Modulating the Cellular Mechanics by Regulating the Cytoskeletal Proteins

A Major Qualifying Report

Submitted to the Faculty

Of the

Worcester Polytechnic Institute

In Partial Fulfillment of the Requirements for the

Degree of Bachelor of Science

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Table of Contents

Authorship

Each team member contributed equally to the development of this report and was essential in providing feedback.

Acknowledgements

We wish to acknowledge those who guided and helped us through our research project. It is with their assistance that we were able to conclude our experiments and fabricate our design.

With help from the grad students and faculty, who have lent their expertise and skillsets, we were able to receive a number of results and fully understand the basis of each experiment. On behalf of the lab headed by Professor Dominko, we thank Alexandra Grella and Denis Kole for performing the qPCR and Western blot analyses used in this report. These experiments allowed us to characterize each cell line and were essential to the project. We thank Professor Albrecht for preparing the mask for our designs, from which we were able to fabricate our microchannels. We also thank Ross Lagoy for further preparing the microchannel devices so that they were ready for use. Gawain Thomas was skilled in Atomic Force Microscopy and ran multiple analyses to give us data. We are very grateful to Victoria Huntress from the Microscopy Core in Gateway Park. Week after week, she set aside hours to give us confocal images of multiple cells from multiple cell lines.

We are especially thankful to the MQP team of Claire Hambright, Melanie Wiater, Christopher Gorgone, and Lindsay Hock. As our seniors in working with PDMS molds, they walked us through the step-by-step process of preparing and handling our designs. In addition, they were kind enough to fabricate a number of our devices for us.

Finally, we wish to thank our advisers, Professor Wen and Professor Ambady. Our advisors were always present to guide us through the difficulties we encountered. They aided us in perfecting this report and informed us of facets of the project we did not previously consider.

Abstract

Vimentin is an intermediate filament protein involved in the structure and motility of cells. The goal of this project was to study the effect of low vimentin levels on the structure, mechanics, and migration/invasiveness of cells. In accordance with that goal, we performed immunocytochemistry, confocal imaging, atomic force microscopy, and wound healing assays on NIH 3T3 cells that had vimentin knocked down by shRNA to characterize vimentin's effects. We also worked to develop an invasion assay that better recreates *in vivo* conditions and could be better observed for any invasive cell types. An assay was developed, which utilized a microfluidic device that utilizes a Matrigel barrier flanked by a cell culture chamber and a chamber containing a chemoattractant of choice. Our system, if refined, would allow direct observation and quantification of cell invasiveness both in the 2D and 3D mode.

Table of Figures

Table of Tables

Chapter 1: Introduction

Vimentin is a cytoskeletal protein that may play a large role in cellular dynamics such as cell to cell adhesion, viscoelasticity, and cell invasiveness/motility. Vimentin is a type III intermediate filament (IF) that is currently being studied in terms of its effects on cell structure and function. Vimentin has already been shown to affect mechanical properties in the cell (Janmey *et al.* 1991), but limited research has been introduced to address how changing properties, specifically viscoelasticity, will ultimately change the functionality of the cell.

A cell's functionality, motility, and its interactions with other cells are linked to cytoskeletal proteins. There are three types of cytoskeletal proteins: intermediate filaments, microfilaments, and microtubules. All three serve a role in cellular dynamics. Microtubules are responsible for intercellular transport and sensing external forces (Karafyllidis and Lagoudas 2007) and microfilaments help create energy for cell motility (Pfaendtner *et al* 2010). Intermediate filaments are the least researched protein of the three. Intermediate filaments are responsible for the structural integrity of the cell and migration (Comley 2013). Vimentin is the most researched IF protein. A correlation has been found between increased levels of vimentin and increased movement of the cells (Gjerdrum *et al*. 2011, Ivaska 2011). This suggests that decreased levels of vimentin can decrease the movement of the cells.

Cellular mobility is an increasingly important topic of research due to its relationship with cell invasiveness. Cellular invasiveness is a phenomenon in which a cell no longer operates as a normal functioning cell. Cytoskeletal proteins within a normal cell allow cells to communicate with each other allowing movement without hindering, or impeding upon neighboring cells (Chung *et al* 2013). It has been shown that when a cell over-expresses vimentin, it becomes increasingly more mobile and no longer operates as a normal cell (Chung *et al* 2013). When this happens, these cells become invasive and begin growing and spreading

without regard for surrounding cells and tissue. This type of movement and cell behavior is most infamously shown among cancerous cells. Cancerous cells lack the ability to operate as normal cells because they no longer acknowledge their neighboring cells and proliferate in an uncontrolled manner. Research has shown that this behavior may be linked to an overexpression of cytoskeletal proteins (specifically vimentin) and, as a result, increased motility (Gjerdrum *et al.* 2011).

It is because of this link between overexpression of vimentin and increased motility that it was chosen as the subject of this project. By knocking down the levels of vimentin in NIH 3T3 cells and observing its effects on cell structure, viscoelasticity, and motility, a better understanding of vimentin's role in the cell could be gained, aiding in our understanding of cancer.

In order to aid in the study of vimentin's effects on cell migration and invasion, it was necessary to develop a new invasion assay. The current standard assay, a Boyden chamber, can only observe invasiveness after the fact, has difficulty maintaining the chemoattractant gradient, and does not include an accurate representation of the barrier to cell invasiveness (Toetsch *et al* 2008). By developing a new assay which can be more easily observed and quantified and better represent *in vivo* invasive conditions, we hope to improve the study of vimentin's effects on invasiveness and all future study of cell invasiveness.

Chapter 2: Literature Review

2.1 Importance of the Field

Vimentin is a type III intermediate filament protein (IF). Intermediate filaments are proteins that provide cells with mechanical stability, cell to cell adhesion, and motility. Vimentin is considered to have the largest effect on the cell than other similar proteins. It has been shown with lowered levels of this protein that mobility and structural integrity will also be reduced (Chung *et al*. 2013). Cells that are considered invasive have been shown to express high amounts of vimentin, forming a correlation between vimentin and cell invasiveness. The most infamous invasive cell type that shows high levels of vimentin are cancer cells (Gjerdrum *et al*. 2011).

To better understand cancer and a goal of this project, it is important to define invasiveness. Cancer displays a unique quality that most cells found in the body lack. Some cells in multicellular organisms, primarily those in the blood and those involved in wound healing, are migratory, which means they migrate in specific ways to specific locations, typically in response to a signal. This can be seen in many tests such as the wound healing assay. A wound healing migration assay or "scratch test" involves taking a well of confluent cells and scratching the surface to vacate the scratch site of cells. Given enough time, the cells will typically fill in the empty space where scratching has occurred. This is analogous to what happens in the body; migratory cells will proliferate to fill the space in which they are confined. The difference between migratory cells and invasive cells is that invasive cells are able to digest through the basement membrane. The basement membrane is a thin layer of fibers below epithelial tissue that lines the bloodstream and organs that most cells are unable to pass through and constrains their movement in the body. The membrane has a number of surface proteins and other molecules that signal to the cells to stop migration (Paulsson 1992). When cancer becomes

metastatic it is able to pass through this membrane and enter the bloodstream, eventually spreading throughout the body (Liotta *et al*. 1980). This act of passing through the basement membrane is what will constitute an invasive cell.

2.2 Relation to the Larger Problem Area

This project is being conducted because there is a large quantity of information that can be gained. There is a dearth of information on how cytoskeletal proteins function *in vivo* and very little research into the function of intermediate filaments in cells, even outside the body. To address this problem, the project goal is to create and develop an assay that will model some facet of intermediate filaments. The team chose vimentin, in the use of an invasiveness assay due to the knowledge that may be gained of cancer. It is intended that the developed assay will stimulate interest in the field of intermediate filament research and will facilitate further study.

2.3 What is Known and Unknown

There has been limited research into intermediate filaments as they pertain to the viscoelasticity and motility of cells. One study found that altering the viscoelastic properties of actin fibers in fibroblasts significantly increased their migration rate. In that study, malignantly transformed fibroblasts with lower viscoelastic moduli, a measure of their stiffness, were found to migrate faster. Their moduli were found to be approximately half that of the control cells and they moved more than twice as fast (Park *et al*. 2005).

There have also been studies relating vimentin to the motility of cells. Correlations have been found between vimentin and epithelial to mesenchymal transition (EMT), a process in which cells change from immobile connective tissue, to mesenchymal cells (a type of less differentiated cell) that are capable of migration. This transition can be a critical point in the progression of cancer towards metastasis, the process in which cancer spreads from one part of

the body to another (Gjerdrum *et al*. 2011). It was found that silencing vimentin in mammary epithelial cells caused down regulation of a number of different migration related factors including tyrosine kinase Axl, integrin β4-subunit (ITGB4) and plasminogen activator urokinase (PLAU) (Ivaska 2011). Up regulation of vimentin has also been found to be involved in the regulation systems required for cell migration lymphocytes, fibroblasts, and epithelial cells, further proving its importance for cellular movement (Chung *et al*. 2013). Studies have noted high levels of vimentin in types of epithelial cancer cells, including prostate and breast cancer (Chung *et al*. 2013, Gjerdrum *et al*. 2011), suggesting that higher levels of vimentin are linked to more aggressive cells. Lowered levels of vimentin expression have been shown to decrease motility and promote a more epithelial phenotype, meaning that the cells are more rigid and stable (Chung *et al*. 2013). Overexpression of vimentin has been shown to enhance prostate cancer cells' invasiveness (Chung *et al*. 2013). The team will specifically be looking at the role of vimentin in the cell because it has been shown to be an important intermediate filament and is the most studied in this field with such limited research.

2.4 State of the Art in this Field

There are many products on the market geared to studying invasive behavior of cells. One such model is a Boyden chamber, which is composed of an upper and lower chamber, separated by a microporous membrane. Media is placed in both compartments and the cells are placed in the upper chamber. The chemotactic agent is placed in the lower chamber and the cells are allowed to move. Cell Biolabs Inc. has developed a few assays that utilize Boyden chambers. The top of the pore membrane is coated with Laminin, Collagen I, or a basement membrane isolated from Engelbreth-Holm-Swarm tumor cells. There are 24-well and 96-well variants of these that cost $195.00 and $515.00, respectively (Cell Biolabs Inc. Appendix B).

Spheroids, or tumor cell aggregates, are also starting to be used in products. Evidence has been growing that spheroids provide a more representative model of tumors *in vivo* than monolayers of cells can provide. This is because spheroids exhibit several traits of cancer such as decreased proliferation or increased cell survival. Adaptation of spheroids provides a more physiological approach for assessing tumor invasiveness (Amsbio Appendix B). TREVIGEN's and AMSBIO's Cultrex® 3D Spheroid Cell Invasion Assay was created through a need for "more complete and physiologically predictive cancer invasion models" (Trevigen Appendix B). It is capable of running 96 samples and contains an invasion matrix that simulates a basement membrane. This matrix "forms a hydrogel network through which invasive cells can travel" (Amsbio Appendix B) at which point invasion modulating agents can be used to evaluate the impact on cell response. Invasion is modeled through microscopy and can be quantified by using image analysis. The retail price is $295.00 (Amsbio Trevigen, Appendix B).

EMD Millipore markets the QCM™ Gelatin Invadopodia Assays. There are two models that affix both red or green labeled gelatin to a glass substrate and that contain reagents to localize actin degradation sites. When cells invade the Extracellular Matrix, actin-rich protrusions are also formed. The kit enables visualization of the degradation through fluorescence microscopy, which can be quantified by image analysis. Both models are sold as single kits with 32 wells and are sold for $459 (Millipore Appendix B).

Nanoco Technologies specializes in the manufacturing of quantum dots, a group of semiconductor nanoparticles for which the company is developing new applications, from biotechnology to electronics. Based on the size of the quantum dot, it will emit photons, perceived as light, of varying wavelengths when under excitation. It is therefore possible to control the size to affect the desired color. The final products, whatever the size, appear as a

powder or in solution. For biological applications, it is possible to target the nucleus of a cell using these particles and then detect the light that is emitted in order to easily image it. Depending on properties such as material, size, and solubility, the price can vary. Ten milligrams can range from $54 to $750, though other quantities are available (Nanoco Group PLC Appendix B).

