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Engineering An In Vitro Metastasis Platform

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ENGINEERING AN *IN VITRO* METASTASIS PLATFORM

by

Justus Carlisle and Mark-Phillip Pebworth

SENIOR DESIGN PROJECT REPORT

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Engineering an *In Vitro* Metastasis Platform

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ABSTRACT

Although metastasis is the primary cause of cancer deaths and results in 90% of all cancer fatalities currently, all attempts to discover anti-metastatic drugs have failed. Many of the traditional methods of studying cell migration, using two-dimensional (2D) platforms, only study two-dimensional migration, which is fundamentally different and more multifaceted than the three-dimensional migration that occurs *in vivo*. To more accurately capture this *in vivo* variation, we developed a two-and-a-half-dimensional (2.5D) cell culture platform to better study three-dimensional (3D) migration. This platform consists of a layer of alginate on top of a monolayer of cells grown on tissue culture polystyrene (TCPS). To test the parameters of the system, experimentation on Human Embryonic Kidney (HEK) 293T cell lines, and linked the 2.5D platform with attachment-independent amoeboid migration. U87 glioblastoma cell line proved to migrate using similar mechanisms, and was used to test anti-metastatic drug candidates. Axitinib, a current chemotherapy drug, blocked migration through pure alginate. Additionally, Cilengitide, a failed anti-metastatic drug candidate, was shown to increase migration in pure alginate. When attachment-mediated migration was induced, Cilengitide did not halt migration, but rather caused the cells to switch to an attachment-independent mechanisms, which corroborates with Phase II Food and Drug Administration (FDA) trials in which Cilengitide failed to stop metastasis. In the study of cancer metastasis, this platform can thus be used to not only to explain the failure of past clinical trials, but also to discover new anti-metastatic drug candidates.

Keywords: Metastasis, Cell Migration, Cancer, Alginate, Drug Testing, Platform

Acknowledgements

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATIONS

2D	Two-dimensional
2.5D	Two-and-a-half-dimensional
3D	Three-dimensional
ATP	Adenosine Triphosphate
DI	Deionized
DMEM	Dubecco's Modified Eagle's Medium
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HEK	Human Embryonic Kidney
MMP	Matrix Metalloproteinase
MTT	MTT (3-(4,5 – Dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide
NEAA	Nonessential Amino Acid Solution
NM II	Non-muscle Myosin II
PDMS	Polydimethylsiloxane
RGD	Arginine-glycine-aspartic Acid Peptide
Rho GTPase	Rho Guanosinetriphosphatase
ROCK	Rho-associated Protein Kinase
SCU	Santa Clara University
TAE	Tris-acetate-EDTA
TCPS	Tissue Culture Polystyrene
VEGF	Vascular Endothelial Growth Factor

INTRODUCTION

Background/Motivation:

Cancer is a leading cause of death in the world, and in 2008 represented 13% of all deaths according to the World Health Organization.[1] The primary cause of cancer deaths is metastasis, or the spread of cancer out of a tumor into another part of the body. In fact 90% of all cancer deaths are the result of metastasis, yet no successful anti-metastatic drugs exist to date.[2,3] Currently, all cancer treatments, including surgery, radiation, and chemotherapy use the strategy of directly attacking the cancer; however, these treatments are often the cause of mortality, and can be more harmful than the disease itself. Past attempts to develop anti-metastatic drugs have failed because the traditional method of studying cell migration, using two-dimensional platforms, only studies two-dimensional migration, which is fundamentally different from the three-dimensional migration that occurs *in vivo*. By developing a tool that can aid cancer researchers develop anti-metastasis drugs, we can help target the primary cause of cancer deaths.

Review of Literature:

Cell Migration

Cell migration is the movement of cells through the combined effort of protrusions of the plasma membrane by the actin cytoskeleton and matrix adhesions formed by adhesion receptors. These two key processes of migration (protrusion and adhesion) consistently rely on a few key proteins. Protrusion is only possible due to actin filaments, which formed by actin subunits.[4] These filaments are formed in a process known as actin polymerization, which starts from an original "pointed" end, where subunits slowly fall off, while at the "barbed" end subunits rapidly bind (causing the filament to grow) using energy from adenosine triphosphate (ATP) hydrolysis.[4] These filaments work in tandem with an actin-binding protein, non-muscle myosin II (NM II).[5] NM II cross-links and contracts actin filaments to aid protrusion.[5] During adhesion a substrate is bound by an adhesion receptor, most of which are integrins, a family of binding proteins consisting of heterodimers of α and β subunits.[4] Adhesion proteins and

protrusion proteins join together at actin-integrin linkages, which have proteins which bind to actin or integrin (or both i.e. talin).[4]

Migration can occur as a random walk or in response to a stimulus, such as a chemoattractant or a stiffness gradient (chemotaxis and durotaxis, respectively). In either case, in order to migrate, cells must first develop polarity by organizing the actin cytoskeleton so that there is a front, or leading edge, and a rear. When a stimulus activates the protein complexes involved in migration and polarization this activation is caused by a family of regulators known as Rho guanosinetriphosphatases (Rho GTPases).[4] For the scope of this paper we need only focus on RhoA and Rac, two of the most common Rho GTPases involved in migration.[4] In general RhoA and Rac act as antagonists, such that when one is highly active the other generally is not; this inverse relationship is best thought of as a sort of switching mechanism (the exact pathway is not fully understood, but this relationship has been experimentally shown).[4,6]

Previous Research Focuses on 2D Rather Than 3D

Traditionally, most cell migration studies have focused on 2D cell migration instead of 3D migration; as a result, 2D migration is a good starting point for further understanding migration. 2D migration occurs through discrete steps during which protrusions are made at the leading edge, adhesions are generated at the front of the cell and eliminated at the back of the cell, and the rear retracts. An apt metaphor for this type of motion would be swinging on the monkey bars: you reach forward with one arm and grasp the bar in front of you and then you let go of the bar behind you. More technically, during 2D migration a flat broad structure at the leading edge known as the lamellum is formed through actin polymerization and antiparallel reorganization of actin filaments into thick bundles which are crosslinked with NM II.[4] At the front of the lemellum is the lamellipodia, which contains a branched network of actin filaments.[4] The lamellipodia is where the primary driving forces of motility occur as new adhesions are made.[6] Activation of the lamellipodia, and by extension the motive force of 2D migration in general, is primary driven by Rac; meanwhile retraction of the end of the cell is mediated by RhoA.[6]

Limitations of 2D Migration

Although 2D migration is comparatively well understood, it is poor approximation of the cell's native behavior inside the organism.[7] The cell's natural environment is the extracellular matrix (ECM): a scaffolding made primarily of a backbone of protein fibers filled with glycosaminoglycans (typically, proteoglycans) at interstitial voids.[7] In the laboratory, *in vitro* experiments on 3D cell migration require the use of ECM mimics, known as hydrogels, which are crosslinked networks of hydrophilic polymers.[7,8,9] Many of these hydrogels are naturally present in the ECM, such as the protein collagen or the gycosaminoglycan hyaluronic acid.[7] Whether it is ECM *in vivo* or hydrogels *in vitro*, the density and stiffness of the environment limit the translocation of the cell's nucleus through narrow pores in the scaffold's structure during 3D migration.[8]

Possible Solutions

There are two ways to handle this physical constraint: either squeeze the nucleus through narrow pores ("path finding"), or degrade the ECM to widen pores so that the nucleus can easily slide through ("path generating").[8,9] These two forms of 3D migration are known as amoeboid migration and mesenchymal migration, respectively.[9] Mesenchymal migration is very similar to 2D migration; the cell is elongated and uses high concentrations of adhesions and bundled actin filaments.[9] In contrast, amoeboid migration is fundamentally different from 2D migration; instead of protrusions resulting from flat or elongated bundles of actin filaments, protrusions result from NM II activity and are spherical.[11,12] These spherical protrusions, or blebs, are formed through hydrostatic pressure created by NM II and maintained by cortical tension from actin filaments.[4,11,12] Finally, unlike 2D migration, which is mediated by Rac, blebbing has been shown to be mediated by RhoA, which activates ROCK(Rho-associated Protein Kinase) which in turn phosphorylates NM II.[5,11,12]

In reality, cells in the body transition between amoeboid and mesenchymal 3D migration, and cells are capable of performing both 2D and 3D migration.[9,13] These forms

of migration can also take place during complex natural phenomena and pathologies, such as cancer metastasis. Metastasis is the movement of cells from a primary tumor in one location of the body to a secondary site. During metastasis tumors vascularize themselves and then tumor cells detach from the surface of the tumor and migrate into ECM degraded by proteolysis from matrix metalloproteinases (MMPs).[3,9] In theory, this means that during metastasis, cells migrate into a softer ECM; however, there are notable exceptions such as breast cancer.[3] It is important to note that stiffness plays a significant role in this detachment step.[3,9] Next, the cell enters the blood stream through invasion, which is migration through a thick barrier (in this case the cells of the blood vessels).[3] Then it circulates through the blood stream and enters the tissue at a secondary site to form a new tumor.[3] Lastly, note that detachment from the tumor involves 3D migration, but that cells often use 2D migration to move along the blood vessels when moving to a new site.[3]

Critiques of Current Literature and Technology:

Obviously, the complex multi-step process of metastasis cannot be fully simulated *in vitro*; however, since metastasis is a linear sequence of steps, blocking one of them could stop this harmful process. Ideally, anti-metastatic drug should target the initial migratory step, the 3D migration during detachment from the tumor. Of primary interest to this cause is amoeboid migration, which is fundamentally different from both 2D and 3D mesenchymal migration. By developing a platform to study amoeboid migration, the knowledge gained in this endeavor could lead to the development of anti-metastatic drugs.

