THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOMEDICAL ENGINEERING

THE EFFECT OF NANOFIBER DIAMETERS ON INTEGRIN REGULATED FAK-SRC
SIGNALLING AND MIGRATION RATES OF HUMAN MESENCHYMAL STEM CELLS

MARY ELIZABETH MCCULLOCH
SPRING 2016

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Bioengineering
with honors in Biomedical Engineering

Reviewed and approved* by the following:

Justin Brown
Assistant Professor of Biomedical Engineering
Thesis Supervisor

William Hancock
Professor of Biomedical Engineering
Honors Adviser

* Signatures are on file in the Schreyer Honors College.
ABSTRACT

Nanofibers play a significant role in modulating tissue growth.[1][2][3] Applications include nerve regeneration, wound healing, hernia repair, and repair of musculoskeletal tissues. Cells sense environments via signal transduction pathways, which can lead to changes in cell shape, proliferation rates, gene expression, and differentiation.[4] Integrins are a type of protein that exists on the cell’s surface and initiate intracellular signaling and play a major role in the adhesion of cells to a surface. These integrins utilize inside-out as well as outside-in communication and are the mediator of the cell’s interaction with surrounding extracellular matrix. Integrins activate other signaling proteins, including Focal Adhesion Kinase (FAK) and Src which exist in many phosphorylation states. FAK and Src act in a mutual activation to amplify existing signal for cellular migration processes.

This research aims to characterize migration of Human Mesenchymal Stem Cells (hMSCs) on poly(methyl methacrylate) (PMMA) nanofibers of five different diameters ranging from 100nm – 1200nm. Relative FAK and Src concentrations of hMSCs were quantified on nanofiber scaffolds of varying diameters. Migration rates across each diameter of nanofiber were also observed at two different time points and focal adhesion lengths were measured. This study shows FAK and Src concentrations differed for substrates of nanofibers with different diameters. Migration rates were found to be significantly different on nanofibers of different mean diameters. A decrease in pFAK-576/577 concentration resulted in faster migrating cells and ultimately revealed a dependency on Src-416 for activation, whose decrease also lead to faster migration.
# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................... iv

LIST OF TABLES ........................................................................................................................ ix

ACKNOWLEDGEMENTS .............................................................................................................. v

Chapter 1 Background ..................................................................................................................... 1

Chapter 2 Introduction ..................................................................................................................... 11

  Significance of Research ........................................................................................................ 12
  Hypothesis and specific aims ................................................................................................. 12

Chapter 3 Nanofiber Fabrication and Human Mesenchymal Stem Cell Culture ........... 14

  Background .............................................................................................................................. 14
  Materials and Methods .......................................................................................................... 14
    Electrospinning ..................................................................................................................... 15
    Nanofiber Sample Preparation ........................................................................................ 15
    Live Cell imaging prior to protein harvest ........................................................................ 17
    Cell Lysates .......................................................................................................................... 17
  Results ..................................................................................................................................... 17
    Nanofiber Morphology ........................................................................................................ 17
    Cell Morphology ................................................................................................................ 19
  Discussion ............................................................................................................................... 20

Chapter 4 Modulating and Characterizing Nanofiber Diameter ..................................... 21

  Background .............................................................................................................................. 21
  Materials and Methods .......................................................................................................... 21
    Modulating Diameter of Nanofibers .................................................................................. 21
    Characterizing Diameter of Nanofibers .......................................................................... 23
  Results ..................................................................................................................................... 24
    Scanning Electron Microscopy ........................................................................................... 24
    Diameter analysis determined manually ......................................................................... 25
    Diameter batch analysis determined automatically ......................................................... 27
  Discussion ............................................................................................................................... 28

Chapter 5 Characterization of Migration ............................................................................. 32

  Background .............................................................................................................................. 32
  Materials and Methods .......................................................................................................... 32
    Time-Lapse Imaging .............................................................................................................. 32
<table>
<thead>
<tr>
<th>Chapter 6 Characterization of Src, FAK, and Vinculin</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>39</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>41</td>
</tr>
<tr>
<td>Coomassie Protein Staining of a PVDF membrane</td>
<td>41</td>
</tr>
<tr>
<td>Dot Blot</td>
<td>41</td>
</tr>
<tr>
<td>Western Blot</td>
<td>42</td>
</tr>
<tr>
<td>Immunofluorescence staining</td>
<td>46</td>
</tr>
<tr>
<td>Statistics</td>
<td>46</td>
</tr>
<tr>
<td>Results</td>
<td>46</td>
</tr>
<tr>
<td>Discussion</td>
<td>49</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 7 Velocity Profiles, Focal Adhesion Length, and Src/FAK Comparisons</th>
<th>51</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>51</td>
</tr>
<tr>
<td>Results</td>
<td>51</td>
</tr>
<tr>
<td>Discussion</td>
<td>59</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 8 Conclusions and Future Experiments</th>
<th>61</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix A Live Cell Images on Various Diameter Fibers</td>
<td>63</td>
</tr>
<tr>
<td>Appendix B Preliminary Experiments using CSB</td>
<td>69</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>73</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1 - Diagram depicting actin arc contraction, a vital piece to cellular migration Figure adapted from reference [8]. .................................................................2

Figure 2 - Diagram depicts nascent and mature focal adhesion and focal complexes, respectively.[9] ........................................................................................................3

Figure 3- Images show distortions in micropatterned substrates due to focal adhesion medicated contractions. Red arrowhead show distortion and magenta dots underline the pinching action of contraction.[10] ......................................................................................4

Figure 4- Fluorescent image of human foreskin fibroblast expressing tagged-vinculin. The forces transmitted at focal adhesion shown with red arrows, while green arrows show displacement.[10] ......................................................................................4

Figure 5- Diagram depicts adhesion maturation and Rho GTPase activation.[9] ......................6

Figure 6 - Diagram depicts a presented mechanism for adhesion. Although quite complex; it encompasses the vast majority of proteins often associated with cellular migration.[12] 7

Figure 7 – Model for FAK and Src signaling involved in cell adhesions.[14] .......................8

Figure 8-shows substrates with varying ECM proteins and their levels of alkaline phosphatase (ALP) activity.[15] ................................................................................................................10

Figure 9- Illustration of an electrospinning apparatus. [30] ..................................................15

Figure 10 – This shows a depiction of the spin coating process. Image source ..................16

Figure 11 - SEM images of samples of various diameters. Diameters were modulating using different concentration of high molecular weight (HMW) of poly(methyl methacrylate) (PMMA) in a 60:40 solvent mix of Dimethylformamide:Tetrahydrofuran. Electrospon nanofibers were spun using a polymer concentration of A) 1.54wt%  B) 3.1wt%  C) 3.7wt% D) 6 wt% E) 8.3 wt% ........................................................................18

Figure 12 - HMSCs on A) Flatt PMMA B) 1.54% HMW PMMA Nanofibers C) 3.1% HMW PMMA Nanofibers D) 3.7% HMW PMMA Nanofibers E) 6% HMW PMMA Nanofibers F) 8.3% HMW PMMA Nanofibers ........................................................................19

Figure 13-This figure shows increased etching and therefore decreased diameter of nanofibers. Notice the size scales are consistent throughout the images.[24] .................................22

Figure 14 – ImageJ manually determined the diameter of PMMA nanofibers .........................23

Figure 15 – A diagram depicting the fiber analysis of DiameterJ after the SEM image has been segmented.[25] .......................................................................................24
Figure 16 – SEM images of samples of various diameters. Diameters were modulating using different concentration of high molecular weight (HMW) of poly(methyl methacrylate) (PMMA) in a 60:40 solvent mix of Dimethylformamide:Tetrahydrofuran. Electrospun nanofibers were spun using a polymer concentration of A) 2.5wt%  B) 3wt%  C) 4.5wt% D) 7 wt% .................................................................

Figure 17 - Relation between mean fiber diameter and PMMA wt% as determined by manual measurement using ImageJ. Graph shows fiber diameter for fibers spun from a 18G and 30G needle. Data was provided by Daniel Bowers.................................................................26

Figure 18 – Relation between mean fiber diameter and PMMA wt% as determined by manual measurement using ImageJ. Graph shows fiber diameter for fibers spun from a 18G and 30G needle. Data was provided by Daniel Bowers.................................................................27

Figure 19- Relation between mean fiber diameter and PMMA wt% as determined by automatic measurement using DiameterJ Plugin. Graph shows fiber diameter for fibers spun from a 18G and 30G needle.................................................................28

Figure 20 – Comparison of fiber diameter analyzation and PMMA wt% as determined by manual and automatic measurement. Graph shows fiber diameter for fibers spun from a 18G and 30G needle. .........................................................................................................................29

Figure 21 – This shows two images that lead insight into discrepancies between DiameterJ and ImageJ .........................................................................................................................30

Figure 22 - Mean velocities from 6-12 hours of MEFs on different diameter PMMA fibers (given in molecular weight concentrations of PMMA). Error bars denote standard error of the mean. N=5 See Table 3 for approximate diameters for a given concentration. Bars with asterisk represents a Pvalue<0.05 as determined by the t-test. Note that the 1.54% PMMA nanofibers are not included due to cells not attaching during the 6-12 hour time period and no migration data was collected. Data provided by Daniel Bowers.........................................................................................34

Figure 23 -Mean velocities from 13.5-19.5 hours of MEFs on different diameter PMMA fibers (given in molecular weight concentrations of PMMA). Error bars denote standard error of the mean. N=5. See Table 3 for approximate diameters for a given concentration. Bars with asterisk represents a Pvalue<0.05 as determined by the t-test. Data provided by Daniel Bowers ........................................................................................................35

