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INFLUENCE OF MECHANICAL CUES AND THE EXTRACELLULAR MATRIX ON PATTERNS OF CELL MIGRATION AND RATES OF PROLIFERATION

by

TARA ALGER

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, MI

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2013

Major: Biological Science

Approved by:

____

Advisor Date

DEDICATION

I would like to dedicate this work to my husband, who is my support, my refuge, and my strength. I can never fully express how thankful I am that you are always by my side.

ACKNOWLEDGEMENTS

I would like to thank the people I work with in the Biological Sciences Department at Wayne State University. First, and most importantly, I am grateful to my advisor, Dr. Karen Beningo, who has encouraged me, educated me, supported me, and given me a range of advice in both a personal and professional light that helped me make it through my degree. I thank all of my labmates, who are helpful and inspiring on a day-to-day basis, who have the excitement for life and patience for my quirks that have made working with them so enjoyable. I would also like to thank people in the biology department, including friends who have walked alongside me during the journey, and the administrative staff who have helped with the frequently overlooked steps behind the scene.

I would like to thank other labs at the University, including Dr. Peter Hoffmann in the Physics Department and his lab for our AFM work, and other labs in Biological Sciences, including Dr. Kang's lab, Dr. Greenberg's lab, Dr. Branford's lab, and Dr. Njus' lab, for lending me equipment for my work.

Lastly I would like to thank my husband, Sean, who has been my pillar of strength and my co-pilot as he has helped in this new adventure we have been experiencing together: parenthood. We have learned and laughed together as our daughter grows. I couldn't imagine our life without either of them.

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CHAPTER 1

Introduction

Cells can sense their mechanical environment and respond by either maintaining or changing their behavior. Information about the mechanical make-up of the environment flows through the multiple physical contacts a cell establishes with its underlying and surrounding environment, as well as its contacts with neighboring cells. The contacts between a cell and its extracellular matrix, called focal adhesions, mediate signaling from the environment to the cell, called outside-in signaling, and from the cell to its environment; called inside-out signaling (Hu 2013). Outside-in signaling receives signals from the environment, including the physical signals that influence cellular behavior (Ginsberg 2005). Biological processes influenced by the physical microenvironment include normal functions such as changing tissue morphology during development and wound healing, as well as disease states such as cancer metastasis and heart disease (Indra 2011, Menon 2011, Chatzizisis 2007).

The Mechanical Environment and Heart Disease

Extracellular rigidity has been shown to affect the maturation of cardiomyocytes (Jacot 2008). The growth and development of these cells is required for normal cardiac function, and abnormalities may lead to congenital heart diseases and cardiomyopathies. It is important to understand the factors influencing these cells because possible therapies involve injecting

undifferentiated stem cells into scarred tissue (Fukuda 2006). Neonatal rat ventricular myocytes (NRVMs) display increased mechanical forces and enhanced maturation on rigidities similar to those seen in native myocardium, whereas this was not observed on substrates with more divergent rigidity (Jacot 2008). The effect of environmental elasticity seen here illustrates the importance of the native stiffness for typical cellular behavior for normal heart development and function.

The Mechanical Environment and Development

In development, embryos are undergoing constant physical rearrangement, and the collective movement of cells contributes to the function of tissues which make up an entire organism (Tada 2012). Physical forces play a role in directing cell movement to shape the embryo as it develops. Many genes involved in development are controlled by mechanical force (Farge 2003). The tension surrounding a cell in its extracellular matrix plays a regulating role not only in migration, but upstream in the transition between the epithelium and the mesenchyme of an embryo and in stem cell differentiation (Fleury 2002). Naïve mesenchymal stem cells have been shown to differentiate into different types of cells in response to varying substrate stiffness (Engler 2007). The cells mirror the cell fate of the tissue with the stiffness in which it has been introduced. Stem cells that differentiate on soft substrates develop neuronal markers, and cells on hard substrates develop bone markers (Engler 2007). Previous to these findings, it was not known that the single applied condition of substrate stiffness could affect

the fate of the whole cell. This implies that environmental mechanics have a large role in a developing embryo, and that a change in the physical conditions could lead to severe consequences in the normal development of an organism.

Environmental conditions such as the makeup of the extracellular matrix (ECM) are tightly regulated although incredibly dynamic during the development process both in a spatial and temporal manner (Reilly 2009). This suggests that ECM properties play a role in morphological development and alterations in cell fate and position (Reilly 2009). Another example that supports this contention is the behavior of mammary epithelial cells (MEC's) on rigid substrates when it was shown that harder substrates promote focal adhesion assembly and interfere with the maturation of the basal lamina. This was found when the MECs failed to express β-casein, a differentiation indicator. The increase in focal adhesion number inhibited the differentiation initiated by rigid substrates (Kass 2007). A third example involves the formation of the notochord in an embryo. The mesoderm extends through net cell movement in response to applied tensional forces (Czirok 2004). Furthermore, a study found that during avian notochord development, the ECM protein fibrillin reorganized in a controlled manner, simultaneously relocating prior to and during gastrulation. The dynamic movement of these ECM components created a mechanical strain upon the cells which was believed to be required for the shaping of the embryo (Visconti 2003, Czirok 2004).

The Response of Cytoskeleton and Focal Adhesions to Mechanical Forces

The cytoskeleton of a cell is altered in response to changes in the composition and mechanics of the ECM. When changing migration direction in response to stimuli, for example, cells must de-polymerize actin fibers in the previous leading edge of the cell and begin polymerization at the new leading edge of its alternate direction, creating new protrusions or lamellipodium. This alteration in actin polymerization requires the deactivation and activation of actin-associated proteins such as the Arp2/3 complex (Pollard 2007).

The focal adhesions, a complex of proteins on the cytosolic side of the plasma membrane form a significant link between the actin cytoskeleton and the underlying ECM. The mediation of signals within focal adhesions is transported through the multitude of proteins associated within this complex. Most notably, integrin is a heterodimer that spans the plasma membrane of a cell, transducing a signal in either direction across it. Integrin-mediated signaling in response to substrate stiffness has been seen in multiple cellular processes. Changes in cytoskeleton assembly due to integrin signaling lead to altered cellular morphologies and to further downstream signaling. For example, a distinct difference in ECM stiffness is known to affect the subsequent structure of focal adhesions and alter their cytoskeleton (Bershadskey 2003). ECM proteins also play a role in the adhesion and migration of cells in an integrin-dependent manner. The increase of force to the $\alpha 5\beta 1$ integrin-fibronectin bond, for example, further activates the interaction and strengthens the bond between these two

molecules (Friedland 2009). The response of the cells to substrate stiffness illustrates the initiation of an intercellular signaling cascade which changes the morphology of the cytoskeleton and ultimately alters the subsequent behavior of the cell.

Collective Cell Migration

When studying cell migration in the attempt to better characterize morphogenesis, it is important to consider the migration of cells as a group as well as each cell individually. Collective cell migration is the movement of multiple cells that are physically connected to one another during migration. While individual cell migration is widely studied *in vitro* and *in vivo*, less is known about the mechanisms underlying collective migration. The attachment of cells in collective migration allow for a degree of organization during biological processes that is not achieved in individual cell migration. Collectively, cells can migrate across a flat surface, creating a two-dimensional sheet as seen in the development of the epithelium lining the small intestine (Friedl 2009). Furthermore, cells have been shown to also move collectively in any direction, enabling the growth in a three-dimensional configuration as seen in the branching morphogenesis that occurs during mammary gland formation (Affolter 2003).

The mechanisms of collective cell migration differ depending on the *in vivo* context where the migration is taking place. A small, finite cluster of cells can move alone through tissues, which is seen with the border cells in the *Drosophila*

melanogaster egg chamber (Montell 1992). Otherwise, a leader cell can direct a larger group with a trailing inner lumen that becomes elongated as cells migrate further. This phenomenon is seen both in the formation of glands or in the process of angiogenesis. A leader cell is the one at the front of the group when the collective is moving in one particular direction. The majority of cells, however, are follower cells that make up the group but do not determine the path or direction of movement. Leader cells and follower cells differ in morphology and gene expression, giving them different functions in the collective movement. Leader cells, for example, have higher levels of expression of CXCR4, a receptor ligand that recognizes chemokines (Aman 2008). This recognition to chemical signals or ECM components and any subsequent reaction directs the migration of the leader cells, causing a net movement in the collective cell mass. This directed movement causes a polarity to the overall structure of the cells, which is a critical step in stages of development, including the initial stray from physical symmetry in a developing embryo (Friedl 2009). This directional movement is also a key step in the controlled process of regeneration after wounding. In this process, the blood vessels in the affected area must be reformed, and the wound must be closed with a new epithelial sheet. The controlled movement of the group of cells ensures immediate coverage, preliminary protection, and eventual regeneration of the tissue after physical injury (Friedl 2009).

Little has been discovered about migratory reactions to a physical stimulus and cellular mechanotransduction mechanisms involved in a group of migratory

cells. Many steps are necessary to understand the reaction of cells to the stiffness of the environment or to any targeted physical manipulation (Friedl 2009). In a wound-healing assay to analyze collective migration of an epithelial sheet, it was found that the migration speed, persistence, and coordination of movement were all increased on a more rigid surface as compared to a softer one (Ng 2012). Collective migration plays a large role in an embryo, so this information can help to understand how some of the physical cues affect the changes that occur during the various developmental stages. This information may also help to find more accurate and efficient ways to engineer tissues.

Durotaxis, the Use of Mechanical Cues by the Cell to Guide its Migration

Previous studies on varied substrate rigidities have shown that 3T3 fibroblasts preferentially migrate toward harder substrates. In these trials, the transition between hard and soft areas was immediate, and there was a considerable difference between the two rigidities. Substrates were created by combining polyacrylamide and bis-acrylamide in increasing concentrations, creating varying degrees of crosslinking of the polyacrylamide, and thus creating multiple controllable compliances. In this study, two separate polyacrylamide solutions were placed in close proximity on a glass surface, and when a glass coverslip was placed on top of the solutions, they created a finite border between the solutions before polymerization. When seeded on soft regions of the substrate, fibroblasts migrated toward harder surfaces, and when seeded on hard surfaces and are facing the softer region, they reverse directions and

migrated back toward the hard region (Lo 2000). This phenomenon was seen under equivalent ECM protein concentrations, therefore varying protein concentration was not a contributing factor. Traction forces produced by the cells on these substrates were significantly weaker on the soft regions than those produced on the hard. In the same study, an applied pushing force which created a decrease in substrate tension caused a cell to reverse direction, while a pull on the substrate, which generated an increase in substrate tension, triggered a cell to move directly toward the stimulated spot (Lo 2000). These results indicate that on substrates coated with equal concentrations of protein, fibroblasts preferentially migrate towards a more rigid surface.

Vascular smooth muscle cells were also shown to migrate toward harder substrates (Wong 2003). In this study, cells were introduced to a surface with a shallow gradient of stiffness instead of the steep transition from a soft to hard surface described in the Lo experiment above. This gradient hydrogel was produced by the addition of a chemical called IRGACURE 2959, a cross-linking substitute that utilizes ultraviolet radiation to establish the extent of polymerization. This chemical replacement allows for a less inhibited degree of polymerization that allows the gel design to be more discriminatory due to the ability to mask UV light in a controlled manner. Not only did the smooth muscle cells seeded on these substrates migrate toward the harder regions of the hydrogel, but they moved with more persistence, or in a more direct path. They also moved more slowly when on stiffer substrates. This difference in migratory

behavior may be different for other cell types studied, and the mechanisms underlying these behaviors are not fully understood. The behavior of these cells in response to environmental compliance has been studied little in the past, so there are many further directions to take to better understand the migratory patterns. Additionally, the presence of a continuous gradient in this experiment is not suitable for studies focused on *in vivo* behavioral patterns because the compliance transitions in tissues are drastic in actuality. It would be more prevalent to use hydrogels that have a robust difference in rigidity between one region and its neighbor.

Quantification of Substrate Stiffness

The measurement of the difference in stiffness is often studied by calculating the Young's modulus, the numerical value representing the elasticity of a surface. This value is expressed in Pascals; a unit of physical force. The Young's modulus is measured in many ways, a common method being atomic force microscopy (AFM), which uses the reflection of a laser to measure the stiffness of the surface (Figure 1.1). In an AFM device, a small cantilever is placed on top of the substrate being measured, and the cantilever head is raised or lowered, depending on the rigidity of the surface on which it is resting. An infared laser is then reflected off the back of the cantilever and targeted onto a photodiode. The reflective point on the photodiode moves according to the angle of reflection off the back of the cantilever. This difference in laser position can be converted mathematically into a value for the Young's modulus. The Young's

modulus within the human body varies from less than one kilopascal (kPa) in brain tissue to about 34 kPa in calcified bone (Reilly 2009). This technique has commonly been used to measure the rigidity of *in vitro* polyacrylamide substrates prepared for migrational studies.