There are several other migration platforms out there, though none that simulate the conditions necessary for amoeboid migration during the detachment step of metastasis. Essentially, the dilemma is that, on one hand, time-tested platforms that could be used to study potential drug candidates do not permit amoeboid migration and solely focus on chemotaxis, rather than stiffness, which is of primary importance during the detachment step.[3,9,14,15,16] On the other hand 3D platforms focus mainly on invasion, rather than detachment amoeboid migration.[17]

Three Classic Migration Platforms

Boyden chambers are used for more motile, non-attached cells, forcing their migration through an ECM mimic filter, which is great for testing the migration of leukocytes and other mobile cells. Since our stated goal is to study migration during the detachment of cells during metastasis, Boyden chambers will not work since they primarily study invasion.[3,14] Dunn chambers allow the use of some attached or unattached cells; these cells migrate through small channels between two chambers along a gradient of migration-inducing chemicals (i.e. chemotaxis).[15] This form is good for studying 2D chemotaxis on highly mobile cells, such as macrophages.[15] Although Boyden and Dunn chambers are commonly used to study migration, their target cell lines (i.e. leukocytes and macrophages) are highly motile and very different from most tumor cell lines. The third form is capillary migration, where cell from a liquid moves through capillary tube up a chemotactic concentration gradient.[16] Ultimately, the primary limitation of these platforms is their focus on chemotaxis rather than ECM stiffness (especially since Dunn chamber and capillary technique lack hydrogels)

3D Platforms

There are 3D migration platforms in existence such as the microfabricated polyacrylamide channel platform; this particular platform has solid benefits over other platforms as it allows for modification of pore size and hydrogel stiffness.[17] However, this platform focuses on invasion (which has already been heavily studied in Boyden chamber), and we are primarily concerned with amoeboid migration during detachment. Finally, we could find no 3D platforms that successfully allowed the abrupt transition form stiff to soft environment,[13,17] as shown in Table 1:

Table 1: Critiques of Current Technologies: This table summarizes the current popular migration platforms and leaves a check mark where desired qualities are met, and an x mark where necessary conditions are not fulfilled.

Critiques of Current Technologies	Can use a low-adhesion 3D environment	Migration away from a group of cells	Abrupt transition from stiff to soft environments
2D Migration Studies	☒	☑	☒
3D Migration Studies	☑	☑	☒
Boyden Chamber	☑	☒	☑
Dunn Chamber	☒	☑	☒
Capillary Technique	☒	☑	☒

All of the current migration platforms lack the three key features for focusing research on metastasis. First, a low adhesion 3D environment enhances expression of amoeboid migration (recall that high adhesion is necessary for mesenchymal migration). Second, during metastasis cells are migrating from the tumor into the ECM. Finally, stiffness is the key factor in the detachment step of metastasis.

Statement of Project Goal, Objectives, and Expected Results:

Herein, we will study amoeboid migration, specifically as an analogue to the detachment step of metastasis, by developing a migration platform that has all the properties described in Table 1: namely, a low adhesion 3D environment, migration from a group of cells, and an abrupt transition from stiff to soft environment. There are three milestones in this project: exploration of the migration platform using the HEK cell line, verification of the mechanism in U87 glioblastoma, and drug candidate experimentation. We expected that the U87 cell line would migrate and that we would be able to accurately generate data on drug candidates.

Backup Plan:

Our backup plans were to use different cell lines such as U251 and MDA if U87 cells failed to migrate. Alternatives to Cilengitide and Axitinib can be found in all three appendices (especially Appendix B).

Significance:

Metastasis is important because it is the cause of >90% of cancer deaths.[3] Unfortunately, metastasis is not fully understood, and there currently are no anti-metastatic drugs on the market.[2] Ideally, this metastasis platform could generate knowledge on amoeboid migration of cancer cells that could be used to select drug candidates or lead to the development of a better platform which could do the same.

Team Management:

After working together to determine the mechanism of migration we used a multipronged approach with a shifting work-load based on individual schedules; by this we mean that often times team members were working on different experiments, but were able to aid in another's experiment if a scheduling conflict existed. The goals of the project were dynamically re-evaluated as research data was generated. For example, if a cell line, inhibitor, or drug candidate showed no results we moved forward to more promising avenues of investigation.

Budget for Project:

We would like to again thank the Santa Clara School of Engineering, Bioengineering Department, and our advisor Dr. Prashanth Asuri for funding this project. At no time did we have difficulty paying for reasonable or necessary elements of the project.

Timeline for Project:

The proposed timeline can be seen in the Gantt chart in Appendix C.

DESIGN DESCRIPTION

Overview:

Our platform is composed of an alginate layer above TCPS; the softness of the alginate and stiffness of the TCPS mimics how a cancer cell moves from the stiffness of a tumor to softer healthy tissue. In addition, as alginate has an approximate stiffness of 1 kPa, our system works well with the specific cancer cell line, U87 glioblastoma, since brain tissue has roughly the same stiffness. In order to tailor our design to the needs of researchers, we mad our platform easily modifiable for a variety of research models. Our choice of alginate allows for future modification with attachment site groups, including arginine-glycine-aspartic acid peptide (RGD). Also our system can allow for variable stiffness of the bottom TCPS layer using polydimethylsiloxane (PDMS). We verified and tested the system using MTT (3-(4,5 – Dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assays for counting the number of cells in the alginate layer (the cells that have migrated or "metastasized") and those still attached to the TCPS layer, the tumor analogue. Furthermore, we applied MTT assays to test inhibitory molecules to study anti-metastatic properties of said molecules to demonstrate the validity of our design.

Media Protocol (for 15% FBS):

1. Weigh out 6.39 g Dubecco's Modified Eagle's Medium (DMEM) [w/ glucose] and 1.85g $NaHCO_3$ and then pour in graduated cylinder labeled "media" with 409 ml deionized (DI) water. Mix thoroughly by inversion with parafilm over top of cylinder.
2. Take 75 ml of Fetal Bovine Serum (FBS) (which was thawed over night) and solution from (1.) and vacuum filter into 500 ml container.
3. Add 5ml penicillin/streptomycin, 5.75 ml of nonessential amino acid solution (NEAA), and 5.75 ml of Na pyruvate.
4. Put in fridge overnight before use.

Alginate Protocol (for 3% stock solution):

1. Fill 50 ml centrifuge tube with 25 ml of DI water and .75g of alginate.

2. Place in water bath for 2-3 hours depending on amount of degradation desired.
3. Autoclave.

Platform Set-Up Protocol (for 48 well plate):

1. Take 100 mm dish with cells, aspirate media, add 1ml phosphate buffer solution (PBS), and tilt flask to cover cells.
2. Aspirate PBS and add 1ml of room temp trypsin, tilt, and place in incubator for 3 min. If cells are still attached use cell scraper.
3. Add 4ml of warm media to dish to wash off cells, place in 15 ml centrifuge tube, and centrifuge for 5 min. at 500 RPM.
4. Aspirate supernatant, and resuspend in 5 ml media. Then take 1ml and place on new 100 mm dish and add 9ml media, and store plate in incubator (wait at least 4 days before repeating process at step 1).
5. With leftover 4 ml of cells use the hemocytometer to count cell concentration per ml by counting the amount of cells in the four corner boxes. The concentration is this value ÷ 4 x 10^4 cells/ml.
6. With the concentration choose the seeding density desired, then multiply by 24 wells. This value divided by the concentration in (5) is the volume of solution to pour in a new 15 ml centrifuge tube. After that, add enough media for a final volume of 7.5 ml.
7. Plate 300µl of solution from centrifuge tube from (6) into 24 wells of the 48 well plate, and then wait approximately 2 days before (8) for cells to attach.
8. For three wells at a time, pipette out media, while trying to leave just a little at the bottom of the well. Then add 300µl alginate solution of the desired concentrations (suggested concentrations .25% - 1%). Finally add equal volume of 100mM $CaCl_2$. Wait between 2-5 minutes as desired (but be consistent per plate) before pipetting out excess $CaCl_2$.
9. Visually inspect plate and mark wells where too many cells may have been pushed up to the top during addition of alginate or $CaCl_2$. If all wells are fine, which is unlikely, as you are bound to have some less than perfect wells, be sure to eliminate one well to leave room for the blank in step 2 of the MTT protocol.

10. Add media with desired drug concentration to top of wells with gelled alginate. For our experiments these concentrations are listed in Table 2:

Table 2: Experimental Drug / Inhibitor Information: This table lists the concentrations used in our experiments, as well as, the company name of the manufacturer.