Figure 24-Mean velocities from 6-12 hrs. and 13.5-19.5 hours of MEFs on different diameter PMMA fibers (given in molecular weight concentrations of PMMA). Error bars denote standard error of the mean. N=5. See Table 3 for approximate diameters for a given concentration. Bars with asterisk represents a Pvalue<0.05 as determined by the t-test. Data provided by Daniel Bowers........................................................................................................35

Figure 25 - Trends for mean velocity rates of MEFs for a given diameter. N=5 for migration velocity. Error bars denote standard error of the mean ........................................................................................................36

Figure 26 - Time lapse of MEFs for 6-12 hours. Picture were taken every 90 second intervals and reported as an average of 15 minutes. N=5 ........................................................................................................37
Figure 27 – Proposed model of Src-induced phosphorylation of FAK and its effects.[26].....40

Figure 28 - This image depicts a typical dot blot which can be further analyzed for intensity, although size information is not known. Image source<http://rsb.info.nih.gov/ij/docs/examples/dot-blot/>.................................................................42

Figure 29 - An overview of the western blotting technique. Some difference between the protocol and the diagram is the proteins were transferred onto a PVDF membrane not a Nitrocellulose sheet. Image Source<http://proteomics.case.edu/proteomics/westernblot.html> ..........43

Figure 30 - Odyssey Infrared Imaging system. Image Source<http://www.brown.edu/Research/CGP/core/equipment/>.........................................................45

Figure 31 – Specific phosphorylation states of pFAK-397 and pFAK-925 intensity levels normalized to Total FAK for a given HMW concentration of PMMA. Total FAK is also shown normalized to Tubulin. See Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Table 3 for approximate diameters for a given concentration. N=3 Error bars denote standard error of the mean. FAK 397 showed a PPMC coefficient of 0.87 and FAK925 PPMCC= -0.65034. ...............................................47

Figure 32 - Specific phosphorylation states of pFAK-576/577 and total FAK intensity levels normalized to Total FAK for a given HMW concentration of PMMA. Total FAK is also shown normalized to Tubulin. See Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Table 3 for approximate diameters for a given concentration. N=3 Error bars denote standard error of the mean. FAK 576/577 showed a PPMC coefficient of -0.60 and Total FAK PPMCC= -0.61. .........................47

Figure 33 – pSrc 416 intensity levels on different diameter PMMA fibers (given in molecular weight concentrations of PMMA). See Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Table 3 for approximate diameters for a given concentration. N=3. Error bars denote standard error of the mean. Src 416 normalized to Tubulin showed a PPMC coefficient of 0.58 and Src 416 normalized to FAK total showed a PPMCC= -0.07. ..........................................................48

Figure 34 -This figure shows an immunofluorescent image of an hMSC on PMMA nanofibers. Green/blue represents the nucleus, yellow represents focal adhesions(vinculin), and red represents actin.................................................................48

Figure 35 - Focal Adhesion length is compared to nominal nanofiber diameter. N>2000. Error bars denote standard error of the mean. .................................................................49

Figure 36 - The mean migration speed compared to the relative concentration of pFAK-397 normalized to total FAK for a certain diameter of fiber scaffold. N=5 for migration velocity N=3 for protein concentration.................................................................52

Figure 37 - The mean migration speed compared to the relative concentration of pFAK-576/577 normalized to total FAK for a certain diameter of fiber scaffold. N=5 for migration velocity N=3 for protein concentration.................................................................52
Figure 38 - The mean migration speed compared to the relative concentration of pFAK-925 normalized to total FAK for a certain diameter of fiber scaffold. N=5 for migration velocity N=3 for protein concentration..........................................................53

Figure 39 - The mean migration speed compared to the relative concentration of pSrc-416 normalized to total FAK for a certain diameter of fiber scaffold. N=5 for migration velocity N=3 for protein concentration..........................................................53

Figure 40 - Relative concentration of pFAK-397 was normalized to total FAK and compared to migration velocity in micrometers per hour. Pearson Product-Moment Coeff. = -0.36 ..54

Figure 41 - Relative concentration of pFAK – 576/577 compared to migration data given in micrometers per hour. Error bars denote standard error of the mean. Pearson correlation test = -0.872 ..........................................................54

Figure 42- pFAK - 925 is compared to migration data given in micrometers per hour. Error bars denote standard error of the mean. Pearson correlation test = -0.12 ..............................................55

Figure 43 – pSrc - 416 is compared to migration data given in micrometers per hour. Error bars denote standard error of the mean. Pearson correlation test = -0.72 ..............................................55

Figure 44 - Focal adhesion length is compared to migration data given in micrometers per hour. Error bars denote standard error of the mean. Pearson correlation test = -0.39 ..................56

Figure 45 - Focal adhesion length is compared to relative intensity of pFAK-397. Error bars denote standard error of the mean. Pearson correlation test = -0.60171........................57

Figure 46 - Focal adhesion length is compared to relative intensity of pFAK-576/577. Error bars denote standard error of the mean. Pearson correlation test =0.09229 ....................57

Figure 47 - Focal adhesion length is compared to relative intensity of pFAK-925. Error bars denote standard error of the mean. Pearson correlation test = -0.38974 ..................58

Figure 48 - Focal adhesion length is compared to relative intensity of pFAK-925. Error bars denote standard error of the mean. Pearson correlation test = -0.38974 ..................58

Figure 49 - Adapted from Nanofiber diameter-dependent MAPK activity in osteoblasts.[31]61

Figure 50 - Specific phosphorylation states of pFAK-576/577 normalized to Total FAK for a given HMW concentration of PMMA. Total FAK is also shown normalized to Tubulin. See Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Table 3 for approximate diameters for a given concentration. N=3 Error bars denote standard error of the mean. FAK 576/577 showed a PPMC coefficient of -0.60 and Total FAK PPMCC= -0.61. ..................................................62

Figure 51 - This figure shows a western blot of samples 99 – 104 and band correspond to the stained Src protein..........................................................70
Figure 52 - This figure shows a western blot of samples 99 – 104 and band correspond to the stained Tubulin protein. ................................................................................70

Figure 53 - This figure shows a western blot of samples 102– 107 and bands correspond to the stained Src protein. ........................................................................................................70

Figure 54 - Those Shows samples 102– 107 and their Tubulin Expression .........................70

Figure 55 - Those Shows samples 108– 113 and bands correspond to the stained SRC protein. 70

Figure 56 - Those Shows samples 108– 113 and their Tubulin Expression ..........................70

Figure 57 - Those Shows samples 113– 116 and their SRC Expression ...............................71

Figure 58 - Those Shows samples 113– 116 and their Tubulin Expression ..........................71

Figure 59 - This figure shows the relative SRC intensities. Intensities were normalized to Tubulin intensities. Error bars are the standard deviation of the sample group..................................71

Figure 60 - This figure shows the relative SRC intensities in the CSB of the samples. Intensities were normalized to Tubulin intensities. Error bars are the standard deviation of the sample group. ........................................................................................................72
LIST OF TABLES

Table 1 – Diameter and standard deviation of nanofibers spun using four different weight percents of PMMA. These values were generated manually through ImageJ. Data was provided by Daniel Bowers………………………………………………………………………………26

Table 2 - Diameter and standard deviation of nanofibers spun using four different weight percents of PMMA. These values were generated automatically through DiameterJ………………27

Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Figure 20………………………………………………………………………………31

Table 4- Concentration of beta-mercaptoethanol solution and protein sample to be loaded into each well for a Western blot…………………………………………………………………43

Table 5 - This table depicts the various primary antibodies that detect various proteins expressed in cells at the varying environments…………………………………………………………45

Table 6 - This table depicts the two secondary antibodies that detect various proteins expressed in cells at the varying environments…………………………………………………………45

Table 7 - This table shows the descriptions of each samples used and their corresponding sample number ………………………………………………………………………………………………………69
ACKNOWLEDGEMENTS

I have been fortunate to work with a supportive advisor, Dr. Justin Brown, who inspired and motivated me to explore the cellular adhesion pathway. I would like to thank his encouraging grad students, Brittany Banik and Pouria Fattahi, who made my time in the lab enjoyable. Thanks to all the Biomedical Engineering faculty who have shaped me into the engineer I am today. I will always cherish the many hours and late nights in the basement of Hallowell, following my mother’s footsteps. This research would not be possible without my collaborator Daniel Bowers, whose patience and teachings made me into the researcher I am today. Thank you for rising to the challenge and taking me as your undergraduate assistant when I knew nothing about lab etiquette. Thanks to the Project Vive team for their patience with my work schedule while I completed my thesis. Lastly, I would like to thank my family and friends for their support and listening ears throughout my college career. My accomplishments all point back to their steadfast support system.
Chapter 1

Background

Nanofibers play a significant role in modulating tissue growth.[1][2][3] Applications include nerve regeneration, wound healing, hernia repair, and repair of musculoskeletal tissues. Cells have been shown to sense their environment via signal transduction pathways. These pathways can lead to changes in cell shape, proliferation rates, gene expression, and differentiation .[4] Integrins are a type of protein that exists on the cell’s surface, initiate intracellular signaling, and play a major role in the adhesion of cells to a surface. These integrins utilize inside-out as well as outside-in communication and are the mediator of cell’s interaction with surrounding extracellular matrix.

