Predicting Metastasis with a Novel Biophysical Cell-Adhesion Force Technique

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1. Abstract

Metastasis accounts for approximately 90% of all cancer deaths, but current methods of metastasis prediction rely on genome-sequence datasets that may not account for the complexity of metastasis. Measuring cell-adhesion force greatly simplifies the system, however, current techniques are expensive and inefficient. This work tests the novel bead-pipette diagnostic to distinguish between control NIH3T3 cells and mutated RasV12 cells (metastasis model) based on cell adhesion strength.

Control cells and RasV12 cells were evaluated with wound healing, spreading area, and focal adhesion (FA) analysis assays to test metastatic potential. Then the cells were tested for adhesion force by the novel bead-pipette assay, which uses a fibronectin-coated bead and a glass micropipette.

The RasV12 cells showed faster migration, polarized cell shape, and smaller FA area than control cells. With this evidence of metastatic potential, the RasV12 cells also exerted higher adhesion forces than control cells.

The RasV12 cells had metastatic potential compared to control. The novel force-quantification assay was able to measure forces that distinguished the RasV12 cells. In addition, this novel force-quantification technique allows for measurement of FA formation rate and is relatively cheap and accessible. In the future, it may develop as a drug or clinical screening trial for metastasis.

2. Introduction

Currently, metastasis is the cause of about 90% cancer patient deaths [1]. The migration of cancer cells to a new tumor location is difficult to predict and so far, no general prognostic marker of metastasis has been identified. Most research is focused on genomic or protein predictors through sequencing or microarray assays, but are usually cancer-type specific and aren't completely comprehensive. Sequencing tests, although getting faster and cheaper, still fail to account for the innate complexity of the gene regulation of metastasis due to factors like alternative splicing, post-translation modifications, and protein processing. Other tests include blood tests, CT scans, bone scans, and MRI, which are largely unspecific. [1,2]

Current literature indicates that cell adhesion force can differ between metastatic and stationary cells, and therefore has potential as a universal, prognostic marker of cancer cells [2,3]. This project investigates the adhesion force of cells to predict metastasis, resulting in a simpler resolution to this complex dilemma, applying a physics solution to a biological problem. There are multiple methods to quantify cell adhesion that vary greatly from population to individual studies to types of equipment and reasoning. Although many have key advantages including specific force observation and standard reproducibility, they also have disadvantages such as low maximum forces and inaccurate modeling due to cell or chamber deformation. [4]

These current cell-adhesion force techniques use a myriad of materials that range from common lab objects to delicate, advanced tools. They include methods such as traction force microscopy, centrifugal force assays, hydrodynamic shear assays, atomic force microscopy, and single-cell aspiration [4,5]. Besides common lab techniques to observe cell-matrix adhesion like wash assays, focal adhesion analyzation, and measuring spreading area, methods which specifically are able to quantify the force of adhesion give new insights into cell migration [6]. However, these methods are often expensive or require large numbers of cells, which make them inapplicable in a clinical setting where they could be assisting the diagnosis of cancer patients [4]. A goal of this research is not only to expand on the idea of using cell adhesion force as a marker for metastasis, but to test a novel method of measuring cell adhesion force that is more applicable in a hospital than current techniques.

Further expanding on cell adhesion, the bead-pipette assay (novel technique) measures each cell in a sample very specifically. Metastasis involves cell adherence to extracellular matrix (ECM) and other cells through focal adhesions, protein complexes that are a primary focus in this research. The first step in cell adhesion is the extension of lamellipodia and binding of integrin receptor proteins to ECM. Next, cytoplasmic proteins and cytoskeleton are recruited to form nascent FAs, generating a pulse of propulsive traction force upon the ECM substrate. The complexes then either dissemble to form new FAs to continue propelling the cell forward, or they will mature into larger, established FAs which provide passive anchorage instead of propulsion [7].