Experimental Drug / Inhibitor	Concentration	Manufacturer
NSC23766	100µM	EMD Biosciences, La Jolla, CA
Blebbistatin	5µM	Cayman Chemical, Ann Arbor, MI
Y27632	16µM	Selleck Chemicals, Houston, TX
Cytochalasin D	1µg/ml	Enzo Life Sciences, Farmingdale, NY
Cilengitide	25µg/L, 2.5µg/L, 1µg/L	MedChem Express, Monmouth Junction, NJ

11. After 2 days, remove excess media and add same solution as in (10) (repeat 11 if longer migration period is desired.

MTT Assay Protocol:

1. After desired migration period has occurred, remove excess media, and add 50X of stock tris-acetate-EDTA (TAE) buffer of equal volume (300µl) to each well and incubate plate for 20-30 min.
2. Place solution from each well into individual microcentrifuge tubes, and immediately add 200µl of media to bottom of wells that had the samples in them. Microcentrifuge the samples for 2 minutes and then remove supernatant and resuspend cells in 200µl of media in the empty wells on the plate. Remember to add 200µl of media to an empty well as a blank.

3. Add 20µl of MTT reagent to each full well and incubate for 4 hours. Then add 100µl of MTT detergent to lyse cells and wait for at least 3 hrs. or overnight (<12 hrs.)
4. When you are ready to take your readings move 100µl of each sample into its own well on a corresponding 96 well plate and then perform an absorbance reading using the spectrophotometer at 570 nm.

Chapter 3: Preliminary HEK Inhibitor Results

Initial experiments with the 2.5D metastasis platform were carried out using the HEK cells, and while they are not cancer-derived, they are an excellent proof-of-concept cell line and were used as such in order to prove and substantiate that quantifiable, literature-backed migration was occurring in our system. In order to prove that an amoeboid mechanism was being used, we sought to inhibit the activity of key proteins related to amoeboid migration. Fortunately, previous experiments beyond the scope of this paper, conducted by one of the authors, had provided more details of exactly which proteins to inhibit by implicating FBS as a key agent for this migration. FBS is well-known to provide a plethora of bioactive small molecules and proteins, including those which activate the RhoA biochemical signaling pathway and lead to enhanced amoeboid migration.[18] Since this pathway had already been implicated in migration mechanisms, we were able to narrow down the list of candidate proteins to those within the RhoA-ROCK pathway, including ROCK, NM II (see figure 1). We inhibited Rac1, a protein down-regulated during amoeboid migration, as a positive control and actin polymerization as a negative one. Figure 2 shows the results of that experiment.

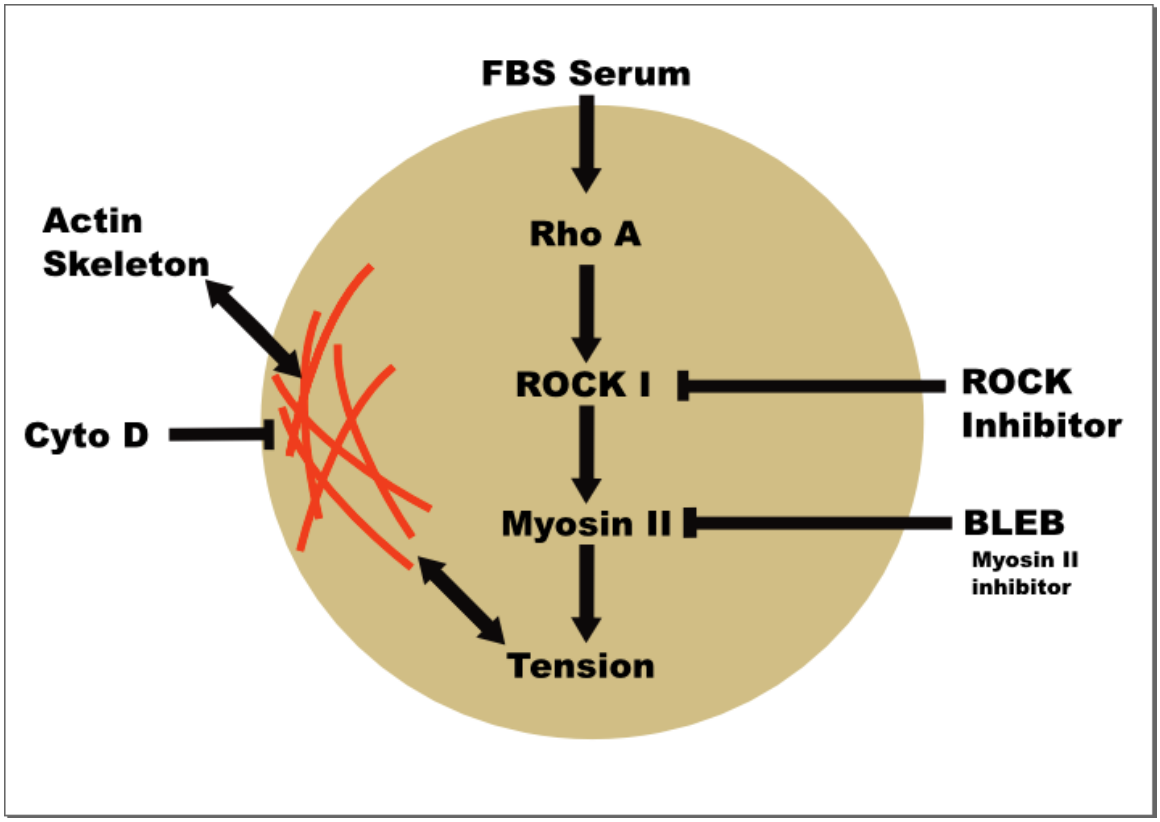

Figure 1: Migration Mechanism: This graphic shows the pathway suggested by the literature and where we inhibited the pathway to verify its accuracy. Pointed arrows imply activation, except for double arrows, which represent equivalence, while the blunted arrows represent inhibition.

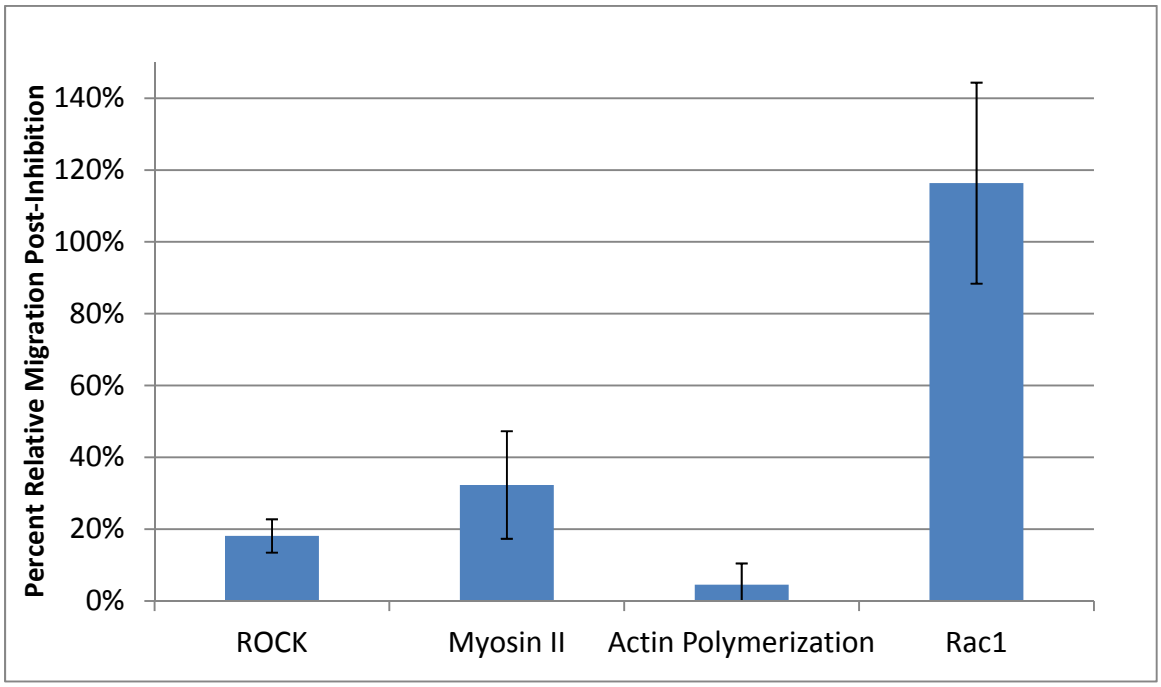

Figure 2: HEK Inhibitor Data: Inhibition of RhoA-ROCK pathway proteins as compared to control. ROCK, NM II, Actin Polymerization, and Rac1 were inhibited using Y27632, Blebbistatin, Cytochalasin D, and NSC23766 respectively. The migration for each experiment was compared with the unhibited control group to see the relative amount of migration left after inhibition. For each of these experiments, n = 3.

The inhibition of ROCK, NM II, and actin polymerization leads to substantial decreases in migration while Rac1 inhibition appears to have either not affected or increased migration; these results match exactly with expectations for amoeboid migration. Recall that ROCK activates NM II contractions and thus both lead to increases in cytoskeletal tension, composed of actin, within the cell, providing it with the internal structure necessary to migration over and through the surrounding environment; thus, inhibiting ROCK and NM II removes tension on the actin network, and prevents

migration. The inhibition of actin polymerization acted as a negative control, since it prevents the actin cytoskeleton from assembling; therefore, without any internal structure whatsoever, mammalian cells are unable to migrate regardless of mechanism. Rac1 acted a positive control, and was not expected to affected migration. Rac1 acts as a key protein activated for attachment-mediated migration and is activated by RhoA, similar to ROCK. However, in amoeboid migration Rac1 activity is down-regulated (i.e. inhibited) while ROCK activity is up-regulated, while the reverse occurs in attachment-mediated migration. Thus the inhibition of Rac1 would be expected to either have no affect on migration or slightly increase it as Rac1 became inactive and ROCK became activated. The results in figure 2 were thus exactly as expected.