Characteristics of the extracellular matrix (ECM), such as porosity, stiffness, and composition, have been shown to regulate cell migration.[5] Current research has shown that integrin expression of a cell depends on the specific parameters of the substrate which allow the cell to mechanically sense their environment.[6] These adhesion proteins and integrins respond to mechanical stress which in turn can promote migration, growth, and stability.[7] Cell movement has been shown to involve actin, myosin II, and is tightly regulated by Rho GTPases and PTKs (Protein Tyr kinases).[8][9] Dorsal Stress fibers (DSFs) are an important class of actin filament-based stress fibers and have been shown to interface actin arcs (top of cell) to focal adhesions (bottom of cell).[8] Myosin II has been shown to be the mediator in actin arc constriction in cells. It has been shown that these adhesion system of DSFs connecting focal adhesion (FA) to actin arc result in flattened lamella and eventually dynamic crawling starting with the stretching of the
leading edge.[8] Figure 1 shows a simplified diagram depicting such association vital to cellular migration. In Figure 1 focal adhesions can be seen to be the start of the entire intracellular network which determines the contractility of cells. The stability of these adhesions and stiffness of the substrate is a significant factor in cellular motility.

For fibroblasts, the ECM that the cell migrates on comes in the form of fibronectin, laminin, or collagen substrate surfaces. These cells attach to substrates via nascent (short lived) or stable (longer lived) adhesions. Previous literature has demonstrated these adhesion types forming in predictable patterns.[9] Nascent adhesions (FA) form closest to the leading edge of the lamellipodium while mature focal complexes reside closer to the cell body yet still in the
lamellipodium.[9] Figure 2 shows more stable focal complexes in green, while the short-lived FAs are shown in yellow.

Figure 2 - Diagram depicts nascent and mature focal adhesion and focal complexes, respectively.[9]

These focal adhesions in turn exert a force on the substrate at which it binds and studies have been done to characterize this force. One innovative method of study came in the form of micro-patterns used and depicted in Figure 3. The unstressed pattern was compared to the stresses exerted by human foreskin fibroblasts.[10] fluorescent microscopy was used to image GFP labeled vinculin in order to spatially categorized the focal adhesions.[10]
Quantization was achieved by measuring displacement from a pipette of a known force exertion. In Figure 3 the force applied by the rat cardiac fibroblast shows both pinching action (3c) and general distortions (3a). Local forces were shown to have a linear correlation between the surface area of the focal adhesion, measured by immunofluorescence, and the magnitude of the force exerted on local space. Further the fibroblasts were shown to indeed be depended on actomyosin contraction by adding 2,3 – butanedione monoxime (BDM) which is a known inhibitor of actomyosin contraction. It was also shown that forces were exerted parallel to the focal adhesions long axis. The forces in fibroblasts were found to be on the order of 10 nN with a stress of 5.5±2 nNμm².

By applying BDM, and hence blocking actomyosin interactions, close correlation to the time dependence of force relaxation and it was further revealed that focal adhesions maintain assembly while the relaxation of actomyosin contraction leads to a decrease in force. As can be seen in , this ultimately leads to the conclusion that integrins indeed do not make up the cytoskeleton utilized in cell contractility, but rather are linked to actin that undergoes actomyosin contractions. The
parts work together through cell transduction pathways and even when actomyosin activity is inhibited the focal adhesions stay intact ready for their role in cell motility to be reestablished.

There are many key proteins in linking integrin to actin. Integrins exist as heterodimers attached to the cell membrane and consist of both $\alpha$ and $\beta$ chains.[11] These proteins include Talin, Vinculin, $\alpha$-actinin, and Kindlins.[9] Talin interacts with vinculin and form antiparallel homodimers. Vinculin is usually associated with cell-to-cell and cell-extracellular matrix junctions. The actin cross-linking protein $\alpha$-actinin links to filaments of actin and finally Kindlins activate integrins although the mechanism is poorly understood.[9] These focal adhesions can be detected by fluorescent labelling of adhesion molecules.

Focal Adhesion Kinase (FAK) is another important protein which recruits Rho Gaps (GTPase-activating proteins) and GEFs (Guanine nucleotide exchange factor) although not clearly understood. [9] The cell signaling cascade for cellular migration has shown to be highly dependent on Rho Gaps and ROCK. This dependency is depicted in Figure 5 where integrins that are associated with the ECM start the cascade. The association between paxillin and FAK are depicted in Figure 5.
FAK and Src are both protein kinases that are activated after adhesion or growth factor simulation. Paxillin is thought to localize to focal adhesion through an association with β-integrin tails. Paxillin is thought to directly affect the organization of actin cytoskeleton, and as discussed, an important feature of cellular mobility. The cascade presented by Turner is depicted in the rather complex Figure 6.
Figure 6 - Diagram depicts a presented mechanism for adhesion. Although quite complex; it encompasses the vast majority of proteins often associated with cellular migration.[12]

Current knowledge of cell adhesion comes primarily from 2D tissue-derived surfaces in vitro. These studies have revealed the specific receptors, regulators, and other machinery which all play important roles in cellular motility.[13] There are many differences between the focal and fibrillar adhesions on 2D substrates vs. 3-D substrates. The dependency of RhoA and ROCK on actomyosin contractility is of primary importance in cellular migration in 3-D cell derived matrices.[13] 3-D matrices serve as a better representation of in vivo cellular activities. By better simulating in vivo environments, the hope is to lead to predictable and desired differentiation and
increased migration. Recently, the importance of 3D ECM to yield normal polarity and differentiation has revealed insight into the future of tissue engineering.

Though there are many proteins that are involved in the mechanism for adhesion, FAK and Src were chosen as the main proteins of study. FAK was chosen because its role is served very early on in the cell signaling cascade: Integrins that adhere to the substrate directly activate FAK.

FAK is a protein-tyrosine kinase localized at focal adhesions and becomes activate by phosphorylation of its 397th amino acid Tryosine (Tyr-397). The interactions of FAK and Src are thought to further stimulate phosphorylation and therefore amplify the signal.[14] Figure 7 identifies which amino acid is phosphorylated in the different states. Note the differences in FAK and Src in their phosphorylated and dephosphorylated states. These specific states of Src and FAK can be targeted using immunostaining, further revealing where they are in the pathway based on if they are active (phosphorylated) or inactive (dephosphorylated). Multiple signals are involved in this binding, and other FAK sites phosphorylate by Src is Tyr-925, which is the most well characterized protein. The autophosphorylation of proteins in Figure 7 is poorly understood.

![Figure 7 – Model for FAK and Src signaling involved in cell adhesions.[14]](image)
Controlling the differentiation of stem cells into osteogenic differentiated cells is another goal that depends greatly on the characterization of interactions with in vivo environments. It has been shown that when Mesenchymal Stem Cells (MSCs) are placed on PLGA thin films they deposit both fibronectin and Collagen I on their environments. [15] These deposits resulted in increased of integrins α5β1 and α2β1, respectively. [15] Using RT-PCR it was also suggested that MSCs on these deposited PLGA substrates expressed osteogenic transcription factors, namely Runx2/CBFA-1. [15] After 28 days the gene expression decreased as the cells were thought to differentiate into the mature osteoblast phenotype. [15] From these studies it was concluded that coating polymeric scaffolds with the ECM proteins present in vivo is a simple solution to encourage specific differentiation. For osteogenic differentiation this includes fibronectin, collagen, vitronectin, and laminin.[16]

Many factors involved in regulating osteogenesis have been well-characterized. Such factors include ascorbic acid, transforming growth factor-β, β-glycerophosphate, bone morphogenetic protein-2, and dexamethasone. [17][18] These factors can lead to the eventual increased expression of osteogenic genes and production of osteocalcin, osteopontin (OPN), alkaline phosphatase (ALP) and type I collagen. Figure 8 reveals a key result of the study and ALP, was shown to significantly increase for ECM-coated PLGA of vitronectin, fibronectin, or type I collagen.
It has been shown that ECM architecture can influence fibroblasts 3D migration and although migratory process for “pulling” the cell across a 3D space is the same for matrices varying in porosity and stiffness the adhesion turnover is significantly different.[19] Specifically it was confirmed that with human foreskin fibroblasts (HFFs) increased porosity in ECM structures resulted in increased cell migration rates.[19] Using the appropriate technology to study the microenvironment of cellular adhesion, Doyle et al. was able to study the Young’s modulus of varying substrates as would be sensed by a focal adhesion.
Chapter 2

Introduction

To date, the relationship between integrin expressions, which initiate signaling, and their 3-D environments has not been well-characterized. Further investigation will lead into much insight into the cell signaling pathway as it pertains to tissue growth and migration on scaffolds.

This research serves to isolate the specific roles of migration regulators by changing their nanofiber environment observing the differences through immunofluorescence microscopy and western blotting. Human Mesenchymal Stem Cells (hMSCs) were placed on Poly(methyl methacrylate) (PMMA) nanofibers of varying diameters. FAK and Src expression involved in the signaling cascade of hMSCs were quantified on scaffolds of varying diameters. Migration rates and focal adhesion lengths for each mean diameter scaffold were also observed.

Specific diameters of nanofibers will be fabricated by two methods: Etching fibers and changing the high molecular weight concentration of PMMA. A method for accurately quantifying the diameter of scaffolds was then identified. Justin Brown’s lab successfully demonstrated the method of etching fibers through the use of 60% propylene glycol methyl ether acetate/limonene solution. FAK and Src in various phosphorylation states were identified using existing protein staining technology: Western blot. These transferred proteins were treated with primary and secondary antibodies that bind to specific known proteins, and visualized as well as quantified using IR Fluorescence blot imager (Odyssey). By using protease and phosphatase inhibitor the phosphorylation states, some shown in Figure 7, of both FAK and Src were identified.
Significance of Research

Nanofibers play a significant role in modulating tissue growth.[1][2][3] Applications include nerve regeneration, wound healing, hernia repair, and repair of musculoskeletal tissues. Focal adhesions provide link to intracellular actin structures and the extracellular matrix and initiate cell signaling. Signals are integrin-induced and result in Focal adhesion kinase (FAK) phosphorylation. FAK is thought to be a point of convergence that goes on to influence many other processes, including Src activation, that lead to migration, growth, and differentiation.[20]

Hypothesis and specific aims

We hypothesize that changes in nanofiber diameters will promote a response through focal adhesion cell signaling pathways, leading to changes in concentrations of different phosphorylation states of FAK and Src, in human mesenchymal stems cells (hMSCs) and mouse embryonic fibroblasts(MEFs). Further we hypothesize that migration rates and focal adhesion lengths are dependent on both FAK/Src concentrations and diameters of their nanofiber environments.