A major component of the project was to design an invasive cell analysis by creating an assay that can differentiate and track cells that are invasive versus cells that are migratory. One of the most important conditions for distinguishing between invasive and migratory cells is the cells ability to pass through the basement membrane. The basement membrane is the joining of two lamina, basal and reticular, that are attached through collagen fibrils. These two lamina make up the basement membrane and are subsequently attached to blood vessels and organs through type IV collagen fibers (Pavlakis *et al*. 2011). There is great interest currently involving the synthesis of basement membranes because of its importance in intercellular dynamics. A major issue encountered by some scientists, such as Kleinman and Martin, in creating the basement membrane is that it is so thin that it is difficult "to study its composition, structure, and function" (Kleinman and Martin. 2005). However, transmission and scanning electron microscopy have successfully been used to analyze surface topography of the epithelial and endothelial basement membrane, inclusive of the many pores and fibers (Abrams *et al*. 2002).

There have been some reports of people who have created a basement membrane that operates similarly to a real one as well as several companies that have gone on to market products as suitable basement membrane replacements. Matrigel is seen as one of the most suitable products that mimic the basement membrane and have been recorded in many papers as the best material for this application (Kleinman and Martin. 2005). Several research groups

reported the use of hydrogels, collagen, and polymers as a basement membrane with varying success (Kvist *et al*. 2008, White and Deen. 2002, Yamada *et al*. 2013).

Chapter 3: Project Strategy

3.1 Initial Client Statement

The team first met with the clients on September 5, 2013 and received the following client statement.

Cytoskeleton provides mechanical support to cells, drives cell migration, and facilitates mechanical interactions between cells and their extracellular matrix (ECM). The major components of cytoskeleton are: actin microfilaments, intermediate filaments, and microtubules. While mechanical properties of networks of each type of cytoskeletal filaments have been studied extensively in vitro, the contributions of intermediate filaments to viscoelasticity of living cells have not been adequately studied. This project will focus on elucidating the contributions of vimentin to cellular dynamics. You will utilize a transgenic mouse fibroblast cell line (NIH 3T3) in which the vimentin RNA levels are knocked down using a lentiviral vector containing short hairpin RNA (shRNA) against mouse vimentin to study the effects of vimentin. The needs of this project are:

1. *Determine vimentin mRNA levels in clonally selected NIH 3T3 fibroblasts stably transfected with vimentin shRNA in order to choose medium and low vimentin mRNA expressing clones for subsequent work.*
2. *Design image analysis technique(s) to quantify and map the distribution of vimentin in control and knock-down cells.*
3. *Explore additional methods to disrupt/destroy vimentin and other cytoskeletal proteins in cells and evaluate the effectiveness of each method.*
4. *Measure the long and short term changes in cell viscoelasticity due to the disruptions in cytoskeletal components.*

5. *Design an assay to characterize and quantify migration rate of normal 3T3 cells and cells with disrupted cytoskeleton within a 3D scaffold in response to a chemo-attractant(s).*

3.2 Objectives

From the client statement and further correspondence with the sponsor, the team proceeded to develop and rank seven key objectives for the project. A pairwise comparison chart was developed to compare the various objectives against each other.

Table 1: Pairwise Comparison Chart

	Encourage Novel Research	Recognize Invasive Cell Behavior	Reliable	Reproducible	Improvement Over Current Assays	Marketable	User Friendly	Score
Encourage Novel Research	X	0	0	0	0	1	1	2
Recognize Invasive Cell Behavior	1	X	1	1	1	1	1	6
Reliable	1	0	X	1	0	1	1	4
Reproducible	1	0	0	X	0	1	1	3
Improvement Over Current Assays	1	0	1	1	X	1	1	5
Marketable	0	0	0	0	0	X	0	0
User Friendly	0	0	0	0	0	1	X	1

Primary Objectives

1. **Recognize Invasive Cell Behavior**
2. **Improvement over current assays**
3. **Reliable**
4. **Reproducible**

Secondary Objectives

5. **Encourage Novel Research**
6. **User friendly cell analysis software**
7. **Marketable**

It was decided that making a design that was able to recognize invasive cell behavior and differentiate it from noninvasive behavior was the most important feature. It was decided that designing a cell invasiveness kit that was an improvement over current products was the next most important objective to be met. There are many kits for analyzing cellular movement and invasiveness sold by many companies. The team came to the conclusion that it was not only important to differentiate our invasiveness kit from other kits that are available, but to make improvements where these other kits are lacking, in order to compete with them. If our assay was not an improvement over other assays, users would have no reason to change from the assay that they are more familiar with to a newer, less established assay. After deciding that the design had to be a novel improvement, we further broke down what needed to be addressed in the objectives. It was decided that reliability and reproducibility were the next most important primary objectives. We determined that the final three objectives, encourage novel research, user friendly cell analysis software, and marketable, were secondary objectives, but weren't necessary to incorporate into the design.

3.3 Functions

After developing objectives for the project, we focused on the design aspect and created functions. The design portion of the project consists of an assay to monitor cell invasiveness and migration as well as image analysis code to be used with popular software, such as MATLAB or Cell Profiler.

The image analysis code will determine the relative vimentin levels in cells. It will also determine changes in cell structure, specifically the viscoelasticity of the cell and the nucleus. This portion will also determine the distribution of vimentin in the cell. The code will be designed to function with the MATLAB software.

The cell invasiveness assay will serve to distinguish cell behavior, determining whether cells are behaving aggressively or passively. The invasiveness assay will mimic the properties of the basement membrane to test for the cells aggressiveness by looking at its ability to penetrate the basement membrane.

3.4 Constraints

After conducting some research and meeting with the clients, it was clear that there were several design constraints in addition to the expected project constraints of time and budget.

- NIH/3T3 cells
- Code is compatible
- Available equipment
- Assay must be at micron scale
- Comparable retail price

The experiments must be compatible with NIH 3T3 cells, because these are what will be

available for the project. Because the code is designed to be used by others, it must be compatible with common operating systems and software, such as Image J, Cell Profiler, MATLAB, and Microsoft Excel. All experiments must be able to be accomplished with the equipment readily available. The laboratories at Worcester Polytechnic Institute have standard microscopes, confocal microscopes, an Olympus ix83 microscope, and an Atomic Force Microscope (AFM). The team is limited in its use of the AFM due to qualification restrictions. Experiments requiring use of the AFM will be performed by trained personnel.

The invasion assay kit is also subject to constraints. Testing must be compatible with the available NIH 3T3 cells and needs to be manufactured at a micron scale. The kit must be competitively priced compared to what is currently available in the market; otherwise researchers will not want to purchase it. After comparing the prices to similar gel kits that are manufactured, a price of 300$ was a reasonable price for the project kit to compare to.

3.5 Revised Client Statement

The team revised the initial client statement several times while going through the process of determining the important objectives, functions, and constraints of the design. The final client statement is presented below.

Design a reproducible procedure to study cellular behavior, such as viscoelasticity of the cell membrane and nucleus, proliferation, migration and invasiveness of normal and vimentin knock-down NIH 3T3 cells. Design a reliable and reproducible 3D gel system for the study of cell invasiveness. Create cell analysis code to be used with existing software for determining levels and dispersion of cellular proteins.

This final client statement significantly narrows the scope of the project compared to the

initial client statement. The initial client statement was very open ended about the direction of the project so it was important to define and set objectives that could be met with the fairly short time period for the project and which stressed the design component of the project.

3.6 Project Approach

3.6.1 Technical

Four major technical steps in the project approach were determined. These steps were to determine vimentin levels in the available cell lines, analyze changes in the mechanics of the structure of the cells, benchmark the migratory abilities of the cell lines, and develop and test a new design for an invasion assay.

Determine Vimentin Levels

Quantitative Polymerase Chain Reaction (qPCR) is the method of choice to determine the relative levels of vimentin mRNA in clonally selected NIH/3T3 cell lines where vimentin RNA has been knocked down at various levels. Western blots will also be used in order to determine vimentin protein levels in the cell lines. The images generated from the western blots will be subjected to densitometric analysis using ImageJ software to perform semi-quantification of the vimentin protein levels.

Analyzing Changes in Structure/Mechanics of Cells

Immunocytochemistry assay was used to determine localization and dispersion of vimentin in the cells. An anti-vimentin primary antibody will be used with an AlexaFluor-568 (AF-568) conjugated secondary antibody for detection. DAPI (4', 6-diamidino-2-phenylindole) stain binds with DNA, and will identify cell nuclei. AF-488 conjugated fluorescence conjugated phalloidin will be used to detect actin and to aid in the determination of cell dimensions.

Fluorescence microscopy and confocal microscopy will be used to determine the 3D distribution and amount of vimentin and to analyze cell dimensions by utilizing the DAPI, FITC, and Rhodamine filters. Confocal imaging will primarily yield the level and distribution of vimentin through each cell line.

Fluorescent images of cell lines in conjunction with their western blot analysis will be used to design the image analysis code.

Atomic Force Microscopy (AFM) is a method to examine the stiffness of the cellular membrane and find a correlation to the vimentin levels in the cells. The microscope uses a cantilever tip to trace the surface of the cell, recording any deformations in the tip as stiffness values. This is repeated on a large sample of 40 or more so as to average out any fluctuations caused by nucleus stiffness in comparison to cytoplasm stiffness.

Migratory Assay

The team will perform two types of migration assays to determine their invasiveness in response to a chemotactic agent and their rate of migration in wound healing conditions. Three counts of the wound healing assay were performed. For the first two, a confluent sheet of cells was prepared in duplicate in 6-well plates for each cell line, in addition to an extra two for the 3T3 control. Each well was treated with Mitomycin-C (MMC) and left for 48 hours except for two of the 3T3 control wells, which served as controls on the effects of MMC. Wounds were made in each well with a P200 pipette tip and imaged over 49 hours in order to determine the speed of cells. For the third attempt, the same conditions and treatments were prepared but instead of creating a scratch, half of the plate was scraped of cells. Wells were imaged at 6, 24, and 55 hours.

Design/Testing Invasion and Migration Assay

The design of the invasion/migration assay must incorporate several components. It is important for the assay to impede the movement of the cells to better mimic an invasive cell test. There were several options for materials, such as hydrogels or collagen to be used as a barrier and will be employing the chemo attractant used in the Boyden chamber invasive assay to encourage the movement of cells through the barrier. Finally it would ideally have real time imaging incorporated in to the assay to produce constant and consistent data for better analysis.

The invasiveness of two cell lines was tested against the Boyden chamber, a standard invasiveness assay. The cells are treated with Mitomycin-C (MMC) to prevent cell proliferation so that the only movement of the cells would be migration. The positive and negative controls cells are glioblastoma and fibroblast 3T3 cells, which are known to be invasive and non-invasive, respectively.

3.6.2 Management

A Gantt chart for the project based on general deadlines given by the school and the teams advisors. Table 2 below shows the timeline from the Gantt chart. The full Gantt chart is available in Appendix A.

Table 2: Timeline of Tasks

Task Name	Duration	Start	Finish
A Term	36 days	Thu 8/29/13	Thu 10/17/13
Introduction	10 days	Fri 9/6/13	Thu 9/19/13
Design – revised client statement, clear objectives	10 days	Fri 9/6/13	Thu 9/19/13
Gantt Chart for the project	10 days	Fri 9/6/13	Thu 9/19/13
1-2 page report detailing how you would reduce design to	10 days	Fri 9/20/13	Thu 10/3/13

practice			
Experimental design & methods	10 days	Fri 9/20/13	Thu 10/3/13
Development of analysis methods	10 days	Fri 9/20/13	Thu 10/3/13
Experiments to test hypothesis	10 days	Fri 9/20/13	Thu 10/3/13
An 8-10 page hypothetical paper	15 days	Fri 9/20/13	Thu 10/10/13
Presentation 1	5 days	Sun 9/1/13	Thu 9/5/13
Presentation 2	11 days	Fri 9/6/13	Fri 9/20/13
Presentation 3	11 days	Sat 9/21/13	Fri 10/4/13
Ch. 1: Intro - complete draft	20 days	Mon 9/2/13	Fri 9/27/13
Ch. 2: Lit Review - detailed outline with references (include patents)	15 days	Mon 9/9/13	Fri 9/27/13
Ch3: Project Strategy - complete draft except Project Approach	11 days	Sun 9/15/13	Fri 9/27/13
Chapters 1-3 complete	13 days	Fri 9/27/13	Tue 10/15/13
B Term	38 days	Tue 10/29/13	Thu 12/19/13
Ch. 4: Design Complete Draft	66 days	Fri 9/20/13	Fri 12/20/13
Ch. 5: Methodology Complete Draft	60 days	Mon 10/21/13	Fri 1/10/14
Maintain cells	38 days	Tue 10/29/13	Thu 12/19/13
Migration assay - Olympus microscope - chemotactic assay	6 days	Tue 10/29/13	Tue 11/5/13
Determine vimentin mRNA levels - qPCR	11 days	Tue 10/29/13	Tue 11/12/13
Analyze the relative vimentin protein levels - immunocytochemistry - microscopy and confocal microscopy and code	11 days	Tue 10/29/13	Tue 11/12/13
Work on code	47 days	Tue 11/12/13	Wed 1/15/14
Design 3D Hydrogel	84 days	Tue 10/29/13	Fri 2/21/14
Look at the effect of the varying levels on the viscoelasticity of the cells - AFM	38 days	Tue 10/29/13	Thu 12/19/13
Look at the effect of the varying levels on the invasiveness of the cells - 3D scaffold and chemoattractant	61 days	Fri 11/29/13	Fri 2/21/14