This experiment helped to solidly validate that the 2.5D migration platform could indeed be used to study amoeboid migration, and potentially open a window to further understanding forms of cellular motility 3D.

Chapter 4: U87 Glioblastoma Inhibition Results

To prove the relevance of the 2.5D platform on cancer cell migration and thus its relevance to metastases, we expanded to use cancer cells in place of HEKs and attained similar results to HEKs. Initial experiments with the U251s, a glioblastoma cell line, and MDAs, a breast cancer cell line, failed to show any migration. However, migration was observed with another glioblastoma cell line, U87s, that contain a more heterogeneous population, including sub-populations with increased motility, which more closely mimics *in vivo* tumors.

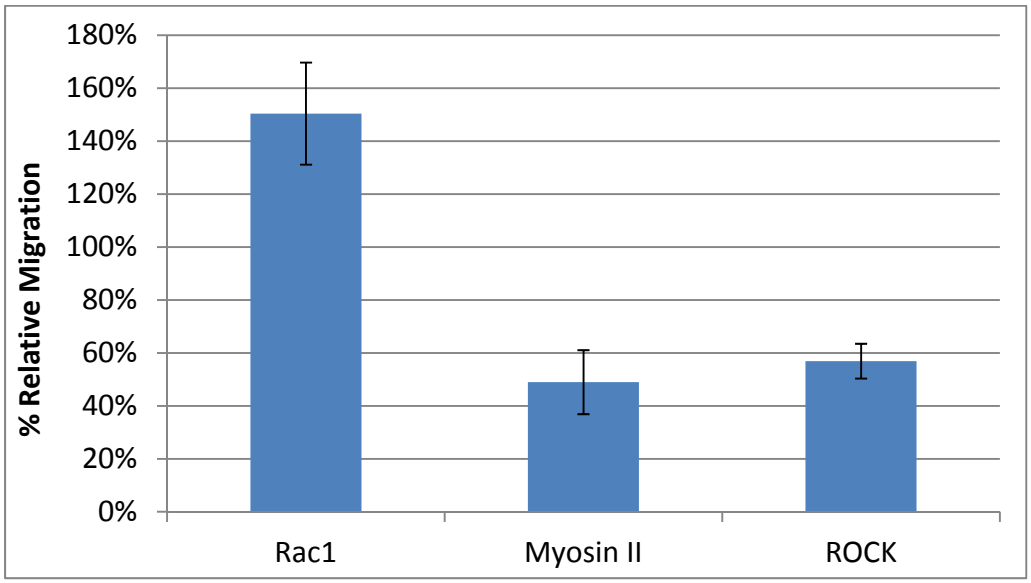

Figure 3: U87 Inhibitor Data: Inhibition of RhoA-ROCK pathway proteins as compared to control. ROCK, NM II, and Rac1 were inhibited using Y27632, Blebbistatin, and NSC23766 respectively. The migration for each experiment was compared with the uninhibited control group to see the relative amount of migration left after inhibition. For each of these experiments, n = 3, and the cells migrated over 4 days.

The inhibitor studies on U87s matched HEK results, and further proved that our system could be used to study cancer amoeboid migration. As with HEK, the inhibition

of NM II and ROCK led to much less migration as compared to control. However, Rac1 inhibition actually increased migration as compared to control, which was not unexpected. As Rac1 must normally be inhibited as ROCK is activated for amoeboid migration, the inhibition of Rac1 most likely lead to more activation of ROCK in cells that may not have activated ROCK otherwise, thereby causing them to migrate. This data thus proves that U87s migrate through a similar pathway to the HEKs as previously shown, and that our 2.5D platform can be used to study cancer cell movement.

Chapter 5: Anti-metastatic Drug Candidate Results

Since we had established that cancer cell movement was indeed occurring and was quantifiable in our system, we screen several promising anti-metastatic drug candidates, including Cilengitide and Axitinib, in our system. Cilengitide, cyclic peptide, binds very strongly to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, both of which are vital attachment proteins that cells use to migrate during traditional, attachment-mediated migration.[19] In laboratory tests, cilengitide had blocked the migration of cancer cells, and it was proven to have few side effects.[20,21] However, cilengitide had little to no effect on cancer progression in large scale FDA trials, including one with glioblastoma multiforme, the cancer from which the U87 cell line was developed.[22] On the other hand, axitinib is already cleared as a chemotherapy drug that prevents the tumor growth and progression.[23] It blocks vascular endothelial growth factor (VEGF) receptors, and so prevents the formation of blood vessels into the tumor, depriving it of nutrients, halting growth.[24] In addition, one study saw that Axitinib inhibited cancer cell migration in cancer cells that up-regulated VEGF receptor.[21] Since U87s had been shown to express VEGF-receptor, we hypothesized that Axitinib might inhibit migration in our system.

When Cilengitide was placed in our system, it greatly increased migration in our system, against our expectations. Additionally, the amount of migration was linearly related to the concentration of Cilengitide present, as seen in Figure 4. As U87 migration had already been proved to follow amoeboid norms, Cilengitide was not expected to have a large effect. Figure 6 shows how nearly all of the cells had moved into the alginate in the presence of Cilengitide, while in the untreated wells, some cells migrated but a base layer of cells remained on the bottom. It appears that blocking integrins in the absence of attachment points actually increased amoeboid migration.

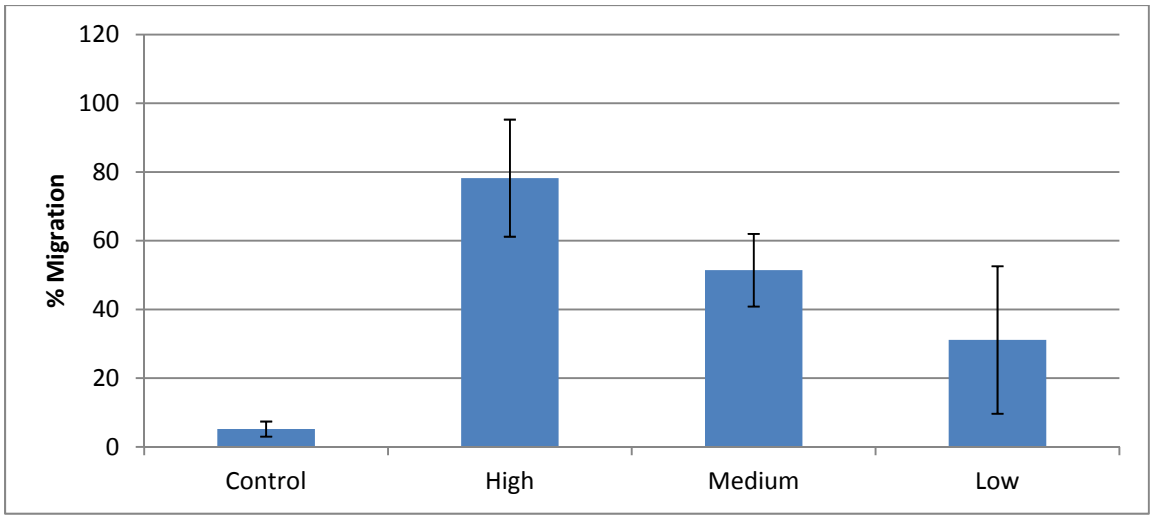

Figure 4: Cilengitide Data: Affect of Cilengitide on Migration after four days. Cilengitide was added in three different concentrations, (25 µg/L), medium (2.5 µg/L), and low (1 µg/L). The percent migration was calculated as the percent of cells that were in the alginate as compared to the total number of cells. For the control and high group, N = 6. For the medium and low group, N = 5.

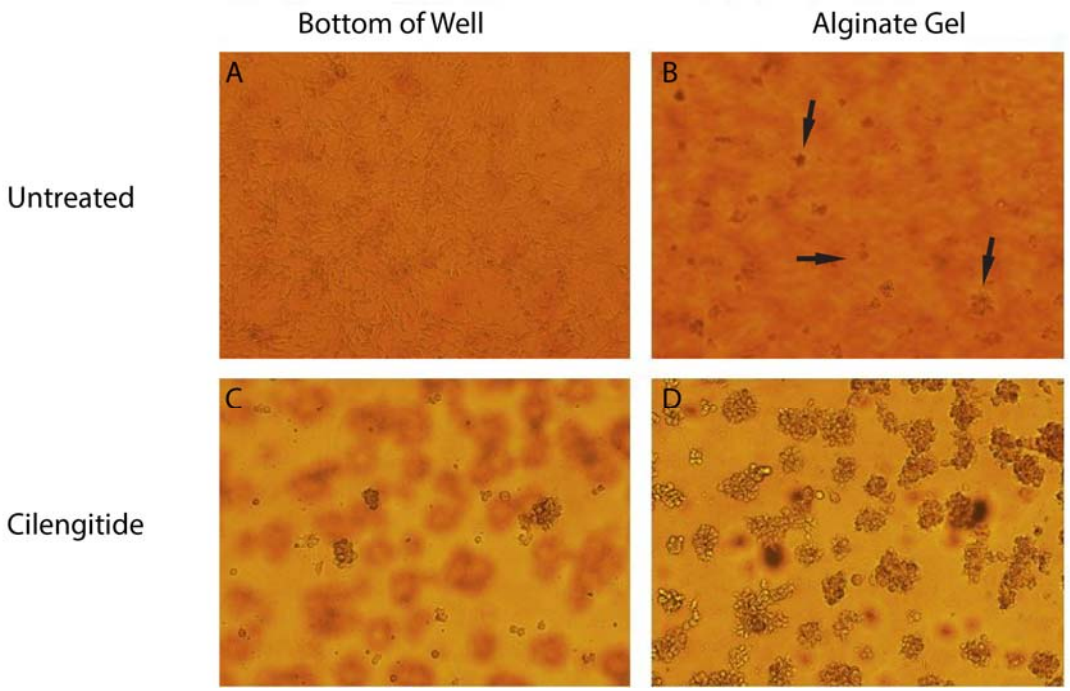

Figure 5: Cilengitide Photos: Pictures of migration through alginate with and without Cilengitide after four days. A) The view of the bottom of the untreated well, showing a healthy monolayer of U87s. B) The view of the cells suspended

in alginate in the untreated well. A few of the multiple migrated cells within the image are marked with arrows. C) The view of the bottom of the Cilengitide-treated well, showing very few, ill-attached U87s. D). The view of cells suspended in alginate in the Cilengitide-treated well, showing the majority of cells suspended and thus migrated into the well.