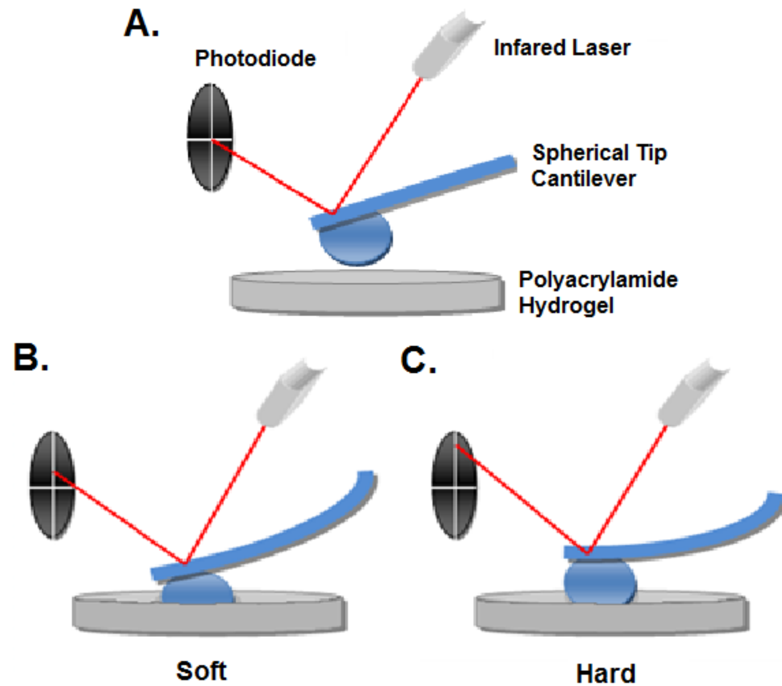

Figure 1.1: Mechanism of Detection of AFM*.* *(A) Infared laser is positioned at the center of the photodiode before sample is introduced. (B) On a softer substrate, cantilever flexes slightly, altering the position of the reflected laser. (C) On a harder substrate, cantilever bends more, resulting in increased movement of reflected laser.*

Previous studies have shown that migratory cells have preferences for different substrate stiffness, but previous experiments have not used compliances relevant to those seen *in vivo*, nor those with stiffness transitions that could mimic those seen in the body. Using the method previously introduced by Wong et. al., we have the ability to create a substrate with multiple compliance regions, tailored with the stiffness values present *in vivo*. This multi-stiffness hydrogel can be used to test the migratory behavior patterns of cells on a

broader scope. We hypothesize that cells faced with multiple *in vivo* tissue rigidities will preferentially migrate to a stiffness that most resembles the endogenous tissue from which it was derived. Additionally, we hypothesize that when two cell types of differing origin are seeded together, that cells would sort onto their prospective stiffness, possibly with more interference, causing slower migration speeds and decreased persistence.

Material and Methods

Tissue Culture

Mouse Embryonic Fibroblasts and Normal Murine Mammary Gland cells were cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM; Sigma) 10% Fetal Bovine Serum (FBS; Hyclone), 1% PSG (2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin), and in Nmumg media, 0.1% insulin. Cells were grown at 37° in 5% CO_2. Passing of cells was performed at confluency with 0.01% Trypsin-EDTA. Cell passages were limited to 10 passages.

Preparation of IRGACURE 2959 Substrates

Solutions for the substrates were prepared to final concentrations of 8% acrylamide, 0.3% bisacrylamide, 0.01M HEPES, and 6.4×10^{-3} M IRGACURE 2959. Solutions were degassed for 20 minutes. A 140 µm thick gel was produced by cutting a 22mm circle into a sheet of transparency film. The film with the hole was centered and adhered with vacuum grease to the surface of coverslips that had been activated as previously described (Wang and Pelham 1998). Unpolymerized acrylamide solutions were pipetted into the central opening, allowed to spread, then a second, unactivated coverslip was placed on top (Figure 1.2). The coverslips were inverted before a circular designed mask was placed on top. The boundary between the lightest and darkest mask regions was labeled on the coverslip outside of the well with marker. Gels were radiated with

UV light for 30 minutes before being immersed in 50mM HEPES for 1 hour. The top coverslip and transparency film were removed and the activated coverslip was mounted to a chamber dish. Substrates were covalently coupled with 0.2 mg/mL collagen Type1 in PBS (BD Biosciences) after Sulfo-SANPAH treatment (Beningo et al. 2002a).

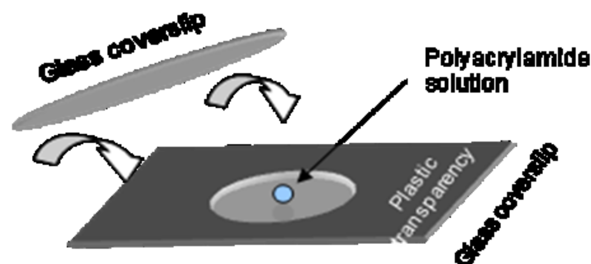

Figure 1.2: Preparation of Polyacrylamide Substrate to Create a 140 µm Thick Hydrogel. *Plastic transparency is mounted onto activated glass coverslip with vacuum grease. Polyacrylamide solution is added to well, and a second, unactivated coverslip is placed on top.*

Cell Migration Assay

Seeded prepared IRGACURE 2959 gels with Mouse Embryonic Fibroblasts (MEF) or Normal Murine Mammary Gland (Nmumg) cells by pipetting 2.5×10^5 cells in center circle of gel with a 10µL aliquot. Cells were allowed to adhere for 20 minutes prior to rinsing and immersing substrates in fresh media. Cultures were incubated at 37^oC /5% CO_2 for five days. Images were captured at 3, 4, and 5 days post seeding.

Checkered Mask Migration Assays

Prepared IRGACURE 2959 solution as described above, making sure to outline mask boundaries prior to UV radiation. Coated gels with 0.2 mg/mL collagen for four hours in 4° C, rinsed substrates twice with PBS, then twice with 50mM HEPES. 0.067 mg/mL Fibronectin was subsequently coated overnight at 4° C, then rinsed three times with 1X Phospho-Buffered Saline (PBS). Seeded cells sparsely and recorded live cell imaging video with 10 minute intervals between images for up to 24 hours.

Spheroid Migration Assay

MEF spheroid was prepared by pipetting 9 $x10^{4}$ MEF cells into a 96 well plate coated with 2% agarose and placing on a rotator overnight at $37^{o}C/5\% CO_{2.}$ The spheroid was pipetted onto the substrate and the cells were allowed to adhere for 1-2 hours before adding media. Images were taken every 30 minutes for 24 hours.

Immunofluorescence

Standard polyacrylamide substrates of 5% acrylamide, either 0.1% (hard) or 0.04% (soft) bisacrylamide, 1M HEPES and crosslinked with 10% ammonium persulfate (APS) and TEMED were made as previously described (Beningo et al. 2002). IRGACURE 2959 polymerized gels were prepared as described above.

Collagen was coupled to the surface at 0.2 mg/mL after Sulfo-SANPAH and treatment with ultraviolet light. Substrates were blocked with 5% Bovine Serum Albumin in 1X PBS for 90 minutes then treated with mouse monoclonal anti-collagen type I (Sigma-Aldrich) (1:2000) at 4° C overnight. 1XPBS was used to rinse substrates 4X with 10 minute intervals. Goat anti-mouse IgG Fluores-brite Carboxylate beads were rinsed 4X in 1X PBS and centrifuged 5 minutes at 10,000 x g after each rinse. Beads were conjugated to substrates at a 1:20 dilution in 1X PBS for 1 hour at 4° C Substrates were rinsed 3 times in 1X PBS.

Microindentation

Standard APS/TEMED crosslinked gels were prepared as described previously (Beningo et al. 2002a). 1% Fluorospheres carboxylate-modified 0.2 µm red fluorescent beads (Invitrogen) were added to the substrates and the coverslips were inverted prior to polymerization. After polymerization, substrates were immersed in 1X PBS. A 0.5 mm ss420 magnetic microsphere (Salem) was placed on the surface of the substrate. Images of red fluorescent beads were taken at 16X magnification. First image was taken of red fluorescent beads in focus under the center of the microsphere. The first z-stack value was recorded at this focal point. Magnetized tweezers were used to remove the microsphere. A second image was taken of red fluorescent beads in focus in the absence of the microsphere. The second z-stack value was also recorded at this focal point

Atomic Force Microscopy

Sample polyacrylamide hydrogels were read with an Agilent 5100 device with a tipless cantilever with a stiffness of 50 N/m. A sphere was mounted to a tipless cantilever with a UV adhesive and irradiated for 15 minutes, and rinsed with 1X PBS prior to mounting in the AFM device. Hydrogels were immersed in room temperature 1X PBS during analysis.

Results

Polyacrylamide Substrate Design

A number of different cell types are known to change their migratory behavior in response to varying substrate stiffness. These cells change their cell morphology, migration speed, persistence, and direction upon the introduction of varying substrate stiffness (Lo et al. 2000, Wong et al. 2003). In order to test if cells will detect, select, and migrate toward different rigidities, we designed a substrate containing six different stiffness values by using IRGACURE 2959 hydrogels. These gels provide the ability to design the regions of rigidity on a single hydrogel by selecting location, size, and extent of polymerization as individual parameters. The substrate has a central circular region of medial stiffness, surrounded by six different rigidities radiating away from the central region (Figure 1.4A). Each stiffness is represented by the numerical value of the shading used during the designing of the mask. The highest value, 240

represents the luminosity value with no shading, and the lowest value, 2 represents the maximum shading the mask provides.

Cells Spreading Varies according to Substrate Stiffness

To test whether the mask used allows for differential polymerization that can be sensed by cells, we performed an assay to measure the percent of cells that spread on each of these six substrate rigidities (Figure 1.3). The percentage of cell spreading for each stiffness shows a linear regression that corresponds to the decrease in stiffness. The substrates that were exposed to the most UV radiation showed the greatest percent of cell spreading, and the substrates that were exposed to the least amount of UV radiation showed the lowest percent of

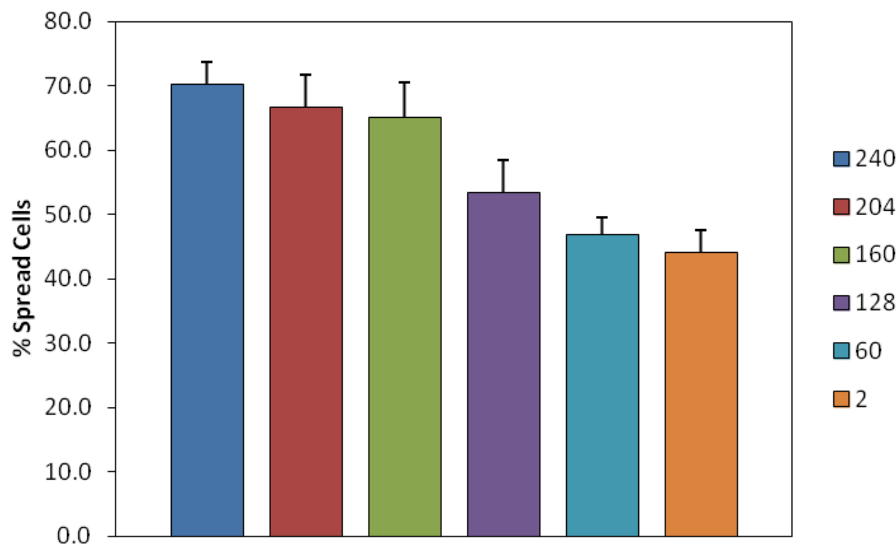

Figure 1.3: Cell Spreading Decreases on Substrates with Decreasing Rigidity. *Percent of cells spread on substrates polymerized with varying stiffness as a value of degree of shading. Percent was determined as the ratio of number of spread cells over total number of cells in frame. Higher rigidities correspond to lighter shading (240), and lower rigidities correspond to darker shading (2).Error bars represent standard error. (n=11, 10, 10, 13, 11, 13 respectively)*

spreading. These results show that using the IRGACURE 2959 as a UV cross-linker allows for a degree of polymerization that is sensitive enough for cells to sense a difference between substrates of varying rigidities.

