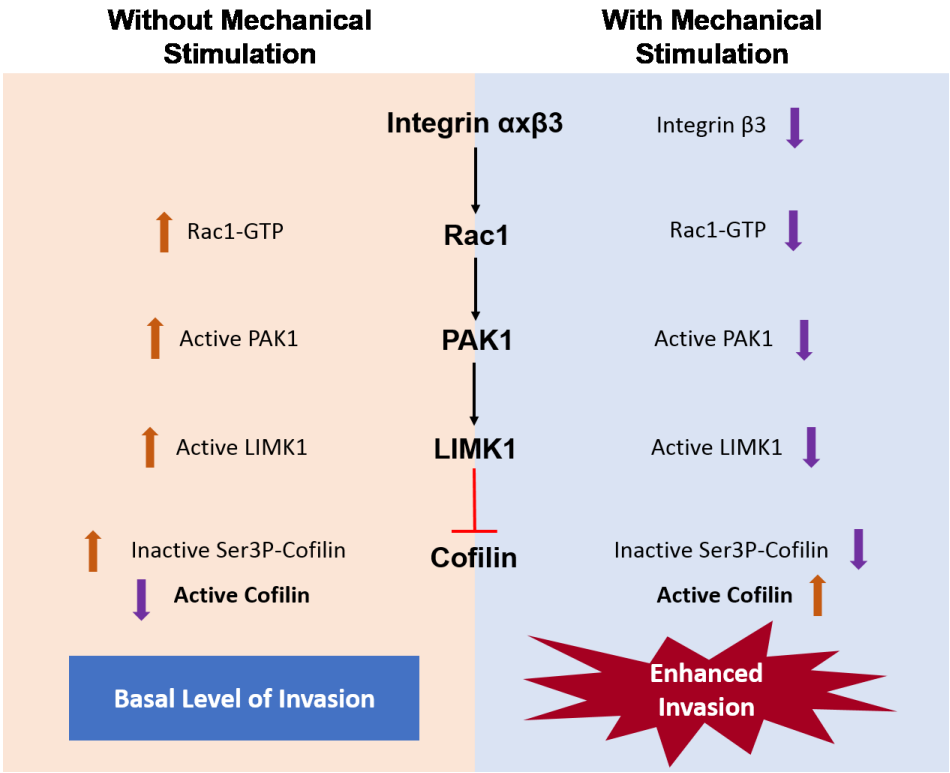


Figure 10. Possible pathway for enhanced invasion upon mechanical stimulation. Signaling down-stream of integrin β3, through sequential activation of Rac1, PAK1 and LIMK1, results in inhibition of cofilin activity by phosphorylation at Ser3. Upon mechanical stimulation integrin β3 gets down-regulated thereby decreasing the levels of down-stream kinases. Decrease in activation of LIMK1, also decreases phosphorylation of cofilin. This leads to increase in levels of active cofilin. Increased cofilin activity probably through formation of more mature invadopodia causes enhanced invasion.

stimulated conditions, the amount of active cofilin is lower than inactive cofilin, yet it is enough to produce basal levels of invasion. When mechanical stimulation is provided, integrin β3 is down-regulated through an unknown mechanism. As the amount of integrin β3 displayed on the surface decreases, the overall signaling down-stream of it also decreases. This leads to a decrease in the amounts of Rac1-GTP, which further causes less activation of PAK1 and LIMK1. Thus, the collective effect of integrin β3 down-regulation is a decrease in the amount of inactive Ser3 phospho-cofilin and an increase in non-phosphorylated active form. This increase in active cofilin upon stimulation leads to the formation of more mature invadopodia which eventually cause enhanced invasion.

In conclusion, this study has uncovered a part of the pathway that leads to enhanced invasion of cancer cells in response to tugging forces in the tumor microenvironment. For future studies, we first need to confirm the involvement of Rac1, PAK1 and LIMK1 down-stream of integrin β3 for controlling activation status of cofilin, as predicted by the model. As cofilin is known to be involved in invadopodia maturation, it will be interesting to test by microscopic studies if stimulated cultures show increased invadopodial activity. Moreover, how cofilin controls the maturation of invadopodia is still unknown. Solving these still unanswered questions will take us one step closer to understanding the mechanosensing pathway causing enhanced cancer cell invasion.

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ABSTRACT

DEFINING THE MECHANISM OF ENHANCED CELLULAR INVASION INDUCED BY MECHANICAL STIMULATION

by

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May 2013

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Metastasis is a multistep process driven by various biochemical and mechanical factors, which eventually leads to formation of secondary tumors. The tumor mass is surrounded by basement membrane (BM) and stroma made of various extracellular matrix (ECM) proteins. During metastasis tumor cells disseminate from the primary tumor, breach the BM, invade the stroma, travel through blood and lymph and colonize tissues distant from the primary tumor. Formation of secondary tumors by metastasis is a leading cause of death in cancer patients. Even though plenty of research has been focused on biochemical factors affecting metastasis, information on role of mechanical factors in this process is very limited. Using our previously developed *in vitro* mechano-invasion assay, we had observed enhanced cellular invasion in response to tugging forces in the stroma during cancer cell invasion. In vivo, such tugging forces would be produced by contractile cells within the stroma as they migrate and remodel the matrix fibers. In addition, we found this mechanically enhanced invasion by cancer cells to be dependent on the presence of fibronectin in the extracellular matrix. The objective of our study is to understand the mechanotransduction pathway leading to enhanced invasion. We hypothesized that in response to mechanical forces in the stroma, tumor cells will show an altered expression

of genes involved in mechanosensing. We performed expression profiling of several genes related to cell migration, adhesion and tumor metastasis by real-time PCR analysis. Six genes were confirmed to be differentially expressed between mechanically stimulated and non-stimulated conditions. Surprisingly, one of the genes found to be significantly down-regulated in the mechanically stimulated invasion culture is a fibronectin specific integrin subunit, integrin β3. Over-expression of this gene resulted in a significant decrease in enhanced invasion, supporting its role in sensing the mechanical stimulus. Furthermore, down-regulation of integrin β3 resulted in decrease in amounts of inactive form of cofilin (Ser3 phospho-cofilin).

AUTOBIOGRAPHICAL STATEMENT

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