Specific Aim 1: Fabricate via electrospinning poly(methyl methacrylate)(PMMA) nanofibers that vary in mean diameter. Specific Aim 1 will be considered a success if nanofiber substrates are created at five unique diameters with minimal deviation among mean diameters, as determined
by analyses of scanning electron microscopy (SEM) and field electron scanning electron microscopy (FESEM) images.

**Specific Aim 2: Culture hMSCs and MEFs on all nanofiber substrates and observe migration.** Specific Aim 2 will be considered a success if cells are seen to migrate into and proliferate on nanofiber scaffolds as determine by bright-field microscopy prior to lysing.

**Specific Aim 3: Quantitatively measure migration rates of MEFs on nanofiber scaffolds that vary in diameter.** Specific Aim 3 will be considered a success if cells are seeded at a low concentration to ensure that scaffolds are not able to excessively proliferate scaffolds and cells are successfully tracked.

**Specific Aim 4: Qualitatively measure length of focal adhesion and concentration of focal adhesion proteins FAK and SRC in their various phosphorylation states.** Specific Aim 4 will be considered a success if immunofluorescent protein detection through Western blotting and immunostaining reveals significant differences in cells on nanofiber vs. controls.
Chapter 3

Nanofiber Fabrication and Human Mesenchymal Stem Cell Culture

Background

Electrospinning has been the primary method for many researchers to create scaffolds from various biomaterials. The phenomenon behind electrospinning was first described by the physician and electrician, William Gilbert circa 1600.[21] In his most famous work, *De Magnete*, he describes an electrically charge piece of amber

“plainly attracts the body itself in the case of a spherical drop of water standing on a dry surface; for a piece of amber held at suitable distance pulls toward itself the nearest particles and draws them up into a cone; were they drawn by the air the whole drop would come toward the amber.”[21]

The process of electrospinning would not be patented until 1900 by J.F. Cooley.[22] However the electrospinning process that has led to many discoveries in the material science field, was performed two decades ago at the University of Akron by Reneker and co-workers to generate 1D polymer structures.[22] Electrospinning is described by some as a “fast-moving front” of material science, as additive manufacturing becomes more precise at smaller scales (<500nm) the lack of spatial and angular control of nanofibers will soon be a problem of the past.

Materials and Methods

Cells will be placed in scaffold environments that vary in diameter. This involves a method for modulating and quantifying the diameter of scaffold, both of with can be found in Chapter 4.
**Electrospinning**

Scaffolds were made with an electrospinning apparatus consisting of a New Era pump system syringe pump, copper targets, super magnet for aligning fiber, and voltage supply. The apparatus is depicted in Figure 9. The solution used for scaffolds was poly(methyl methacrylate) (PMMA) solution which dried to make nano-scale fibers when extracted using a high voltage. Voltages used were in the 5-20 kilovolt range. The fibers were collected on four types of material: aluminum foil, 3-D printed grids, glass coverslips, and Poly(2-hydroxyethyl methacrylate) (pHEMA)-coated glass cover slips. Nanofiber scaffolds were then sterilized with UV for 20 minutes before seeded with cells.

**Nanofiber Sample Preparation**

**Spin Coating**

Spin coating was used to form a uniform thin film of both Poly(2-hydroxyethyl methacrylate) (pHEMA) and poly(methyl methacrylate) (PMMA) on glass cover slips. The spin coater used was the Laurel Technologies - Spin Coater WS-400-6NPP-LITE (Manual Spin Processor).
Spin coating creates a uniform film of a desired polymer, that can have a thickness of microns to nanometers. The glass cover slip is placed inside the spin coater, covered with a small quantity of polymer, and then subject to a large centripetal force by rotating at high speeds. The spin coating process, depicted in Figure 10, results in a uniform film spread out over the glass cover slip.

Based on the strongly polar hydroxyl and ester group of pHEMA, proteins from the media do not bind to the surface and therefore hMSCs do not adhere to coverslip coated with this polymer. This ensures that cells seeded on electrospun nanofibers do not adhere to the coverslip, but remain in the scaffold. Cover slips were both single and coated on both sides in pHEMA.

**Incubation and seeding of scaffolds**

Nanofibers spun on grids were incubated with alpha-MEM media containing fetal bovine serum (FBS) overnight for 24 hours. Nanofibers spun on pHEMA cover-slips were not incubated in media prior to seeding. The scaffolds were seeded with human mesenchymal stem cells (hMSCs) and passed before confluency.
**Live Cell imaging prior to protein harvest**

After hMSCs were seeded on scaffolds for 24 hours, media was replaced with 1 mL PBS containing phosphatase inhibitor cocktail and imaged using a Leica DM5500 B-Automated Upright Microscope.

**Cell Lysates**

Cell were lysed by using a lysis buffer which included phosphatase and protease inhibitors to ensure that integrins were not altered post lysing. Lysis buffer included 150mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (Sodium dodecyl sulfate, and 50 mM Tris pH 8.0.

Nanofibers were inverted into the lysis buffer solution. The contents were vortexed carefully and frozen in -80C six-well plate sealed with parafilm. Once frozen the lysate was completed by extracting to a micro-centrifuge tube on ice and sonicated to shear DNA. This was centrifuged at 12,000xg for 10 minutes and the supernatant collected and pellet discarded.

**Results**

**Nanofiber Morphology**

Nanofibers were imaged using a scanning electron microscope (SEM) and field emission scanning electron microscope (FESEM) at various magnifications. A description of these imaging methods can be found in greater detail in Chapter 4.
Figure 11 - SEM images of samples of various diameters. Diameters were modulating using different concentration of high molecular weight (HMW) of poly(methyl methacrylate) (PMMA) in a 60:40 solvent mix of Dimethylformamide:Tetrahydrofuran. Electrospun nanofibers were spun using a polymer concentration of A) 1.54wt%  B) 3.1wt%  C) 3.7wt%  D) 6 wt%  E) 8.3 wt%
Cell Morphology

Cells were also imaged using the laboratory microscope, as previously described in the Live Cell Imaging section. A total library of the live cell images can be found in Appendix A.

A) Flat PMMA

B) 1.54% HMW PMMA

C) 3.1% HMW PMMA

D) 3.7% HMW PMMA

E) 6% HMW PMMA

F) 8.3% HMW PMMA

Figure 12 - hMSCs on A) Flatt PMMA B) 1.54% HMW PMMA Nanofibers C) 3.1% HMW PMMA Nanofibers D) 3.7% HMW PMMA Nanofibers E) 6% HMW PMMA Nanofibers F) 8.3% HMW PMMA Nanofibers
Discussion

Nanofiber fabrication was successful and characterization of diameter can be found in Chapter 4. Beading was prevalent in nanofiber scaffold for diameters <400nm or 3.7% HMW PMMA. Nanofibers that were spun with a 3.7%, 6%, or 8.3% HMW PMMA solution showed no beading. (Figure 11). Small fibers (1.54% and 3.1% HMW PMMA) seeded with hMSCs for 24 hrs. showed cells aggregating and forming ball-like masses, seen in Figure 12(B, C). The smallest fibers (1.54% HMW PMMA) affected all hMSCs to form aggregates Figure 12(B). Nanofibers approximately 200nm (3.1% HMW PMMA) displayed much less cell aggregates Figure(C). This observation was otherwise not seen of hMSCs for larger diameter scaffolds. Figure 12(A, D, E, F).
Chapter 4

Modulating and Characterizing Nanofiber Diameter

Background

The use of nanofiber scaffold for tissue engineering applications has rapidly grown over the past twenty years. Fiber properties, including diameter, are an important part to creating a specific environment for which cells interact. Nanofiber diameter was synthesized using two types of methods: Etching and modulating high molecular weight of PMMA. Fiber properties were assessed using two methods: manual assessment using ImageJ and automated assessment using DiameterJ.

Materials and Methods

Modulating Diameter of Nanofibers

Experiments have shown that PMMA fiber diameter can be modulated using different needle gauge, distance from needle tip to collector, concentration, molecular weight, voltage, and feed rate.[23] Among these concentration and feed rate have shown significant effects on fiber diameter and bead formation.[23] A feedrate of 0.7 ml/hr., voltage of 15kV, collector distance of 15cm, and humidity of approximately 30% was used across all samples. The concentration and needle gauge were found to be the most effective at controlling the fiber diameter.
**High Molecular Weight PMMA Concentration**

High molecular weight PMMA concentrations were initially chosen as 2.5wt%, 3wt%, 4.5wt%, and 7 wt% based on those reported in literature.[23]

**Etching**

Etching is a technique used to decrease the diameter of the electrospun nanofibers. A common problem in research today is systematically varying the diameter of nanofibers. Using the previously established techniques and stock polymers, it is not possible to vary the diameter of the PMMA by large amounts, especially under 500nm diameter.

In Brown’s Lab the method of etching fibers has been successfully achieved through the use of 60% propylene glycol methyl ether acetate/limonene solution. The fibers were first immersed in the limonene solution at various time points. Increased time in limonene solution results in a decrease in diameter. Once the desired time is reached the fibers undergo two water bath to stop the decrease in PMMA fiber diameter. This can be seen in the SEM image of fibers with increased etching from A to D in Figure 13.