Cell Migration is Uniform on Variable Stiffness Substrate

To test whether cells migrated toward any particular substrate stiffness, we seeded cells in the central region of the substrates and monitored cell migration over a period of several days to observe any preferential migration to any outlying regions. Preliminary data showed that Nmumg cells migrated toward the region with the luminosity value corresponding to 204, indicating a preference for intermediate substrate stiffness (Figure 1.4B). Unfortunately, these data were not reproducible and subsequent experiments showed little directional migration (Figure 1.4C), as cells migrated similar distances in all directions. These migrational studies also show that Nmumg cell migration seemed to occur passively, only moving to cover a larger surface area as cells proliferated. This could indicate a growing cell mass; not a collectively migrating sheet of cells. We also used live cell imaging to view the migration of MEF cells within the center of the substrate (Figure 1.4D). Although these cells seem to be adhering to the substrate and proliferating, they do not cover any significant distance during the course of 35 hours, as would have been seen with active cell migration. These migrational studies tell us that neither Nmumg nor MEFs prefer a particular

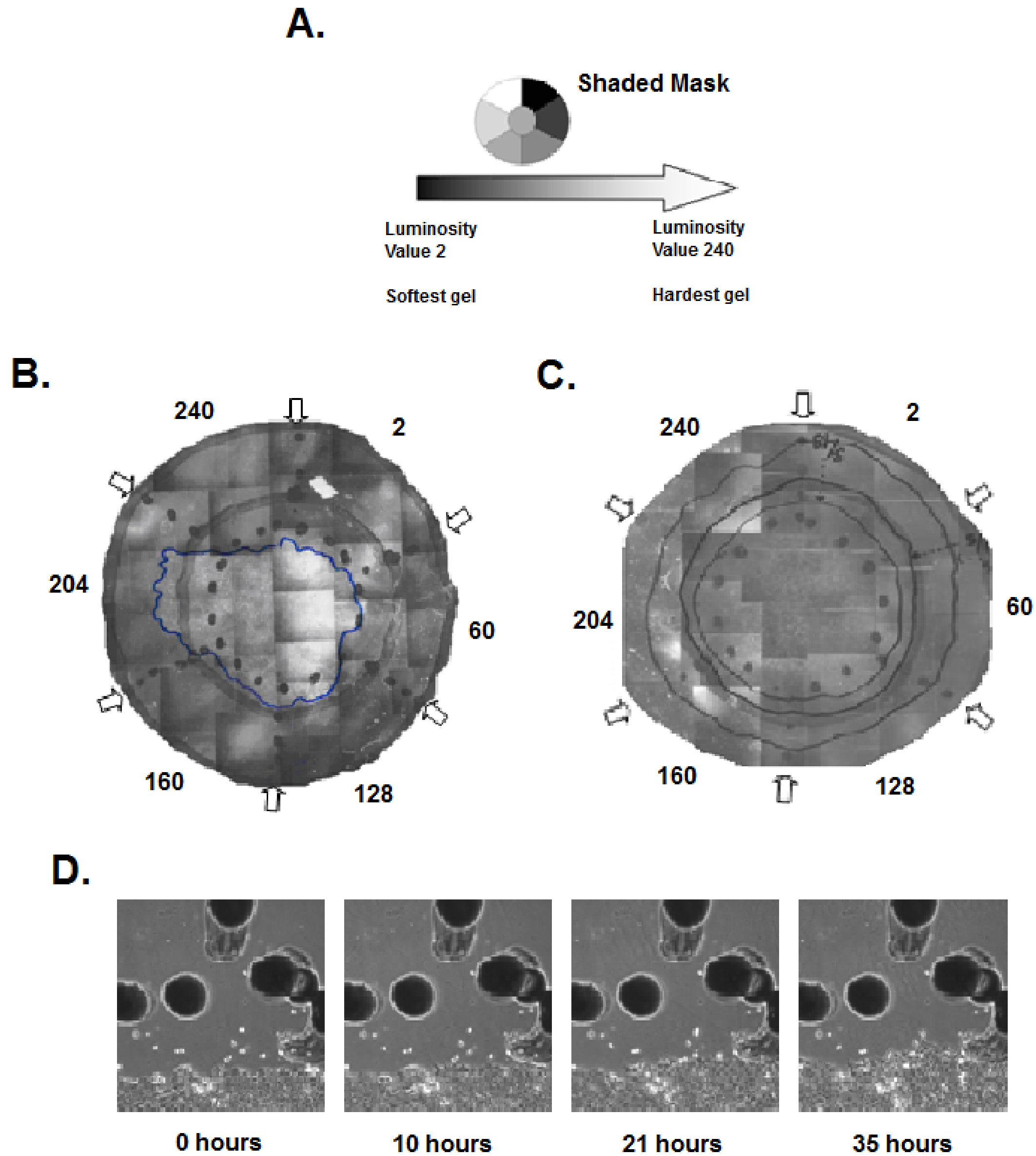

Figure 1.4: Cells Do Not Actively Migrate nor Sort onto Specific Rigidities on IRGACURE Spherical Mask-Created Substrate*. (A) Design of substrate created with Microsoft Paint software. Mask contains six stiffness regions with various luminosity values which depict amount of shading. (B) Nmumg cell migration after 72 hours (blue line) after seeding. Migration trend showed directionality toward region with luminosity value of 204. (C) Nmumg cells 3, 4, and 5 days (Solid black lines) after seeding. Cells showed uniform radial movement from center region (D) Time lapse images of MEF cells from 3 to 4 days after seeding.*

substrate stiffness, and that in many cases they do not actively migrate on IRGACURE 2959 polymerized substrates.

Direction of Migration is Not Affected by Close-Range Stiffness Variation

Due to the size and design of the IRGACURE 2959 substrate, distance between varying stiffness regions may have been too large for cells to be able to sense difference in their immediate surroundings. The absence of nearby environmental differences would give the cells no mechanical cue to respond to, hence no behavioral or migrational changes would take place. To test whether cells could respond to changes in stiffness located in closer spatial proximity, we redesigned the mask for substrates to create smaller regions of varying rigidities allowing for a better exposure for cells to more than one rigidity (Figure 1.5A). These “checkerboard” substrates contain the highest and lowest rigidities possible, with each region masked with either a luminosity value of 240 or 2, respectively. MEFs were sparsely seeded on these gels to minimize interference due to cell-cell communication and monitored with live cell imaging to see if they migrated to either the hard or soft regions (Figure 1.5B). Surprisingly, many cells migrated toward and along the borders between the hard and soft regions (Figure 1.5C, red circles). Few migrated within one region (blue circles), and some migrated very little at all (yellow circles). These results lead us to conclude that on these hydrogels, cells do not migrate to any regions of specific stiffness.

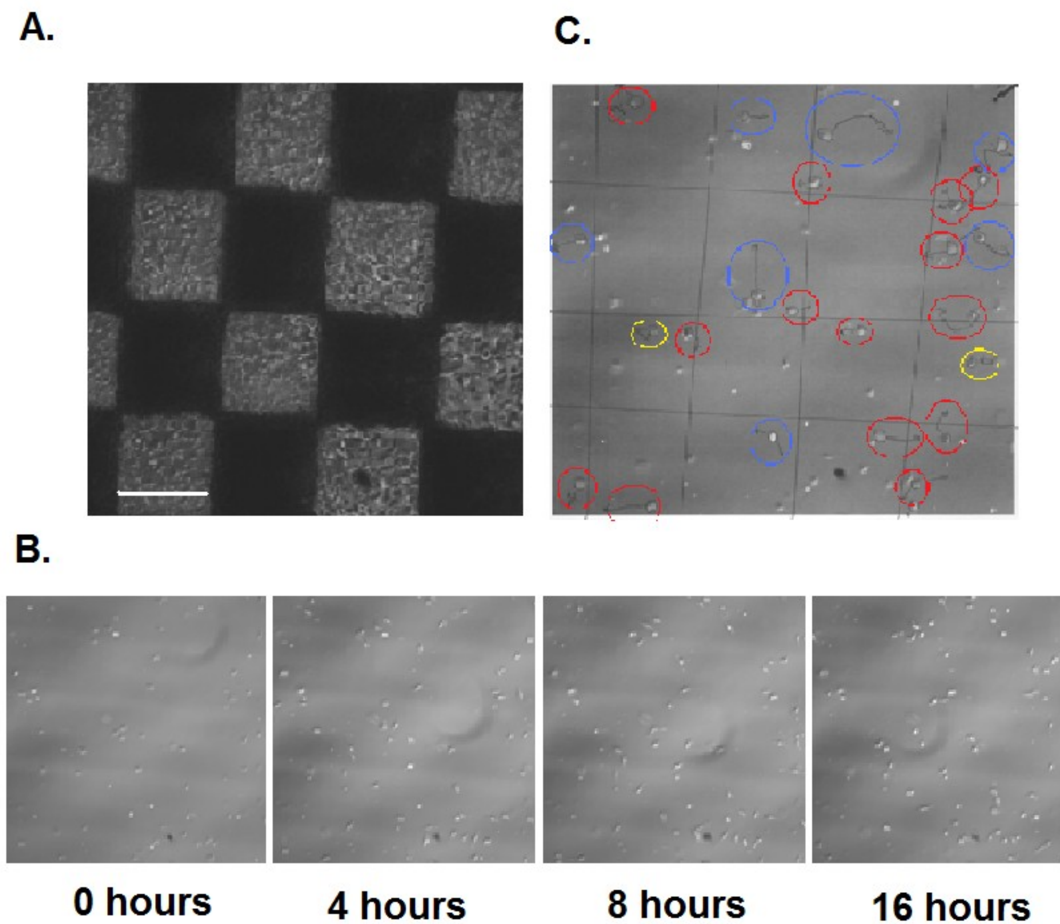

Figure 1.5: Mouse Embryonic Fibroblasts do Not Show Controlled Directional Migration When Given Close-Range Diversity in Substrate Stiffness. *(A) Substrate mask designed in a checkered pattern. Scale bar is equal to 500 µm(B) Time lapse images of MEF cells seeded on a substrate with mask pattern from (A). Images were taken every 10 minutes for 16 hours. (C) Overview of cell migration from (B). Blue circles indicate cells that migrate within a shaded region. Red circles indicate cells that migrate toward or along the border between two shaded regions. Yellow circles indicate cells that only migrate a short distance with a direction that is inconclusive.*

Cells do not Migrate Collectively toward any Particular Stiffness

We used the checkered design substrates to seed a spheroid of MEF cells in order to test whether cells would migrate collectively to varying substrate

rigidities given close proximity variation in substrate stiffness (Figure 1.6A)A). Using time lapse imaging, we monitored the leading edge of a spreading sheet of MEF cells away from a spheroid over 24 hours. The position of the cells from the spheroid at the 24 hour time point showed no obvious difference in distance between the hard and soft regions (Figure 1.6B). These migration trends along with those from the individual cell migration studies lead us to believe that cells do not alter their migrational behavior in response to different rigidities when seeded on hydrogels produced by IRGACURE crosslinking.

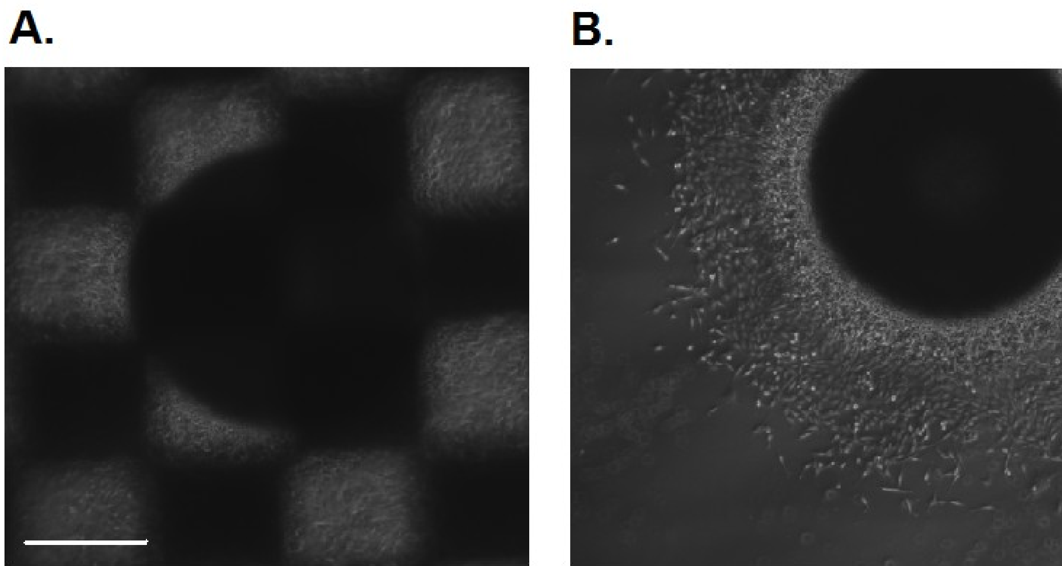

Figure 1.6: Mouse Embryonic Fibroblasts do not Migrate Collectively Toward Any Particular Substrate Stiffness When Given Close-Range Diversity in Substrate Stiffness. *(A) Spheroid positioned on masked substrate relative to checkerboard pattern (B) MEF cells 24 hours after seeding. Images taken with 2X objective lens.Scale bar is equal to 500 μm.*

<u>*ECM Coating is Insufficient and Uneven on IRGACURE 2959 Substrates*</u>

To determine if the migration behavior seen on IRGACURE gels could be due to improper ECM coating, we used immunofluorescence of the ECM protein collagen. Using a primary antibody against collagen and a secondary antibody linked to fluorescent microbeads, we were able to visualize the collagen coated to the surface of the substrates (Figure 1.7). We used substrates crosslinked with APS/TEMED as a positive control to compare the efficiency of collagen coating

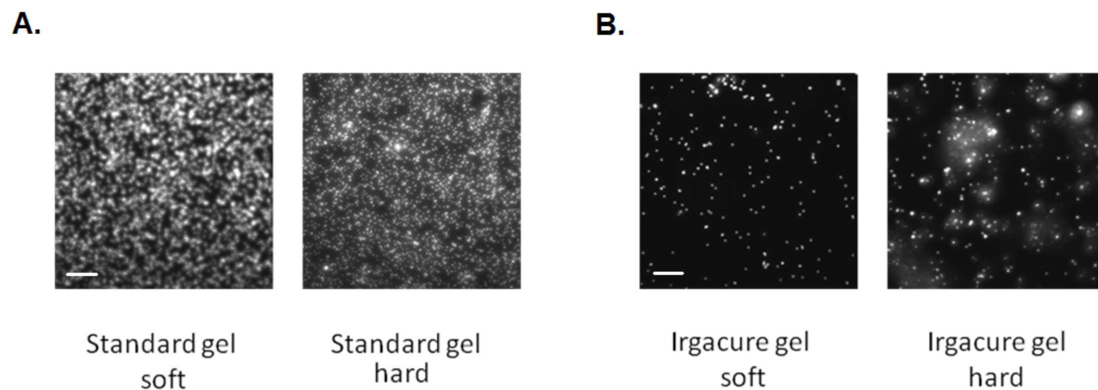

***Figure 1.7: Extracellular Matrix Coating is Inefficient and Inconsistent Across the Surface of IRGACURE Hydrogels**. Immunofluorescence images of anti-collagen antibody coupled to fluorescent anti-IgG-coated microbeads on (A) hydrogels made from standard APS/TEMED previously used method and (B) IRGACURE UV polymerization method for hard and soft gels. Scale bar is equal to 25 μM.*

(Figure 1.7A). Our results show that although coating seems to be similar between the hard and soft substrates, IRGACURE hydrogels have significantly less collagen coated to the surface as compared to the control (Figure 1.7B). Also, collagen appears to be coated unevenly, with clusters of beads in some regions. These results lead us to believe that the ECM proteins are not being

sufficiently coated onto the surface of the IRGACURE gels, and likely explains the poor migration efficiency we observed on these gels.