C Term	37 days	Thu 1/16/14	Fri 3/7/14
Ch. 6: Results Complete Draft	61 days	Fri 12/20/13	Fri 3/14/14
Ch. 7: Analysis and Discussion Complete Draft	66 days	Fri 12/20/13	Fri 3/21/14
Ch. 8: Conclusions Complete Draft	66 days	Fri 12/20/13	Fri 3/21/14
Ch. 9: Recommendations Complete Draft	66 days	Fri 12/20/13	Fri 3/21/14
D Term	37 days	Mon 3/17/14	Tue 5/6/14
First Draft of Paper	12 days	Fri 3/21/14	Mon 4/7/14
Project Presentation	29 days	Mon 3/17/14	Thu 4/24/14
eCDR			Thu 5/1/14

In A-term, the team focused mainly on background research for the project and planning for the terms ahead. Chapters 1 through 3 of the MQP report were also completed. The focus of B-term is on drafting chapters 4 and 5 of the MQP report, completing much of the testing of the effects of varying levels of vimentin on the cells, designing the invasiveness assay, and beginning work on the code to determine the relative levels of vimentin in the cells. The testing of the assay and the code will continue into C-term. Also during C-term, the results of the project will be completed and chapters 6 through 9 of the MQP report will be completed. In D-term the MQP report will be completed and as well as preparing, as well as giving a PowerPoint presentation regarding the project.

3.6.3 Financial

A budget of $624 was allocated for necessary materials. The project advisors had made the decision to deduct $100 from each of the groups in order to pool together a group of common supplies for all the teams and to procure a few specific items. Although this reduced the budget to $524, the team will have access to the NIH 3T3 cell line, media, cell plates, the use of

microscopes, cantilevers for use with the AFM, a DAPI stain, and a FGF chemoattractant.

There are multiple options of what material will be used as the scaffold in the invasiveness assay. Collagen costs $350 for 100 ml, but fibronectin may also be used which costs $300 for 5mg. The team has also considered purchasing a similar product to test against the designed invasion assay, which typically cost $300. In order to determine properties of the nucleus, quantum dots will be used to determine its viscosity. Quantum dots can range from $54 to $750 for 10 mg. The team will need to better understand the relevant problems and their solutions in order to maximize the budget and obtain everything necessary.

Chapter 4: Alternative Designs

4.1 Needs and Wants

The materials used in the creation of this assay must not be cytotoxic. The Matrigel barrier must completely isolate the cell suspension from the chemoattractant. The barrier must be on the micron scale. If possible, we want the invasion assay device to be reusable.

The code must be able to determine the average vimentin level in the cells in an input picture. It must be able to be used with an existing platform such as MATLAB, ImageJ, etc. It would be beneficial to have some level of automation with the code, so that it may be run on a folder of images and output the necessary data for all at the same time.

4.2 Functions/Specifications

There are two design portions of this project, each with their own functions and specifications, the invasion assay and the image analysis code.

4.2.1 Invasion Assay

The primary function of the invasion assay is to distinguish between invasive and noninvasive cells. This will be accomplished by incentivizing the movement of the cells with a chemoattractant and blocking their path with a barrier that only invasive cells will be able to pass.

The secondary function of the assay is to mimic the properties of an *in vivo* tissue. This is important as cellular behavior is heavily influenced by their environment and by recreating the *in vivo* conditions; we ensure that the cells behave as they would in a living organism for more accurate results and observations. This will be done by suspending the cells in a collagen hydrogel to recreate the extracellular matrix of a tissue, surrounded by a Matrigel layer. The Matrigel layer recreates the basement membrane that separates tissues and is the primary barrier

that invasive cells cross. Since Matrigel is derived from actual basement membrane proteins, it will help with cell signaling and proper reaction by the cells.

The tertiary function of the assay is to be easily observable and duplicated on a large scale. By making it easily observable, it makes it easier for the researcher using the assay to get results. The large scale use is important because researchers work with large sample sizes to get statistically significant results. This will be done by making the assay small enough to fit in a well of a multi-well plate and thin enough that it can be easily imaged by a microscope. It also will be simple enough to set up the assay so that it could be quickly done many times in a row in a short amount of time.

4.2.2 Image Analysis Code

The primary function of the code is to measure the relative concentrations of vimentin in cells using immunocytochemistry images. This is important to correlate the vimentin levels to the other data that we obtain through our experiments. This will be done by measuring the intensity of the color from the fluorescent microscopy and correlating that to concentration.

The secondary function of the code is to automate the process of analyzing the image. This is important to increase the throughput of the analysis so that it can be used with more images in less time. This will be done by making the code so that it can be given a folder of images and automatically go through all of them and output the data all at once.

4.3 Conceptual Designs

In designing our invasion assay, we went through several iterations. At first we just came up with a basic idea, based on the principles that we wanted to incorporate into our design. The cells would move from a starting position, through a barrier, into a region with chemoattractant. This initial design was a placeholder until we developed newer designs.

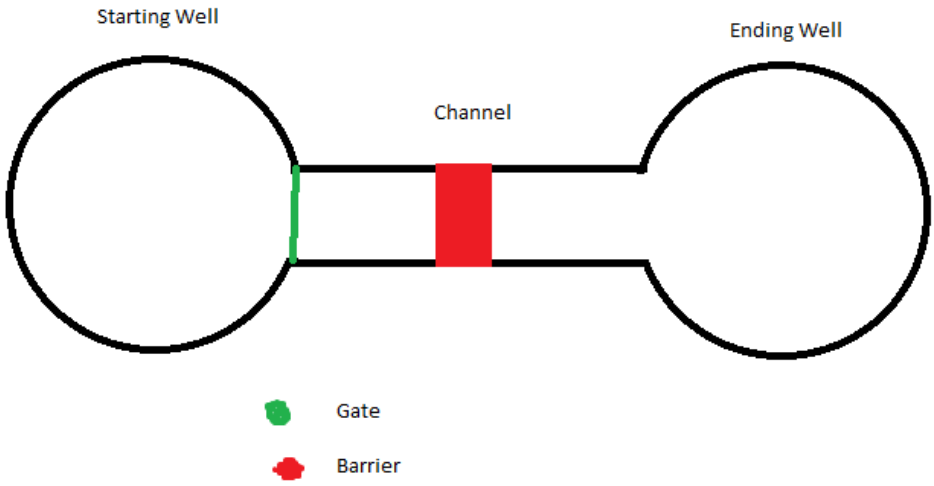

Figure 1: Initial Design

The first theoretical design developed

The first design had the cells suspended in a collagen hydrogel surrounded by an outer layer of Matrigel. The idea of this design was to put the cells into a 3D matrix to better recreate *in vivo* conditions. With this design, the outer Matrigel layer would be formed by setting it in a mold of two hemispheres. Unfortunately, this design had several issues that made it a non-viable option. We discovered that in order for the cells to be able to traverse the barrier within a reasonable amount of time (less than a week) the assay would need to be on the scale of micrometers rather than the previously thought millimeters. This meant that the mold strategy would not work as we could not make the molds small enough to work.

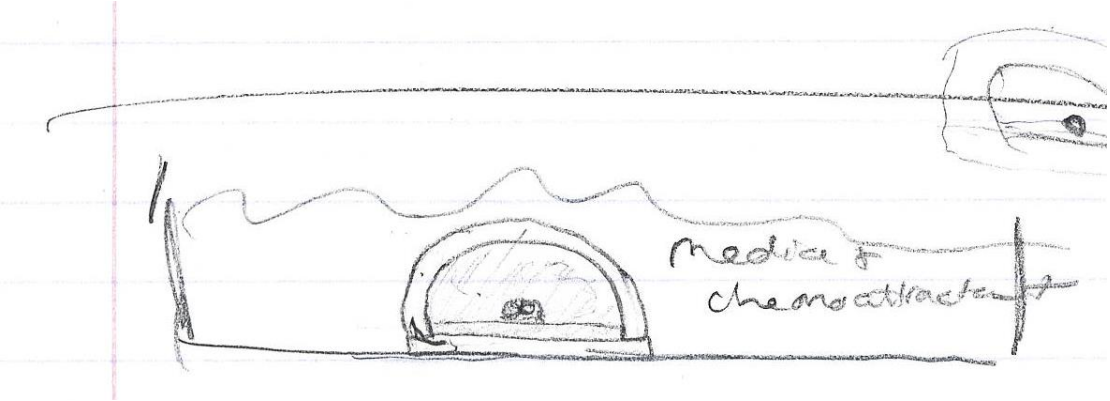

Figure 2: Design 1

A conceptual sketch of the first major design idea

Once we took the scale into consideration, we developed a new design. This design was very similar to the original design, using gates to keep the Matrigel in the center until it solidifies. This design was made to work on a much smaller scale, but it wouldn't work as the length of the microchannel would be too short for a gate system to work effectively.as the gates would be so close together that they would be difficult to use.

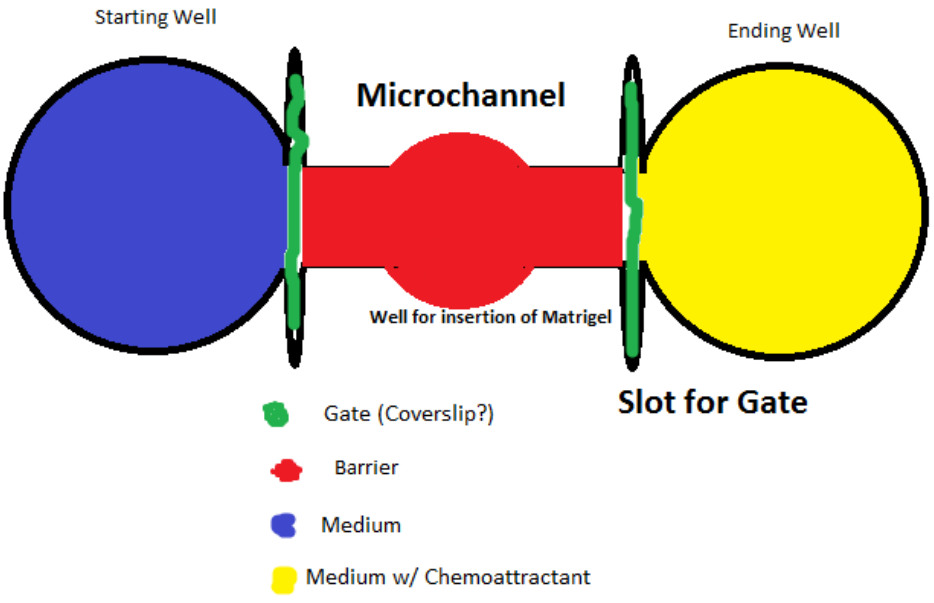

Figure 3: Design 2

A computer created sketch of our second major design.

The final design that we decided to actually fabricate utilized microfluidics. The sketch of this design can be seen in Figure 4. The inlet holes for the tubing have a diameter of 1mm with channels coming from them with widths of 200 microns. The central channel is for the Matrigel barrier. The design was sketched in CAD multiple times with the central channel width measuring 10, 20, 30, 40, 50, and 60 microns. The two squares on either side of the channel are for the cells and chemoattractant to flow through. These chambers are 1x1 mm. The gap in the central channel for the cells to pass through the Matrigel barrier is 100 microns wide. The Matrigel barrier would have the width of the channel. The cells in the chamber would sense the chemoattractant and migrate toward it. If the cells were invasive, they would be able to digest through the barrier and travel into the chemoattractant chamber.