![SEM images of etched fibers](image)

**Figure 13**-This figure shows increased etching and therefore decreased diameter of nanofibers. Notice the size scales are consistent throughout the images.[24]
Characterizing Diameter of Nanofibers

**Manually via ImageJ**

ImageJ was used to calculate the mean fiber diameter and standard deviations. The measure bar used to determine the actual length of diameter. A measurement of an SEM image is displayed in Figure 14.

![ImageJ manually determined the diameter of PMMA nanofibers.](image)

**Automatically via DiameterJ**

DiameterJ is an open source program that was developed by the National Institute of Standards and Technology to automate analyses of nanofiber scaffolds. It serves as a plugin for the popular software ImageJ. Its initial release was February 2015 has since been validated by laboratory groups globally. DiameterJ works as a digital imaging-processing package by segmenting SEM images through threshold techniques existing in the ImageJ program. After images are segmented, images undergo fiber analysis depicted in Figure 15.
DiameterJ outputs many useful parameters including mean pore area, mean fiber diameter, number of intersections, as well as standard deviation for most parameters.

**Results**

**Scanning Electron Microscopy**

After fabrication nanofibers were visualized using scanning electron microscopy. Figure 16 shows images at different magnification for electrospun fibers from a 2.5%, 3%, 4.5%, and 7% HMW PMMA solution.
Figure 16 – SEM images of samples of various diameters. Diameters were modulating using different concentration of high molecular weight (HMW) of poly(methyl methacrylate) (PMMA) in a 60:40 solvent mix of Dimethylformamide:Tetrahydrofuran. Electrospun nanofibers were spun using a polymer concentration of A) 2.5 wt%, B) 3 wt%, C) 4.5 wt%, D) 7 wt%.

Diameter analysis determined manually

The SEM images of nanofibers were then analyzed to determine the mean diameter for each HMW PMMA solution. The results from manual determination of diameters can be seen in Bowers. Table and in.
Table 1 – Diameter and standard deviation of nanofibers spun using four different weight percents of PMMA. These values were generated manually through ImageJ. Data was provided by Daniel Bowers.

<table>
<thead>
<tr>
<th>wt% of PMMA</th>
<th>Nanofibers spun with 18G (nm)</th>
<th>Stdev (nm)</th>
<th>Nanofibers spun with 30G (nm)</th>
<th>Stdev (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>194.266134</td>
<td>35.2950811</td>
<td>158.03266</td>
<td>22.9113739</td>
</tr>
<tr>
<td>3</td>
<td>279.93916</td>
<td>136.080298</td>
<td>199.195252</td>
<td>34.2094234</td>
</tr>
<tr>
<td>4.5</td>
<td>533.873628</td>
<td>158.740496</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>977.107091</td>
<td>213.900373</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 17 - Relation between mean fiber diameter and PMMA wt% as determined by manual measurement using ImageJ. Graph shows fiber diameter for fibers spun from a 18G and 30G needle. Data was provided by Daniel Bowers.
Diameter batch analysis determined automatically

The plug-in DiameterJ was used as an automated process of determining the mean diameter of the nanofiber samples. The results are displayed in Table 2 and graphically in Figure 19.

Table 2 - Diameter and standard deviation of nanofibers spun using four different weight percents of PMMA. These values were generated automatically through DiameterJ.

<table>
<thead>
<tr>
<th>wt% of PMMA</th>
<th>Nanofibers spun with 18G (nm)</th>
<th>Stdev (nm)</th>
<th>Nanofibers spun with 30G (nm)</th>
<th>Stdev (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>185.46</td>
<td>48.04</td>
<td>146.70</td>
<td>47.18</td>
</tr>
<tr>
<td>3</td>
<td>318.30</td>
<td>108.48</td>
<td>157.44</td>
<td>38.86</td>
</tr>
<tr>
<td>4.5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>1194.54</td>
<td>175.54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 18 – Relation between mean fiber diameter and PMMA wt% as determined by manual measurement using ImageJ. Graph shows fiber diameter for fibers spun from a 18G and 30G needle. Data was provided by Daniel Bowers.
Discussion

Comparison of Analyzation Methods.

Automated fiber diameters determined through DiameterJ values showed an increased value as compared to values determined manually. Figure 20 shows the slope of the linear trend of DiameterJ values is greater at a value of 222.25nm/wt% as compared to 174nm/wt% for manually determined values.
For 7 wt% PMMA DiameterJ yielded a mean diameter of 1194.54nm, a 122% increase from the manual mean diameter at the same concentration of 977nm.

Figure 21 leads insight into the inflated values of DiameterJ as compared to the manual values. In (A) a bead is shown. Diameter J determines the length of the fiber and iteratively slices it consistent with all other fibers. When determining manually, one value may be taken from the bead while other more “reflective” fibers may be taken at a higher frequency. However, a systematic error also exists in DiameterJ. DiameterJ successfully identifies intersection during its and excludes them from any calculations. However, DiameterJ cannot determine if two fibers are superimposed as can be seen in (B). The red arrow shows two fibers that are superimposed.
DiameterJ ultimately analyzed this fiber as one. Visual assessment clearly shows that two fibers exist and no measurements would be taken along that area.

![Image A](image1.png) ![Image B](image2.png)

Figure 21 – This shows two images that lead insight into discrepancies between DiameterJ and ImageJ

Overall DiameterJ is a very convenient piece of software and can do batch imaging of fifteen SEM images in less than 10 minutes. Mean diameter values were comparable to those determined through manual assessment.

**HMW concentration effect on nanofiber diameter.**

From the linear trend between PMMA wt% and nanofiber diameter, displayed in Figure 20, the wt% was calculated for five unique diameter sizes that were used for studying velocity profiles (Chapter 5) and protein analyses (Chapter 6). The concentrations and corresponding mean diameters are displayed in Table 3.
Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Figure 20.

<table>
<thead>
<tr>
<th>Diameter approx.</th>
<th>Needle</th>
<th>Wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>30G</td>
<td>1.54</td>
</tr>
<tr>
<td>200</td>
<td>30G</td>
<td>3.1</td>
</tr>
<tr>
<td>400</td>
<td>18G</td>
<td>3.7</td>
</tr>
<tr>
<td>800</td>
<td>18G</td>
<td>6</td>
</tr>
<tr>
<td>1200</td>
<td>18G</td>
<td>8.3</td>
</tr>
</tbody>
</table>
Chapter 5

Characterization of Migration

Background

Cell migration has great importance in both physiological and developmental process, ranging from cancer metastasis to wound healing. The 3D environment has shown to greatly affect cell motility and initiate signaling from the microenvironment. MEFS were tracked over a period of six hours in environments of mean nanofiber diameters of 100nm, 200nm, 400nm, 800nm, and 1200nm.

Materials and Methods

Time-Lapse Imaging

FluoroBrite™ DMEM was used as a media to image the migration of mouse embryonic fibroblasts (MEFs). Cells are time lapse imaged in FluoroBright basal media supplemented with FBS, HEPES, GlutaMAX, and TROLOX. FluoroBrite™ is phenol red-free which results in 90% lower background fluorescence emitted, and is designed to preserve cell health outside of an incubator. Cells and scaffolds are held in P30 culture dishes custom prepared with a glass coverslip attached to the bottom of the dish. A heated stage with a layer of white mineral oil over the media is used. Images using a Leica DM5500 B-Automated Upright Microscope were taken at intervals as quick as 90s with bright-field illumination. Differential interference contrast microscopy was used to image cells.
Cell Tracking Method

MEF’s were tracked by loading the 240 frames from time lapse, and using a manual tacking plugin in ImageJ. Five cells were tracked for each type of diameter sample by clicking in the middle of each cell in every frame.

Statistics

The standard error of the mean was calculated to estimate the variability among samples. To visualize how precise the mean of the sample estimated the population mean, error bars were included in the results. The formula used for standard error of the mean is $\sigma_M = \frac{\sigma}{\sqrt{N}}$, where $\sigma$ is the standard deviation and $N$ is the sample size.

Results

MEFs were seeded on scaffolds with five different diameter scaffolds corresponding to the PMMA concentrations 8.3%, 6%, 3.7%, 3.1%, and 1.54%. Cells were tracked for a two time periods 6-12 hours and 13.5-19.5 hours. Cells were not visualized after 19.5 hours due to over population of scaffolds. Note that in Figure 22 the 1.54% PMMA nanofiber data is not included. Cells did not attach to the surface within the 6-12 hour timeframe and therefore, no tracking data was collected.
Figure 22 - Mean velocities from 6-12 hours of MEFs on different diameter PMMA fibers (given in molecular weight concentrations of PMMA). Error bars denote standard error of the mean. N=5 See Table 3 for approximate diameters for a given concentration. Bars with asterisk represents a P value < 0.05 as determined by the t-test. Note that the 1.54% PMMA nanofibers are not included due to cells not attaching during the 6-12 hour time period and no migration data was collected. Data provided by Daniel Bowers
Figure 23 - Mean velocities from 13.5-19.5 hours of MEFs on different diameter PMMA fibers (given in molecular weight concentrations of PMMA). Error bars denote standard error of the mean. N=5. See Table 3 for approximate diameters for a given concentration. Bars with asterisk represents a P-value<0.05 as determined by the t-test. Data provided by Daniel Bowers.
Figure 24 - Mean velocities from 6-12 hrs. and 13.5-19.5 hours of MEFs on different diameter PMMA fibers (given in molecular weight concentrations of PMMA). Error bars denote standard error of the mean. N=5. See Table 3 for approximate diameters for a given concentration. Bars with asterisk represents a $P$ value $<$ 0.05 as determined by the $t$-test. Data provided by Daniel Bowers.