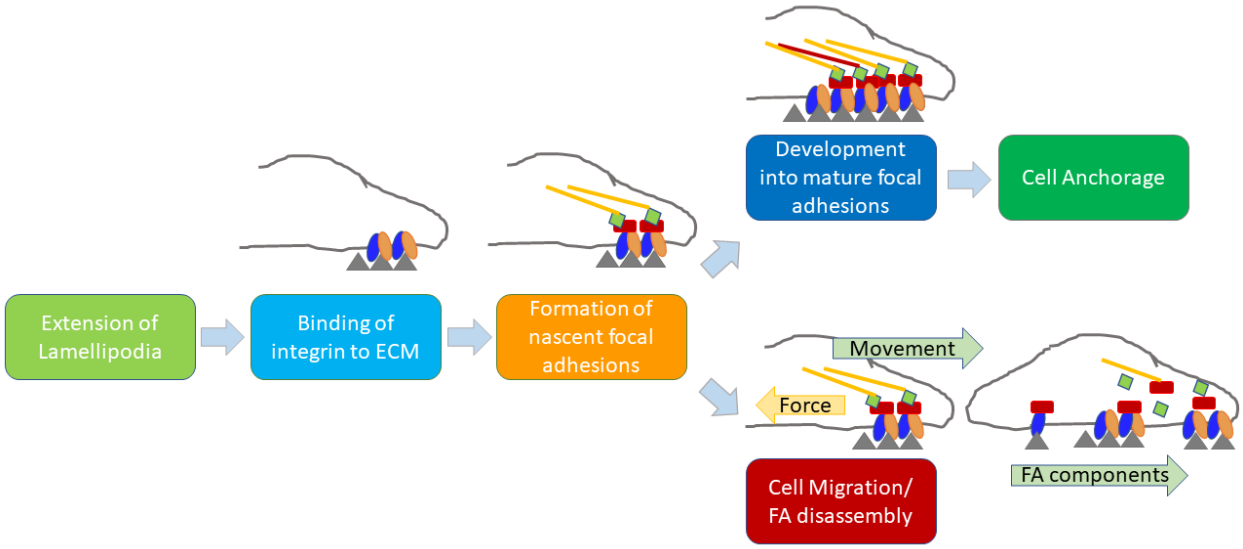

Figure 1. The development of focal adhesions (FAs). FAs differ in morphology between anchored cells, which have large, mature FAs, and motile cells, which have nascent, constantly assembling and deconstructing FAs.

These motile cells are polarized and have distinct leading and trailing edges. The leading edge is in the direction of movement and is formed by actin-polymerization-controlled-protrusion. A feature of the leading edge in addition to the formation of nascent FAs is the adhesion turnover. This refers to the rate that which FAs assemble then quickly dissemble to form new FAs, and tends to be high at the leading edge of highly motile cells. In addition, adhesions also must dissemble at the leading edge of the cell so that it may continue forward in its direction. [8]

Integrin is the transmembrane protein that is the coordination point of an FA to extracellular matrix. Integrin $\alpha_5\beta_1$ is the integrin of interest in this project. Its binding sites have been explored through x-ray crystallography, and it retains three metal binding sites in the β_1 subunit. An example of this integrin binding to our ligand of interest, fibronectin, is on the RGD sequence, the most well-known of the fibronectin binding sites, pictured in figure 2. In the complex, the β_1 subunit's MIDAS coordinates with the RGD aspartic acid, and the α_5 subunit's glutamine 221 and aspartic acid 227 retain hydrogen bonds with the RGD arginine. These hydrogen bonds and metal coordination are an example of molecular interactions that would form during cell

adhesion and are the types of bonds whose strength is tested through the novel bead-pipette assay. [9,10,11]

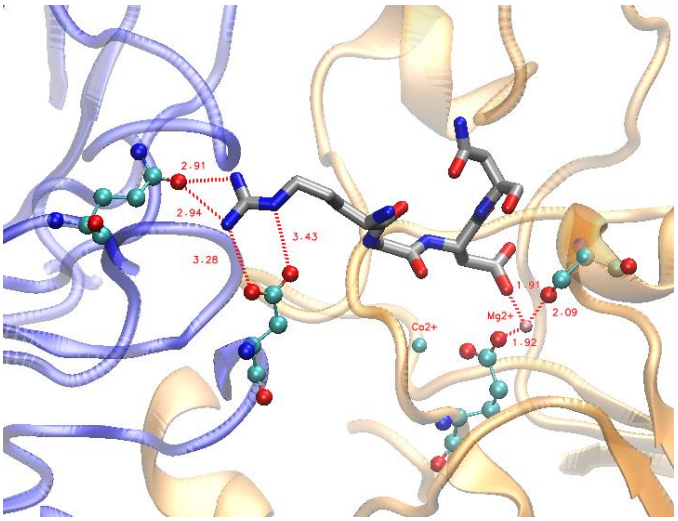

Figure 2. An example of a common interaction between an integrin and fibronectin section at the edge of a focal adhesion. Pictured here is integrin $\alpha_5\beta_1$ (PDBID 3VI4)[9] bound to an RGD peptide (fibronectin repeat) through hydrogen bonds and metal coordination. RGD peptide is depicted in grey licorice style and integrin subunits are depicted in NewCartoon style, where α_5 is in blue and β_1 in orange.

The purpose of this project is to test the ability of a novel cell-adhesion force quantification technique to predict metastasis. The bead pipette assay is a single-cell manipulation technique for quantifying cell adhesion and give insight into the force exerted by metastatic model cells. The RasV12 cancer model is used here to imitate metastatic behavior in mouse fibroblast NIH3T3 cells [12]. The bead-pipette assay may be ultimately aimed as an inexpensive, efficient, and simple way to identify metastasizing cells in patient samples or screen drugs through cell adhesion force.

3. Methods

3.1 Wound Healing Assay

The wound healing assay was performed to gage the initial metastatic potential of the RasV12 cells vs. the NIH3T3 cells. The cells used throughout the project were p53 knockout mouse embryonic fibroblasts of the NIH3T3 cell line that were RasV12-transformed. The wound healing assay was performed by seeding the cells on collagen-coated glass with dividers for 24 hours until 100% confluency, then removing the dividers and imaging the cells for another 24 hours afterwards. The data was measured at the eight-hour mark in the corresponding videos, and the distance migrated was calculated in micrometers through the Fiji ImageJ visualization program. [13]

3.2 Spreading Area

The spreading area of the cells was calculated through imaging the cells with a normal light microscope at 20x magnification. Cells were photographed at a low confluency and their area was quantified by carefully outlining the cell membranes by hand in Fiji ImageJ visualization program. [13]