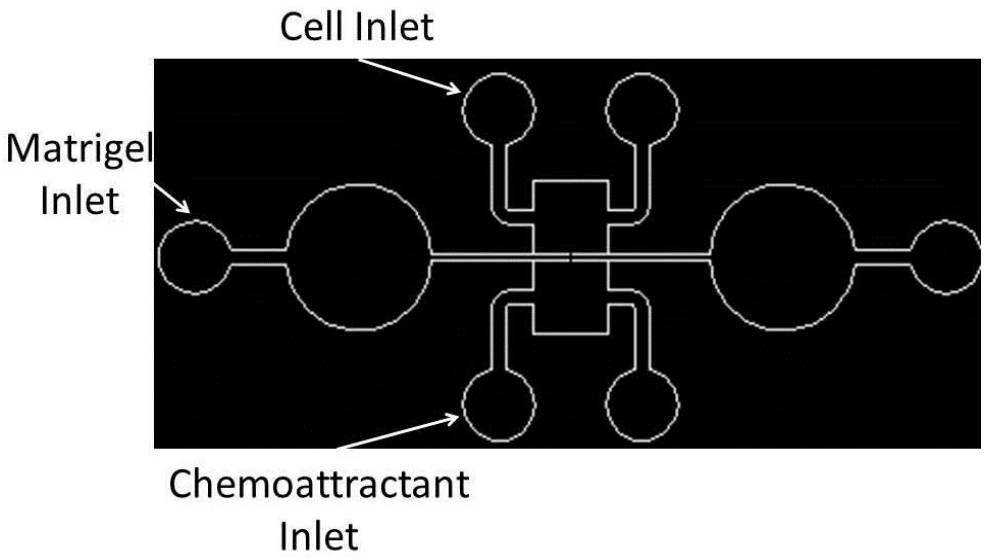

Figure 4: Final Design CAD Sketch

A CAD drawing of our final design that was used as the basis for creating our mold mask

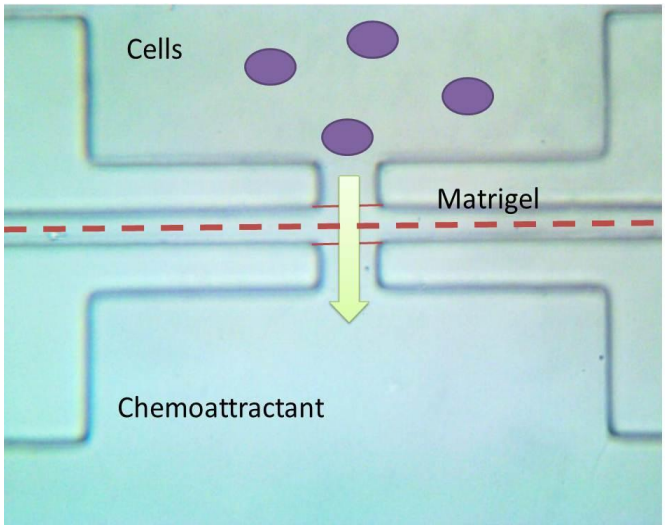

Figure 5: Final Design Function Schematic

Our final design, imaged with a microscope at 10X magnification. The cells start in the upper chamber and pass through the Matrigel, towards the chemoattractant.

4.4 Design Fluidics Testing

Matrigel has a viscosity of 10-15 cP (Albrecht *et al*. 2007). To model the flow of Matrigel in the microfluidic device, a mixture of glycerol and water was created with a viscosity of 12 cP. Blue dye was added to make the mixture more visible under the microscope. Yellow dye was added to DPBS. Tubing was put in each of the inlets and outlets. The ends of the tubing in the outlets were placed in a beaker. The tubing for the inlets of the cell and chemoattractant chambers was attached to 5 mL syringes filled with the yellow DPBS. These syringes were placed in a syringe pump. A syringe containing the glycerol-water mixture was attached to the Matrigel inlet tubing. The DPBS was pumped through while the glycerol-water mixture was injected by hand. This method for pressurizing the chambers was not working as the DPBS or glycerol-water mixture would get outside of their intended channels. We then tested the setup with air in place of the DPBS. Through a combination of starting and stopping the syringe pump and varying force applied to the glycerol-water mixture syringe, we were able to contain the

mixture in the channel.

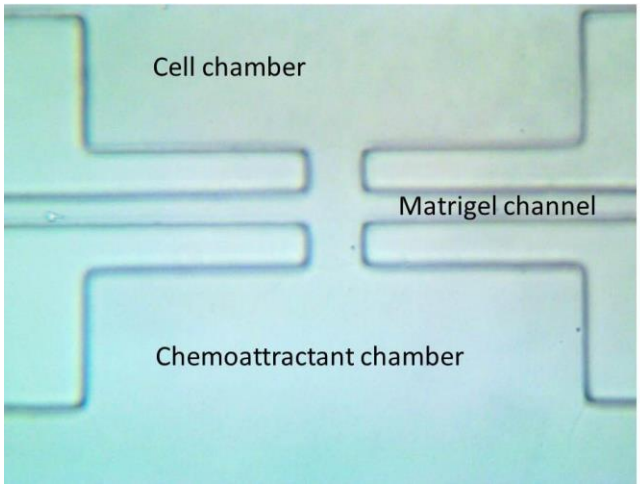

Figure 6: Central image of the empty microfluidics design

An image of the central region of the unloaded microfluidics design at 10X magnification.

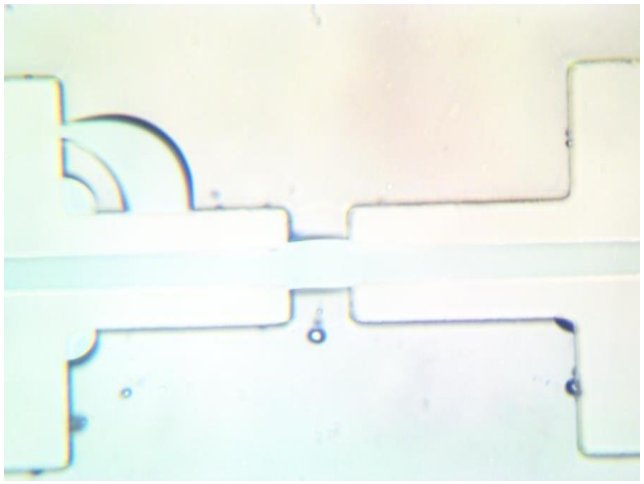

Figure 7: Image of loaded design for fluidics testing

The fluidics design has been loaded with a glycerol-water mixture to simulate the Matrigel insertion.

4.5 Decisions

The design utilized PDMS to house the microchannel because it was a readily available, biocompatible, and easily fabricated polymer. The tubing was necessary to deliver fluids into the microchannels, as the scale was very small. Hollow metal rods were used in conjunction with the

tubing in order to attach it to the PDMS and provide more stability at the junction. Syringes were used with the tubing as it was readily available, and could be manipulated by hand or by syringe pump. The FGF chemoattractant was used because it was readily available and stimulated both of the cell lines being tested.

A decision was made to utilize only the control 3T3 cells as a negative control. NIH 3T3 cells are non-invasive and a knockdown would not alter this behavior; the testing was solely to test if the design could differentiate between invasive and non-invasive cells. The flow study was conducted using a mix of glycerol and water at the same viscosity as Matrigel because there was no desire to consume the limited resource.

4.6 Optimization:

We developed microchannels in several sizes to test the design. The channel widths were 10, 25, 50, and 100 µm. The 10 and 25 µm channels were found to be too narrow, as the Matrigel could not flow through them to form the barrier. The 100 µm channel was too large for the cells to sense the chemoattractant across and migrate through. The larger channel also created deformations in the channel walls, having a negative effect on the overall design. The 50 µm channel was found to be the optimal size for use in the design, as a useable medium between the 100 µm channel that was too large and the 25 µm channel that was too small.

Chapter 5: Design Verification

5.1 Quantification of vimentin RNA and protein in normal and vimentin knockdown NIH 3T3 cells

Vimentin levels in control and vimentin knockdown NIH3T3 cells and cell lines were determined by quantitative PCR (qPCR) and Western blotting following standardized protocols followed in Dominko lab. Among the 3 knockdown cell lines, clones 1.16, and 1.17 expressed the lowest RNA and protein levels; clone 4.13 expressed moderate levels and control 3T3 and transfection control 3T3 cells expressed similar levels of RNA and protein (data not presented).

5.2 Immunocytochemistry

Cells were fixed and stained on by adapting the Vimentin Immunocytochemistry protocol (Ambady 2013). Cells were plated at ~40% confluency and allowed to attach. Following this, cells were fixed with 3.7% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, rinsed, blocked with 1% BSA (in H_2O) for 30 minutes, and then incubated with a 1:50 dilution of primary mouse anti-canary vimentin IgM antibody (DHSB, U of Iowa) at . 4°C overnight. The following day, goat AF-568 conjugated anti-mouse IgM secondary antibody was added and incubated in dark for 30 minutes. Counter-staining was done with 400 ng/mL DAPI for 10 minutes in darkness. Pictures were taken using the appropriate filters at 5x, 10x, 20x, and 40x.

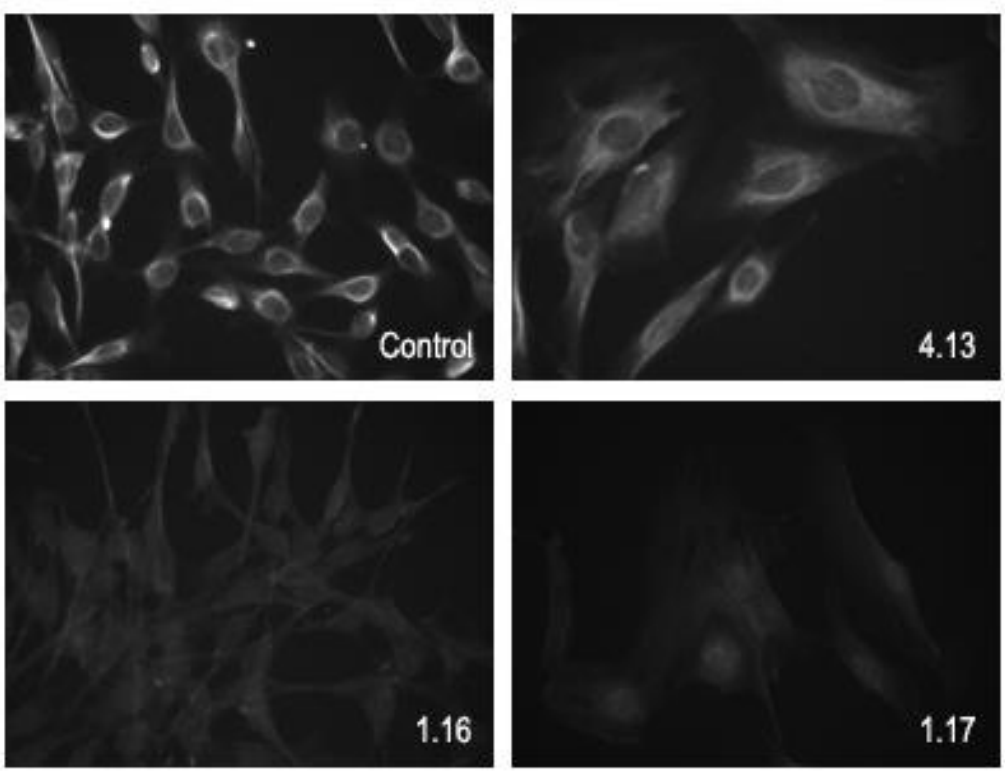

Figure 8 – Brightfield Images of Vimentin

The 3T3 control and knockdown cell lines were stained with DAPI and an AF-568 conjugated fluorescence conjugated secondary. Pictures were taken at 40x as a grayscale of the vimentin fluorescence

5.3 Confocal Imaging

Confocal images were taken of each cell line, having been stained with AF-568 (red) and DAPI (blue). Representative images of each line are shown in Figure 9. These pictures display how the vimentin protein is dispersed throughout the cell.