Figure 25 - Trends for mean velocity rates of MEFs for a given diameter. N=5 for migration velocity. Error bars denote standard error of the mean.
Discussion

A general trend of increased velocity with fiber diameter (increased wt%) can be seen in the 6-12hr period (Bowers Figure). The control showed a significant difference compared to both the 8.3% and the 3.1% spun fibers, according to the t-test (pvalues<0.05). The 8.3% electrospun fibers showed a significant difference in MEF velocity compared to both 6%, and 3.7% PMMA electrospun fibers. The positive trend can be seen in Figure 25 with a good fit of $R^2=0.7134$.

A general trend of increased velocity with fiber diameter (increased wt%) can also be seen in the 13.5-19.5hr period (Bowers Figure), but at a much poorer fit than that of the 6-12hr period, $R^2=0.2098$ (Figure 25). Figure 23 reveals a peak migration velocity at 6% PMMA
electrospun fibers and a significant decrease of migration velocity for 8.3% electrospun fibers. The control also showed a significant difference compared to both the 8.3% and 6%, according to the t-test (p-values<0.05). Figure 24 shows a side-by-side comparison of the two time periods and reveals a higher overall velocity for the later period of 13.5-19.5 hours.

Velocities over the 6-12 hour period were average of 15min intervals and graphed in Figure 26. Differences in velocity waveforms can be attributed to navigation through nanofiber pores, proliferation, and cell-to-cell collisions. Further analysis of this data is needed to determine if any relationships exist between nanofiber size and velocity waveforms.
Chapter 6

Characterization of Src, FAK, and Vinculin

Background

Focal adhesions provide link to intracellular actin structures and the extracellular matrix and initiate cell signaling. Signals are integrin-induced and result in Focal adhesion kinase (FAK) phosphorylation. FAK is thought to be a point of convergence that goes on to influence many other processes that lead to migration, growth, and differentiation.[20] An important function of FAK is its interaction with Src homology 2 (SH2) domains of the Src-family tyrosine kinases. Evidence suggests that after an adhesion event, FAK is autophosphorylated at its amino acid Tyrosine-397 (pFAK-397) which recruits and ultimately phosphorylates Src.[26][14] FAK-Src activation is pictured in Figure 7 where inactive FAK is phosphorylated to pFAK-397 after integrin-cell clustering from a cell adhesion. Src is then converted from its inactive state pTyr-527 to its active form of pTyr-416. Src than participates in many other signaling cascades, a few depicted in Figure 27.
Src-induced phosphorylation of FAK has shown to dynamically regulate focal adhesion disassembly, and important part of cellular migration.[26] Focal adhesion turnover by calpain also requires Src-induced phosphorylation of FAK. Src-induced phosphorylation of FAK has been shown to suppress apoptosis, an interesting area of studying regarding cancer cells.
Materials and Methods

Coomassie Protein Staining of a PVDF membrane

Coomassie blue was used to determine if proteins existed on gel after transfer. This proved a useful diagnostic tool when larger proteins that were predicted to be present in cells did not show up on the analyzed PVDF membrane. The stain solution was made of 50% of Methanol (30mL), 7% Acetic Acid (4.2mL) and Q.s. to 60 mL with ddH2O (25.8mL) and finally 0.1% Coomassie Brilliant Blue R stain 0.09g. N.B: 65% dye composition, therefore, in 1g there is 0.65g dye. The destain #1 solution was made with 50% methanol 15mL, 7% Acetic Acid (2.1mL) and 12.9mL of ddH2O. The destain #2 solution was made with 27mL of 90% Methanol and 3mL of 10% Acetic Acid.

The blot was washed in 1x TBST for 5 min at room temp and stain solution was added. The stained PVDF was incubated with agitation on rocker for 5 minutes at room temperature. Stain solution was removed and destain solution #1 was added and again the PVDF membrane was incubated with agitation on rocker for 5 minutes. The destain solution #1 was removed and destain solution #2 was added until bands were of appropriate intensity. Blot was dried with Kim wipe and analyzed on Li-Cor Odyssey infrared Imaging System.

Dot Blot

Dot blots prove useful for quickly identifying if a protein is present without wasting hours doing an electrophoresis just to find you never had the protein in the first place. Although you cannot characterize it according to size, it serves as an effective preliminary test. It is often thought
of as the simplified version of the western blot. Dot blots were made by dotting the sample on the activated PVDF membrane which was then stain according to the protocol of Immunofluorescence staining described below and followed the remainder of the Western Blot procedure. A typical dot blot is shown in Figure 28.

Western Blot

Electrophoresis

Buffers were prepared before the running gel electrophoresis. TBST made by 10X TBS diluted with Tween 10 (1mL per 1L of TBS). Running Buffer was made by combining 500mL of DI water with 8.7g of Tris-Glycine Buffer and 10mL of 10% SDS solution. Transfer Buffer (1) was made by combining 400mL of DI water with 8.7g of Tris-Glycine Buffer and 25 mL of methanol, then qs. to 500 mL with water. Transfer Buffer (2) was made by Combining 850mL of DI water with 17.4g of Tris-Glycine Buffer and 50 mL of methanol, then qs. to 1000mL with water. Running gels and stacking gels were made ranging from 6% to 12% depending on the size of the proteins of interest. Proteins were thawed and beta-mercaptoethanol to 4X loading solution according to Table 4. A general overview of Western Blot can be seen in Figure 29.
Table 4- Concentration of beta-mercaptoethanol solution and protein sample to be loaded into each well for a Western blot.

<table>
<thead>
<tr>
<th></th>
<th>1 lane(uL)</th>
<th>2 lanes(uL)</th>
<th>4 lanes(uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-ME solution required</td>
<td>7.5</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>protein sample</td>
<td>22.5</td>
<td>45</td>
<td>90</td>
</tr>
</tbody>
</table>

A sample with a volume of 22.5uL was combined with 7.5uL of solution from table one to make a total of 30uL. This 30uL solution was then vortexed and heated at 95C for 10 minutes. The 4uL of a protein ladder was added where seen appropriate and the 20uL of the sample were added to the wells of the lanes. The gel was run at 120V for 3-4 hours or as needed. The leading edge of the gel can be easily visualized by a bright yellow color and once this line neared the end of the gel it could be removed from the voltage.

Figure 29 - An overview of the western blotting technique. Some difference between the protocol and the diagram is the proteins were transferred onto a PVDF membrane not a Nitrocellulose sheet. Image Source<http://proteomics.case.edu/proteomics/westernblot.html>
Transfer

Filter papers and PVDF membrane were used in the transfer. PVDF membrane was activated with methanol for 5 minutes. SDS was only added to Transfer buffer if absolutely necessary (i.e. large proteins) Transfer was run for 80V for 80 minutes or 240mA for 2 hours. The 240mA for 2 hours seemed to have better results in fully transferring proteins. Once complete the membrane was washed with TBST for 5 min at room temperature while rocking. The membrane was then blocked overnight in TBST with 5% BSA at 4C.

Immunofluorescent Protein Detection

Primary Antibodies of appropriate concentration in 3mL of solution at room temperature were added to the membrane and rocked for 1 hour. Washes followed (3X with >5mL TBST), 5min each, at 100 RPM on orbital shaker. Secondary Antibody at appropriate concentration in 3mL Washes followed (3X with >5mL TBST), 5min each, at 100 RPM on orbital shaker. There are many types of integrins that can be stained for using primary antibodies. The integrins of interest and where they can be found is displayed in Table 5, while the corresponding secondary antibodies are displayed in Table 6. These integrins were chosen based on their involvement with focal adhesion and the cell-transduction pathway involved with migration. By targeting these specific integrin one can gain insight into the proteins expressed at varying environments as described earlier in the methods.
Table 5 - This table depicts the various primary antibodies that detect various proteins expressed in cells at the varying environments.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-FAK (Tyr-397)</td>
<td>Cell Signaling Technology</td>
<td>#85</td>
</tr>
<tr>
<td>Phospho-FAK (Tyr576/577) Antibody</td>
<td>Cell Signaling Technology</td>
<td>#3281</td>
</tr>
<tr>
<td>Phospho-FAK (Tyr925) Antibody</td>
<td>Cell Signaling Technology</td>
<td>#3284</td>
</tr>
<tr>
<td>α-Tubulin Antibody</td>
<td>Cell Signaling Technology</td>
<td>#2144</td>
</tr>
<tr>
<td>Phospho-Src Family (Tyr416) (D49G4) Rabbit mAb</td>
<td>Cell Signaling Technology</td>
<td>#2101</td>
</tr>
</tbody>
</table>

Table 6 - This table depicts the two secondary antibodies that detect various proteins expressed in cells at the varying environments.

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Source</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse IgG, HRP-linked Antibody</td>
<td>Cell Signaling Technology</td>
<td>#7076</td>
</tr>
<tr>
<td>Anti-rabbit IgG, HRP-linked Antibody</td>
<td>Cell Signaling Technology</td>
<td>#7074</td>
</tr>
</tbody>
</table>

**Membrane visualization**

Membranes were analyzed using a Li-Cor Odyssey infrared Imaging System. Using the Odyssey Western blot software, intensity of bands could be better contrasted, quantified, and exported to excel for further analyzation.
**Immunofluorescence staining**

HMSCs on nanofibers were fixed in a 4% formaldehyde solution diluted with PBS for 15 minutes. The hMSCs on nanofibers were then rinsed three times with PBS for 5 minutes each. All samples were blocked with blocking buffer for 60 minutes, and then probed with a primary antibody for vinculin and actin. After one-hour incubation at room temperature, the slides were washed with PBS for five minutes three time and followed by addition of the secondary antibody. DAPI was added as a final step to visualize the nucleus.

