UNIVERSITY OF CALIFORNIA SAN DIEGO

Physical Selection of Murine Astrocytes to Study Intra-tumoral Heterogeneity in Glioblastoma Multiforme

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Bioengineering

by

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2021

The thesis of Divya Lakshmanan is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

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ABSTRACT OF THE THESIS

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Divya Lakshmanan

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Professor Adam Engler, Chair

Glioblastoma multiforme is one of the most aggressive tumors, with an extremely low five-year survival rate of 10%. Vast inter- and intra-tumoral heterogeneity in GBM tumors leads to aggressive phenotypes and poor prognosis in most patients. One source of heterogeneity stems from variance in adhesion strength of cells to extracellular matrix, which leads to differences in migration and invasion. We hypothesized that a cell line with a set of mutations that are common in GBM will have a subpopulation of cells that will drive invasion based on their adhesion strength. In order to identify and characterize the cells that lead to aggressive GBM outcomes, we optimized and utilized a parallel plate flow chamber assay to expose

wtEGFR murine astrocytes to shear stress and sort the population based on adhesion strength. Sorted populations were described either as weakly or strongly adherent and analyzed through various downstream assays such as the spinning disk shear assay to quantify adhesion strength and immunofluorescence staining to elucidate focal adhesion formation. While phenotypic differences between sorted populations and unsorted control populations were not easily reproduced between replicates at 48 hours post exposure to shear stress, differences between the populations were more easily identified 16 hours post exposure to shear stress. This indicated that phenotypic differences that are revealed as a result of physical selection are not permanent and that the underlying mechanisms of these differences need to be uncovered.

Introduction

Glioblastoma multiforme (GBM) is the most commonly occurring malignant primary brain tumor, accounting for 45.6% of malignant primary brain tumors and 16% of all primary brain tumors (Tamimi et al., 2017). It is classified as a grade IV glioma, which are defined as intra-axial tumors derived from the glial cells of the central nervous system. Histological analysis of GBM indicates hypercellularity, nuclear abnormalities, proliferation of microvascular structures, and necrosis of healthy tissue (D'Alessio et al., 2019). Apart from histopathological features, one of the hallmarks of GBM is its ability to invade surrounding brain tissue. Further classifications of GBM which can help to choose the most appropriate course of treatment can be defined based on genetic and epigenetic profiles of the tumor. Current treatment strategies for GBM include surgical tumor resection, radiation, chemotherapeutic agents aimed at various targets, and temozolomide therapy (Rajaratnam et al., 2020). Despite the multimodal approach to treatment of GBM, the average survival time is only 12 to 18 months and the five-year survival rate remains extremely low at 10% (D'Alessio et al., 2019). Poor prognosis for GBM can partially be attributed to poor tumor margins which complicate surgical interventions. In fact, more than 90% of GBM tumors recur within 2-3 cm of the original lesion. Another significant factor in poor prognosis comes from extreme inter- and intra-tumoral heterogeneity, which often makes available therapies unsuccessful due to their ability to attack varied tumor signatures.

One of the key sources of heterogeneity in GBM is the presence of a wide variety of intra-tumoral genetic mutations. Typically, gliomas that are classified as grade IV tumors (ie. GBM) lack the Ink4a-ARF tumor suppressor locus, meaning that normal cell cycle arrest mechanisms in the G1 and G2 phases are dysfunctional (Holland et al., 1998). In addition, about 60% of GBM tumors notably possess alterations in the transmembrane cell receptor tyrosine kinase, epidermal growth factor receptor (EGFR) (Francis et al., 2014). In healthy glial cells, EGFR is activated upon binding to a signaling ligand (Rajaratnam et al., 2020). Activation

causes a conformational change of EGFR into a homodimer and autophosphorylation of tyrosine residues in the C-terminal domain of EGFR. This autophosphorylation results in the initiation of downstream signaling cascades that lead to DNA synthesis, cell proliferation, migration, and adhesion. Two of the most common EGFR alterations include EGFR overexpression (wtEGFR) and EGFR truncation of exons 2-7 (EGFRvIII) (Banisadr et al., 2020). In particular, the EGFRvIII alteration leads to constitutive autophosphorylation of tyrosine residues, resulting in heightened downstream pathway activation and pronounced effects of these pathways including reduced apoptosis.