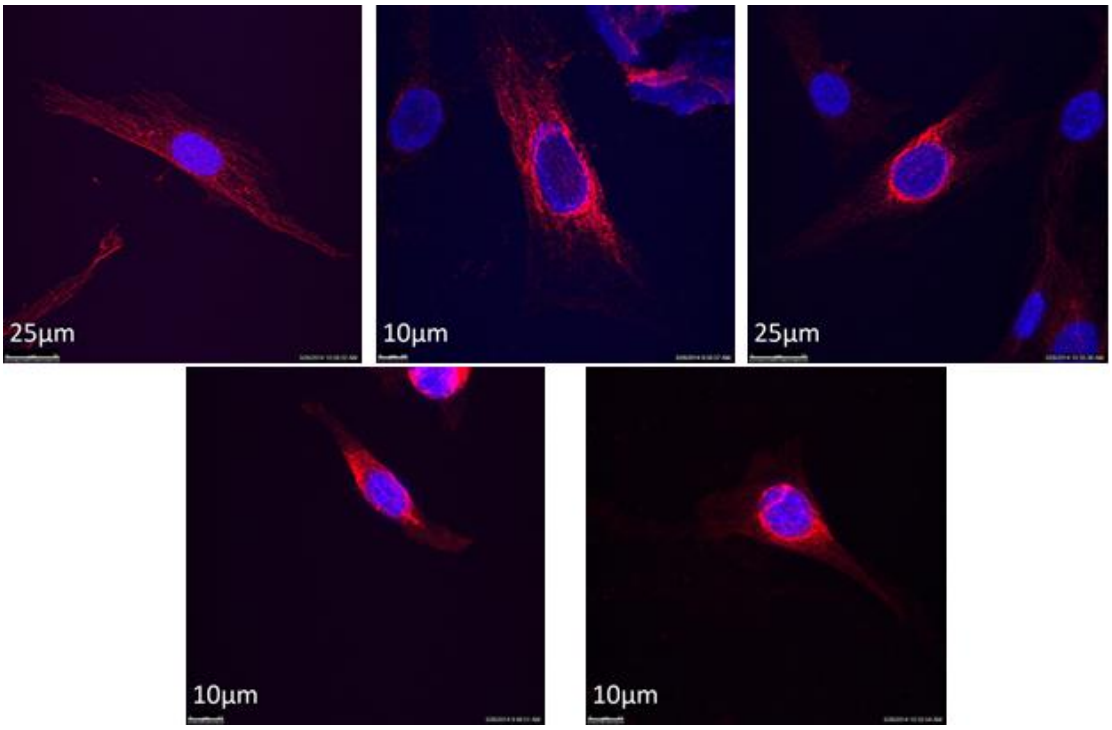

Figure 9 – Confocal Images of Cell Lines (Max Projections)

From top left to top right, and bottom left to bottom right- Max projections of cell lines 1.16 (Zoom 1.5), 1.17 (Zoom 2.1), 4.13 (Zoom 1.6), 3T3 control (Zoom 2.5), and GFP control (Zoom 2.5). Vimentin was stained red with AF-568 and the nucleus was stained blue with DAPI. Cells were imaged on a Leica TCS SP5 Spectral Confocal Microscope using a HCX PL APO lambda blue 63.0x 1.40 OIL UV objective.

5.4 Atomic Force Microscopy [AFM]

Cell stiffness is a very important mechanical characteristic of cells since it greatly affects cell characteristics such as motility, wound healing, tumor formation, and cell differentiation. Since vimentin is a structural protein within the cell, it is reasonable to assume that the cell stiffness will be affected by changes in vimentin protein levels. In order to determine average cell stiffness atomic force microscopy was used. Measurements were taken by having the cantilever tip approach the cell from a few micrometers above. The tip is then lowered until it makes contact with the cell. The cell is then indented until the cantilever tip begins to deflect and reaches a preset value. Information is recorded regarding the indentation of the cell as well as the force applied by the tip. The data was then analyzed using MATLAB to gain a list of elastic modulus for multiple cells per cell line (n=40). The modulus values were then analyzed further

using Excel to determine average cell stiffness for each cell line. Statistical analysis was performed using a 2-tail t test to see how the averages related to one and other. The AFM protocol and MATLAB code was provided by Gawain Thomas. (Thomas *et al*. 2013)

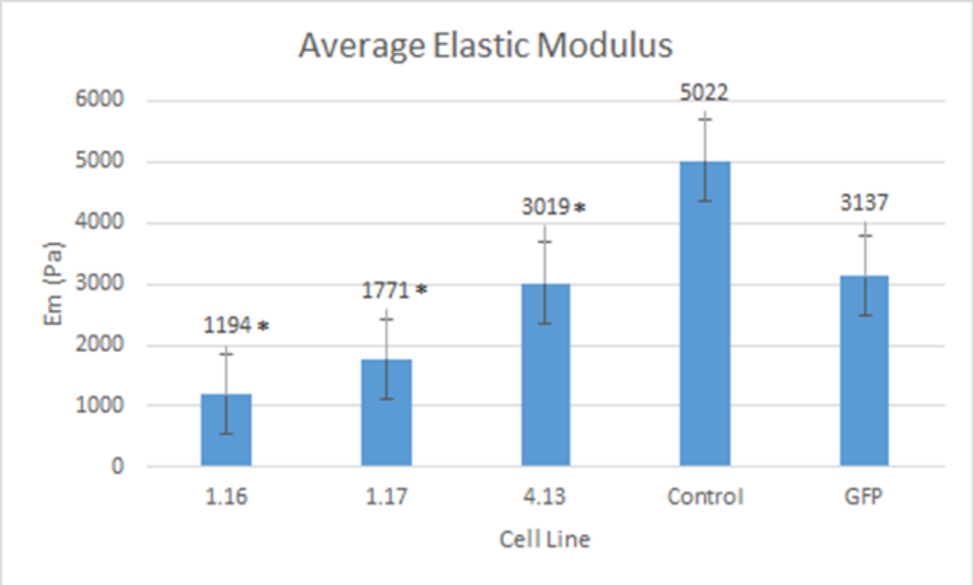

Figure 10 – Atomic Force Microscopy Cell Stiffness Results
The average Em was calculated and plotted on the bar graph. 5 Cell lines were analyzed in total, 3 knockdown cell lines (1.16, 1.17, 4.13) as well as a control and GFP-ON cell line.

5.5 Wound Healing Assay

Data from the third attempt of the migration assay is shown in Table 3. Images from the MMC-treated 3T3 control and 1.17 are shown in Figure 11 and Figure 12, respectively, taken at 6 hours and 55 hours.

Table 3: Wound Healing Assay Results

Cell Line	**Avg. Rate (Microns/hour)**
3T3 Control w/o MMC	16.241
1.17	12.490

1.16	12.444
4.13	7.466
GFP Control	4.559
3T3 control w/MMC	5.866

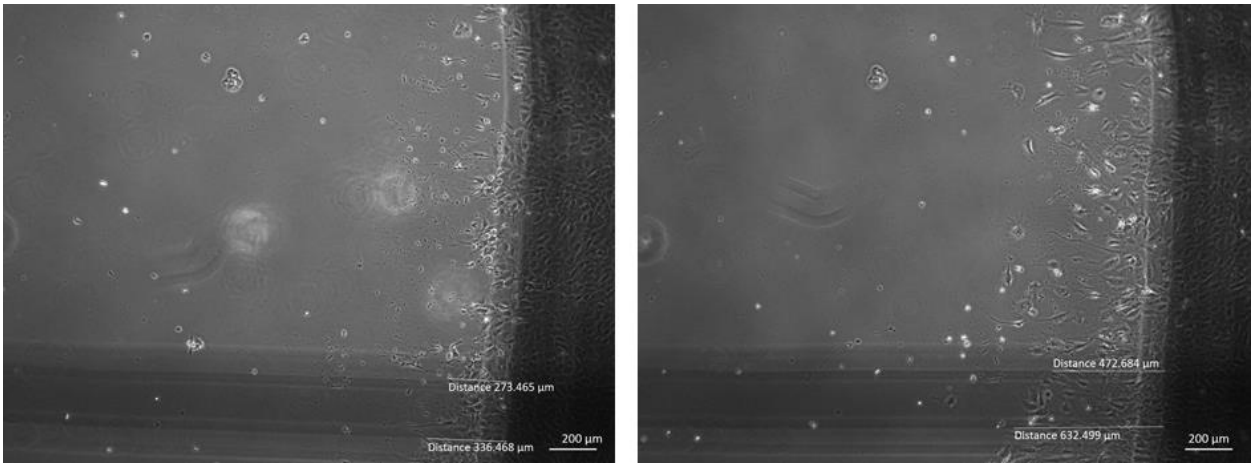

Figure 11 – Wound Healing of MMC-Treated 3T3 Control

Images taken at 5x magnification of the MMC-treated control at 6 hours (left) and 55 hours (right).

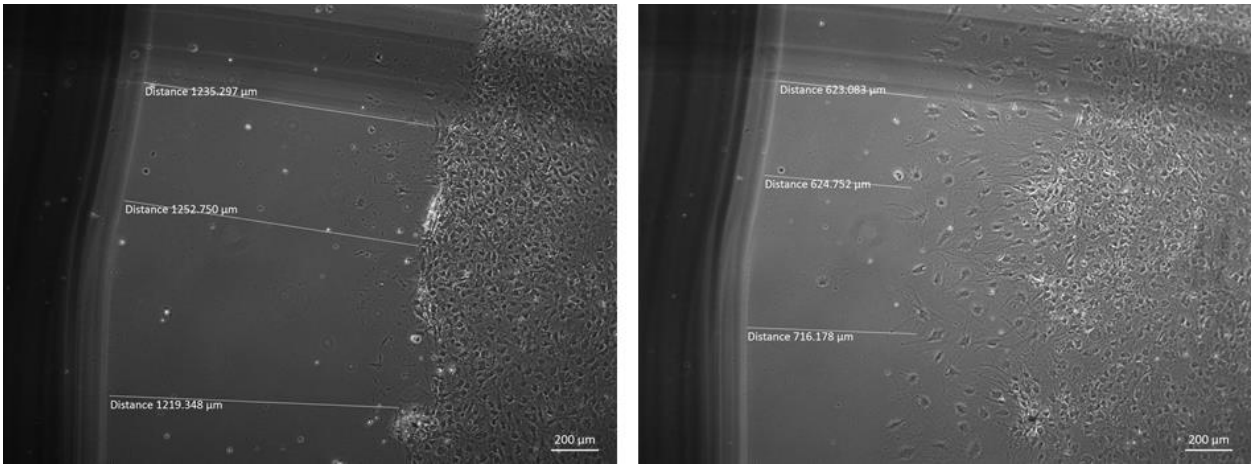

Figure 12 – Wound Healing of Cell Line 1.17

Images taken at 5x magnification of the 1.17 cell line at 6 hours (left) and 55 hours (right).

5.6 Boyden Chamber Assay

In order to validate our assay and to test the invasiveness of the cells and the effectiveness of our chosen chemoattractant, we used a Boyden chamber assay. Cells were seeded, at a density of 250,000 cells/mL in a medium of DMEM with 0.1% BSA, into the upper chamber (Kang 2004). The lower chamber was filled with the same BSA media with an added 10% FBS as a chemoattractant (Muinonen-Martin *et al* 2010). The cells were incubated in the chamber for 14 hours and then fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton-X 100 and stained with Giemsa (10%, diluted in distilled water) stain, giving the cells a pink/purple color. The cells inside the chamber (non-migrated cells) were scraped off of the upper compartment side of the porous membrane using a cotton swab, and the cells remaining on the bottom layer were imaged. Some cells were found to pass through the membrane for both lines, with significantly more glioblastoma cells passing through the barrier.

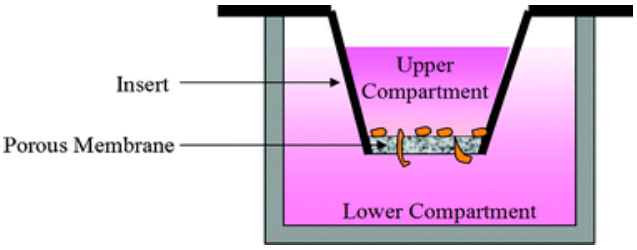

Figure 13 Sample Image of Boyden Chamber Assay

The cells are placed in the upper compartment and migrate down through the membrane in response to the chemoattractant (Toetsch *et al* 2008).