3.3 Focal Adhesions

The focal adhesion area was measured through immunofluorescence visualization. Cells were seeded on small petri-dishes with a fibronectin-coated glass well. Paraformaldehyde was used to fix the cells, then Triton was added to make perforations in the cell membrane, and finally a blocker for unspecific binding was added. A primary antibody for paxillin – a focal adhesion protein – was added and incubated at 4 ºC overnight. Secondary antibodies for the primary antibody and nuclear staining were added, and fluorescence was visualized under a microscope. Focal adhesion area was quantified through setting a brightness threshold of 3320 minimum in visualization program Fiji ImageJ. [13]

3.4 Force Quantification – Bead-Pipette Assay

The force quantification bead assay is a novel force-measurement method that utilizes ECM-coated beads and a glass micropipette to calculate the force with which a cell can adhere to ECM. Cells were seeded overnight in a chamber composed of glass slides and PBMS and coated in collagen. Then, the cells were supplied with fibronectin-coated beads. 1 mm diameter glass micropipettes were pulled to a fine point of about 2 µm diameter, and were attached to a small water reservoir which could control suction into the pipette by fluid pressure. The pipette was brought into the cell chamber and using the fluid pressure at different positions relative to the microscope stage, the micropipette was positioned in front of a bead and made to hold one tightly on its tip by the suction.

Using the micropipette to manipulate the FN-coated bead, cells were tested for adhesion force by bringing the bead in contact with the cell surface and letting them have contact for 1 minute. After focal adhesions form at the contact site at 1 min, the cell was moved away at a speed of 10 µm/sec until the bead broke contact with the cell. The process was photographed at every 5 µm. After the bead broke contact with the cell surface, the rebound distance of the pipette was measured in Fiji ImageJ and the force was calculated through the displacement and the spring constant of the micropipette. [13]

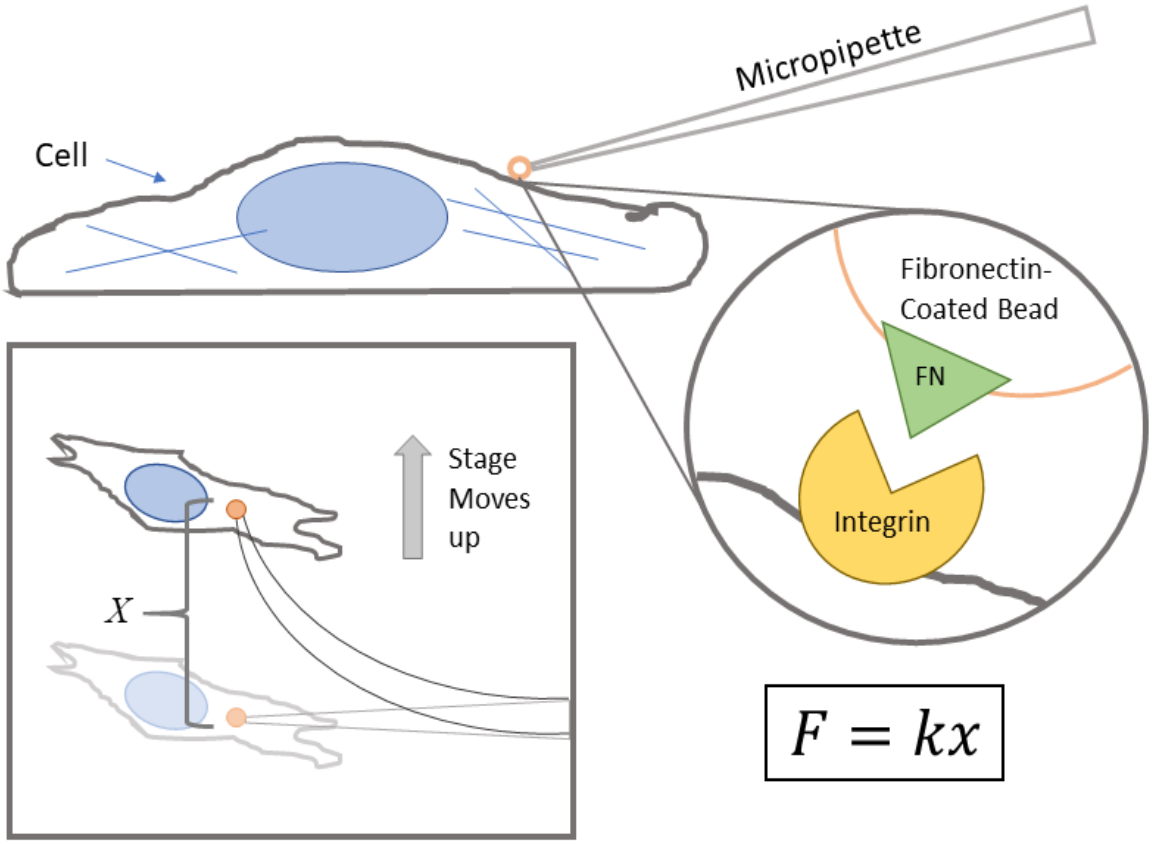

Figure 3. The bead-pipette method, using a flexible micropipette and fibronectin-coated bead. The force is quantified by moving the bead to come in contact with the cell, then measuring the deflection of the micropipette when the cell is moved away at 10 µm/sec. Multiplying the deflection distance (x) by the spring constant (k) of the pipette gives the force needed to break the integrin-fibronectin interaction.