**Statistics**

The standard error of the mean was calculated to estimate the variability among samples. To visualize how precise the mean of the sample estimated the population mean, error bars were included in the results. The formula used for standard error of the mean is $\sigma_M = \frac{\sigma}{\sqrt{N}}$, where $\sigma$ is the standard deviation and N is the sample size. The Pearson product-moment correlation coefficient (PPMCC) was also calculated to determine the linear correlation between variables.

**Results**

A Western blot was performed using a 10% separating gel for each sample of hMSCs on substrates after a 24-hour incubation. The membranes were stained for pFAK-397, pFAK-925, pFAK-576/577, and total FAK. All phosphorylated FAKs were normalized to total FAK.
Immunostaining was also performed for SRC-416 and α-Tubulin. SRC-416 was normalized to α-Tubulin and total FAK.

Figure 31 - Specific phosphorylation states of pFAK-397 and pFAK-925 intensity levels normalized to Total FAK for a given HMW concentration of PMMA. Total FAK is also shown normalized to Tubulin. See Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Table 3 for approximate diameters for a given concentration. N=3 Error bars denote standard error of the mean. FAK 397 showed a PPMC coefficient of 0.87 and FAK925 PPMCC= -0.65034.

Figure 32 - Specific phosphorylation states of pFAK-576/577 and total FAK intensity levels normalized to Total FAK for a given HMW concentration of PMMA. Total FAK is also shown normalized to Tubulin. See Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Table 3 for approximate diameters for a given concentration. N=3 Error bars denote standard error of the mean. FAK 576/577 showed a PPMC coefficient of -0.60 and Total FAK PPMCC=-0.61.
Figure 33 – pSrc 416 intensity levels on different diameter PMMA fibers (given in molecular weight concentrations of PMMA). See Table 3 – Five diameters were used to determine the corresponding weight percent based up linear trend in Table 3 for approximate diameters for a given concentration. N=3. Error bars denote standard error of the mean. Src 416 normalized to Tubulin showed a PPMC coefficient of 0.58 and Src 416 normalized to FAK total showed a PPMCC= -0.07.

HMSCs on varying sized nanofibers were also probed for vinculin and actin and visualized using immunofluorescence. The focal adhesions were measure by taking the major axis length measurement of vinculin. Figure 34 shows the immunofluorescent image of a hMSC. The focal adhesion lengths were calculated with N>2000 and displayed in Figure 35.

Figure 34 - This figure shows an immunofluorescent image of an hMSC on PMMA nanofibers. Green/blue represents the nucleus, yellow represents focal adhesions(vinculin), and red represents actin.
Preliminary results of western blots using the etching method are displayed in Appendix B. Pictures of PVDF membranes, and relative intensity graphs are included. Appendix B

Preliminary Experiments using CSB

Discussion

FAK397 levels revealed a positive relationship with increased fiber diameter from 100 to 1200nm. Figure 32 shows a strong positive correlation between FAK-397 and increased nanofiber diameter PPMCC of 0.87. As discussed in the background and depicted in Figure 7, the phosphorylation pFAK-397 appears to be a critical contributor to the beginning to the rather complex processes that ultimately lead to the promotion of cell migration. [27] From Figure 32 it can be predicted that cellular migration rates will be significantly different among nanofiber scaffolds of varying diameters.
Western blotting showed pFAK-925 activity vs. fiber diameter had no appreciable trend although the PPMCC was -0.65. pFAK-925 activity peaked at the diameter of 3.7% PMMA fibers and maintain lower levels at diameters both larger and smaller (Figure 31). pFAK-576/577 activity negatively correlated with increased fiber diameter with good fit (PPMCC=-0.6). Overall total FAK normalized to tubulin increases with larger diameter (PPMCC=0.61). pSRC-416 activity showed inconsistent trends against fiber diameter when normalized to either alpha-Tubulin or FAK total. Although both revealed poor fits with PPMCC of 0.58 and -0.07, respectively.
Chapter 7

Velocity Profiles, Focal Adhesion Length, and Src/FAK Comparisons

Background

Focal adhesion kinase has been shown to directly affect fibroblast migration speed.[27] Fibroblasts with a transfected FAK gene, controlled by a tetracycline repression system, migrated at a decreased speed compared to their controls.[27] Furthermore, cells expressing a mutant 397-FAK also showed a decrease in migration speed, similar to those FAK-null cells.[27] FAK at Tyr-397 has shown play a key role in FAK’s control of focal adhesion turnover.[28] In this study the concentrations of pFAK-397, pFAK-576/577, and pFAK-925 are compared to the average mean migration speed for a certain diameter of fiber.

Results

Migration rates were then compared to relative protein concentrations for each group of fibers of different mean diameters. The standard error of the mean was calculated to estimate the variability among samples. To visualize how precise the mean of the sample estimated the population mean, error bars were included in the results. The formula used for standard error of the mean is \( \sigma_M = \frac{\sigma}{\sqrt{N}} \), where \( \sigma \) is the standard deviation and \( N \) is the sample size. The Pearson product-moment correlation coefficient was also calculated to determine the linear correlation between variables.
Figure 36 - The mean migration speed compared to the relative concentration of pFAK-397 normalized to total FAK for a certain diameter of fiber scaffold. N=5 for migration velocity N=3 for protein concentration.

Figure 37 - The mean migration speed compared to the relative concentration of pFAK-576/577 normalized to total FAK for a certain diameter of fiber scaffold. N=5 for migration velocity N=3 for protein concentration.
Figure 38 - The mean migration speed compared to the relative concentration of pFAK-925 normalized to total FAK for a certain diameter of fiber scaffold. N=5 for migration velocity N=3 for protein concentration.

Figure 39 - The mean migration speed compared to the relative concentration of pSrc-416 normalized to total FAK for a certain diameter of fiber scaffold. N=5 for migration velocity N=3 for protein concentration.
Migration in hMSCs was better represented in 13.5 – 19.5 hour time point and was further analyzed and the Pearson product-moment correlation test was performed.

![Graph showing correlation between pFAK-397 and migration velocity. The Pearson product-moment correlation coefficient is -0.36.](image)

Figure 40 - Relative concentration of pFAK-397 was normalized to total FAK and compared to migration velocity in micrometers per hour. Pearson Product-Moment Coeff. = -0.36

![Graph showing correlation between pFAK-576/577 and migration velocity. The Pearson correlation coefficient is -0.872.](image)

Figure 41 - Relative concentration of pFAK – 576/577 compared to migration data given in micrometers per hour. Error bars denote standard error of the mean. Pearson correlation test = -0.872.
Figure 42 - pFAK - 925 is compared to migration data given in micrometers per hour. Error bars denote standard error of the mean. Pearson correlation test = -0.12.

Figure 43 – pSrc - 416 is compared to migration data given in micrometers per hour. Error bars denote standard error of the mean. Pearson correlation test = -0.72.
The focal adhesion length was then compared to migration data based for a given nominal diameter of nanofibers.

Figure 44 - Focal adhesion length is compared to migration data given in micrometers per hour. Error bars denote standard error of the mean. Pearson correlation test = -0.39.

The focal adhesion length was then compared to relative protein concentrations based for a given nominal diameter of nanofibers.
Figure 45 - Focal adhesion length is compared to relative intensity of pFAK-397. Error bars denote standard error of the mean. Pearson correlation test = -0.60171.

Figure 46 - Focal adhesion length is compared to relative intensity of pFAK-576/577. Error bars denote standard error of the mean. Pearson correlation test = 0.09229.
Figure 47 - Focal adhesion length is compared to relative intensity of pFAK-925. Error bars denote standard error of the mean. Pearson correlation test = -0.38974.

Figure 48 - Focal adhesion length is compared to relative intensity of pFAK-925. Error bars denote standard error of the mean. Pearson correlation test = -0.38974.
Discussion

Focal adhesion kinase has been shown to directly affect fibroblast migration speed.[27] FAK and Src have been thought to directly simulate each other’s kinase activity. Specifically, the autophosphorylation of pFAK-397 subsequent to integrin engagement interacts with Src and ultimately leads to an increase of FAK-dependent signaling. However, a mutation to prevent FAK-397 from phosphorylating did not show complete loss of ability to migrate, but a decrease as compared to wild-type control.[27] This highlights that fact that FAK and Src act in a mutual activation to amplify existing signal, not an “on/off” activation for cellular migration. This enhancement leads to FAK catalytic activity that is otherwise not seen at a normal rate in cells expressing mutated FAK-397.

shows a positive trend between mean velocities in cellular migration compared to relative concentrations of pFAK-397 with $R^2 = 0.1159$ for 6-12 hours after seeding and $R^2=0.1593$ for 13.5-19.5 hours after seeding. This is consistent with literature reports that FAK at Tyr-397 has shown play a key role in focal adhesion turnover which results in a decrease of migration speed.[28] However a Pearson product-moment correlation test yielded a coefficient of -0.36 and is displayed in Figure 40. No conclusion for pFAK-397 effect on velocity could be made.