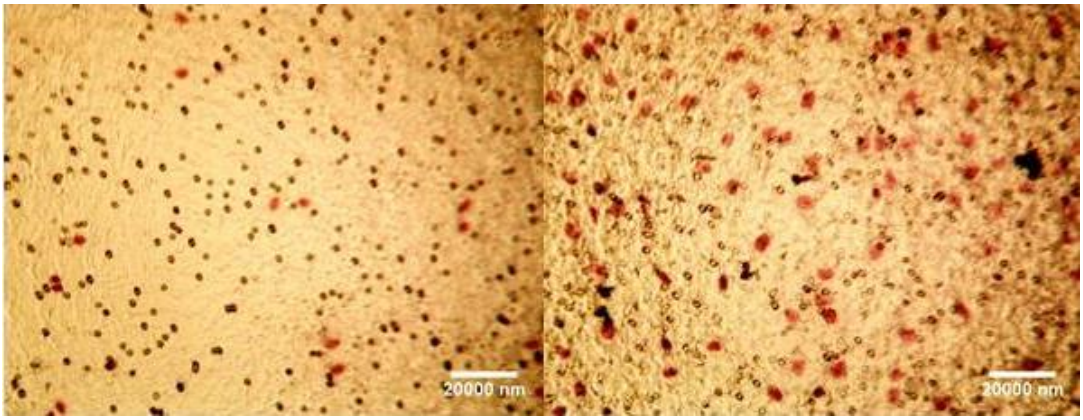

Figure 14 Results of Boyden chamber assay

Boyden Chamber membrane for Control 3T3 cells (left) and the Glioblastoma cells (right) at 20X magnification. The pink spots are the cells and the black spots are the pores in the membrane.

5.7 Cell Shape

Each of the cell lines were plated in one well of a four well plate. They were fixed and stained according to the protocol for Immunocytochemistry staining for Actin cytoskeleton following the manufacturer's protocol (Life Technologies). The cells were stained with AlexaFluor-488 Phalloidin for actin and DAPI for DNA, specifically the nucleus. Images were taken using a Zeiss Axiovert 40 CFL microscope.

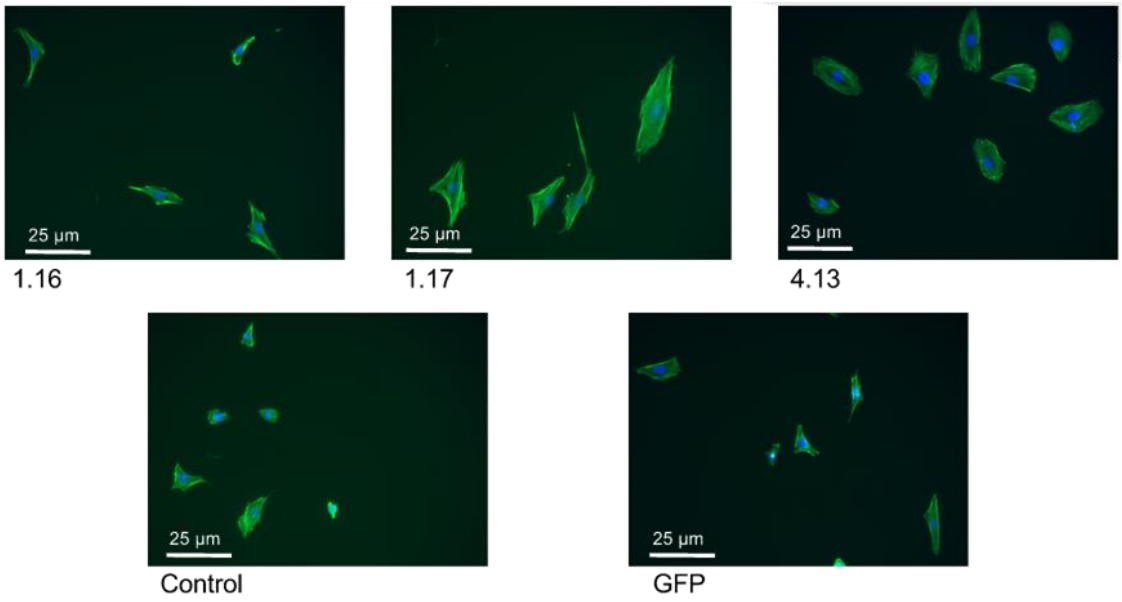

Figure 15 Sample cell images that were used in cell shape calculations

Cell lines stained with DAPI for nuclei (blue) and AlexaFlour-488 Phalloidin for actin (green). Cells were imaged at 20X magnification.

The images were analyzed in ImageJ using the Analyze Particles feature. Data for the images was compiled and is presented in the graphs below.

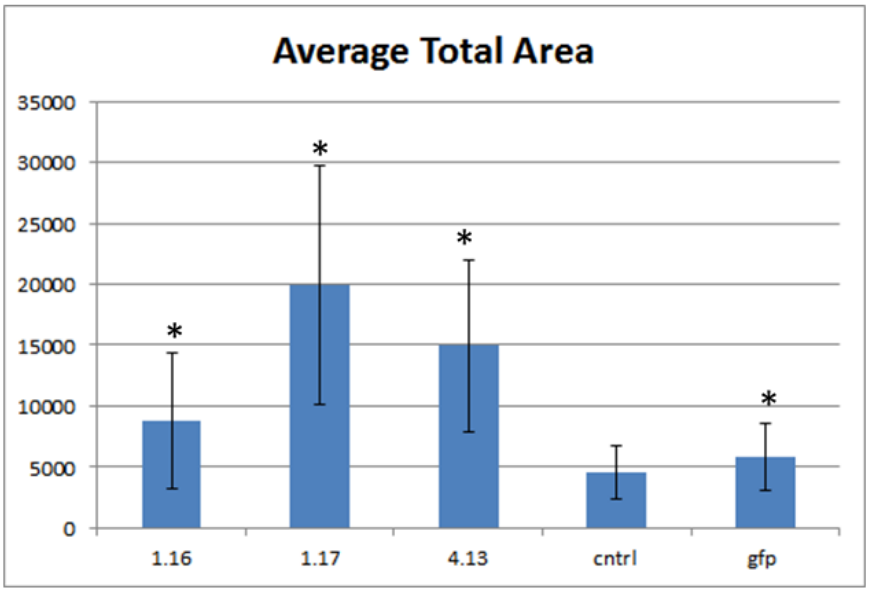

Figure 16 Average calculated total cell area

This graph shows the average total area in pixels for each of the cell lines. Area of each cell in each picture was determined by using the Analyze Particles Feature in Image J. * Statistically significant relative to control

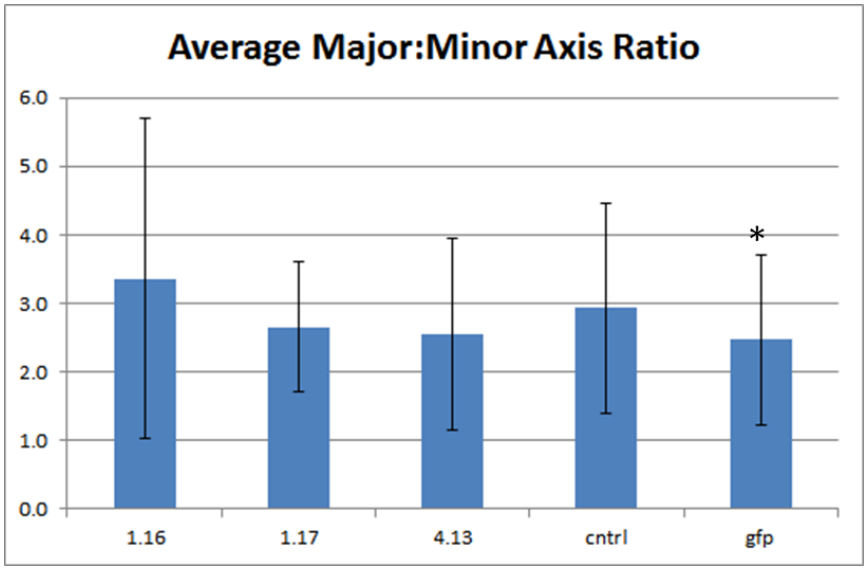

Figure 17 Average calculated major:minor axis ratios of cells

This graph shows the average major to minor axis ratio for each of the cell lines. Ellipses were fit to each cell in each image by the Analyze Particles feature. The major and minor axis dimensions were output.
* Statistically significant relative to control

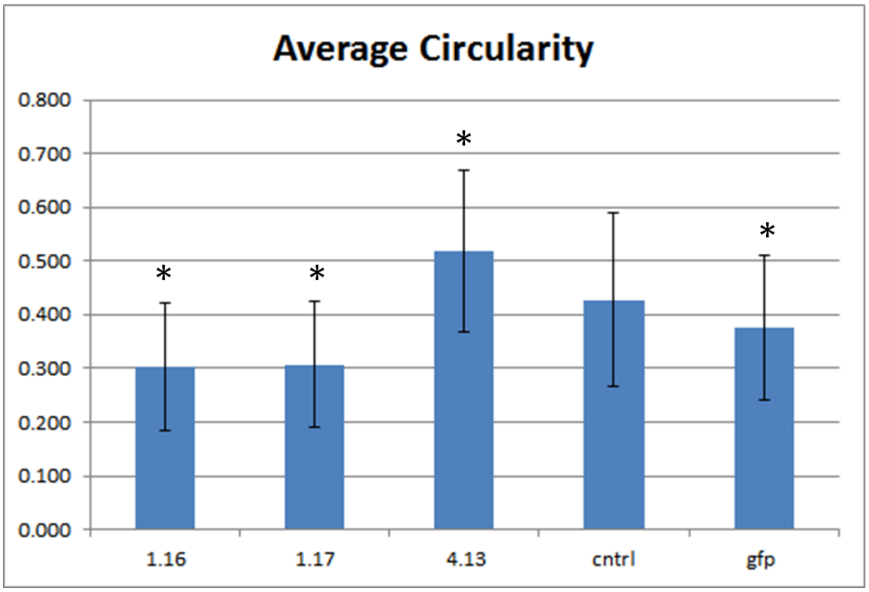

Figure 18 Average circularity of cells

Circularity was analyzed using the equation $4\pi(\text{Area})/\text{Perimeter}^2$. A perfect circle would have a circularity value of 1. The smaller the number, the more branches the object has. * Statistically significant relative to control

Chapter 6: Discussion

6.1 Vimentin quantification - Immunocytochemistry

The ICC results from Figure 8 were taken with a Zeiss Axiovert 40 CFL microscope using a red filter, and the red fluorescence was then set to black-and-white. All cell lines were imaged with the same settings, and this yields data on the true intensity of vimentin. The control and 4.13 cells have the largest vimentin levels and are also the brightest. The amount of vimentin in 1.16 and 1.17 is very low and so is very faint. It is also interesting to note that the control cell line has a particularly bright ring of vimentin around the nucleus. Based on ICC, qPCR and Western blot, vimentin knockdown clones 1.16 and 1.17 were found to have the lowest vimentin expression; clone 4.13 with moderate levels; and the untransfected 3T3 and transfection control 3T3 cells expressing similar vimentin levels (data not presented).

6.2 Confocal Imaging

The pictures from Figure 8 were adjusted for scale and brightness according to each individual cell, so as to view the detail of the dispersion of the vimentin protein. As seen in cell lines with higher levels, such as the 3T3 and GFP controls, the protein focuses around the nucleus. Conversely, when looking at the image of 1.16, which has one of the lowest levels of the protein, the vimentin is spread further and more evenly throughout the cell. We acknowledge that we did not take pictures at standardized settings, which would make a comparison across each line easier. However, for the purpose of determining the distribution of vimentin, the pictures shown sufficed.

6.3 Atomic Force Microscopy

The results of the AFM assay showed that the 1.16 and 1.17 had lowered elastic moduli that were statistically significant compared to the control. The 4.13 line had a moderate modulus which is consistent with its vimentin levels. The results are all consistent with the level of

vimentin that each cell line expressed. These results are directly related to the knowledge that vimentin is a cellular mechanic protein and provides the cell with mechanical stability. Therefore a reduction in vimentin levels will result in reduced mechanical integrity which can be correlated to the stiffness of the cell.

6.4 Wound Healing Assay

Measurements were taken for each condition in duplicate using ZEN software. Measurements were taken from a static reference point for each well and the difference across time points, divided by the time difference and averaged with the duplicate, was used to calculate migration rates. Understandably, the 3T3 control without MMC had the fastest rate, as the cells were still able to proliferate. However, the rest of the results address contradictions in current research, which supports the claim that increased migration rates result from increased vimentin levels (Gjerdrum *et al*. 2011, Ivaska 2011) or decreased vimentin levels (Park *et al*. 2005). The results obtained support the latter argument. Cell lines 1.16 and 1.17, which display the lowest levels of vimentin, also displayed the highest migration results while the controls exhibited the slowest migration rates. This may be due to the methodology used. Because there was no gap to close, cells were migrating into empty space rather than closing a wound; as a result, there was little to no stimulus for the cells to migrate. In addition, the results themselves follow separate patterns. While the MMC-treated 3T3 control migrated slowly, it exhibited bulk movement. Cell line 1.17, however, migrated as individual cells; close inspection of Figure 12 shows that the starting point of the cells from 6 hours is still visible after 55 hours.