4. Results and Discussion

4.1 Wound Healing Assay

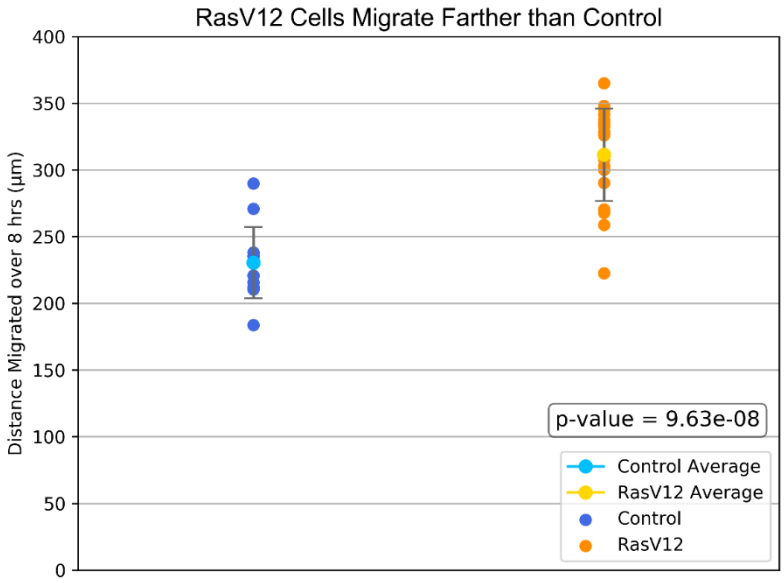

Figure 4. Wound healing assay shows that the RasV12 cells had a higher migration potential than the nonmutated NIH3T3 cells. Over a period of 8 hours the RasV12 cells migrated farther, with a p-value of 2.42E-05. The control cells averaged at 230.45 µm with ±26.76 error, and the RasV12 cells averaged at 311.35 µm with ±34.62 error.

The wound healing assay revealed that the RasV12 cells migrated farther than nonmutated NIH3T3 cells over a period of 8 hours, and farther migration is a characteristic of metastatic cells. In addition to the significant difference in migration distance between the control and RasV12, the cell behavior shown in the wound healing assay photos are distinct of their type.

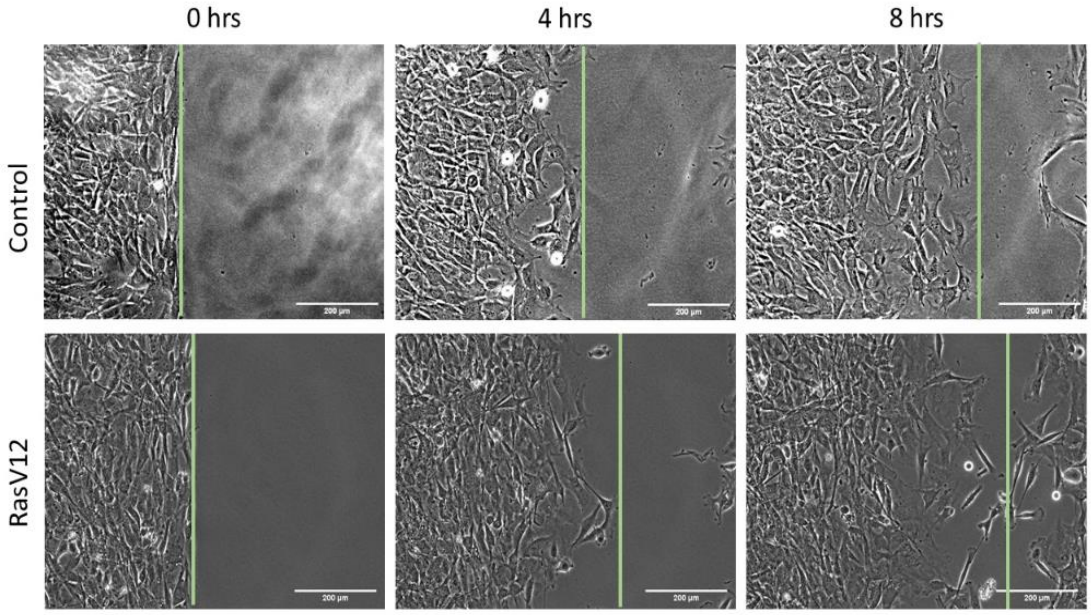

Figure 5. Wound healing assay. The RasV12 phenotype moves farther than the control phenotype over 8 hours. In addition, the control cells tend to stay together, having stronger cell-cell adherence vs. cell-ECM adherence. However, the RasV12 cells are just the opposite, seeming more individualized and having weak cell-cell adherence, instead focusing on migrating away from other cells.

In figure 5, the RasV12 cells travel farther than the control cells. However, an interesting phenomenon that can be observed in the images is the clustering of the control cells vs. the detachment of the RasV12 cells during migration. The control cells tend to move together while the RasV12 cells move independently. The control cells appear to have stronger cell-cell adhesion through proteins such as cadherin, whereas the RasV12 cells are more separated and seem to have weaker cell-cell adhesion, which is another characteristic of metastatic cells.