The migration speed compared to relative concentration of pSrc-416 showed an interesting relation (Figure 39). For the 6-12 hour period after seeding, a positive correlation can be seen although with an extremely poor fit, $R^2 = 0.5282$. The period of 13.5-19.5 hours after seeding showed an unexpected negative correlation with a significantly better fit of $R^2 = 0.5282$. Comparing this to the FAK-Src activation model of Figure 7, the opposite would be expected: an increase of pFAK-397 would lead to an increase into phosphorylation of pSrc-416.
The concentration of pFAK-576/577 shows a negative correlation to mean migration velocity, depicted in Figure 37. The comparisons show for mean velocity for 6-12 hours vs. pFAK-576/577, \( R^2 = 0.3087 \), and for 13.5-19.5 hours after seeding \( R^2 = 0.5057 \). At first glance this disagrees with Figure 7 when comparing pFAK-397 levels with phosphorylated FAK-576/577. However, FAK-576/577 has been identified as a Src-specific site and is dependent on phosphorylation from Src kinases which is shown after step 3 in Figure 7.[14][29] Increased levels of Src416 leads to decrease in mean velocity and the same applies to increased concentrations of pFAK-576/577. Figure 41 compared FAK – 576/577 and Velocity. A Pearson product-moment correlation(PPMC) test yielded a coefficient of -0.872, revealing a strong negative correlation. Src – 416 also showed a strong negative correlation when compared to velocity, with a PPMC coefficient of -0.72.

pFAK-925 concentration did not show a significant trend with mean migration velocity. Figure 38 shows a mean velocity for 6-12 hours vs. pFAK-576/577, \( R^2 = 0.2052 \), and for 13.5-19.5 hours after seeding \( R^2 = 0.0515 \).

Figure 44 showed no strong correlation between focal adhesion length and migration velocity using the Pearson product-moment correlation coefficient. Figure 45 - Figure 48 also showed no strong correlation between focal adhesion length and relative protein concentrations.

Overall, the initiator for the FAK-Src activation pathway, pFAK-397, showed positive correlation with migration velocity, which was expected. The relationship between pFAK-576/577 and pSrc416 concentrations showed a comparable decrease for the period of 13.5-19.5 hours with increased migration velocity.
Conclusions and Future Experiments

In general, this research found that FAK and Src concentrations differed for hMSCs based on the mean diameter nanofiber scaffolds. Migration rates were found to be significantly different on nanofibers of different mean diameters. Focal adhesion length showed no significant difference when compared to migration velocity (Figure 44). Focal adhesion length showed no significant difference when compared to relative protein concentration of pFAK-397, pFAK-576/577, pFAK-925 or pSrc – 416. A decrease in pFAK-576/577 resulted in faster migrating cells. A Pearson product-moment correlation (PPMC) test yielded a coefficient of -0.872, revealing a strong negative correlation between pFAK-576/577 concentration and velocity. (Figure 41) Src – 416 also showed a strong negative correlation when compared to velocity, with a PPMC coefficient of -0.72. (Figure 43) This ultimately revealed a dependency of pFAK – 576/577 on Src-416 for activation, which is seen in literature.[14]

Previous research by Jaiswal and Brown in 2012 showed interesting similarities to pFAK-576/577 expression of hMSCs on nanofibers, Figure 32. The trend present in Figure 49 is comparing ERK ½ activity with etching time. The etching time of 15 minutes corresponds to an approximate fiber diameter of 2um, an etching time 35 minutes corresponds to 800nm, and finally an etching time of 55 minutes corresponds to a diameter of 600nm. FAK 576/577 is thought to activate ERK1/2. Figure 49 shows minimum ERK activity at an etching time of 35 minutes or an approximate diameter of 800nm.

Figure 49 - Adapted from Nanofiber diameter-dependent MAPK activity in osteoblasts.[31]
The relative concentration from this research that overlap with Figure 49 are displayed in Figure 50. A similar minimum trend can be seen in FAK 576/577 concentrations where a minimum is seen at a 6% HMW PMMA which also corresponds to a diameter of 800nm, Table 3. Figure 32 shows the pFAK-576/577 intensities for substrates of nanofibers with a diameter of less than 400nm. For diameter under 400 nm the “bowl-shaped” trend does not continue.

Continuation of this research will involve repeats of the above experiments to increase reliability of the results. All reported Western blots were complete with N=3 and more experiments will reduce errors and/or anomalous results. Further, other types of materials will be used for the substrate. Silk fibroin, derived from Bombyx mori cocoons, has strong mechanical properties, controlled degradation rates, and overall biocompatibility. The procedure for electrospinning silk is still in the troubleshooting process. Lastly, alpha- and beta-integrins will be quantified using the Western blot procedure previously used. Integrin concentration will lead insight into the initiation of the cell migration pathway that activate the FAK and Src amplification loop.
Appendix A

Live Cell Images on Various Diameter Fibers

Flat PMMA
1.54% HMW PMMA
3.1% HMW PMMA
Appendix B

Preliminary Experiments using CSB

Table 7 - This table shows the descriptions of each samples used and their corresponding sample number

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>99</td>
<td>Fibers w/o etching</td>
</tr>
<tr>
<td>100</td>
<td>Fibers w/o etching</td>
</tr>
<tr>
<td>101</td>
<td>Fibers w/o etching</td>
</tr>
<tr>
<td>102</td>
<td>30 second Etched Fibers</td>
</tr>
<tr>
<td>103</td>
<td>30 second Etched Fibers</td>
</tr>
<tr>
<td>104</td>
<td>30 second Etched Fibers</td>
</tr>
<tr>
<td>105</td>
<td>Five minute etched fibers</td>
</tr>
<tr>
<td>106</td>
<td>Five minute etched fibers</td>
</tr>
<tr>
<td>107</td>
<td>Five minute etched fibers</td>
</tr>
<tr>
<td>108</td>
<td>10 minute etched fibers</td>
</tr>
<tr>
<td>109</td>
<td>10 minute etched fibers</td>
</tr>
<tr>
<td>110</td>
<td>10 minute etched fibers</td>
</tr>
<tr>
<td>111</td>
<td>Smooth Untouched</td>
</tr>
<tr>
<td>112</td>
<td>Smooth Untouched</td>
</tr>
<tr>
<td>113</td>
<td>Smooth Untouched</td>
</tr>
<tr>
<td>114</td>
<td>Smooth etched 10 minutes</td>
</tr>
<tr>
<td>115</td>
<td>Smooth etched 10 minutes</td>
</tr>
<tr>
<td>116</td>
<td>Smooth etched 10 minutes</td>
</tr>
</tbody>
</table>
Figure 51 - This figure shows a western blot of samples 99 – 104 and band correspond to the stained Src protein.

Figure 52 - This figure shows a western blot of samples 99 – 104 and band correspond to the stained Tubulin protein.

Figure 53 - This figure shows a western blot of samples 102 – 107 and bands correspond to the stained Src protein.

Figure 54 - Those Shows samples 102 – 107 and their Tubulin Expression

Figure 55 - Those Shows samples 108 – 113 and bands correspond to the stained SRC protein.

Figure 56 - Those Shows samples 108 – 113 and their Tubulin Expression
Figure 57 - Those Shows samples 113–116 and their SRC Expression

Figure 58 - Those Shows samples 113–116 and their Tubulin Expression

Figure 59 - This figure shows the relative SRC intensities. Intensities were normalized to Tubulin intensities. Error bars are the standard deviation of the sample group.
Figure 60 - This figure shows the relative SRC intensities in the CSB of the samples. Intensities were normalized to Tubulin intensities. Error bars are the standard deviation of the sample group.


38. Available from: http://dx.doi.org/10.1016/j.biomaterials.2015.05.015


ACADEMIC VITA

Mary Elizabeth McCulloch

Permanent Address: 11139 Runville rd, Bellefonte, PA 16823
Phone Number: 814-360-3517 | Email: mxm5344@psu.edu

Education

The Pennsylvania State University
Biomedical Engineering
Schreyer Honors College
University Park, Pa
Graduation: May 2016

Leadership Experience

Member of Penn State Altoona’s Varsity Swim Team
Women’s Captain
Led a twenty person team with the help of two other co-captains

Menajero de La Paz Orphanage
Activities Director
Spent the summer volunteering and planning events for a special needs orphanage of forty children and adults

Learning Resource Center
Math and Physics Tutor
Women of Engineering
Physics Tutor
Tutored a group of 30 engineering students weekly in mechanical physics

Medical Experience

Hershey Medical Center
Volunteer
Various tasks in both the emergency department and sterile processing

Christian Medical Society Medical Missions Trip
Interpreter
Interpreted for doctor patient consultations and directed triage in the Spring of 2012, 2013, and 2014

Gastroenterology at Penn State Hershey
Sharlowing
Sharlowed Dr. Matthew Moyer and residents of the gastroenterology department

Projects and Research

Project Vive
Founder/CEO
Project Vive is an initiative to give a voice to those with severe speech disorders by providing low cost and robust speech assistive devices. Check us out at projectvive.com

Musculoskeletal Regenerative Engineering Lab
Undergraduate Research assistant
Integrin expression of mesenchymal stem cells to aid in the intelligent design of biomaterial scaffolds

2015 - Current

2014 - 2016

Awards and Honors

Penn State Altoona Sportsmanship award - 2012
ECAC Division III Female Scholar-Athlete - 2013
NL3 Pitch Competition Finalist
D2PA State Grant(5,000) - 2015 Project Vive
Happy Valley Launchbox Space Recipient - 2016

Penn State Altoona Scholar-Athlete of the Year - 2013
AMCC Swimmer of the Year - 2013
First Place- Undergraduate Research Fair -2013
Summer Founders Grant(10,000) - 2015 Project Vive
INCU Funding Award(17,000) - 2015 Project Vive

Societies and Clubs

The Tau Beta Pi Engineering Honor Society
Officer, Initiation Chair

Christian Student Fellowship and Student About Living Truth
Pianist and Vocalist

Suicide Prevention Committee
Student-Athlete representative

University Park, Pa
2014 - 2016

Altoona & University Park, Pa
2012 - 2013

Altoona, Pa
2012 - 2013

Skills

Spanish Speaking and Writing - Advanced
Social Entrepreneurship

Matlab
Public Speaking