Previous studies have shown that cell populations with various EGFR alterations have phenotypic differences in terms of adhesion strength to extracellular matrix proteins and migration potential. When isogenic mouse glioma cells with various combinations of Ink4a/ARF deletion and EGFR alterations were subjected to a spinning disk shear assay in which they experience radially increasing shear stress, the EGFRvIII cell population displayed a lower adhesion strength to a fibronectin coated coverslip than the wtEGFR population and the basal population without EGFR alterations (Banisadr et al., 2020). Furthermore, EGFRvIII cells had a longer pathlength than wtEGFR cells while wtEGFR cells had a greater average displacement than EGFRvIII cells. This result emphasized a relationship between adhesion strength and migration potential and showed that EGFRvIII cells are more invasive than wtEGFR cells since they are able to travel longer distances in random directions. Taken together, these data indicate that EGFR alterations play a significant role in adhesion strength and migration potential of glioma cells, which eventually contributes to the degree of aggressiveness in glioblastomas. GBM tumors can possess any combination of these mutations and many others, making them hard to treat with any given therapy. For example, EGFRvIII cells which are usually diffusely spread throughout a tumor can communicate with wtEGFR cells in order to confer their more aggressive phenotype to the wtEGFR cells (Banisadr et al., 2020). While heterogeneity between cell populations with different combinations of relevant mutations has

been characterized, biophysical heterogeneity within a cell population with a given set of mutations has not been well defined.

Since one of the driving factors behind the poor prognosis of GBM is the invasiveness of GBM, biophysical properties of glioma cells including cell adhesion and migration are important to characterize. In order to invade healthy tissue, cancer cells must modulate interactions with the extracellular matrix (ECM). Specifically, at the leading edge of a cluster of migrating cells, existing adhesions in a protrusion must disassemble as new adhesions form to support migration (Ridley et al., 2003). Therefore, migration speed depends on cells' ability to turnover focal adhesions, which ultimately depends on adhesion strength to the matrix. Consistent with this relationship, it has been shown that invasive cancer cells are able to turnover focal adhesions at a higher rate than their noninvasive counterparts (Bijian et al., 2013). Given that adhesive signature plays an important role in tumor aggressiveness, previous studies in the lab have developed and utilized a parallel plate flow chamber to study adhesive and invasive heterogeneity within several cancer cell lines (Beri et al., 2020). They were able to sort cancer cell populations based on their adhesive signature and found that the more weakly adherent subset migrated for longer distances, had higher contractility, and exhibited higher focal adhesion disassembly. In the case of GBM, heterogeneity within individual cell lines with a given set of EGFR alterations has not yet been characterized. Given that EGFR alterations play a significant role in modulating cell adhesion and migration, identifying and studying distinct populations based on adhesive strength within cell lines can help to further understand the vast heterogeneity in GBM.

In order to study the biophysical heterogeneity within GBM cell lines, a parallel plate flow chamber (PPFC) was used to sort cells into a weakly and strongly adherent population. The PPFC assay had to be optimized in terms of various inputs for optimal function using astrocytes. The wtEGFR murine astrocyte cell line (with Ink4a-ARF deletion and EGFR amplification) was used for this optimization process. Once cells were sorted using the PPFC, various downstream

assays including the spinning disk shear assay and immunofluorescence staining were used to try to pinpoint differences between these sorted cell populations. Eventually, phenotypic differences between these populations can be correlated with transcriptomic changes upon selection and the permanence and effect of these differences on tumor progression can be identified.

Materials and Methods

I. Cell Culture

Astrocytes were cultured in high-glucose Dulbecco's modified eagle medium (DMEM) supplemented with L-glutamine, which was combined with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% 200 mM L-glutamine. Cells were incubated in an incubator containing 5% CO_2 and at 37℃. They were grown in tissue culture treated polystyrene flasks. They were typically passaged about once every two days, depending on confluency. 0.25% trypsin was used to cleave cellular adhesions with their substrate and resuspension of cells in cell culture media at the appropriate ratio neutralized the trypsin.