4.2 Spreading Area

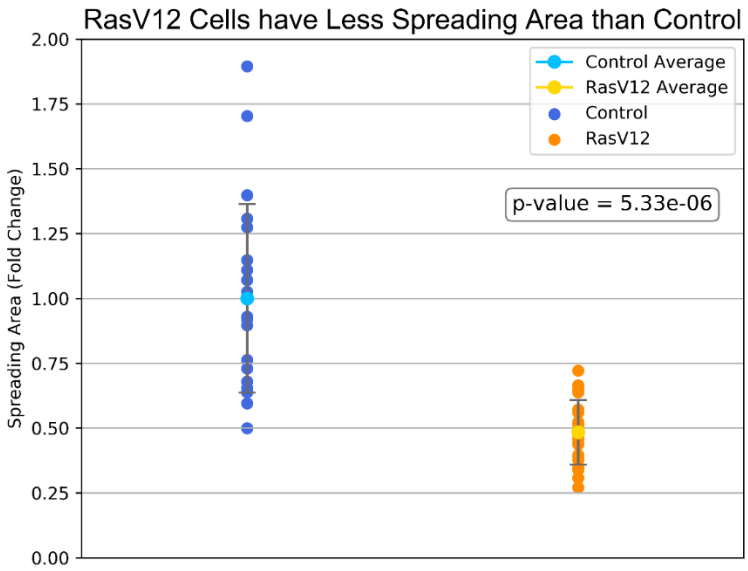

Figure 6. The RasV12 cells show less spreading area than the control cells. The data is normalized to the mean of the control cell area. The RasV12 phenotype cells have significantly smaller spreading area than the control cells with a p-value of 5.33E-06.

The RasV12 phenotype cells have significantly smaller spreading area than the control cells. The control cells are larger and more spread out, with tendrils of lamellipodium anchoring it over an extensive area. In contrast, the RasV12 cells are thinner and tapered, showing polarization of the focal adhesions to a leading edge, a characteristic of motile cells. This leading edge typically has many nascent focal adhesions forming, and as the cell moves, the cell directs a force backwards and pulls itself along the extracellular matrix, perpetually forming new FAs at its leading edge and dissembling them at the trailing edge. [8,9]

4.3 Focal Adhesions

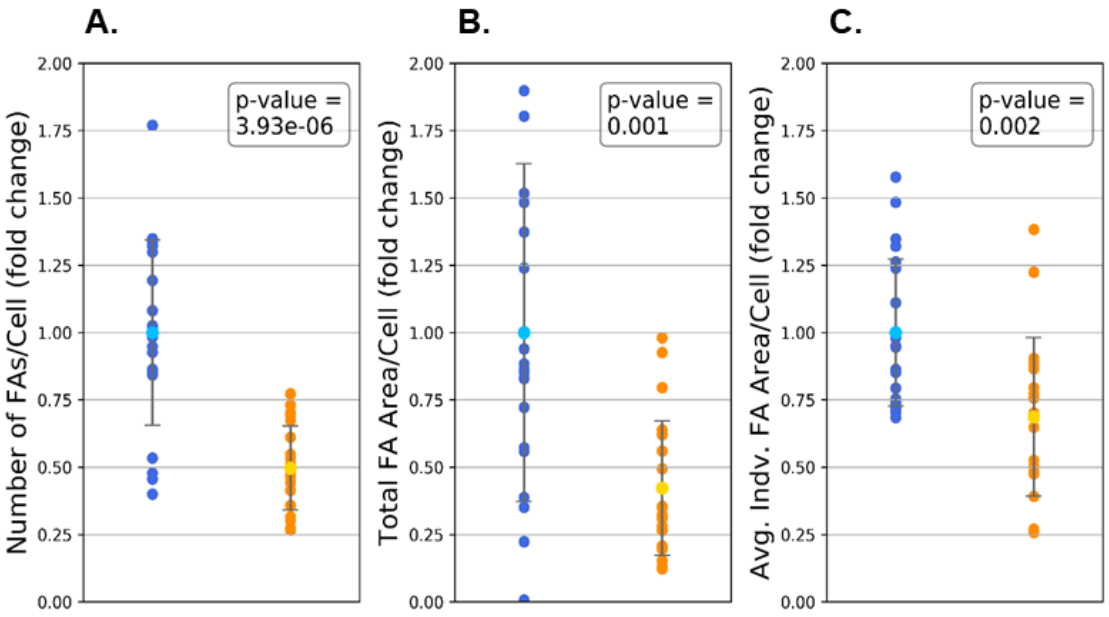

Figure 7. Focal adhesion quantitative analysis shows that RasV12 cells have less and smaller focal adhesions than control. Control cells are indicated in blue and RasV12 cells are indicated in orange. **A.** RasV12 cells have less total FAs than control cells. Less FAs -in addition to smaller FAs from previous data- indicate less anchorage of the RasV12 cells, in comparison to the control cells which have more FAs and larger individual FAs. The data is normalized to the mean of the control # of FAs per cell. The p-value is 3.92661E-06. **B.** RasV12 cells have less total FA area than control cells. More FAs may imply a more secured cell to the ECM due to more connections, whereas less FAs imply cell motility due to less FAs to assemble/dissemble. This data is normalized to the mean of the control cell focal adhesion size. The p-value is 0.001. **C.** RasV12 cells have smaller individual FA area than control cells. In addition to smaller total FA area per cell, average individual FA size per cell was also measured. RasV12 individual FAs are smaller and may be considered nascent; they are just budding and can more easily be synthesized and taken apart than mature FAs and are also a characteristic of metastasizing cells. This data is normalized to the average individual FA size for control cells. The p-value is 0.002.