Moving forward, we repeated the experiment using RGD-Alginate to verify the effect of Cilengitide in the presence of attachment points. RGD repeat peptides mimic the proteins that integrins bind to, and thus by covalently attaching these RGD repeat peptides to alginate, we can create a comparable alginate matrix with integrin attachment points, which Cilengitide would then be able to inhibit.

While it is difficult to state with certainty that Cilengitide increased migration through RGD-alginate, the cells did seem to switch from the attachment mediated migration that took placed in untreated wells to the attachment-independent (or mesenchymal), amoeboid migration that was more typical of regular alginate. Though migration percentages, shown in Figure 6A, did seem to increase in the presence of Cilengitide, all treated wells fell within a standard deviation of the control group. Microscopic analysis from Figure 7 are similarly inconclusive in attempting to discern a difference in percent migration. Nevertheless, Cilengitide was still affecting the cells, as shown by its anti-proliferative effects (Figure 6B).

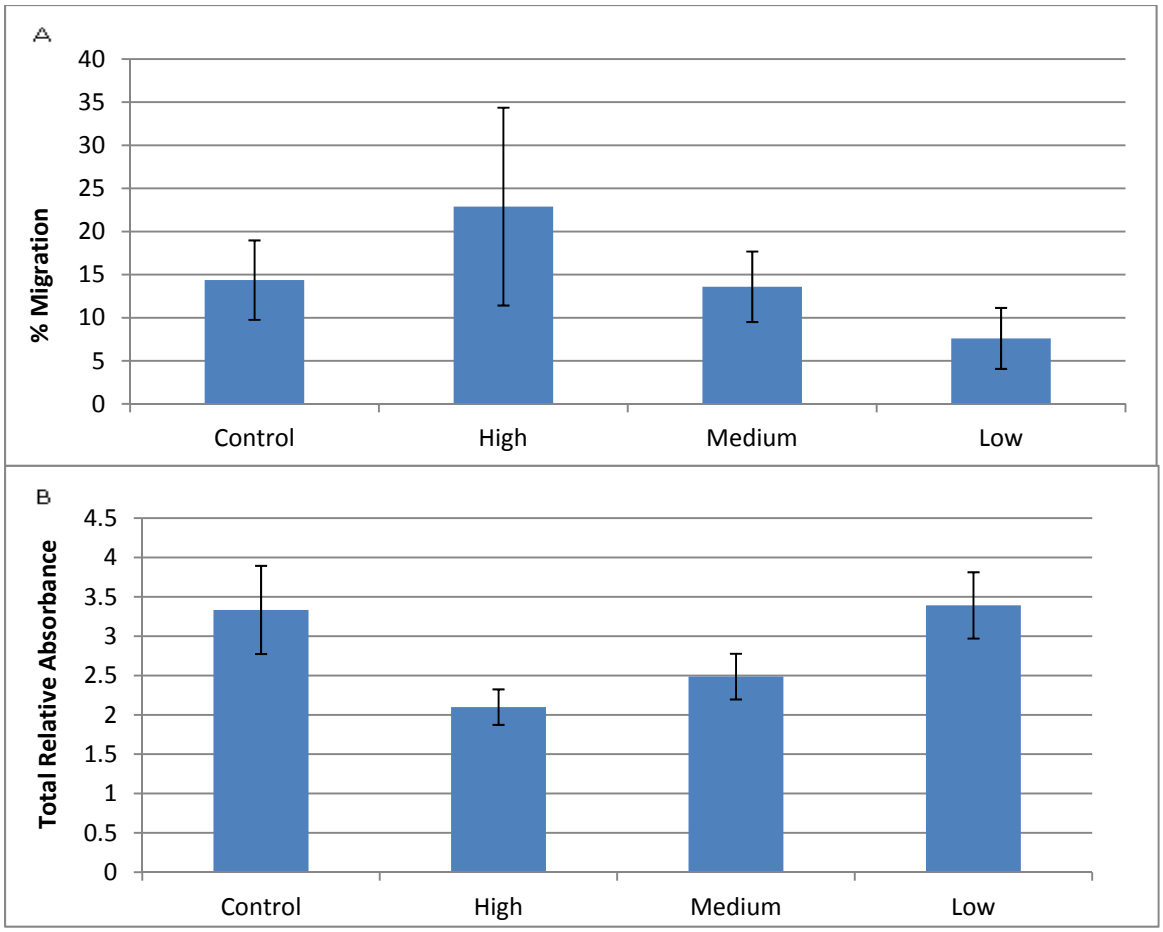

Figure 6: Cilengitide with RGD Data: The effect of Cilengitide on Migration through RGD-Alginate. A). Percent migration in RGD-Alginate across high (25 µg/L), medium (2.5 µg/L), and low (1 µg/L) with N=3. B). Relative number of cells, as measured by total relative absorbance through the MTT assay, for the same wells as in A.

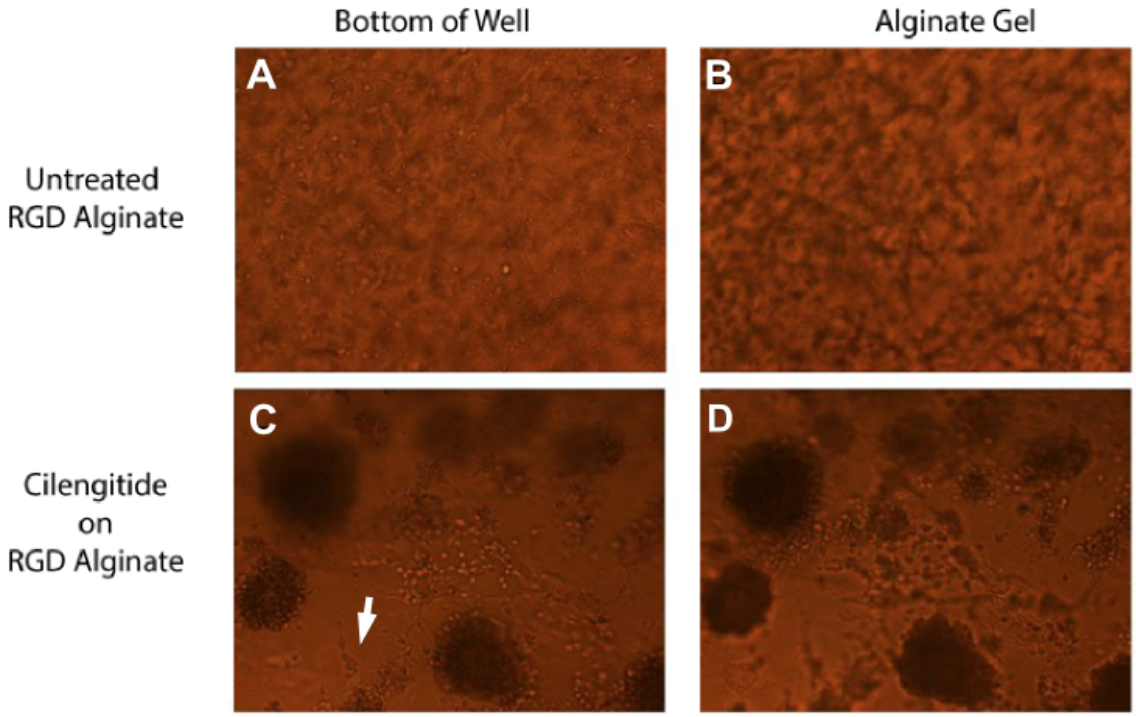

Figure 7: Cilengitide with RGD Photos: Pictures of migration through RGD-Alginate with and without Cilengitide. A) The view of the bottom of the untreated well, showing a healthy monolayer of U87s. B) The view of the cells suspended in RGD-Alginate in the untreated well. Cells have migrated throughout the gel, spreading out, attaching, and generating so many shadows they render the picture so blurry. C) The view of the bottom of the Cilengitide-treated well (25 µg/L), showing a few attached cells with an arrow. D) The view of cells suspended in alginate in the Cilengitide-treated well. Most cells are group in large clusters at multiple levels.