Even when analyzing the scratch methodology attempts, the same general trend follows. Over a time span of about 49 hours, the knockdown lines closed, and closed more tightly, than the MMC-treated 3T3 control and GFP control did.

6.5 Boyden Chamber Assay

Based on the fact that at least a few cells were visible in each membrane, we can conclude that the FBS chemoattractant was successful in attracting the different cell types. It can be difficult to determine an exact number of cells that passed through each membrane as the unmigrated cells were removed by scraping a q-tip across the membrane which can potentially leave behind cells that would then seem to be migrated. It is also possible that the cells were able to pass through the membrane without being invasive as a porous polymer membrane is not the same as a basement membrane, lacking the signaling proteins and other factors that tell a cell not to pass through it. Invasive cells must also be able to digest through the basement membrane rather than squeeze through it. It is thus possible for cells attracted to the FBS to be able to squeeze through the pores of the membrane without any invasive properties. This assay is also lacking in that the cells can only be observed after they have passed through the membrane and not during the process. That is why newer invasion assay designs need to be developed that can better represent and observe invasive cell behavior are necessary.

6.6 Average Total Area

The control and GFP lines had about the same average area. The average total area of 1.16 was about twice that of the control and GFP. 1.17 and 4.13 were about four and three respectively times the average area of these lines. 1.16 had about half the average area of 1.17 and about two thirds the area of 4.13. While 1.16 and 1.17 were the lines with the lowest vimentin content and 4.13 had moderate vimentin levels, the average total are of 4.13 fell in between that of 1.16 and 1.17. Each of the knockdown lines had cells with greater average total area than those of the control lines. This suggests that cells with lower vimentin have a greater area. It is possible that this has nothing to do with vimentin content as the cell lines are clones of separate, individual cells. The selected cells may have been different sizes, resulting in their

clone lines having different areas for this reason rather than because of the vimentin knockdown.

6.7 Average Major: Minor Axis Ratio

The major to minor axis ratio for all of the lines were similar. Only the GFP line was statistically significant compared to the control line. There does not seem to be a correlation between vimentin content and the cell's major: minor axis ratio. While the cell size may be different between the lines because of vimentin content or the initial cells selected for the clone lines, the major to minor cell axis ratio does not seem to be affected.

6.8 Average Circularity

Circularity was analyzed using the equation 4π(Area)/Perimeter^2. A perfect circle would have a circularity value of 1. The smaller the number, the more branches the object has. Ellipses were fit to each cell in each image by the Image J Analyze Particles feature. The major and minor axis dimensions were output. 1.16 and 1.17 have noticeably lower numbers than the other lines. 4.13, which is also one of the knock down lines, has a noticeably greater value than the control and GFP lines. 1.16 and 1.17 have the lowest vimentin content of all the lines. The fact that 1.16 and 1.17 have substantially lower circularity values than the other lines and also have very similar vimentin content levels suggests a relationship between the knockdown level and circularity. It is possible that there is some sort of threshold relationship between cell circularity and vimentin content. Vimentin levels below a certain value may cause more branching in the cell, while levels above this threshold may not have this effect.

6.9 Microfluidics Assay Discussion

For a discussion of the final microfluidics assay, please refer to Chapter 7.3.

6.10 Overall Impact

By creating an invasive assay that more accurately models invasive cell behavior as well as studying vimentin's effect on cell characteristics this MQP has furthered research on cell

metastasis and invasiveness. This project will potentially influence future cancer research and thus have a larger impact on society and the health care industry.

In completing the project, we were able to keep within our constraints. The assay was indeed at the micrometer scale and was tested with NIH 3T3 cells. Also, all the equipment available to us was sufficient to accomplish our tasks. However, the design needs to be refined further, so it is too soon to place a price point on it, or the code. Given the outcome of the testing, we were unable to achieve some of our objectives. While we were able to easily reproduce a reliable design and barrier, neither cell line invaded through the barrier. As a result, it is not possible to say that it succeeded in recognizing invasive cell behavior or in improving over current designs. It is also too early to state whether the design will encourage novel research or be marketable.

6.10.1 Economics

The economic impact of this project would be limited to primarily cancer researchers and manufacturers of invasion assays. This design provides a more accurate and more easily studied model of cell invasiveness which is important in the study and prevention of cancer metastasis. This could potentially help expedite the development of cancer therapies in pharmaceutical companies, reducing their costs and development time. If this assay were to become popular enough, it could have a negative impact on other manufacturers' sales of invasion assays.

6.10.2 Environmental Impact

Our design is comprised of a cross-linked PDMS mold and a glass slide. Inside of the design is media containing growth factors and, most notably, cells. As a result, the device cannot be disposed of normally, and needs to be treated as biohazardous waste, which will properly handle disposal. The device should not be disposed of normally. The media can dry out and kill the cells before they leave the device, but the PDMS and glass slide are not biodegradable and

will lead to pollution.

6.10.3 Societal Influence

Cancer is a serious and life threatening disease that affects a large portion of the U.S. population and presently has limited treatment options such as surgery and/or radiation therapy, both of which have no guarantee of "curing" affected individuals and can often lead to a recurrence of cancer as well as have several severe side-effects. Cancer research is an important field of interest to find new treatment options that are an improvement over the industry standard. Finding novel ways to experiment and observe cancer cell behavior can lead to new treatment options that can improve the lifestyle of many individuals affected by this condition.

6.10.4 Political Ramifications

The development of this device would have a minimal impact on the local market, as the design has very little fame or reputation, which will minimize the audience it will reach. It is also unlikely to affect the global market. Design refinements aside, the inherent cost of making our product available globally would be large and in competition against companies such as Life Technologies™, our product would be unlikely to raise enough funds or to shift the current balance of market power.

6.10.5 Ethical Concerns

The microfluidic device does not have any direct impact on daily life. It can indirectly improve it, however. Our hope is for this design to be used in many labs to further research and to provide more reliable results. For example, in researching cancer metastasis and various conditions affecting it, scientists may use this design. The results obtained from testing on the design could then be used to create an easier lifestyle for those affected by the subject of research.

6.10.6 Health and Safety Issues

Our assay will hopefully be able to aid in the health and personal safety of people as the assay will give cancer researchers a better understanding of metastasis and be able to better develop therapies. This will ensure that the probability of a therapy that makes it to clinical trial being successful will be higher, helping to save more lives.

6.10.7 Manufacturability

Our design is fairly easy to replicate, and was theorized to be an improvement over a standard assay, such as a Boyden chamber; because there are no pores in the Matrigel barrier, noise in the data from non-invasive cells squeezing through is eliminated. The process of creating the microfluidics channels is very straightforward. The variability lies in the formation of the Matrigel barrier. We were unable to create the barrier with the desired width. Future work must be done to improve on this portion of the process, likely by optimizing the design.

6.10.8 Sustainability

In addressing sustainability, it is important to note that the Matrigel barrier can be solubilized by leaving it at 4°C and then set again, if needed. However, cells will be used in these devices and even with extensive cleaning, it is possible that not every cell will be washed out, skewing future experiments on the same device. For this reason, each device is only usable for one attempt, which will vary in duration based on the cell type used. As of now, the media needs to be replenished within every hour, which is important as experiments may last in excess of this.

Chapter 7: Final Design and Validation

7.1 Fabricate Assay

After obtaining the mask, production of the PDMS molds begin. A 1:10 solution by weight of siloxane and a curing agent are mixed to form a PDMS solution. The solution is then placed in a vacuum and sits for one hour to remove any bubbles that formed during the mixing process to obtain more uniform and air tight molds. The PDMS solution is then poured on top of the mask and allowed to set for 5 minutes. The mask and PDMS are then baked in a 65-70°C oven for 45 minutes or up to 1 hour. After baking the mask is removed from the oven and sits for an additional 5 minutes to cool and set in place. The PDMS should now be a gel/solid and is cut away from the mask using a razor. The individual molds being used for testing are then cut away individually from the PDMS disc and placed in cell culture discs until they are plasma treated. In order to prepare the molds for testing they are bound to glass slides using plasma treatment methods to create the microfluidic channels. The glass/mold construct is then used for invasiveness testing.

7.2 Determine Cell Density to Place in Chamber

In order to determine how many cells to put in the chamber, we performed some calculations. First the volume of the chamber was calculated.

1mm*1mm*120μ**m=120*10-12 m3=1.2*10-4mL**

We had decided that a cell plating density of 5,000 cells/cm2or 50 cells/mm^2 (the area of the chamber). Using the plating density and the volume of the chamber, we determined the cell concentration/mL to inject into the chamber.

50 (cells/mm^2)*1mm/1.2*10-4mL=420,000 cells/mL

Using this cell density, we injected the cells into the chamber, but found that the density was not enough to fill the chamber. The cells were redispersed at a density of 1.68*106cells/mL which appeared to be satisfactory. For future tests, a cell density of 2*106cells/mL was used as it provided more than enough cells and was easier to work with in calculations.

7.3 Test of Design with Matrigel and Cells

To fully assemble the devices, a set-up was prepared involving the microfluidic channel, syringes, tubing, metal tips, a syringe pump, and a microscope. Before filling the chambers, the Matrigel barrier was formed. The metal rods were attached to the ends of the tubing and then inserted into each inlet and outlet. To the tubing in the three inlets, three syringes filled with DPBS (-) were attached. The interior of the device was gently wet with DPBS (-), and then the syringes were replaced with empty syringes to flow air through the device. Matrigel was thawed and aliquoted, making sure to keep all instruments cold. A syringe was filled with a 500µL aliquot of Matrigel, connected to the channel inlet, and kept on ice. Empty syringes were attached to the inlets and outlets of the two chambers, so as to establish a pressure differential that would isolate the Matrigel barrier to the channel. This was only marginally successful, so the Matrigel was gently released through the channel inlet, air was expelled through the chamber inlets, and air was sucked in from the channel outlet to keep the Matrigel from bleeding into the chambers. This was incubated at room temperature for 5 minutes to soft-gel. The device was then incubated at 37°C to fully gel the barrier.

To test the functionality of the device, glioblastoma and 3T3 cell lines were used as positive and negative controls, in separate devices. Each cell line was ultimately suspended at $2.0*10^6$/mL DMEM w/ 0.1% BSA. This was loaded into a syringe and connected via tubing and metal rod to the inlet of the cell chamber, while the outlet emptied into a waste beaker. The

syringe was manually ejected until cell suspension filled the entire chamber and the tubing was carefully detached. The same set-up was used for the chemoattractant chamber, only to load DMEM w/ 0.1%BSA and 10% FGF. These chambers were imaged at 0 hours and at 90 minutes. At this point, the control device and glioblastoma devices had dried out, and some air entered a chamber or two, ejecting it of fluid.

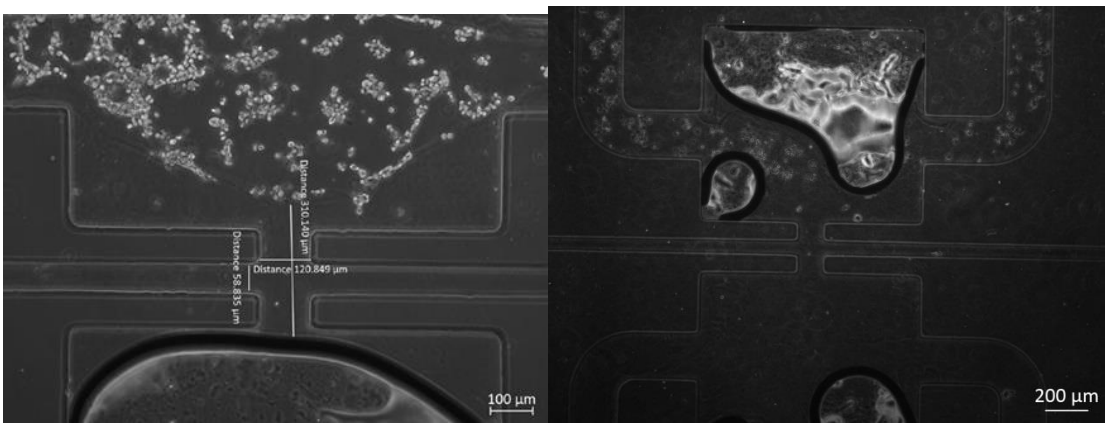

Figure 19 Final design loaded with cells

Above are images of the final loaded design with Glioblastoma cells at 10X magnification (left) and Control 3T3 cells at 5X magnification (right).