The number of total FAs was quantified with Fiji ImageJ (Figure 7a). Using a consistent threshold to define fluorescence, the individual FAs were identified and counted for each cell of each type. The RasV12 cells overall showed less FAs, and coupled with smaller individual size, the RasV12 cells have less anchorage to their substrate than control cells. Control cells tended to show more FAs with more mature individual FAs, indicated by their size, and therefore were more strongly attached. Less FAs in general imply that the cell will be required to disassemble

less FAs if it's in the process of migrating. Motility will likely require fewer and smaller FAs, as a moving cell will need to swiftly synthesize and deconstruct FAs.

In figure 7b, the total focal adhesion area of a cell was measured through immunofluorescence assays. In previous results of the spreading area of the cell, the control cells were much larger than the RasV12 cells, and their total focal adhesion area naturally reflects that. More focal adhesions imply that the cell is more tightly anchored to the surface, as in order to move the cell requires less focal adhesions to deconstruct should it decide to migrate. In relation to the RasV12 cells, these results implicate that they are more likely to migrate because of less total focal adhesions.

Average focal adhesion size per cell was measured next (Figure 7c). In these results, the RasV12 cells have smaller individual focal adhesion size, indicating that many of the focal adhesions are nascent and may be just forming or dissembling. Nascent focal adhesions can easily disperse and reform, a behavior associated with motile and metastasizing cells. On the other hand, the control cells have larger, mature focal adhesions, becoming more established instead of transient. [8]

In figure 8, the sizes and fluorescence assays of the control and RasV12 cells are shown. The original scale of the cells was intentionally kept to show the difference in size between the control and RasV12 cells. It's clear that the control cells show many branching lamellipodia which are in no particular direction, and from the identification of the Fiji ImageJ software, they also have much larger individual FAs. In contrast, the RasV12 cells are smaller in area and their number and area of FAs also tend to be less than that of the control. Another interesting feature shown by figure 8 is the congregation of nascent FAs in the RasV12 cells towards a particular side of the cell, indicating the formation or establishment of a leading edge for motility.