II. Parallel Plate Flow Chamber Assay

About 16-20 hours before running the parallel plate flow chamber assay, glass slides were cleaned, coated with fibronectin, and seeded with cells. Glass slides were sonicated with 70% ethanol for 10 minutes and subsequently with DI water for 10 minutes. They were then autoclaved. Once cleaned, the slides were coated with fibronectin in a tissue culture hood at a concentration of 0.1 μg/cm^2 of fibronectin. Once the coating solution was allowed to sit for an hour, the solution was aspirated and the slides were blocked in the culture media used to passage astrocytes. Once the plates were allowed to block for an hour, cells were seeded onto them at a density of 6,250 cells/cm^2 in order to minimize cell-cell contacts. The cells were allowed to incubate for about 16-20 hours.

At the time that the cells were ready to be sorted, the components of the parallel plate flow chamber were assembled. Tubing, polypropylene luer fixtures, and the silicone gasket were

autoclaved prior to being used, while the actual chamber was exposed to ultraviolet light in the tissue culture hood for an hour in order to be sterilized. Once the components were assembled and the fibronectin-coated glass slide with cells was clamped into the chamber, the tubing connected to the inlet was connected to the syringe pump on the other side and the appropriate flow conditions were specified. Dulbecco's phosphate buffered saline (DPBS) with the addition of magnesium and calcium and with 4.5 g/L of dextrose was used as the buffer to shear cells. In order to isolate the weakly adherent fraction of wtEGFR astrocytes, cells were sheared at 8 dynes/cm^2, which translates to fluid flow through the chamber at a rate of 11 ml/min for 2 minutes total at 25℃. Once the fluid flow was finished, the cells that remained attached to the glass slide, or the strongly adherent population, were retrieved through trypsinization. Both populations were reseeded and allowed to incubate before being used for downstream applications.

III. Spinning Disk Shear Assay

Cells that are analyzed using spinning disk assay are seeded onto 25 mm coverslips. Prior to the assay, these coverslips were sonicated in 70% ethanol for 10 minutes, DI water for 10 minutes, then autoclaved. These sterile coverslips were coated with fibronectin at a concentration of 10 g/ml, consistent with previous spinning disk experiments performed in the lab. After being coated with fibronectin for one hour, they were blocked in cell culture media for another hour. After this, the sorted cells that collected from the PPFC were seeded onto the coverslips and allowed to incubate for a given amount of time according to the experiment.

At the time of the assay, the fibronectin-coated coverslips containing cells were mounted onto the spinning disk device. They were then submerged into a large volume of buffer maintained at 37℃, made of PBS containing magnesium and calcium and 4.5 g/L of dextrose. The coverslip was then spun at a given speed measured in rotations per minute (rpm), during which cells on the coverslip experienced a radially increasing shear stress profile. After the spin, the cells on the coverslip were immediately fixed in 3.7% formaldehyde. Cell nuclei were stained

with 4′,6-diamidino-2-phenylindole (DAPI) at a 1:2000 dilution and the coverslips were mounted onto glass slides using Fluoromount-G. Each coverslip was imaged at about 1500 different positions using the Nikon Eclipse TI fluorescent microscope and a custom Matlab script was used to stitch these images together and quantify the number of nuclei that were present at each radial position, in order to generate the desired shear stress curve.

IV. Immunofluorescence Staining

Cells of interest that were seeded on coverslips were fixed in 3.7% formaldehyde for 10 minutes. They were then washed with blocking buffer three times and then blocked for an hour. The blocking buffer was made of 0.3 M glycine, 10% goat serum, 1% bovine serum albumin (BSA), and 0.1% saponin, which served as the permeabilization agent. Post blocking, cells were incubated with the primary antibody of interest, rabbit anti-paxillin, at a 1:250 dilution for 1.5 hours at room temperature or overnight at 4℃. Subsequently, cells were washed thrice with blocking buffer and then incubated with secondary antibody Alexa Fluor goat anti-rabbit 488 at 2 g/ml for an hour. Certain samples were additionally incubated with rhodamine phalloidin at this stage. After secondary antibody incubation, cells were again washed thrice with blocking buffer. In order to stain nuclei, cells were incubated with DAPI at a 1:2000 dilution for 15 minutes. Finally, the coverslips were mounted onto slides using Fluoromount-G and allowed to dry overnight. The slides were imaged using a 60x magnification lens on the Nikon Eclipse TI fluorescent microscope to visualize focal adhesions, actin fibers, and nuclei as appropriate. Images were analyzed using ImageJ.