7.4 Final Design Discussion

The final design was not successful in distinguishing invasive cells, but we believe we understand the reasons. The main issue was that as the Matrigel barrier was unable to stay within its channel and flowed into the other chambers, it became too thick. This added thickness prevented the cells from sensing the chemoattractant across it, so they did not attempt to migrate across it. There was also the issue that the cells seemed to dry out, suggesting that we need to continue adding media to the design once the test has begun. There were also issues with bubbles forming in the channels, most likely caused by a flaw in the procedure to load the device. Overall, while this design did not work, we believe that we understand why and would be able to improve upon the design further in the future.

Chapter 8: Conclusion and Recommendations

8.1 Conclusions

Vimentin is a structural protein. We anticipated a decrease in vimentin content to affect the mechanics, structure, and behavior of the cell. Based on the results from our experiments we concluded that vimentin levels affected stiffnesses, cell area, circularity, and protein dispersion. Cells with lower vimentin content were less stiff. Lower expressing vimentin clones showed increased cell area and decreased circularity. It is possible that with the decreased stiffness, cells with lower vimentin content spread out more, resulting in a greater total area. In cells with lower vimentin content, the protein is dispersed throughout the cell.

Our results for migration were inconclusive and require further research in order to understand the relationship between lower vimentin and cell motility. Our research into vimentin suggests that it does have an effect on motility with an increase causing increased movement, giving evidence that our research is going in the right direction. Vimentin's effects on cell migration and invasion make it a good target for cell therapies to prevent metastasis. With further research into vimentin we believe that we will find that knocking down vimentin will inhibit metastasis. This would make using siRNA to knockdown vimentin in cancer cells a valid cancer therapy.

8.2 Recommendations

In order to build upon the achievements of this project, the team offers a series of recommendations. The microfluidic design must be improved, such that the barrier remains in the confines of the channel. This will isolate the barrier to the intended width so that the chemoattractant can signal the cells. Additionally, the process of filling the design needs to be optimized so that the barrier is not broken, so that liquid is not ejected due to pressure

differentials, and so that the chambers do not dry out. It would also be beneficial to remove the sharp angles of the chemoattractant and cell chambers to improve flow. After successful 2D testing of the modified design, it should be tested by filling the cell chamber with a 3D matrix to better resemble *in vivo* conditions.

Results from our wound healing assays suggested that lower vimentin levels led to higher migration rates, which contradicts current research. Further tests will be run to validate this. Results were, however, all obtained through the knockdown of a non-invasive fibroblast cell line. While this yielded data of the effects on non-cancerous cells, the team advises that the project be repeated with an invasive cell line. This will elucidate the potential role of vimentin in cancer therapy. A final recommendation for future research is to study proteins that associate with vimentin in cell signaling pathways, in order to better understand vimentin's specific role.

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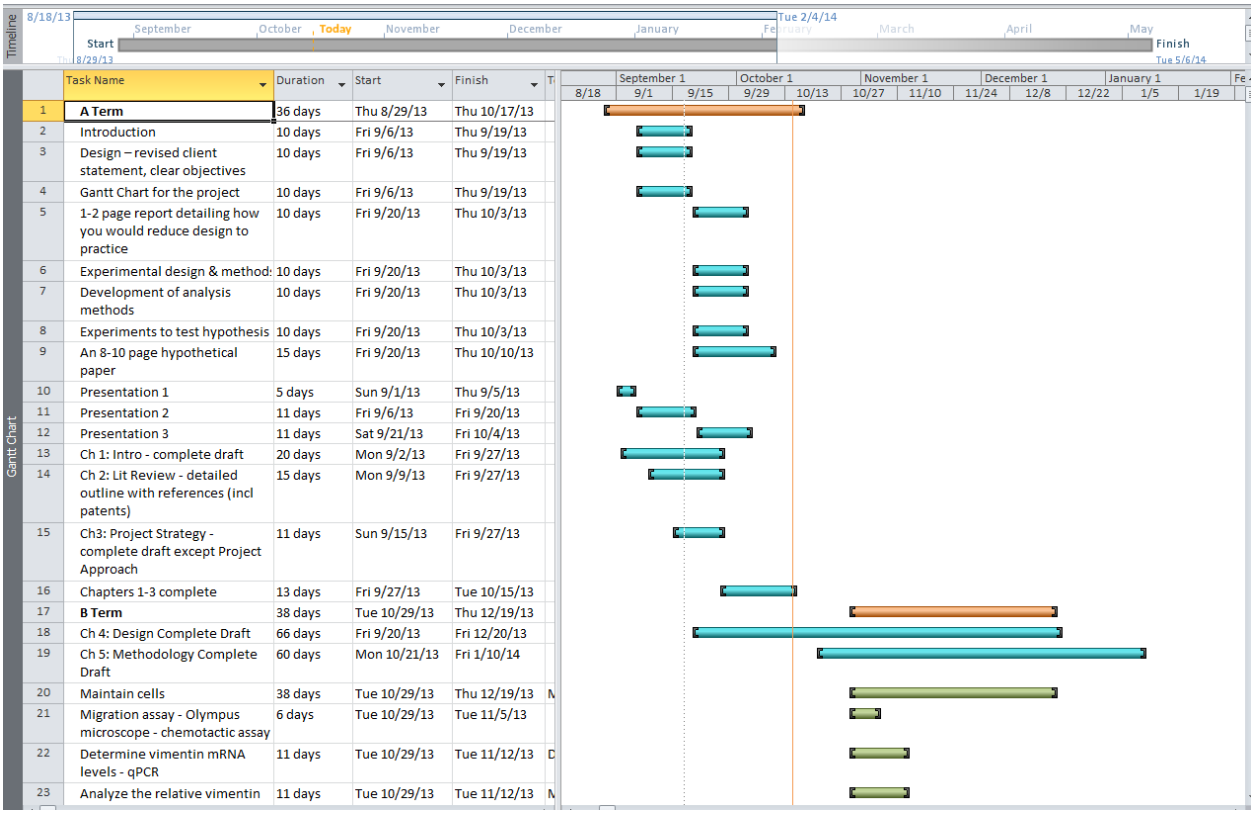
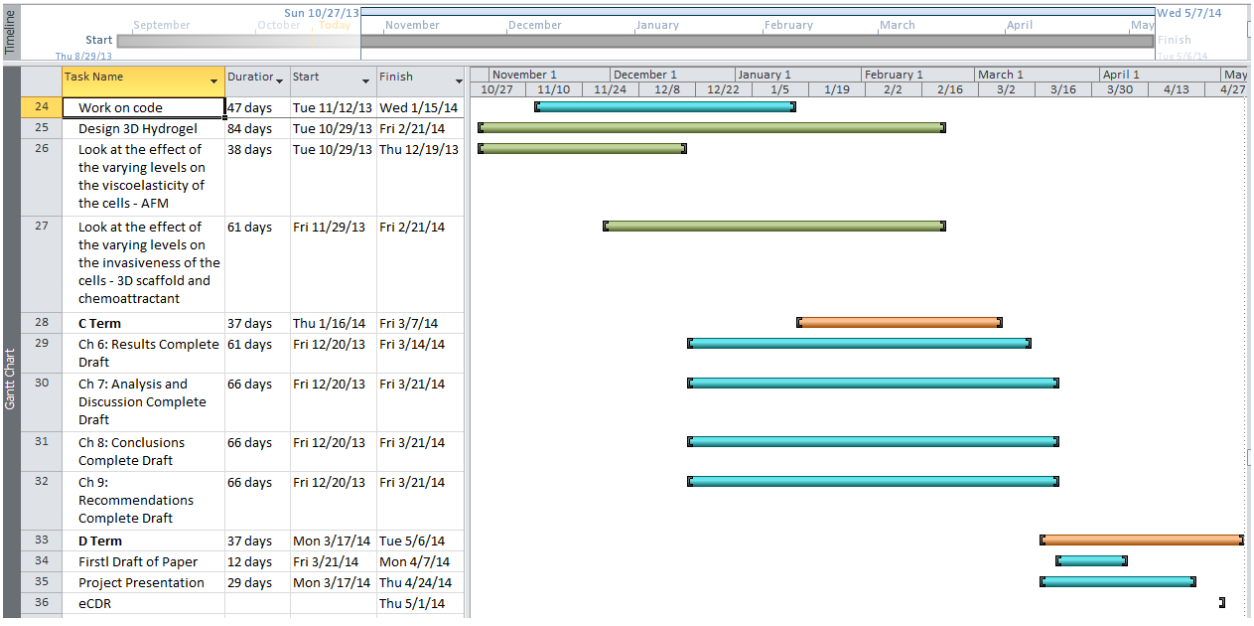
Appendix A : Gantt Chart



	Task Name	Duration	Start	Finish
1	**A Term**	36 days	Thu 8/29/13	Thu 10/17/13
2	Introduction	10 days	Fri 9/6/13	Thu 9/19/13
3	Design – revised client statement, clear objectives	10 days	Fri 9/6/13	Thu 9/19/13
4	Gantt Chart for the project	10 days	Fri 9/6/13	Thu 9/19/13
5	1-2 page report detailing how you would reduce design to practice	10 days	Fri 9/20/13	Thu 10/3/13
6	Experimental design & method	10 days	Fri 9/20/13	Thu 10/3/13
7	Development of analysis methods	10 days	Fri 9/20/13	Thu 10/3/13
8	Experiments to test hypothesis	10 days	Fri 9/20/13	Thu 10/3/13
9	An 8-10 page hypothetical paper	15 days	Fri 9/20/13	Thu 10/10/13
10	Presentation 1	5 days	Sun 9/1/13	Thu 9/5/13
11	Presentation 2	11 days	Fri 9/6/13	Fri 9/20/13
12	Presentation 3	11 days	Sat 9/21/13	Fri 10/4/13
13	Ch 1: Intro - complete draft	20 days	Mon 9/2/13	Fri 9/27/13
14	Ch 2: Lit Review - detailed outline with references (incl patents)	15 days	Mon 9/9/13	Fri 9/27/13
15	Ch3: Project Strategy - complete draft except Project Approach	11 days	Sun 9/15/13	Fri 9/27/13
16	Chapters 1-3 complete	13 days	Fri 9/27/13	Tue 10/15/13
17	**B Term**	38 days	Tue 10/29/13	Thu 12/19/13
18	Ch 4: Design Complete Draft	66 days	Fri 9/20/13	Fri 12/20/13
19	Ch 5: Methodology Complete Draft	60 days	Mon 10/21/13	Fri 1/10/14
20	Maintain cells	38 days	Tue 10/29/13	Thu 12/19/13
21	Migration assay - Olympus microscope - chemotactic assay	6 days	Tue 10/29/13	Tue 11/5/13
22	Determine vimentin mRNA levels - qPCR	11 days	Tue 10/29/13	Tue 11/12/13
23	Analyze the relative vimentin	11 days	Tue 10/29/13	Tue 11/12/13



	Task Name	Duration	Start	Finish
24	Work on code	47 days	Tue 11/12/13	Wed 1/15/14
25	Design 3D Hydrogel	84 days	Tue 10/29/13	Fri 2/21/14
26	Look at the effect of the varying levels on the viscoelasticity of the cells - AFM	38 days	Tue 10/29/13	Thu 12/19/13
27	Look at the effect of the varying levels on the invasiveness of the cells - 3D scaffold and chemoattractant	61 days	Fri 11/29/13	Fri 2/21/14
28	**C Term**	37 days	Thu 1/16/14	Fri 3/7/14
29	Ch 6: Results Complete Draft	61 days	Fri 12/20/13	Fri 3/14/14
30	Ch 7: Analysis and Discussion Complete Draft	66 days	Fri 12/20/13	Fri 3/21/14
31	Ch 8: Conclusions Complete Draft	66 days	Fri 12/20/13	Fri 3/21/14
32	Ch 9: Recommendations Complete Draft	66 days	Fri 12/20/13	Fri 3/21/14
33	**D Term**	37 days	Mon 3/17/14	Tue 5/6/14
34	Firstl Draft of Paper	12 days	Fri 3/21/14	Mon 4/7/14
35	Project Presentation	29 days	Mon 3/17/14	Thu 4/24/14
36	eCDR			Thu 5/1/14

Appendix B: Non-Peer Reviewed Sources

Cell invasion assays: Cell biolabs, inc. Retrieved 10/13, 2013, from http://www.cellbiolabs.com/cell-invasion-assays?gclid=CMeU49HOrbkCFeh9OgodZCYAnQ

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