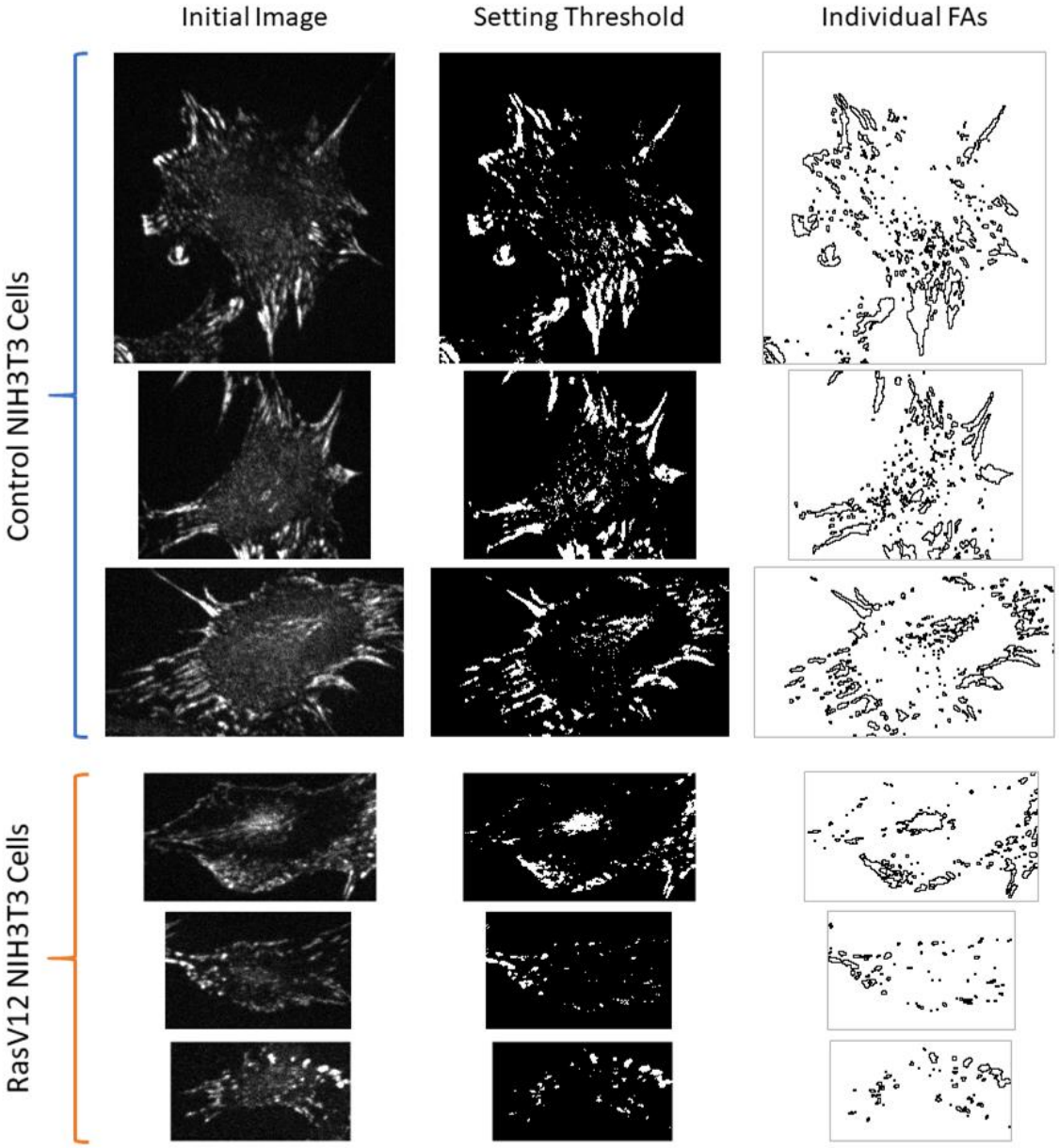

Figure 8. Representative cells are shown for each condition and each step in the process of calculating FAs. The cells are at their original scale. The RasV12 genotype cells are generally smaller and their focal adhesion numbers and area also reflect that characteristic. In addition, in

contrast to the control cells with many branching focal adhesions all around, the RasV12 cells were observed to be polarized with leading and trailing edges in terms of focal adhesions.

4.4 Force Quantification – Bead-Pipette Assay

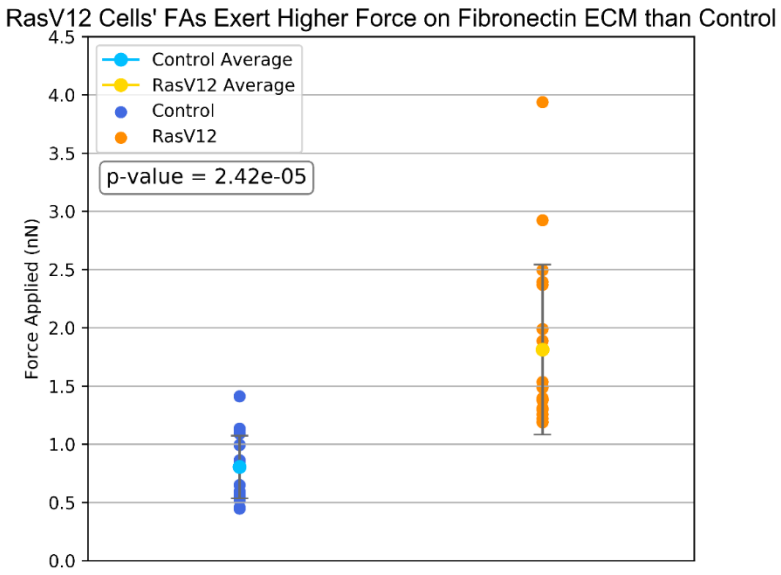

Figure 9. RasV12 Cells' FAs exert higher force on fibronectin ECM than control cells. The RasV12 phenotype cells have significantly higher force than the control cells with a p-value of 2.42E-05.

In the force quantification bead assay, the RasV12 cells adhered to the FN-coated beads much stronger than the control cells. On average, they adhered about twice as strong. A higher force indicates that the cells will cling stronger to ECM in the same amount of time. This indicates that the RasV12 cells are able to swiftly establish young FAs which are smaller in individual area and quantity but can more quickly adhere. The fast turnover time is an indicator of metastatic potential as cells that move need to be able to quickly form and dissemble FAs as they migrate. On the other hand, the control cells are likely synthesizing FAs that form to maturity and may be more established, however these are more slowly assembled and deconstructed. [7]

As detailed earlier, there are many different and varied methods of force quantification of cell adherence, and many have their advantages and disadvantages. Most techniques are population-based such as traction force microscopy, hydrodynamic shear, and centrifugal force, in which the percentage of cells or the net movement is measured. On the other hand, single-cell manipulation techniques use probes to push the cell from its position, or sometimes in AFM the probe is used to lower the cell onto a lawn of ECM proteins. In general, most techniques – with

the AFM exception – are detaching cells with already established FAs. In contrast, this bead-pipette assay controls the contact time and is primarily invested in measuring *nascent* FAs; in addition to quantifying the force of the FA attachment, this experiment takes into account turnover time for formation of FAs.