Quantification of Substrate Stiffness with Two Microindentation Techniques

The characterization of cell behavior as a result of substrate stiffness in the past has utilized in vitro hydrogel substrates with stiffness values irrelevant to those seen in vivo. While stiffness levels in the body range from less than 1 kPa in brain all the way to more than 100 kPa in bone, substrates previously used in vitro only range between 1 and 8 kPa, depending on the concentrations of acrylamide and bis-acrylamide (Engler et al. 2007, Guo et al. 2006) In the process of adjusting the elasticity of hydrogels polymerized with IRGACURE, we utilized two previously established methods to quantify the stiffness of each substrate.

The first method used to quantify the elasticity of the hydrogels was a microindentation technique using microspheres (Figure 1.8A). In this method, the substrates are embedded with fluorescent microbeads and inverted to ensure beads are aligned on the surface of the gel. A steel microsphere is placed on top of the substrate submersed in buffer to prevent drying. The z-stack value is recorded for the fluorescent microbeads in the region immediately below the sphere, then the sphere is removed with a magnet. The subsequent z-stack value for the surface of the unindented surface microbeads is measured. The indentation distance is calculated from these two values. In our experiment, we

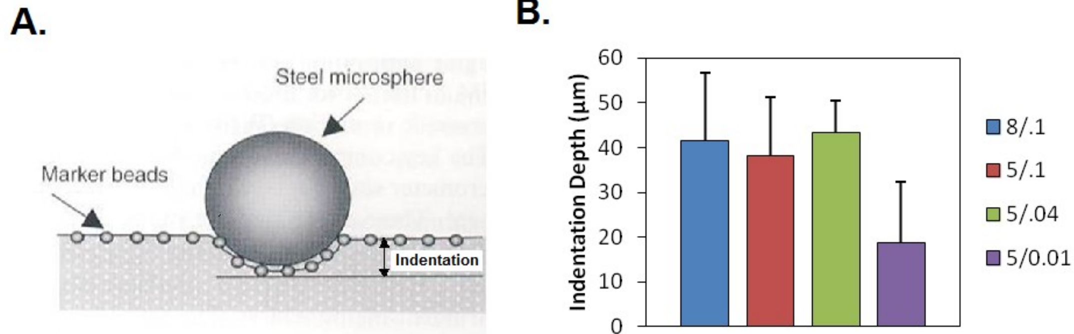

Figure 1.8: Quantification of Substrate Stiffness is Inconsistent with Microsphere Method. *(A) Microsphere technique showing microsphere placed on a substrate containing fluorescent beads. The in-focus z-stack value is recorded for beads at the lowest indentation position and at the unindented substrate surface. Indentation distance is quantified by the difference of these two values. (B) Focus depth for four decreasing rigidities using standard polyacrylamide substrate protocol. Each substrate represents the percent acrylamide and corresponding percent of bisacrylamide in each solution. Magnification at 10X objective (n=9, 12, 12, and 8, respectively)*

tested this microindentation technique using hydrogels polymerized chemically with APS/TEMED crosslinkers, gels with stiffness variation established previously. These four gels, 8/.01, 5/.1, 5/.04, and 5/.01 represent the percentage of acrylamide and bisacrylamide in each substrate solution. In the concentration ratio of acrylamide to bis-acrylamide, increased concentrations of bis-acrylamide in relation to acrylamide represent harder gels. With this in mind, it is expected to see a larger indentation depth for softer gels compared to harder ones, however we see no differences in the three hardest gels, which indent 41.5, 38.1, and 43.3 µm, respectively, and very low indentation depth for the softest gel, which only indented 18.6 µm (Figure 1.8B). Also, the deviation between one gel and another for each rigidity value is very high, with standard deviation values of

15.0, 13.1, 7.2, and 13.7, respectively (Figure 1.8B). These results indicate that there is very little consistency in the results for this method. Although this method is well established in literature, we believe that our application of the procedure does not yield favorable results, possibly due to minor differences in substrate production or measurement. Nevertheless, we have concluded from these data that in our hands the microsphere technique is not reliable for measuring the stiffness of our IRGACURE substrates.

A second method of microindentation used to test the stiffness of our substrates was Atomic Force Microscopy. Although we tested both gels polymerized with APS/TEMED as well as IRGACURE, the rigidity of the IRGACURE-crosslinked hydrogels was indeterminable. A force-curve was not attainable, we suspect due to the chemical makeup of these gels. The process of measurement involves lowering a cantilever down on to the surface of the hydrogel, then lifting it back up in the process of determining the extent of indentation, similar to the microsphere method. The photodiode detector gave unreadable force curves, we believe due to the fact that the cantilever would attach to the gel, causing the cantilever to bend upon separation (data not shown). Because we could not obtain a force curve, we could not calculate the stiffness of these hydrogels.

Discussion

The behavior of cells in response to their physical environment is a major contributing clue to understanding morphogenesis during development, during tissue repair, and in the engineering of tissues (Benhardt and Cosgriff-Hernandez 2009). Although previous studies have shown a mechanosensitive response for both NIH 3T3 cells and VSMCs, in which cells migrate faster, with more persistence, and with directional cues, there are still many gaps to fill in understanding the mechanisms of behavioral responses as well as the extent of any response.

Previous studies *in vitro* saw changes in behavioral migration with a difference in stiffness that only spans a portion of the rigidities found *in vivo*. Also, gradual gradient changes used previously are not comparable to differences in stiffness found *in vivo* which would have a more abrupt transition. We attempted to create an *in vitro* polyacrylamide hydrogel that would forego both of these issues. The design of our hydrogel would allow for cells to interact with multiple varying rigidities comparable to those found *in vivo*, with a drastically changing boundary between regions of stiffness. The *in vitro* design of these polyacrylamide gels would also permit us to control outlying variables that may affect cell behavior such as ECM proteins, growth factors in the media, etc., allowing us to exclude these as contributors to our results.

Preliminary studies of the hydrogels using our circular design showed that substrates polymerized with IRGACURE did have varying rigidities with each of the six shaded masks. Cell spreading is a well established indicator of substrate stiffness, as many previous studies show that cells spread more readily on hard substrates as compared to soft ones. Our cell spreading assay showed that the gels polymerized to different degrees with each shade, and that the variation is drastic enough for MEF cells to be able to respond accordingly. These studies did not test whether cells could sense differences between neighboring rigidities, however. All studies testing this mechanosensing response showed that cells did not perceive differences in the neighboring substrate rigidities. Both on a macroscale in the millimeter range, as well as microscale in the micrometer range, cells did not migrate toward any specific substrate stiffness. It is unclear, however, the reason for the lack of response. The defect could lie in the absence of mechanosensing, in which the cells could not perceive the differences, or in the response, in which cells sensed the differences, but could not respond accordingly.

Because cells did not seem to migrate with much activity on the circular design substrates, we tested whether the substrates were being properly coated with extracellular matrix proteins with immunofluorescence. When compared to hydrogels chemically crosslinked with APS/TEMED, IRGACURE UV crosslinked gels showed uneven coating of collagen, as well as significantly less protein present on the substrate surface. This lack of ECM explains the poor migration of

cells on the circular design substrates, and possibly the absence of the mechanosensing response. Although there appeared to be sufficient ECM for cell spreading, and the degree of coating did not change among varing stiffness, there may not have been sufficient ligand available for integrin to become active, and to formulate new focal adhesions. The presence and maturation of focal adhesions at the cell surface are required for the production of traction forces to propel a cell forward, and this process is dependent on the ECM rigidity (Pelham and Wang 1997). Inefficient ECM coating is a practical reason for the mechanosensing problems, as studies have made apparent the importance of cell-ECM interactions for normal cell function and tissue homeostasis (Parker and Ingber 2007).

We believe the ECM coating was inefficient due to an issue in the UV activation of Sulfo-SANPAH prior to protein addition. The process of Sulfo-SANPAH activation conjugates one end of a linker arm to the polyacrylamide while the other end acts as a binding partner to the amine groups of the ECM proteins. Our rationale is that the Sulfo-SANPAH did not undergo a colormetric change during UV exposure as is seen with gels polymerized chemically (data not shown). The unique chemical makeup of the IRGACURE gels likely accounts for the absence of color change. A viable option would be to try one of the alternative methods for protein coating established in previous studies, such as the use of 1-ethyl-3-(3dimethylaminopropyl) carbodiimide-HCL or N-Hydroxysuccinimide ester (Beningo and Wang 2002b, Pelham and Wang 1997).

Because the chemistry of the reaction that may be responsible for the inefficient coupling is not understood, it is not known whether an alternate approach to conjugating protein would be any more efficient than Sulfo-SANPAH.

Many previous studies using polyacrylamide substrates have stiffness values ranging from one to eight kPa, although more recently polyacrylamide substrates reached stiffness values of up to 55 kPa in focal adhesion studies (Plotnikov et al. 2012). In order to compare cell rigidity preferences to those *in vivo*, it is necessary to tailor the stiffness values to more closely resemble those found in the body. We endeavored to quantify the elastic modulus of our substrates by two different microindentation methods. Using a steel microsphere, we found that simple gravitational indentation measurement was inconclusive and inconsistent. We expected to find increasing degrees of indentation with decreasing substrate stiffness, but instead there was no trend in indentation depth in correspondence with known rigidities. In addition, depth of indentation varied significantly even within the same hydrogel. Because the results for the chemically crosslinked indentation depths were unpredictable, we determined that this method of measurement to further determine substrate stiffness was unreliable for IRGACURE gels in which we had even less expectation for gel elasticity.

The second method of microindentation we used for measuring the substrate elasticity was atomic force microscopy. We utilized a device for reading

the IRGACURE substrates in buffer solution to prevent substrate drying. Because the cantilever tip seemed to stick to the substrate surface, we believe that the hydrogel was either too soft, causing the cantilever to sink into the substrate, or it contained chemical properties that caused it to bond to the cantilever tip. To test the first theory, we used cantilevers with spherical tips in place of pyramidal ones to prevent possible piercing of the surface. The spherical tip did not prevent the prolonged interaction with the substrate, but it could still be adhering to the surface of the gel due to the stickiness of the substrate.

The production of a substrate with a UV crosslinker would enable us to design the substrate with any number of rigidities while giving us the capability of changing the value of each stiffness at will. The IRGACURE 2959 added as the catalyst, however, seems to alter the chemical composition of the gel to subsequently affect the ECM binding capabilities and possibly renders the hydrogels unreadable by atomic force microscopy.

A possible explanation for both the ECM coupling and AFM issues we have faced is that the IRGACURE 2959 hydrogels were not fully polymerized. Another study that used IRGACURE 2959 found multiple variables that affected the degree of polymerization. Some of these variables include the concentration of IRGACURE, the distance from the light source during exposure, and the wavelength of UV light source (Sunyer et al. 2012). While the results of our morphology assay show that our gels polymerized enough for cells to sense the

relative degree of polymerization, the gels may have been too soft to affect directionality.

Given that the ECM could be properly adhered to the gel surface, and that the rigidity of the substrate could be selectively measured and adjusted, this substrate would be tremendously useful for *in vitro* studies of cell migration. We would use these substrates to determine if cells migrate to a substrate *in vitro* that mimics the *in vivo* stiffness, thus indicating that cells maintain a mechanical memory post-extraction and immortalization. Also, these substrates would be useful for studying mechanisms of collective cell migration, competition in migration between multiple cell types, and even for cell behavioral changes in a three-dimensional *in vitro* environment. The results of these studies would help to better understand the effects of physical microenvironment on cell behavior, specifically in embryonic development and tissue engineering.

CHAPTER 2

Introduction

The Extracellular Matrix

The extracellular matrix (ECM) of the cellular microenvironment is chiefly composed of a network of proteins bound to one another to form a functional scaffold for cells. This scaffold is comprised of multiple protein components, commonly made up of collagen, fibronectin, and laminin. The composition of proteins is tissue specific, each consisting of a diverse arrangement customized to its functional needs. The scaffolding proteins of the ECM provide a physical structure important for cell functions, including morphogenesis and migration, and maintaining tension required for cell-cell communication (Parker and Ingber 2007, Kumar and Weaver 2009). Studies have shown that the stiffness of a tissue can affect cellular behavior, and the protein composition directly affects its rigidity (Provenzano et al. 2009, Schedin and Keely 2011). In normal tissue, the structural support provided by the ECM is important for maintaining tissue homeostasis (Paszek and Weaver 2004).