However, the migration that occurred in RGD-Alginate without migration was decisively different from that occurring in Alginate. While a similar monolayer of cells remained on the bottom (Figure 7A), the cells that migrated into the RGD Alginate (Figure 7B) migrated uniformly throughout the gel and spread out, showing a morphology indicative of attachment points. Because of this attached, spread out

morphology and the fact that the cells were uniformly distributed throughout the gel, the resulting picture (Figure 7B) had a large amount of shadows, which made the image appear blurry. This attached morphology also indicates that the cells moved via mesenchymal migration mechnaisms. In the presence of Cilengitide, the migrated cells appeared rounded with fewer attachment points (Figure 8D), while very few cells remained attached on the bottom of the well (Figure 8C). This migration pattern in the presence of Cilengitide was strongly reminiscent of the migration through regular alginate as seen in Figure 6, where most cells left the bottom of the well and traveled into the alginate in rounded clumps. Thus we surmise that Cilengitide, while it inhibits attachment-mediated migration, only causes the cancer cells to switch to attachment-independent amoeboid migration.

As for in the presence of Axitinib, migration through regular alginate was strongly inhibited, indicating that it may be a good anti-metastatic drug candidate.

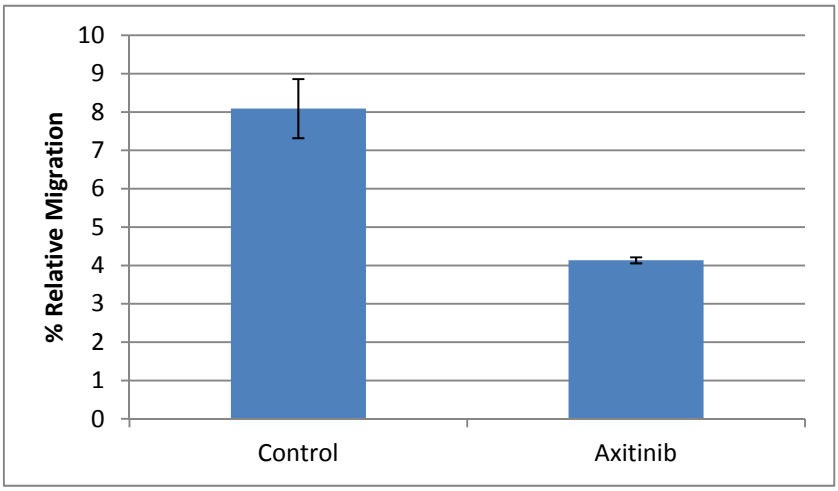

Figure 8: Axinitib Data: Effect of Axitinib on migration through regular alginate after six days. Due to cell counts below MTT detection limits, the migrated cells were pooled into two wells for the cell counting assay, and the bottom wells N=6 was averaged to calculate a relative percent migration between the control and axitinib groups.

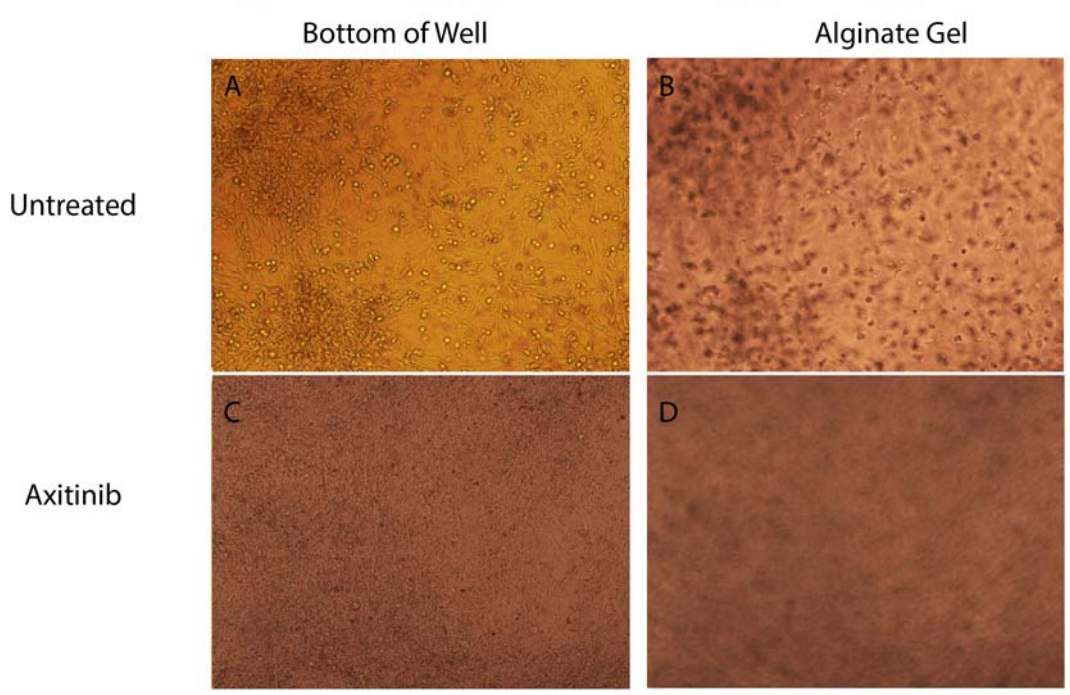

Figure 9: Axinitib Photos: Migration in the Presence of Axitinib through alginate after six days. A) Bottom of untreated well. A healthy, dense monolayer is showing, with single cells out of focus since they have begun migrating into the alginate gel and are on a different plane. B) Alginate of untreated well with many single cells that have spread throughout the gel. Some cells are out of focus because they are above or below the focal plane. C) Bottom of well in the presence of Axitinib with a clean monolayer of cells. D) Picture of alginate gel without any cells in the presence of Axitinib. Patchy look comes from light reflecting through the algiante and the bottom monolayer of cells being out of focus.

While the migration measured through cell counting assays as shown in Figure 8 reveals that Axitinib caused an approximate 50% decrease in migration, microscopic analysis (Figure 10) revealed that there was essentially no migration in the presence of Axitinib. In comparison, the U87s migrated and spread out throughout the untreated alginate as shown in Figure 10B, and have a rounded morphology, indicative of amoeboid attachment-independent migration. Thus, we believe this platform was able to prove that Axitinib does indeed inhibit amoeboid migration, and may prove to be a more

successful anti-metastatic drug candidate than Cilengitide, which, despite previous *in vitro* tests to the contrary, failed to halt migration in our system.

Chapter 6: Summary & Conclusion

Conclusion:

Through this project, we have verified the forms and types of migrations occurring in our platform, proven that multiple cell types migrate therein, and tested multiple anti-metastatic drug candidates with results matching similar *in vivo* or *in vitro* experiments from literature, thus proving that our system is indeed an *in vitro* metastasis platform that can help elucidate novel forms of migration as well as screen for novel anti-metastatic drug candidates. Initial inhibition experiments with HEK cells first proved that we were indeed dealing with amoeboid migration when results matched literature-based expectations. Experiments fell within expectation again for inhibition experiments with U87 glioblastoma cell line, thus proving that cancer cells could also migrate in our platform via the same mannerism. Finally, experiments with U87 in the presence of Cilengitide and Axitinib tested how our platform could be use to screen for anti-metastatic drug candidates.

The use of RGD-alginate further expanded our platform, proving that multiple migration mechanisms could be tested therein. Furthermore our system helped provide indications of why Phase II trials of Cilengitide have failed in the past; our platform allowed us to compare the effect of Cilengitide on both amoeboid and mesenchymal forms of migration to show how Cilengitide only inhibits one form. Experiments with Axitinib showed that our system could be used to find anti-metastatic drug candidates as well, providing much deeper support to the few reports of anti-metastatic activity and potentially ensuring that a repeat of the Cilengitide failure does not occur. Thus, we proved that our in vitro metastasis platform is not only applicable for *in vitro* cancer migration studies, but provides a much more comprehensive means of screening for anti-metastatic drugs.

Engineering Standards and Realistic Constraints

Aesthetics:

The audience who might use this platform consists entirely of scientists, and thus all aesthetic considerations focus on the technical aspects, involving the simplicity and

effectiveness of the system itself, as well as the elegance needed in a persuasive presentation of information.

Table 3: Aesthetic Evaluation Criteria: The aesthetics criteria for our design was based on three major criteria: simplicity, effectiveness, and elegance. These criteria are further broken down into subcriteria in this table.

Simplicity	**Effectiveness**	**Elegance**
Of Design	In Qualitative Measurement	In Persuasive Presentation
Of Use	In Quantitative Measurement	
	In Systematic Methodology	

Simplicity primarily relates to the overall simplicity of design, as well as the simplicity with which the system can be. The effectiveness of the system can be evaluated by the nature of the qualitative as well as quantitative measurements that combine to create a systematic methodology to ensure the accurate results. Since this platform needs to be proven, its elegance relies upon the presentation of results and data that support its future use.

Simplicity

The system is about as simple as possible.