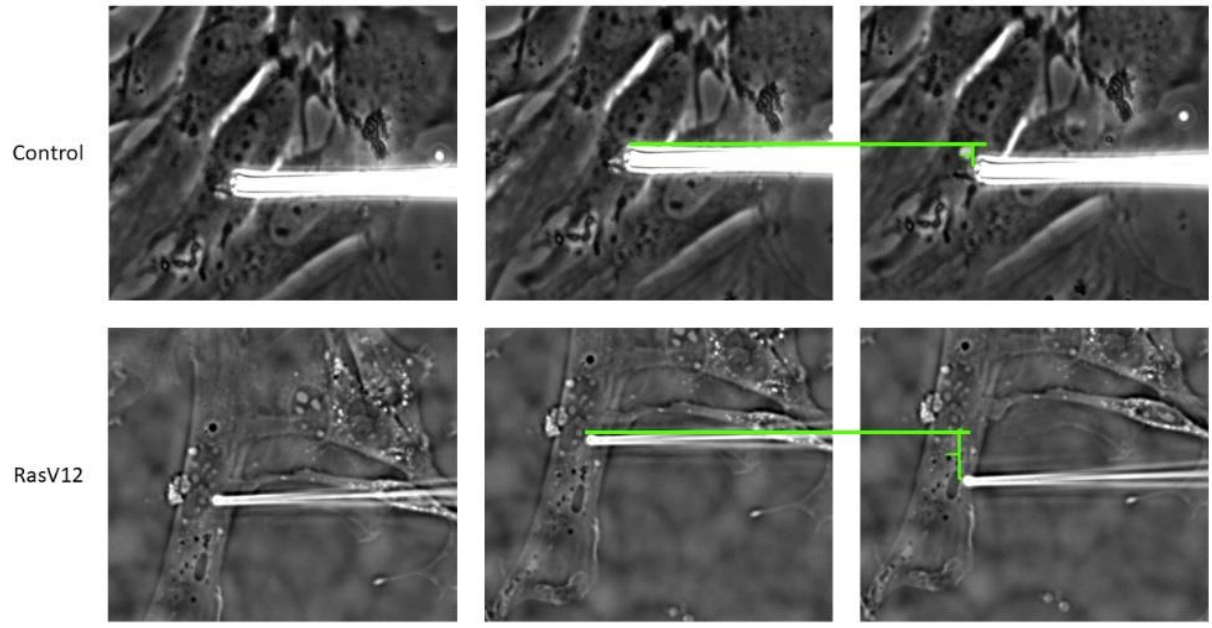

Figure 10. Difference in pipette deflection between control and RasV12 cells in the bead assay. A larger distance contributes to a larger force exerted, in combination with the measured stiffness, or spring constant.

Turnover time is an important factor to consider when viewing FA formation and measuring FA force, because it is a distinguishing feature between nascent and mature FAs. Cells that have a fast turnover time and can quickly form nascent FAs with strong force may be metastatic due to the nature of the cell motility. In contrast, cells with low turnover time that slowly build up mature FAs are more anchored and would not easily migrate because the pace of assembling and deconstructing FAs is too small.

In addition to the measurements, the novelty of the bead-pipette assay also lies in its widespread and cheap laboratory materials. Although methods of AFM have shown to also consider FA formation time by measuring developing FAs over a short period of time, AFM cantilevers and microscopes are very expensive and require specialized training. In addition, AFM force measurements have specific drawbacks due to positional and time-based complications.

Lastly, the bead-pipette assay has an advantage in sample size of cells. Force quantification methods -especially those that detach cells from their seeded plates, such as centrifugal or hydrodynamic shear assays- often require multiple samples of cells to test. However, this method

is on individual cells, and therefore it only requires a single seeding of cells in a cell chamber. From a single cell chamber, a sample size of about 20 or more cells can be taken in a single sitting.

This novel force quantification is unique in its turnover time measurement, inexpensive lab materials and efficient sampling, and still can effectively quantify the force difference between control and RasV12 cells which indicate metastasizing potential.

5. Conclusion

In conclusion, the novel force quantification assay is able to distinguish between the control and metastatic cells based on force. The RasV12 cells have displayed multiple characteristics of metastasizing cells such as faster cell migration, polarized cell shape, smaller FA area, and less FA numbers compared to control cells. Unlike other methods, the bead-pipette assay is able to also account for turnover time of FA synthesis, and has shown that the RasV12 cells have faster turnover time to account for cell motility. Due to the simplicity of the technique and the novelty of the measurement, the bead-pipette assay is an effective and accessible method of force quantification that applies a physical solution to a biological problem.

6. Recommendations and Application

The experiment was limited mostly by the time available to work in the lab, as the research was performed at an international location. Because the data quantification was limited by the brief time at the lab, continuation of the project would allow for many other types of assays and experiments to be tried.

For example, the first recommendation for the project would be to measure the adhesion force of the cells over various time periods. Much literature indicates the constant formation and disassembly rate of focal adhesions; therefore, it would be interesting to use the bead-pipette's ability to vary attachment time to supplement this research. We would expect to see a curve that would represent the deconstruction of a focal adhesion as time goes on due to the nature of metastatic cells' focal adhesions.

In addition, quantifying the adhesion strength of the cells on a different substrate, such as cadherin, the connective protein between cells, is also a further recommendation. The cadherin interaction between cells should be different for metastasizing cells and control cells; as shown in the wound healing assay photos, the cell-cell adhesion was much less in the RasV12 cells than the control cells. Other substrates which could be used include collagen or laminin, or other types of ECM depending on the cells. Cell lines can also be experimented with, like specific cancer cell lines.

For the application of the bead-pipette assay, the force with which a cell exerts on its extracellular matrix can be used as a technique for clinical screening. Drugs that are being tested

for metastatic prevention can be evaluated for their relative effectiveness with the adhesion force on certain extracellular substrates. As the assessment of such drugs must be thorough, the bead-pipette assay would be an informative addition to a comprehensive examination of the effect of these small molecules on cancer cells.

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