V. Western Blot

Cell Lysis: Cells of interest from an 80% confluent plate were washed thrice with ice cold PBS. 200 L of mRIPA buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Triton, 10% glycerol, 25 mM sodium deoxycholate, 0.1% SDS, Roche Complete Protease Inhibitor, and PhosSTOP) was added to the plate and cells were lysed using a cell scraper. The lysed cells were transferred into an Eppendorf tube on ice and briefly vortexed every 5 minutes

over a period of 30 minutes. Finally, the suspension was centrifuged at 15000 rpm for 15 minutes and the supernatant was transferred to a clean tube and saved at -80°C.

Protein Concentration Determination: The Pierce Bicinchoninic Assay (BCA) kit was used to determine concentration of the lysate. In a 96 well plate, 10 µL of each standard was loaded into three wells next to 10 µL of the lysate sample. A multichannel pipette was used to dispense 200 µL of working reagent (50 Reagent A: 1 Reagent B) into each well. The plate was centrifuged for 5 minutes to remove any bubbles and then incubated at 37°C for 25 minutes. A plate reader was used to measure absorbance at 562 nm with the plate lid removed. A standard curve was generated using a 4^{th} order polynomial fit and protein concentration was determined from this fit.

Size Separation/Gel Electrophoresis: Based on the concentrations determined from the BCA assay, 12 µg of the desired protein was combined with 50 mM DTT, 4x Laemmli Sample Buffer, and mRIPA to create a total sample volume of 30 µL. The samples were heated to 95°C for five minutes in order to denature the proteins. Samples were immediately carefully removed from the heating block with forceps and placed on ice. Simultaneously, 4-12% Bis-Tris gels were removed from storage, rinsed with DI water, and allowed to equilibrate to room temperature. The electrophoresis chamber was then assembled with the gel and the chamber was filled with 1X MOPS buffer to the indicated height. Protein samples were loaded into the channels along with a chameleon duo ladder on either side of the samples in order to visualize molecular weight bands. The gel was run at 140 V for an hour.

Membrane Transfer/Blotting: Once the size separation of the proteins was complete, the gel was removed from the chamber and the gel comb/edges of the gel were removed. The iBlot 1 semi-dry transfer system was assembled using the appropriate membrane cassettes and the contents of the gel were transferred to a nitrocellulose membrane.

Antibody Staining/Analysis: The membranes were blocked with Azure fluorescent blot blocking buffer for one hour on a shaker. Then, antibodies of interest (mouse anti-EGFR at 1:750 dilution and rabbit anti-beta actin at 1:10000 dilution) were diluted in blocking buffer and

added to the membranes overnight at 4°C on a shaker. The membranes were then washed thrice in tris-buffered saline supplemented with Tween (TBS-T, 150 mM NaCl, 15 mM Tris-HCl, 20 mM Tris Base, 0.1% Tween). Next, membranes were incubated with the appropriate secondary antibodies (goat anti-rabbit Alexa Fluor 790 and goat anti-mouse Alexa Fluor 680) for one hour on a shaker at room temperature. Finally, membranes were washed thrice with TBS-T, once with water, and imaged using the Odyssey CLx Imaging System and ImageStudio (Licor) software.

VI. Statistical Analysis

All statistical analyses were performed using GraphPad Prism software. Experiments were performed with the number of replicates indicated in each section. Error bars are shown as mean $\pm$ standard deviation. Comparisons between groups of data were performed using one-way ANOVA and p-values were calculated as indicated on graphs. The threshold for significance was $p < 0.05$.

Results

I. Optimization of Parallel Plate Flow Chamber Assay

A. Description of Parallel Plate Flow Chamber

The parallel plate flow chamber is an apparatus that provides a constant and laminar shear stress profile to cells. When the chamber is connected to a syringe pump, fluid flows from the pump into the chamber through an inlet, through the chamber over a fibronectin-coated glass slide with cells seeded on it, and through an outlet into a collection tube. The cells that detach from the fibronectin coated glass slide as a result of fluid flow and travel through the outlet into the collection tube are classified as the weakly adherent population, while the cells that remain attached to the slide are classified as the strongly adherent population. Fluid flow characteristics can be modulated on the syringe pump by setting the desired flow rate and volume to be dispensed. Figure 1 shows a schematic of the parallel plate flow chamber.