Fibronectin Structure

An important component of the extracellular matrix, fibronectin, is a soluble dimer secreted by cells prior to polymerization and scaffold formation. Loss of fibronectin is embryonic lethal, and it is required for both development and tissue regeneration (Mao et al. 2005). mRNA splice variants result in protein

sizes ranging between 230 to 270 kDa (Singh et al. 2010). Each arm of the peptide dimer contains repeats of three types of globular domains, Type I, Type II, and Type III (Figure 2.1A) (Hynes 1990). Type III domains consist of 7-stranded β-barrels (Leahy et al. 1996). The two arms of the fibronectin chains

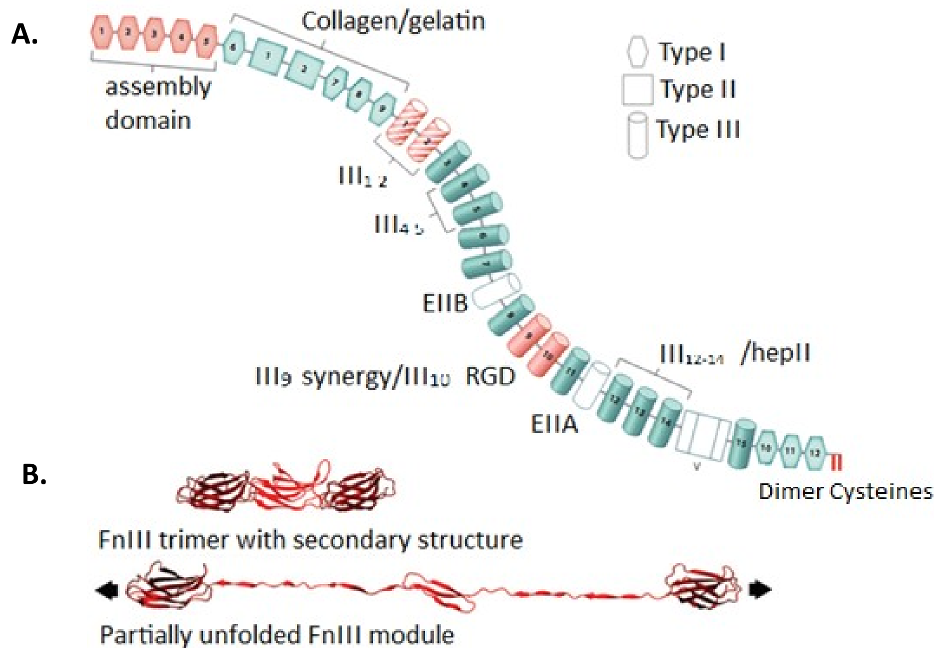

Figure 2.1: Structure of Fibronectin *(A) Type I, II, and III domain repeats on a single strand of the fn dimer. Fn assembly region, collagen binding sites, synergy site, RGD site, heparin binding site, and other common binding site are labeled along the strand (Singh 2010) (B) Structure of β-barrels before and after domain unfolding. Center domain unfolds at each end of the β-barrel sequentially (Antia. 2008).*

connect at their C-terminal ends through two disulfide bonds, arranging the dimer in an antiparallel configuration (Singh et al. 2010). The physical makeup of fibronectin allows it to physically stretch without permanently damaging or altering its structure, a process that influences cell proliferation and differentiation (Kubow et al. 2009).

During stretch, the arms of the protein separate first, followed by the domain separation and unfolding of its secondary structure (Figure 2.1B). Characteristically, the Type III domains unfold at the first and last strand of the β-barrels first (Baneyx et al. 2002, Smith et al. 2007). When physical strain is decreased, the fibronectin can refold into a compacted conformation (Klotzsch et al. 2009). Similar to the stretching of a spring, the process of unfolding of a fibronectin strand increases the tension within the ECM, so that the more it becomes stretched, the more tension it contains. This is supported by the rapid contraction of the fiber after it is released or broken. This increase in tension created by fibronectin stretch subsequently increases the rigidity of the tissue (Klotzsch et al. 2009).

To fully understand the physics of fibronectin unfolding, it is important to study the complete ECM structure in context. *In vivo*, the ECM is composed of the scaffold of protein components and is interlaced with cells of various makeup such as migrating fibroblasts, myopepithelial cells, adipocytes, and white blood cells (Frantz et al. 2010). The process of cell migration requires the attachment to the ECM, the cellular contraction to move, followed by detachment of its posterior end (Wolf and Friedl 2009). The force generated by these cells during migration is sufficient to alter the conformation of fibronectin, leading to enhanced fibronectin fibrillogenesis and reorganization of the extracellular matrix (Baneyx et al. 2002). These two processes of fiber remodeling are dependent on integrin binding and actomyosin contractility of the cell (Sechler and Schwarzbauer 1997,

and Zhong et al. 1998). Conformational changes in fibronectin caused by cells are responsible for its unfolded state (Klotzsch et al. 2009). During the process of unfolding, intermediate conformations remain stable, while others are quickly lost, indicating that native fibronectin may exist in several conformational states *in vivo* (Gao et al. 2002). The study that found this, however, tested single molecule extensibility, without taking additional interactions, such as cell-attachment or fiber formation into account.

Cryptic Binding Sites Within Fibronectin

The process of fibronectin unfolding alters its structure to such a degree that multiple sites become available for new protein interactions. These newly exposed sites, called cryptic binding sites, have been categorized for multiple Type III domains (Klotzsch et al. 2009). So far, domains III_1, III_2, III_5, III_7, III_9, III_{10}, and $III_{13\text{-}15}$ have been found to present new binding capabilities during physical strain (Gao et al. 2003, Hocking et al. 1994). The initial force applied to fibronectin exposes only a few cryptic binding sites, but as those sites are exposed, less strain is required to unfold the remaining sites (Klotzsch et al. 2009). Domain 10 requires the least amount of force to unfold, so is usually the first cryptic binding site to be exposed (Gao et al. 2002).

The potential binding partners for fibronectin are numerous and diverse, and differ dependent on the extent of physical strain (Ingham et al. 1997, Singh et al. 2010). Collagen fibers, cell surface proteins, and other fibronectin

molecules are only some examples. Each domain of fibronectin has specific target proteins to bind, such as the synergy site on domain 9 that binds to various integrins, heparin-sulfate binding sites for syndecan interaction, and domains 7 and 15 which bind to Type I domains of fibronectin (Leiss et al. 2008, Ingham et al. 1997). One prevalent protein segment that can bind to many components is the Arginine-Glycine-Asparagine (RGD) sequence on domain 10. This three peptide sequence binds to multiple integrin isoforms, such as $\alpha5\beta1$, $\alpha8\beta1$, $\alpha IIb\beta3$, and all αv integrin subunits (Pytela et al. 1985, Hynes 2002). The RGD site on fibronectin is a key factor in cell attachment, spreading, contractility, and migration (Prieto et al. 1993, Geiger et al. 2001). Studies have shown that the migration rate and metastasis of tumor cells was inhibited to some extent after the introduction of RGD fragments, which hints at new therapeutic possibilities for cancer treatment (Sanchez-Cortez et al. 2010).

Integrin Interaction

Of all of the cell surface proteins known to interact with the ECM, integrins predominate in the study of fibronectin binding. Not only do integrins combine to form multiple isoforms between their two subunits, but the isoforms can also target multiple binding sites on the fibronectin chain. For example, $\alpha5\beta1$ interacts simultaneously with the RGD on III_{10} and the synergy site on III_9 (Singh et al. 2010). The magnitude of possibilities for binding profiles between integrin and fibronectin could explain the diverse functionalities the stretching of fibronectin

may cause. Activation of integrins upon binding with fibronectin initiates the aggregation of integrin proteins in the plasma membrane, followed by the "outside-in" response of newly active downstream signaling cascades.

Downstream Signaling Effects

Signaling cues that affect cellular behavior take the form of multiple triggers, including chemical cues, electrical impulses, neighboring cell-cell interactions, and also mechanical cues. The physical forces that play a role in cellular behavior present themselves as environmental, as its native compliance, and as a transient force applied to a cell. Studies show that the stiffness of a cell's environment alone can affect behaviors such as migration, differentiation, and invasion (Lo et al. 2000, Engler et al. 2006, Provenzano et al. 2009). The stiffness of tissue is closely associated with cancer, as an increase in stiffness has been correlated to transformation of malignant breast cells in addition to its increase in invasion (Paszek and Weaver 2004). This elevation in the tumorigenesis is also due to a boost in the rate of proliferation in a more rigid matrix. This phenomenon is found to have been coupled to the FAK→Rho→ERK signaling pathway in mammary epithelial cells, and is known to be dependent on the surface receptor protein, α5β1 integrin (Figure 2.2) (Assoian and Schwartz 2008, Levental et al. 2009, Paszek and Weaver 2005, Roovers et al. 1999). However, the complete cellular pathway affected by the substrate compliance is not fully understood. The means of signal transduction is complex, as numerous

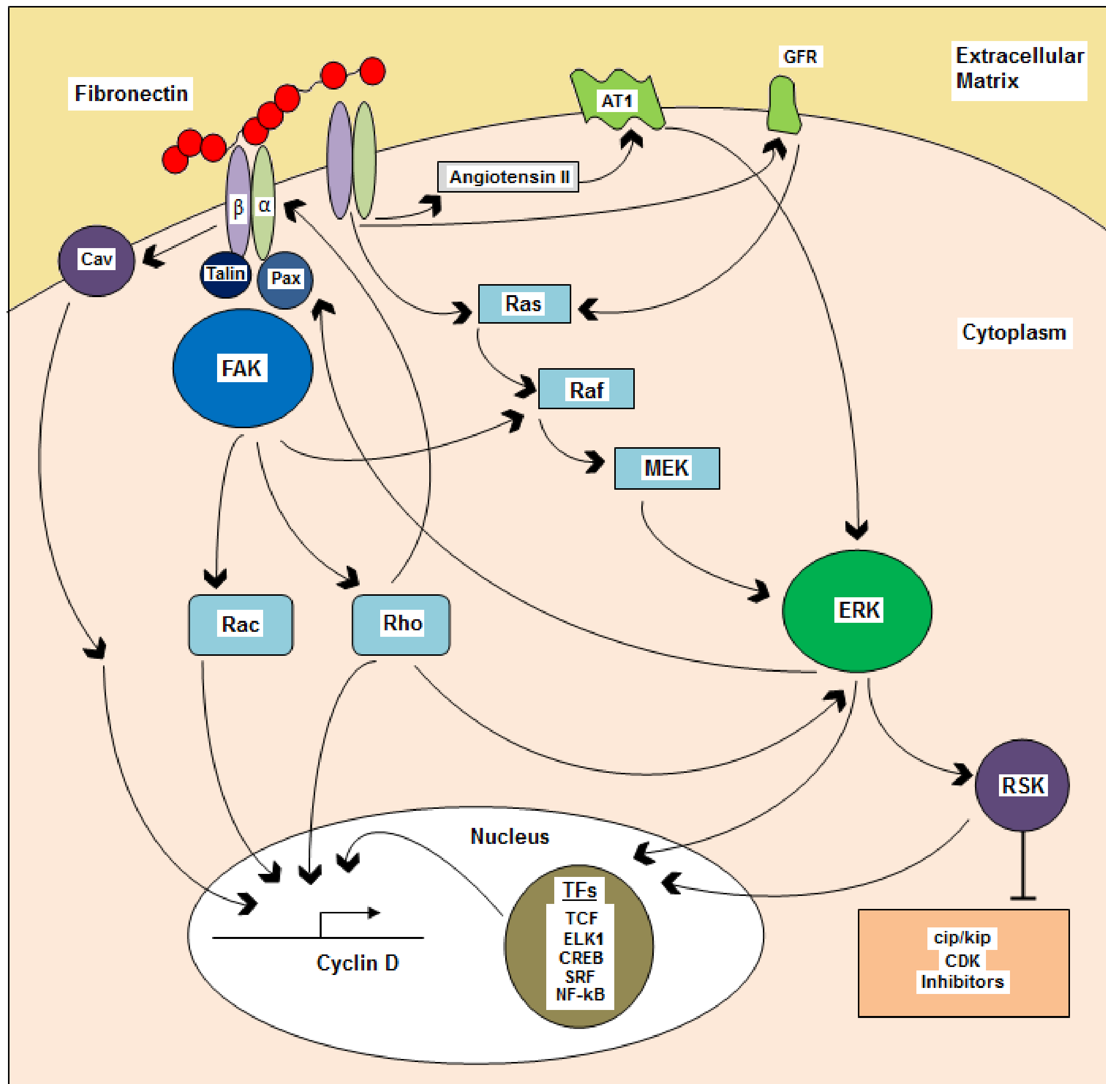

Figure 2.2: Cellular Signaling Cascades Triggered by Integrin Activation via Fibronectin Binding. *Integrin heterodimers bind extracellularly to the fibronectin strand in its partially unfolded state. Talin and Paxillin (Pax) recruit FAK on the cytosolic face of integrin, within the focal adhesion. FAK activates Rac and Rho, GTPases that influence the actin cytoskeleton as well as increase the transcription of cyclin D. Integrins also upregulate G protein-coupled receptor-AT1, through angiotensin II and Growth Factor Receptor (GFR) proteins. Activation of both of these receptors leads to ERK activation, either directly or through the Ras-Raf-MEK signaling pathway. ERK thereby either induces transcription factor (TF) activation leading to cyclin D transcription, or upregulates RSK, another kinase. RSK downregulates CDK inhibitors as well as upregulates transcription factors, both leading to an increase in the cell cycle. Caveolin (Cav) leads to an increase in DNA replication independently of FAK and ERK.*

pathways have been shown to be affected by compliance alone. For example, cells become more sensitive to epidermal growth factor on hard matrix *in vitro* as compared to soft (Kim and Asthagiri 2011). Also, on softer substrates that resemble the physiological stiffness of mammary cells or vascular smooth muscle cells (VSMCs), cyclin D1 induction is inhibited while cdk inhibitor expression remains the same (Klein et al. 2009). It has become clear that the multitude of downstream signals are triggered depending on the rigidity of a cell's microenvironment, but gaps remain to be filled in this very complicated pathway.