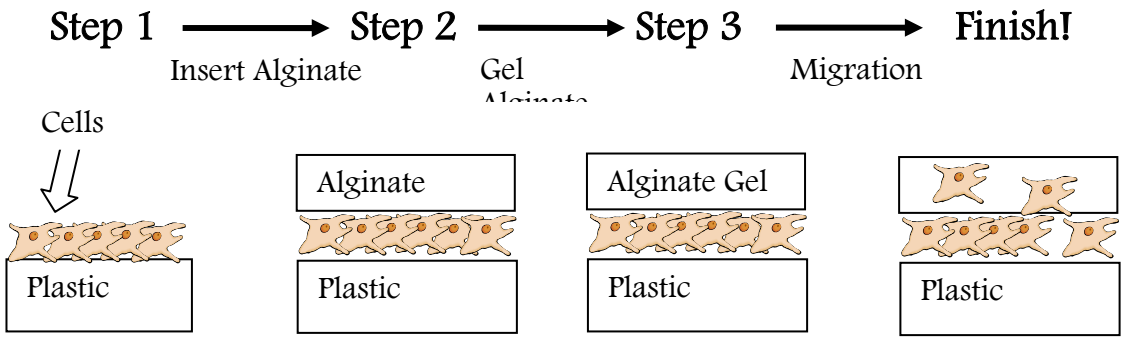

Figure 10: Simplicity of Design: Step by step process describing the protocol for creating our *in vitro* metastasis platform.

To begin the cells are plated onto the typical plastic cell culture material. In step two, alginate is added on top as a liquid and then gelled in step three. After step three, the plate is left until cells migrate into the top alginate gel after several days. Each step of the process is quite simple; the gelation process itself takes only a matter of minutes with a addition of a very easily acquired and prepared chemical. Alginate can also be easily modified to introduce biochemical factors or to change the stiffness of the gel. The process of gelation can also be undone easily, though not as quickly, with the addition of another easily attained chemical. Additionally, the entire process is scalable from 512-well plates to 10 cm Petri dishes. Finally, all reagents involved in this process are non-toxic, making chemical handling and safety extremely simple.

Effectiveness

The platform is quite effective as a low-budget method to record an approximation of three dimensional cell movement. It allows for an ease of *1) qualitative measurement* as well as *2) quantitative measurement*, which creates a *3) systematic method* and easily track the progress and an experiment and compare results.

1) Qualitative measurement. The migration occurs off of a fixed surface and is clearly visible in a microscope of sufficient magnification. Typically migration is tracked using programmable microscopes that can find the exact same point repeatedly over several days to photograph and track migration. While these expensive microscopes would help in quantifying migration, they are not required since a stark difference can be seen in the plate after simply three days.

2). Quantitative measurement. That gelation can be reversed means that the gel can be easily removed after three days, and an analysis of the number of cells now suspended in the dissolved gel can be run. This allows for a quantitative measurement of the migration that is independent of any user-bias inherent in qualitative measurements.

3) Systematic Method. Thus, without the need of any advanced microscopes or machinery, easily obtained qualitative observations through a microscope can inform the

user, a scientist, when the quantitative measurements should take place. Thus quality assurance is inherently a part of the protocol since quantitative measurements are only taken when qualitative measurements are indicative of results first. If migration fails to initiate, it can be seen immediately in the control group through qualitative observation, and if quantitative measurements give a false negative or positive where qualitative measurements indicate the opposite, the user can easily know if the experiment needs to be repeated or if the variables need to be tweaked to get more accurate quantitative results. These quantitative measurements can thus be used to track the effect of various factors on the migration, allowing for the screening of various anti-migration drug candidates or the analysis of alginate hydrogel-cell interactions.

Elegance

Since this metastasis platform remains has been, the elegance of our system is similarly be vested through the precise evaluation of its effectiveness, as proven by our results. For the rapid and elegant presentation of our results, this evaluation was made largely in the form of data graphics, with some photographs. According to Edward Tufte, the eminent graphical design scholar, "Graphical elegance is often found in simplicity of design and complexity of data."[25] Following this vein of thought, our goal was to present the complexity of our system and our results via the simplest graphical means possible, while eliminating bias. We have attempted to follow some of Tufte's principles of graphical design, including: greater length than height, no legend is necessary, precision and clarity in short messages that explain the data, and no coloring/shading/crosshatching.

Furthermore, since we are working with live cells, the most direct method of showing our results will be to use some before and after pictures to conclusively show that the cells have indeed migrated. Although it would be tedious and unnecessary to show photographs for each and every experiment, the selective usage of before and after photographs can clearly illustrate the effective nature of the platform to a general audience. Our experiments will attempt to correlate our current findings with past experiment results, as well as verifying the effectiveness of anti-metastatic drug candidates. We will then try to synthesize the data from these experiments into a series of

graphs that illustrate the elegance of our system, the continuity between the scientific literature and our results, and thus the platform's utility in investigating metastasis.

Ethics:

During the design phase of our senior design project, we will be finalizing research using HEK 293 cells in an alginate hydrogel. Later in the year we will move onto a cancer cell line, specifically the glioblastoma U87.

As members of Santa Clara University (SCU) we have the responsibility to uphold its values which are best stated in the three C's: competence, compassion, and conscience. In competence we will seek to work effectively as a team and uphold professional standards. With regards to compassion the primary focus of our design project is to study cancer metastasis, and as such we feel we are following the call of compassion. In terms of conscience we will seek to address the complex social issues involved with our project.

In discussing the ethical considerations for this senior design project, we will explore five main categories:

- Legal concerns
- The platform/product under development
- The social considerations of the research
- The team dynamics for the senior design project
- The materials and methods used in the research process

The potential ethical risks involved in this project have been assessed to range from no risk, to a medium-low risk. The assessment scale ranges from high risks, which are classified as those that involve serious ethical breaches, personal harm, and the dissolution of personal relationships; to low risks, which involve no possible ethical breaches, personal discomfort, or interpersonal agitation. Table 4 shows the potential risk, the potentially impacted audience, and ethical goal and issues for each of the five categories.

Table 4: Ethical Risk Assessment: A brief analysis for the risk, audience, and detailed ethical considerations for each of the five main categories of ethical importance to this senior design project.

Ethical Category	Risk Assessment	Audience Potentially Impacted	Considerations/Ethical Goal
Legal Concerns	Little/None	Project team, lab, & SCU	Avoidance of potential patent and copyright conflicts
Platform/Product: Health and Safety	Very Low	Project Team / Potential future researchers	Little / no personal harm possible
Professional Considerations	Low	Potential future researchers / Research Community	Documentation of methods to allow for replication of designed platform and subsequent analysis; Honesty in research; Analysis published in good faith.
Team Dynamics	Low/Medium	Project team	Proper communication and work sharing
Methods/Materials	Medium	Project team, Lab, & SCU	Ethically-acquired materials & cell lines

Legal Concerns

Notably, legal concerns are particularly small. All information will be published for the scientific community, and thus public knowledge. Additionally, the system is entirely experimental, and no involved parties have any interest in investing the number of years required to potential develop this platform to the stage of possible deployment or company interest. The project is through Dr. Asuri's lab, which is planning to shift the focus of this research following the completion of this project because his lab at SCU does not have the facilities necessary to push this research further. Additionally, the

nature of this system makes even eventual product development highly unlikely; if the platform proves to be useful in the broad schemes of scientific research, it will still likely not be possible to generate a consumer product just because of the nature of the methods involved.

Furthermore, no involved parties have any business connections, and the senior project advisor only has interest in publishing. Beyond a publication, which enhances SCU and supports a professor's bid for tenure, SCU's interest stop, especially since the professor has not interests in patents. In conclusion, the potential for legal conflicts are near none.

Platform/Product and Health and Safety

The platform/product under development has a very low risk of breaching any health and safety standards. Little to no personal harm is possible; the proper health and safety protocols for any scientific environment in which this platform might be used would mitigate any possible personal harm. To elaborate, use of fume hoods, aseptic technique, closed toe shoes etc. should eliminate almost all health risks.

Professional Considerations

The professional considerations for this project engage mainly the scientific research community. Ethically, the team has a responsibility to properly document the methods to allow future researchers to recreate and perhaps develop further this platform, given that the purpose of this research is for further development of humanity's knowledge and not for the creation of profits. Following the ethical guidelines for Biomedical Engineering Research Obligations as posited by the Biomedical Engineering Society in 2004, all research must be published with results of research activity clearly delimitated, and research influences properly credited. The senior design team has a responsibility to others in the field to accurately and fairly report its findings, to cite sources so as to not plagiarize others, and to live up to the highest standards of the field. These standards include but are not limited to avoiding research misconduct, which is defined as “gross negligence leading to fabrication of scientific message” and

"intentional distortion of the research process."[26] Furthermore, the team has a responsibility to not deviate from promises to sponsors or declared purposes made during applications to grants or additional funding.

The risk was marked as low since attempting to get this material published will require properly credited and clearly marked research information, and since the team project will be striving to adequately test research conclusions to the best of our ability. Potential negative side effects of not following this goal include misleading the scientific community, which delays the progress of science, and not providing adequate citations, thus denying the achievements of other researchers and inadvertently plagiarizing their ideas. Both these issues provide clear negative impacts to individuals and society, and so must be avoided.

Team Dynamics:

The ethical goals involved in team dynamics are to maintain proper relationships between team members, such that work is shared equitably, and friendships built. A dissolution of amicable relationships between team members can hobble the success of a senior design project, harming both students after their friendship is damaged. Using a Kantian ethical model, the dissolution of friendships on the whole would be extremely damaging for society, and thus ethically, the dissolution of friendships within the senior design team must be avoided, through proper communication of expectations and regular meetings to track progress. Additionally, each team member has an ethical responsibility to keep the appropriate agreed-upon deadlines and attend team meetings. The project should be abandoned under ethical grounds if it exposes team members to harm or requires them to perform actions against their moral principles.