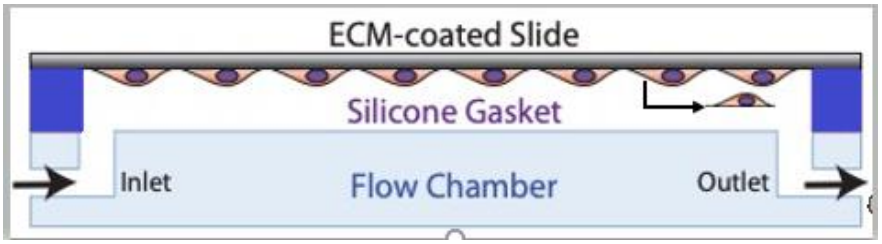


Figure 1: A: Schematic of parallel plate flow chamber.

The shear stress experienced by cells in the chamber can be calculated by simplifying the Navier Stokes equation according to relevant assumptions and simplifications. By assuming that the fluid is Newtonian and incompressible, the flow is laminar, and that the no slip boundary condition applies, the Navier Stokes equation can be simplified to the following:

$$-\frac{\vartheta P}{\vartheta x} = \mu\left(\frac{\vartheta^2 V_x}{\vartheta y^2}\right)$$

In the equation above, $\frac{\vartheta P}{\vartheta x}$ refers to the pressure gradient along the plates in the x direction, μ is the fluid viscosity, V_x is the velocity of the fluid in the x direction, and y is the position on the y axis with respect to the center of the plates or the bottom plate. By integrating this equation and applying appropriate boundary conditions, the following equation is obtained:

$$V_x = \frac{1}{2\mu}\left(\frac{\vartheta P}{\vartheta x}\right)\left(\left[\frac{h}{2}\right]^2 - y^2\right)$$

Finally, by integrating over the height of the chamber and substituting $\tau = \mu\left(\frac{\vartheta V x}{\vartheta y}\right)$, the shear stress τ experienced in the chamber is defined as:

$$\tau = \frac{6\mu Q}{wh^2},$$

where μ is the fluid viscosity, Q is the volumetric flow rate, w is the width of the chamber, and h is the height of the chamber. Therefore, for a given fluid viscosity, the volumetric flow rate can be modified in order to expose cells in the chamber to a range of shear stresses. In this system, cells experience a constant and laminar fluid flow, which easily allows us to study the effects of

shear stress on cell adhesion over a defined period of time and sort cells according to their adhesion strength.

B. Establishing Sort Conditions

Before beginning to use wtEGFR cells for PPFC experiments, Western blot was performed on them as well as the parental cell line (Ink4a-ARF deletion and no EGFR alteration) and EGFRvIII cell line (Ink4a-ARF deletion with EGFRvIII variant) in order to confirm that the correct population of cells was being used. As predicted, the parental cell line showed no bands for EGFR, the wtEGFR cells showed bands for EGFR, and EGFRvIII cells showed bands for the truncated variant of EGFR at a slightly lower molecular weight.

Ink	-	-	-	-	-	-
EGFR	N/A	N/A	wt	wt	vIII	vIII

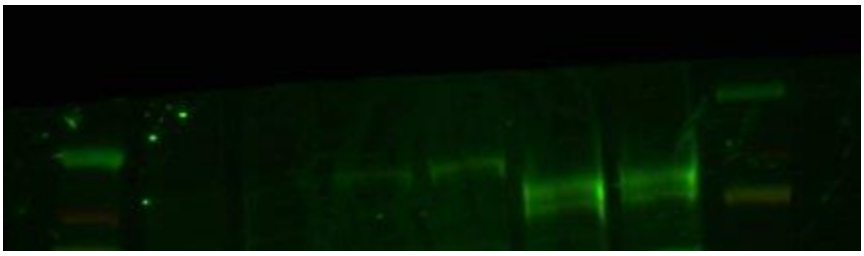


Figure 2: Western blot for EGFR of three different cell lines. wtEGFR cells are shown in the middle two lanes.