On the surface of the cell during outside-in signaling, integrin clustering induces the assembly of a large complex of proteins on the cytoplasmic face of the plasma membrane. These focal adhesion complexes are each a hub of activity for multiple types of proteins, including a tyrosine kinase, called Focal Adhesion Kinase (FAK) that is activated upon integrin-extracellular matrix binding. FAK is a crucial player in the mechanosensing function of cells, which is illustrated when cells seeded on mutated fibronectin, as well as those seeded on soft substrates, have lower levels of phosphorylation (Sechler and Schwarzbauer 1997, Klein et al. 2009). The effects of ECM compliance on the cell cycle are mediated through FAK activity at the focal adhesion. The initial action of this kinase upon integrin activation and clustering is to autophosphorylate at the tyrosine residue 397 (Klein et al. 2009). Upon phosphorylation, FAK incorporates into several separate signaling cascades, initiating a chain reaction for multiple behavioral outcomes, including cytoskeletal reorganization, invasion, and

proliferation (Provenzano et al. 2009). FAK is involved in the activation of both Rho and Rac, two GTPases that alter cell shape via the actin cytoskeleton. The activation of both of these GTPases also increases the expression of cyclin D1, contributing to a boost in proliferation (Klein et al. 2009). The activation of Rho leads to the subsequent triggering of Extracellular Signal-Regulated Kinase (ERK), also known as Mitogen-Activated Protein Kinase (MAPK), a kinase that has many target substrates.

ERK is another significant player in the pathway from extracellular signal to cell division and proliferation. Many proteins activate ERK, and it has over fifty known cytoplasmic substrates (Roskoski 2012). In regards to the cell cycle, transcription factors such as the Ternary Complex Factor (TCF) family and ELK1 are also affected (Roskoski 2012). Ultimately, the transcription of cyclin D1 is increased upon ERK activation, downstream of FAK.

Multiple other signaling cascades are influenced by integrin activation that also influence the cell cycle independent of FAK and ERK. The α5, αv, and α1 subunits of integrin associate with Shc and caveolin to promote DNA synthesis, for example, and integrin signaling also contributes to blocking cdk inhibitors as well via cip/kip downregulation (Assoian and Klein 2001, Klein et al. 2009). The phenomenon of cell cycle enhancement occurs in adherent cells under specific environmental conditions, and the cytosolic proteins influenced by physical force are proving to be widespread and complex.

Linking an Applied Stimulus to Cell Cycle Control

Previous studies have linked together the process of mechanosensing and cellular response to help understand the consequences of environmental changes on cellular signaling, but it is not yet fully understood how a cell perceives a change in its environment. The interaction with the extracellular matrix plays a role in the activation of integrins, but the mechanism of mechanotransduction, specifically in response to an applied physical stimulus is not understood. We have built an experimental assay that delivers a repetitive force to the cell substrate, simulating the pulling forces neighboring cells have on one another *in vivo*. When this mechanical stimulus was applied to cells a significant increase in proliferation was observed. Furthermore, the proliferation response was dependent on the presence of the ECM protein fibronectin. We hypothesize that the cryptic binding sites present on fibronectin are exposed upon experimental stimulation. This exposure then enhances integrin binding and activation, leading to an increase in downstream signaling causing a boost in the cell cycle. Our studies show that the ability of the ECM to be stretched and the interaction between cells and the RGD fragment on integrin domain 10 are both required for the proliferation changes seen in vitro, supporting the idea that cryptic binding sites are responsible for the mechanosensing response that leads to cell proliferation.

Materials and Methods

Tissue Culture

Mouse Embryonic Fibroblasts were cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM; sigma) 10% Fetal Bovine Serum (FBS; hyclone), 1% PSG (2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). Cells were grown at 37° in 5% CO_2. Passing of cells was performed at confluency with 0.01% Trypsin-EDTA. Cells were maintained no more than ten passages.

Substrate Preparation

Polyacrylamide substrates were created with 5% acrylamide, 0.1% bisacrylamide, 1M HEPES as previously described (Beningo et al. 2002). Carboxylated Paramagnetic beads (Promag 3 series Polysciences, Inc.) were sonicated and added at 1% volume. Gels were inverted during polymerization to ensure bead placement at gel surface. ECM proteins collagen and fibronectin were coupled to the surface of the substrate after Sulfo-SANPAH and treatment with ultraviolet light. Collagen type I (BD Biosciences) in PBS was coated at 0.2 mg/mL for four hours at 4° C, followed by rinsing with 1X Phospho-Buffered Saline (PBS) twice, and 50mM HEPES twice. Fibronectin was then added at 0.067 mg/mL in 50mM HEPES overnight at 4° C. Substrates were then rinsed three times with 1X PBS.

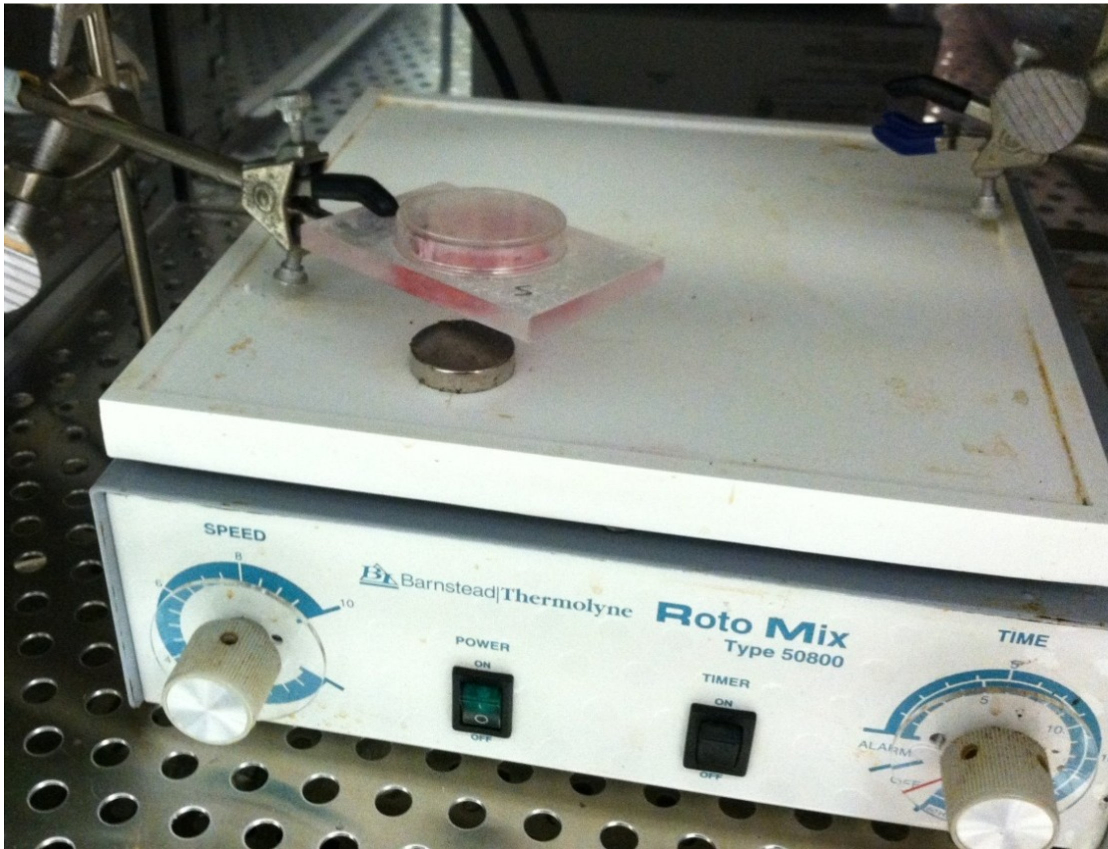

Figure 2.3: Experimental Setup for Applied Stimulus Assay. *Substrates are prepared, seeded with cells, and placed 1" over a magnet rotating on a mechanical stage. Magnet is oscillating approximately 110 rpm for 24 hours. The apparatus is positioned in a* 37° *incubator.*

<u>*Proliferation Assay*</u>

ECM-coated polyacrylamide substrates were UV sterilized, incubated in culture media for 30 minutes, and seeded with MEFs at a concentration of 3.5 x 10^5. Cells were allowed to adhere for two hours, and media was replaced to remove unbound cells. Chamber dishes were placed one inch above a 12,100

Gauss (25 mm diameter x 5.5 mm thick) magnet positioned on a revolving stage and incubated at 37° C for 24 hours (Figure 2.3). Non-stimulated control substrates were incubated at 37° C for 24 hours without magnet exposure. Ten images were taken of each substrate immediately before and after stimulation, individual cells were counted, and the average number for each substrate was used to calculate the fold increase as the ratio of cells before and after stimulation.

Cryptic Binding Site Analysis

For ECM fixation, substrates were treated with 25% paraformaldehyde 2X for 10 minutes each, followed by multiple rinses with 1X PBS for 1 hour at room temperature to ensure all formaldehyde was removed from the substrates. Substrates were then seeded with cells and analyzed for proliferation changes as described above.

For RGD competitive binding studies, 10 mg/mL GRGDS peptide (BACHEM) suspended in water were added to media at 2.6% v/v in media and added to the cultures as a full media change prior to stimulation. Control substrates include water in place of RGD fragment in media. Cells were again analyzed for proliferation changes as described above.

Protein Extraction

Substrates were increased to 25mm in size and coated with 0.2 mg/mL collagen and 0.067 mg/mL fibronectin. Cells were seeded at 7.0 x 10^5 and subject to magnetic rotation for 24 hours. Substrates were then washed 2X with cold 1X PBS, then treated with 100 μL cold Triton-X lysis buffer (0.1% triton-X, 0.15 M NaCl, SigmaFAST Protease Inhibitor (Sigma), and 0.028% Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific) for 30 minutes. Protein samples were stored at -20° C for future use.

Western Blotting

5-20 μg whole cell protein extracts were run by gel electrophoresis on 4-20% Precise™ Protein Gels (Thermo Scientific) at 100 volts for 1.25 hours in the case of β1 integrin and FAK y397 analysis, and 2.5 hours for ERK blots. Proteins were transferred for 30 minutes at 20 volts to a PVDF membrane and subject to blocking for 1 hour. 5% Bovine Serum Albumin (Fischer Scientific) in 0.1% TBS/T was used for blocking FAKY397 and ERK membranes, while 5% Milk blotting grade blocker (BioRad) in 0.1% TBS/T was used for β1 integrin and 5% milk in 0.1% PBS/T was used for GAPDH and Tubulin. Primary antibody probing was performed overnight at 4°C for each membrane. The following conditions and concentrations were used for antibodies; Rat monoclonal anti-active β1 antibody (BD Biosciences)(1:700) in 5% milk in 0.1%TBS/T; rabbit polyclonal anti-FAK pY^{397} antibody (Invitrogen)(1:1000) in 1% BSA in 0.1% TBS/T; mouse

monoclonal anti-ERK1/2 (pT202/pY204) antibody (BD Biosciences) (1:5000) in 5% BSA in 0.1% TBS/T; rabbit monoclonal anti-Glyceraldehyde-3-Phosphate Dehydrogenase antibody Clone 6C5 (Millipore) (1:700) in 5% milk in 0.1% PBS/T; and mouse monoclonal anti-αTubulin antibody (abcam) (1:1000) in 5% milk in 0.1% PBS/T were used. Membranes were washed 3X ten minutes in their prospective buffers, and probed for secondary antibody for one hour at room temperature. Secondary antibodies used in these studies were Goat polyclonal anti-rat (abcam) (1:20000) for β1, sheep anti-mouse IgG Horseradish peroxidase-linked antibody (GE Healthcare) (1:10,000) for ERK, GAPDH, and Tubulin, and donkey anti-rabbit IgG Horseradish peroxidase-linked antibody (GE Healthcare) (1:15000) for FAK Y397.

Results

Mouse Embryonic Fibroblasts Proliferate Faster when a Mechanical Stimulus is Applied

Multiple behavioral patterns such as migration, invasion, and proliferation are influenced by the environment surrounding a cell. The physical environment specifically can alter these cellular processes, both in the form of static compliance as well as an applied transient stimulus. Our lab has created an apparatus that has the ability to deliver a repeating applied stimulus by way of a magnetic force. This apparatus delivers a tugging and pulling motion on a polyacrylamide hydrogel that mimics the forces cells have upon their ECM and on one another. Using this apparatus, we have the ability to study the proliferation rates of adherent cells on controlled rigidities with consistent exposure to chemical influences in the media, while maintaining the ability to utilize the cells after stimulation for additional analysis.