Social and Political Implications of Materials & Methods:

The acquisition and nature of all materials used in lab was not involved, either passively or actively, in generating harm to individuals or society. The methods for the research do not involve living animals, and so are unlikely to ever generate ethical issues. As for the materials, the SCU guidelines on material acquisition ensure that for the most part all materials used in lab are ethically acquired.

However, as a Jesuit institution of learning, SCU has a responsibility to live according to certain moral standards. One salient ethical point that arises in our research revolves around the use of an old, and extremely common cell line that was original taken from a donated legally-aborted fetus over forty years ago. It is known that HEK 293 cells were taken from the kidney of a "completely normal" aborted fetus; however, it is not known whether the fetus was aborted for natural or elective purposes.[27] This leads to several ethical dilemmas, but the chief concern must be the ethical nature of elective abortions. Recently, contention on campus grounds has been rising over the decision to drop elective abortion treatment from faculty and staff health insurance on campus. Tensions have risen so high that in protest Stephen Diamond, associate professor of law and ethics scholar, has resigned from his position as ethics scholar at the Markkula Center for Applied Ethics and roughly 600 members of the community have signed a petition.[28] Regardless of an individual's moral stance on abortion, this leads to several important questions including whether the institution places a higher premium on the pursuit of knowledge and compassion towards others, or whether tradition and conscience rule the campus grounds.

With regards specifically to our project, some might argue that we should simply assume, or rather act as if, the HEK 293 cells were part of an elective abortion. Such an assumption would go against the general principle, "innocent until proven guilty"; however, one may reasonably apply "the well known analogy of a hunter having to be sure of his game before firing a shot."[28] If we then apply this analogy and if life begins at conception (which would lead us to conclude that all abortion is immoral) then strong arguments against the use of HEK 293 cells in research must be considered.

The strongest of these arguments boils down to a claim that such research appropriates benefits from the evil committed by the hypothetical abortion. The primary weakness of appropriation of evil arguments is that they tend to argue for a slippery slope. In other words, if hypothetically, we learned important knowledge from Nazi experiments and we used this knowledge to cure malaria opponents might argue that we

may slide into performing immoral experiments ourselves in order to cure future diseases. Although slippery slope is a formal fallacy, it is also an important example of a human bias, and as such deserves a response. The primary difference between slippery slope applied to individual human evils – such as a person who, for example, starts the manufacture and sale of illegal drugs to provide for their family and then starts killing other drug dealers to protect his territory – and slippery slope applied to societal constructs, is that societal constructs tend to have internal self-regulating mechanisms. Science, as seen as a large organization of human activity over history, for this reason has refrained from falling down the slope.

Other than the slippery slope fallacy, the main weakness of the appropriation of evil arguments is their static deontological stance. Deontology, or "rule-based ethics," is popular among the Catholic Church, and by extension this university, due to its dogmatic nature. The difficulty with this form of ethics is that it ignores the consequences of one's actions, which in this case would be to perform medical research efficiently or inefficiently and possibly not at all. Furthermore, such a form of ethics ignores the intent of the moral agent. As bioengineering students at SCU, we are committed to the pursuit of knowledge while being driven by a desire to compassionately help suffering fellow humans.

Furthermore, this project has moved away entirely from this cell line, as well as any other that might have been acquired under similar conditions, in order to address any possible ethical objections. For the research, cancer cell lines were only used if they were taken legally from cancer cell tumors. In this case, it could potentially be argued that the removal of these cells even aided the patient. Thus, the primarily cell lines used present no ethical problems.

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APPENDIX A

Table 5: Drug Categories: This table defines the four potential anti-metastatic drug candidate categories.

Type	**Details on mechanism for anti-metastatic properties**
VEGF Receptor Inhibitor	Inhibits blood stream supply *in vivo*, inhibits chemicals related to growth and migration.
Integrin Inhibitor	Blocks integrins, a key protein used when cells move through the body *in vivo* and *in vitro*.
Ruthenium-based	Ruthenium-based molecule selectively impairs certain cellular process, including cellular processes.
Anti-inflammatory	Inflammation and invasive cancer recently linked. Anti-inflammatory seem to inhibit some cell migration.

These four classes of chemical represent lines of established or developing anti-metastatic drug research. VEGF receptor inhibitors are commonly used to prevent cancer tumors from receiving blood, halting growth. However, recent research has VEGF to be related to migration mechanisms as well. Integrin inhibitors have been known to halt migration, since integrin proteins on the cell surface are one of the most integral proteins necessary for cell migration. Ruthenium-based chemotherapy drugs have come to replace previous platinum based drugs, and have shown unique activities that allow more selective targeting of cellular processes, including growth and migration. Anti-inflammatory are the most novel, and only recently discover candidates in the search for anti-metastatic agents. Recent research has shown that chronic inflammation can lead to the migration and subsequent development of metastatic cancer; furthermore, certain anti-inflammatory drugs have been shown to inhibit the key proteins involved in this migration, strongly indicating that anti-inflamatory agents may have anti-metastatic affects.

APPENDIX B

Table 6: Comprehensive Drug List: This is a fully comprehensive list of future potential drug candidates that our platform could test.

Compound	**Biochemical Affect**	**Current Uses**	**Type**
Y27632	Inhibits ROCK, stops Rho-ROCK migration ,echanism	Laboratory reagent	Organic molecule
Blebbistatin	Inhibits Myosin, stops contractions necessary for migration	Laboratory reagent	Organic molecule
NSC23766	Inhibits Rac protein, stops Rho-Rac-mediated migration	Laboratory reagent	Organic molecule
Cilengitide	Inhibits integrins, stop certain types of migration	Potential anti-metastatic drug	Cyclic peptide
NAMI-A	Chemotherapy drug, general	Potential anti-metastatic drug	Ruthenium-based molecule
RAPTA-T	Chemotherapy drug, general	Potential anti-metastatic drug	Ruthenium-based molecule
Diosgenin	Chemotherapy drug, general	Potential anti-metastatic drug	Organic molecule
Axinitib	Chemotherapy, VEGF Receptor inhibitor, anti-angiogenic	Potential anti-metastatic drug	Organic molecule
Vatalanib	Chemotherapy, VEGF Receptor inhibitor, anti-angiogenic	Potential anti-metastatic drug	Organic molecule
Ibuprofen	Anti-inflammatory	Potential chemotherapy agent	Organic molecule

APPENDIX C

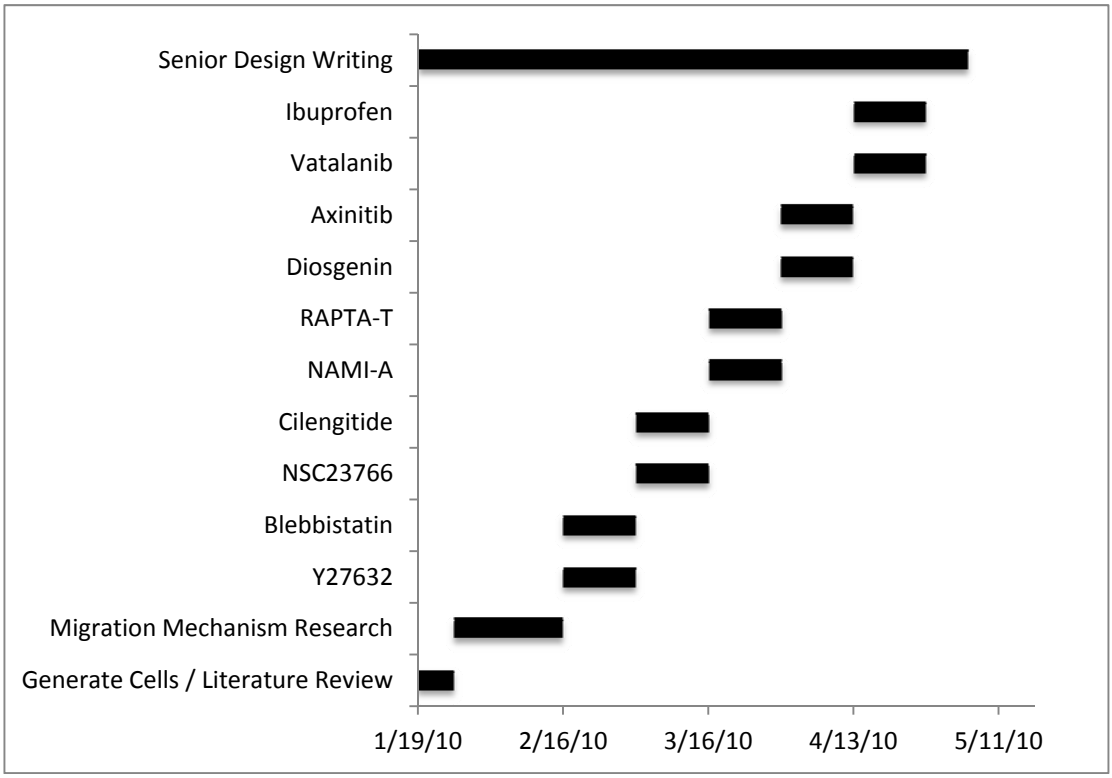

Figure 11: Gantt Chart: This Gantt chart was the original research timeline for our project. Unfortunately, we were unable to experiment on all of these drug candidates, but they are potential areas for future research.