The application of a mechanical stimulus to mouse embryonic fibroblasts resulted in an increase in the rate of proliferation over a twenty-four hour time period. To determine the extent of proliferation changes, images were taken of substrates before and after stimulation, and the cells were counted for each image (Figure 2.4A). The average number of cells for each time period is then used to calculate the normalized value of fold increase as a value of cells after stimulation over cells before stimulation. Our results show that the fold increase for non-stimulated substrates was 2.89, and for stimulated was 3.60, a twenty-

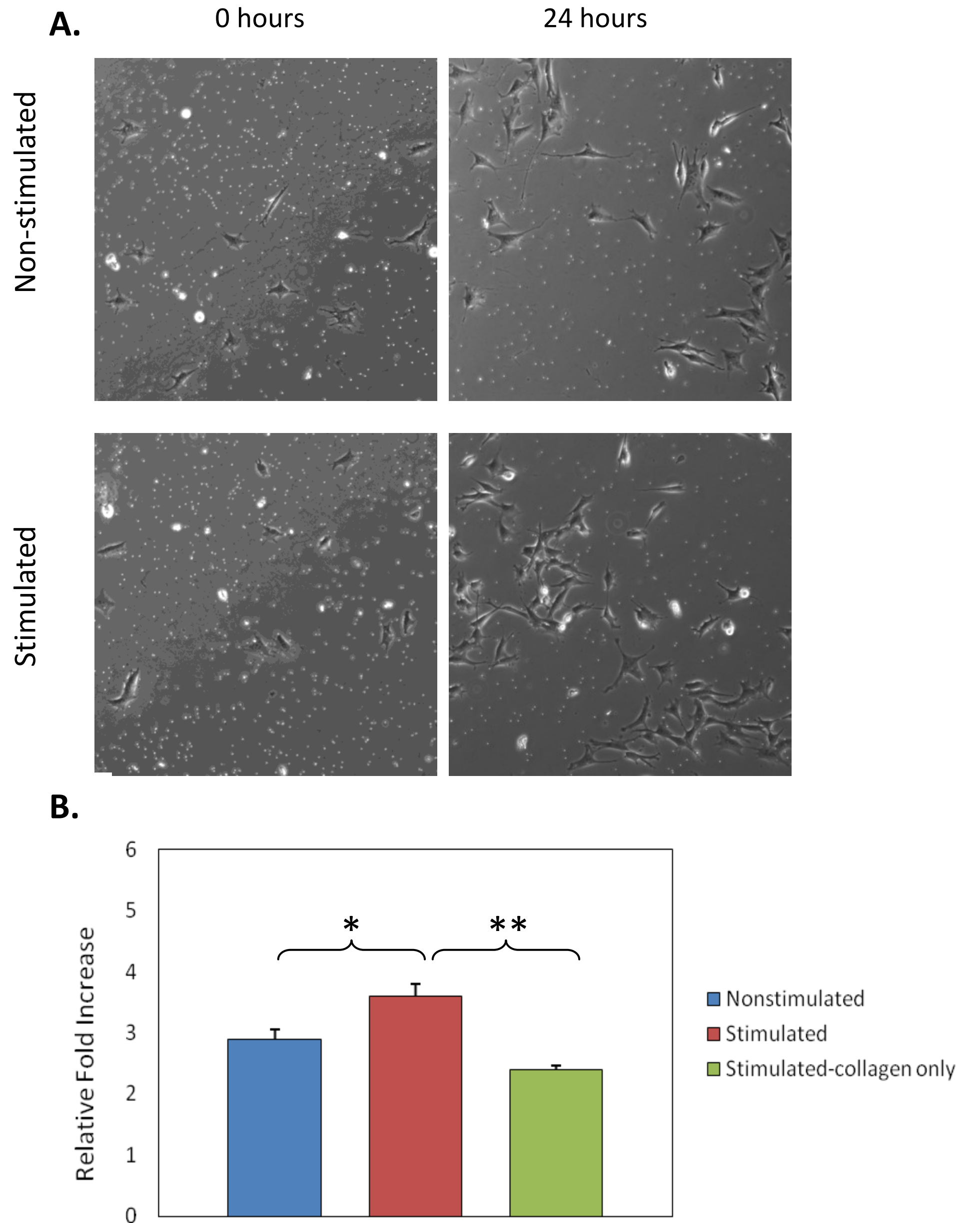

Figure 2.4: Proliferation of Mouse Embryonic Fibroblasts is Increased in the Presence of an Applied Mechanical Stimulus*. (A) 10X objective lens was used to capture images of cells prior to stimulation (0 hours) and after stimulation (24 hours). Non-stimulated control cells were incubated outside the magnetic field. (B) The relative fold increase in proliferation as determined by average number of cells per image taken at 24 hours over average number of cells per image taken at 0 hours. n=16 gels, 14 gels, and 6 gels, respectively (*p=0.0079, **p=0.0022)*

four percent increase upon stimulation (Figure 2.4B). In addition, we compared collagen coated substrates, to substrates coated with both collagen and fibronectin. Under these conditions, when collagen was used alone, the proliferation rates significantly decreased, both for the stimulated as well as the non-stimulated hydrogels. From our results we can conclude that when a transient mechanical stimulus is applied to cells in culture, fibroblasts increase the rate in which they divide, and that fibronectin is required for this process to occur.

Loss of Cryptic Binding Site Availability Interrupts Changes in Proliferation

The mechanism of how fibronectin influences cellular behavior is not fully understood. While much is known about the structure and binding partners of this ECM component, the exact mode of action and interplay of cryptic binding sites on mechanosensing and behavioral changes has not been determined. To test whether the cryptic binding capabilities of fibronectin affect cellular proliferation in our applied stimulus apparatus, we have utilized two methods to prevent the binding of cells to these newly exposed sites. First, we added the RGD peptide to the media just prior to stimulation. This fragment should pose as a competitive ligand for the RGD sequence on domain 10 of fibronectin, an integrin binding partner that is exposed with the least amount of force. Over the 24 hour time period, the proliferation of MEFs was not affected in the non-stimulated hydrogels, but decreased in the stimulated hydrogels in the presence of the RGD fragment by twenty five percent (Figure 2.5). This strongly suggests the

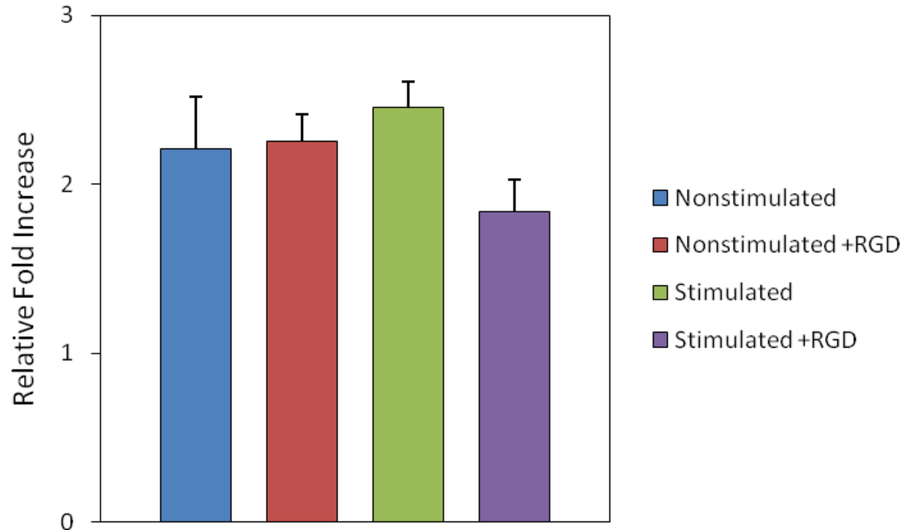

Figure 2.5: Proliferation Increases Seen Upon Stimulation are Lost When RGD Fragments are Introduced*. RGD fragments were added to the media prior to stimulation. Average fold increases for nonstimulated hydrogels were 2.21 without RGD and 2.25 with RGD fragments added. For stimulated hydrogels, average fold increases were 2.45 without RGD and 1.83 with RGD added. n=3 for nonstimulated and 4 for nonstimulated+RGD, Stimulated, and Stimulated+RGD.*

importance of this binding sequence to the mechanical sensing needed for increased proliferation.

As a secondary method for testing the activity of cryptic binding, we reasoned that if we could lock the fibronectin into an inflexibile conformation preventing stretching and access to the cryptic binding sites, we would lose our mechanical response. To test this theory we chemically crosslinked the collagen/fibronectin ECM. Hydrogels coated in ECM proteins were treated with paraformaldehyde prior to stimulation. In this proliferation analysis, the increase in proliferation seen upon stimulation is lost in the ECM crosslinked samples, yet the cells proliferated normally. We observed a 2.15 fold increase in proliferation

on stimulated as well as non-stimulated substrates (Figure 2.6). In both assays where the binding to cryptic binding sites was prevented either by RGD peptide or through chemical crosslinking, the downstream effect of proliferation changes upon mechanical stimulation was lost, supporting our hypothesis that the cryptic binding site exposure upon stretching of fibronectin is a reasonable explanation for the increased proliferation we observe in our applied stimulus assay.

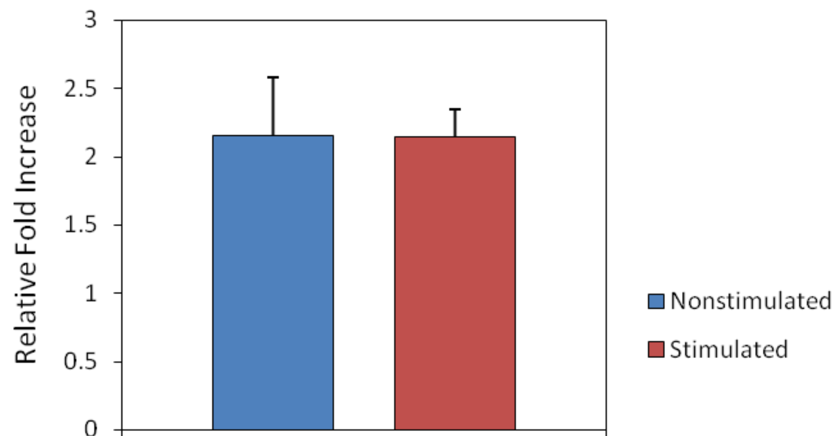

Figure 2.6: Proliferation Increases During Stimulation are Lost When the Extracellular Matrix is Chemically Crosslinked*. Collagen and Fibronectin coated on the substrate were crosslinked with paraformaldehyde prior to seeding the cells and stimulation. Over a 24 hour period, a 2.15 average fold increase was seen for both nonstimulated and stimulated hydrogels. n=8.*

β1 Integrin Activity Does not Change Given an Applied Stimulus

To test whether or not the integrin activity on the cell surface is influenced by the physical manipulation of the substrate, we did Western blot analysis using an antibody specific to the active form of β1 integrin. Surprisingly, the levels of active β1 integrin remained the same in the stimulated cells as compared to the

non-stimulated (Figure 2.7). There are many possible explanations for this phenomenon, including the difference in integrin profiles among the many heterodimers and subunit isoforms found on the cell surface.

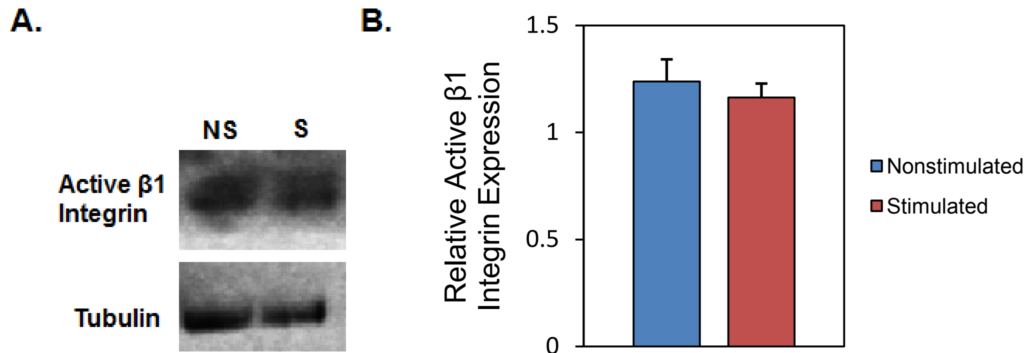

Figure 2.7: The Active Form of β1 Integrin Does Not Change Upon Stimulation. *(A) Western blot analysis of whole cell lysates using an antibody to the active form of β1 integrin, and alpha-tubulin antibody serves as a loading control (B) Quantification of Western intensity readings showing relative change in band intensity of protein samples from stimulated and nonstimulated MEFs. n=12*

Focal Adhesion Kinase Phosphorylation Levels Increase upon Stimulation

The interaction of the cryptic binding sites on fibronectin with the integrin proteins on the cell surface triggers an outside-in signaling response that opens up a multitude of downstream effects (Assoian and Schwartz 2001, Antia et al. 2008). Those downstream effects that influence the cell cycle have been thoroughly researched, as previous studies have shown many factors influenced by integrin activity (Assoian and Schwartz 2001). One such protein, Focal Adhesion Kinase, is known to be phosphorylated upon integrin activation (Klein et al. 2009). With this in mind, we asked whether the stimulation of cells that increases proliferation has an effect on the phoshorylation state of FAK. Using an

antibody specific to FAK's phosphorylated form we performed a western blot, comparing lysates from stimulated and unstimulated cultures. Our analysis finds that the phosphorylation of FAK on its tyrosine 397 residue is increased 66% upon stimulation (Figure 2.8). Reasonably, these results led us to believe that the FAK activation is likely due to an increased level of integrin activation upon stimulation and thus we should test both upstream and downstream pathways of FAK activation.

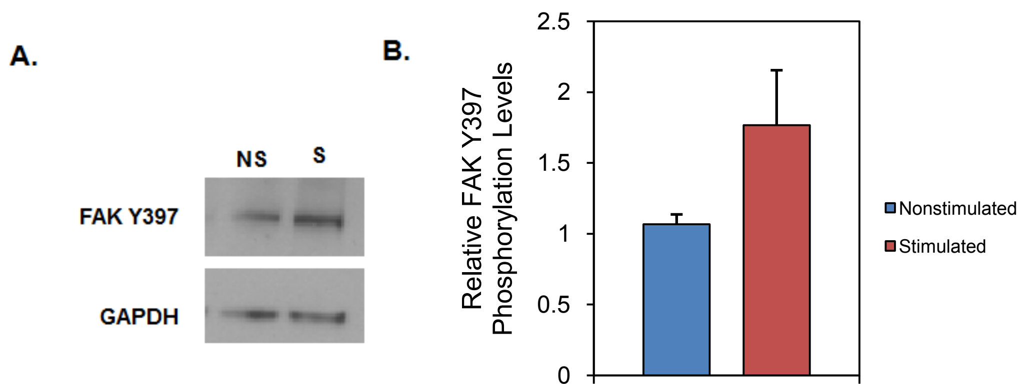

Figure 2.8: Focal Adhesion Kinase Increases in Phosphorylation at Tyrosine 397 Upon Stimulation. *(A) Western Blot image of FAK phosphorylation states with anti-pY397 antibody. Anti-GAPDH was used as a loading control. (B) Quantification of western intensity readings. Relative intensity increased from 1.06 for nonstimulated to 1.77 for stimulated cells. n=3 independent experiments.*

The Phosphorylation of Extracellular Signal-Regulated Kinase is Increased upon Stimulation

Downstream of FAK, many pathways are activated to affect the cell cycle. One protein in particular that is known to affect the transcription of cell-cycle

dependent genes is ERK. To determine if ERK is a possible contributing factor to the proliferation effects seen in our experiments, we again used an antibody specific to phospho-ERK in western blot analysis to test the phosphorylation state of ERK with and without stimulation. Upon stimulation we observed a 40 percent increase in phosphorylation on the p204 residue of ERK (Figure 2.9). This increase in phosphorylation supports the hypothesis that the mechanically stimulated increase in proliferation flows through FAK to ERK.

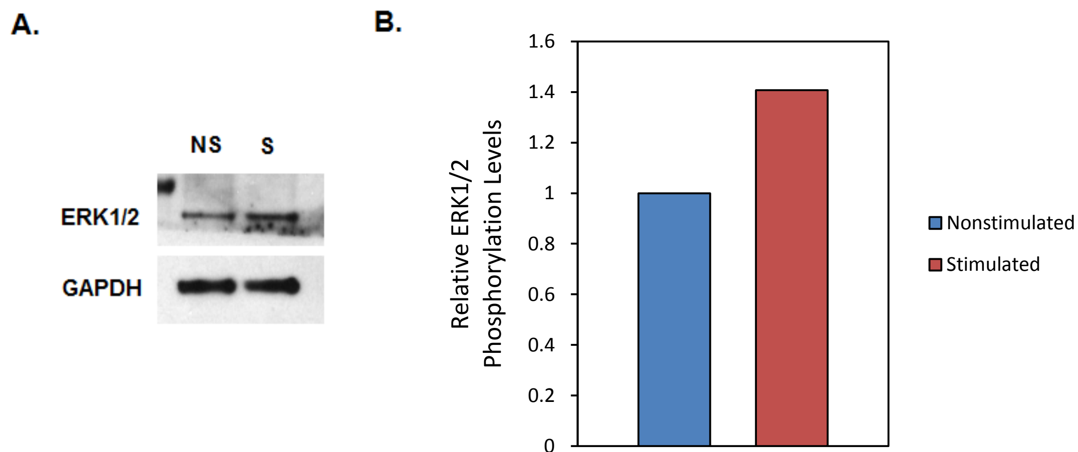

Figure 2.9: Extracellular Signal-Regulated Kinase Phosphorylation State Increases Upon Stimulation. *(A) Western Blot with an anti-ERK1/2 p202/y204. Anti-GAPGH antibody was used for loading control. (B) Intensity readout of blot from A. Relative intensity values were 1.0 for nonstimulated cells and 1.4 for stimulated. (n=1)*

Discussion

The mechanical microenvironment has a significant impact on cells, which is becoming increasingly apparent in recent studies. One such impact is the effect of the mechanical environment on the cell cycle and thus the rate at which cells proliferate. Much is known about the surface proteins responsible for relaying signals across the plasma membrane, as well as the complex intracellular activation cascade that is subsequently activated leading to cell cycle effects, but the relationship between mechanical force and the interacting surface receptors is not well understood.

The physical link between the cell and its underlying substrate, the ECM, is crucial for cellular mechanosensing via focal adhesions (Galbraith et al. 2002, Tee et al. 2009). Our proliferation studies indicated that fibronectin specifically is important, as proliferation rates were identical for cells on stimulated and nonstimulated gels seeded with collagen alone. The conformational changes in fibronectin brought on by physical forces unleash numerous potential binding partners for cells and for other ECM components. Preventing the exposure of the cryptic binding sites by chemically crosslinking the proteins negates any changes in proliferation seen previously. The fibronectin in this state remains available for cell binding, so that cells can continue to adhere, migrate, and proliferate on these substrates, however proliferation rates were slower for cells on crosslinked ECM for both stimulated and non-stimulated as compared to untreated substrates. It is possible that the harsh chemical treatment could have

inadvertently affected the cells, causing unwanted damage. Alternatively, the crosslinking of the ECM also prevents ECM fibrillogenesis by the cells to a certain degree, as movement is required for fiber rearrangement, and cryptic binding sites are also important for the process of fibrillogenesis (Vakonakis et al. 2007, Ingham et al. 1997). The lack of fibrillogenesis would stunt cell division due to a decrease in the available ligand binding partners for integrin receptors. Although proliferation rates were lower for both substrates, the stimulated and nonstimulated cells showed similar proliferation rates, supporting the mechanism proposed that cryptic binding site exposure is the cause of mechanically induced proliferation changes.

Surface-receptor binding to cryptic binding sites was also prevented by the addition of RGD fragments to the cell culture media. In stimulated cells these short 3-amino acid peptides decreased the rate of proliferation to levels lower than in non-stimulated cells, indicating that integrin-RGD interactions are important for mechanosensing. The RGD fragments present also occupied the integrin binding sites in place of the RGD sequence present on $FNIII_{10}$, one of the cryptic sites that is likely to be exposed during stretching (Krammer et al. 2002). One alternative method to further test the exposure of cryptic binding sites is to add antibodies that would recognize specific domains of the fibronectin. These antibodies would potentially act as a competitive binder for the cryptic site as it is exposed, preventing any integrin interaction and indicating which domains play a role in increasing the rate of the cell cycle.

Both chemical crosslinking and the addition of competitive binding partners support our hypothesis that the exposure of cryptic binding sites on fibronectin causes the mechanosensing response in cells. To further test our hypothesis, the next step is to visualize the stretching of fibronectin on the substrates of our magnetic apparatus. To do this, we have utilized a method of Fluorescence Resonance Energy Transfer (FRET) established by Vogel et. al. that measures the stretching of fibronectin as a loss of the fluorescent signal. In this application, the dimer molecule is labeled with a donor fluorophore on the cysteine residues and an acceptor fluorophore is labeled on the lysine residues. This labeled protein would have the highest state of emission in its most compacted form, and decrease as the protein extends. Using this method of FRET, we would be able to quantify the degree of protein extension during the magnetic stimulation in our experimental setup. We could also use this method to confirm the altered conformation state of fibronectin in our experimental applications. For example, we could also use this analysis to visually confirm the loss of conformational change after the paraformaldehyde treatment, and the retention of flexibility upon RGD addition.

Surprisingly, the surface-receptor protein we expected to be a major player in the mechanosensing cascade, β1 integrin, does not appear to change in the number of active β1 subunits. Numerous explanations could account for this result. For instance, because integrins cluster within the membrane to form focal adhesions as well as alter their secondary structure upon activation, it is

possible that localization effects could be altered instead (Stewart and Hogg 1996). Localization studies would indicate whether the level of total integrin subunits or the location of the integrin proteins is affected by stimulation. Alternatively, other integrin subunits besides α5 or β1 could be acting as mechanosensors in these cells. Multiple alpha subunits have been linked to mechanosensing, as well as the β3 subunit (Ayala and Desai 2011, Mao and Schwarzbauer 2012). Also, the RGD sequence has multiple integrin binding partners (Hynes 1992). The abundance of integrin combinations present at the cell surface, expression profiles among various cell lines, and redundant mechanosensing functions among subunits all support the theory that β1 integrin is not acting alone, or possibly not acting at all in linking applied force to transcriptional changes for proliferation.

To confirm the cytosolic players typically involved in the proliferation increase during mechanical stimulation, we tested the activity of known proteins involved in the mechanosensing response of integrins. After western blot analysis of the two cytosolic kinases FAK and ERK, we have found that the force applied to fibroblasts causes an increased level of activation, indicated by their phosphorylation states. Because both of these proteins have been previously found to activate transcription factors, both in conjunction and working separately, we begin to see the link between focal adhesion components and the cell division targets they activate.

Our study of cell proliferation has led us to believe that cryptic binding sites on fibronectin are important for the mechanosensing function required for changes in proliferation rates. We have confirmed the changes in cellular signaling cascades responsible for the enhanced rate of cell division, but the surface-receptor proteins involved still eludes us. Also, our ECM studies support our hypothesis for cryptic binding site involvement, but additional confirmation with FRET would further validate it as well as be a useful tool in future cryptic binding site analysis with our magnetic stimulation assay.

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ABSTRACT

INFLUENCE OF MECHANICAL CUES AND THE EXTRACELLULAR MATRIX ON CELL MIGRATION PATTERNS AND PROLIFERATION RATES OF CELLS

by

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May 2013

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The mechanical environment of a cell and its tissue can impact multiple biological processes including development, wound healing, and metastasis. Specific cellular behaviors influenced by the mechanical microenvironment include differentiation, morphology, apoptosis, migration, and proliferation. In this thesis I have focused specifically on the effect of environmental stiffness and applied mechanical forces on cellular migration and proliferation. Using two different applications, both tailored to evaluate the mechanical forces alone on cellular behavior, I attempted to simulate the mechanical composition of the *in vivo* tissue microenvironments *in vitro* using polyacrylamide hydrogels. To test whether cells maintain a mechanical memory for a specific stiffness *in vitro*, we utilized a substrate that differentially polymerizes with variant levels of UV exposure and analyzed the directional migration patterns upon different rigidities. These substrates did not show any particular directional preference for migration, however cells did seem to be able to sense variation in stiffness based on the

results of a morphology assay. It is unknown whether the cells were unable to sense differences in neighboring stiffness due to the extracellular matrix or to the hydrogel itself. To examine the proliferation rates of cells given an applied mechanical stimulus, we created hydrogels embedded with magnetic microbeads that provided a tugging and pulling motion mimicking the effects of adherent cells on their neighboring environment. The observed increase in proliferation upon mechanical stimulation was dependent on the presence of fibronectin coated to the hydrogel surface, indicating that this protein is essential for the mechanosensing response of cells. I hypothesized that compacted conformations of fibronectin are released during mechanical stimulation, opening cryptic binding sites for cells to adhere to. I tested the presence of these cryptic binding sites by chemically crosslinking the ECM prior to stimulation, as well as adding the competitive peptide arginine-glycine-aspartic acid (RGD). Both of these resulted in a decrease in the proliferation rate during stimulation but had no effect in control cells. The surface receptor protein responsible for activating these cascades is still unknown. After testing the activity level of β1 integrin, a known mechanosensor and binding partner to fibronectin, there was no difference in the activity of this particular integrin subunit, strongly suggesting this is not the integrin activated by our mechanical stimulus. Protein activity studies found that the phosphorylation states of both Focal Adhesion Kinase (FAK), as well as, Extracellular Signal-Regulated Kinase (ERK) are increased upon stimulation, indicating that these two signaling cascades lead to an increase in

the cell cycle activity. Further studies are required to determine the link between the fibronectin cryptic sites and the downstream signaling cascades activated during stimulation. Both of these cell behavioral studies will help to better understand the extent of impact the mechanical environment has on living tissue systems.

AUTOBIOGRAPHICAL STATEMENT

Education

Master of Science (May 2013)
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Bachelors of Science (May 2007)
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Experience:

Graduate Teaching Assistant (2008- 2013)
Courses Taught:
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Graduate Research Assistant (2011)

Activities:

Guest Lecturer for Undergraduate Level Cell Biology (BIOL 2800) and Graduate Level Cell Biology (BIOL 6000) at Wayne State University
Poster Presentations at WSU Biological Sciences Annual Retreat: 2009, 2010, and 2012
Participant in Scientific Teaching Focus Group for Anatomy and Physiology Lab 2011

Awards:

Exceptional Service as a Graduate Teaching Assistant from Graduate School (August 2012)
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Publications:

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