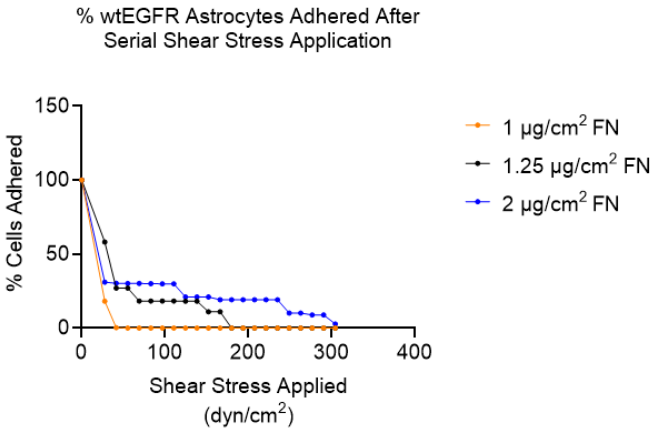
In order to successfully sort the wtEGFR astrocytes into distinct weakly and strongly adherent populations, the first step was to choose an appropriate shear stress value. The shear stress value chosen needed to be able to detach a significant number of cells that could be reseeded for various downstream applications, while preventing high degrees of cell apoptosis. Based on previous data generated in the lab from the spinning disk shear assay to determine the adhesion strength of astrocytes, the Tau50 value, or the shear stress at which 50% of the cell population remains attached to the fibronectin coverslip, was determined to be about 400 dynes/cm^2 for wtEGFR astrocytes. In addition, previous experiments in the lab that used the parallel plate flow chamber assay to sort breast and lung cancer cell lines used 2 μg/cm^2 of fibronectin to coat the glass slides onto which cells were seeded. However, when this shear

stress and fibronectin concentration were applied to the wtEGFR astrocytes using the parallel plate flow chamber, most of the cells apoptosed during the process. The shear stress value that was able to detach 50% of cells in the spinning disk assay likely did not translate to the PPFC assay because both assays present a completely different flow profile to the cells. A significant population of cells could not be collected from the assay for downstream applications using these conditions, driving the need for assay optimization.

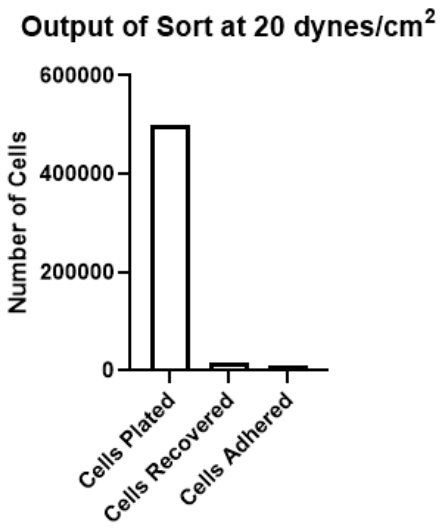
In order to understand and visualize how astrocytes behaved in the presence of fluid flow and to test a range of shear stress values for the assay, the imaging parallel plate flow chamber was used. Compared to the regular PPFC, this chamber was designed based on the same engineering principles and provided the same flow profile, but it was small enough such that it could be secured on the microscope stage. This allowed for visualization of fluid flow through the chamber and subsequent cell detachment in real time. With the chamber set up on the microscope, shear stress values ranging from 0 - 304 dynes/cm^2 were serially applied to the cells. A set of images were taken after each shear stress value was applied, allowing for the determination of the number of cells that remained on the slide after each round of fluid flow. Furthermore, given that high magnitude of shear stress led to high rates of cell death, various fibronectin concentrations ranging from 1 - 2 μg/cm^2 were tested on the glass slides onto which cells were seeded. If cells can attach to glass slides coated with lower fibronectin concentrations, they would likely form adhesions with lower strength, thereby enabling the cells to be sorted into distinct populations using a lower shear stress value. When using the imaging parallel plate flow chamber to test this range of shear stress values and fibronectin concentrations, cells that were seeded on lower concentrations of fibronectin detached from the slide at a lower shear stress than cells that were seeded on higher concentrations of fibronectin. As shown in Figure 3B, the shear stress at which about 50% of cells remained on the slide was 27.7 dynes/cm^2, 41 dynes/cm^2, and 166 dynes/cm^2 in the 1, 1.25, and 2 μg/cm^2 of fibronectin conditions respectively. This supported the hypothesis that using lower concentrations of

fibronectin to coat the glass slides would allow for astrocytes to be sorted at lower shear stresses, thereby reducing the chance for cell apoptosis. This curve generated from this assay also provided a clearer picture of the shear stresses at which specific percentages of cells detached from the slide, leading to a more educated selection of shear stresses to use in the PPFC assay.

A



C



B

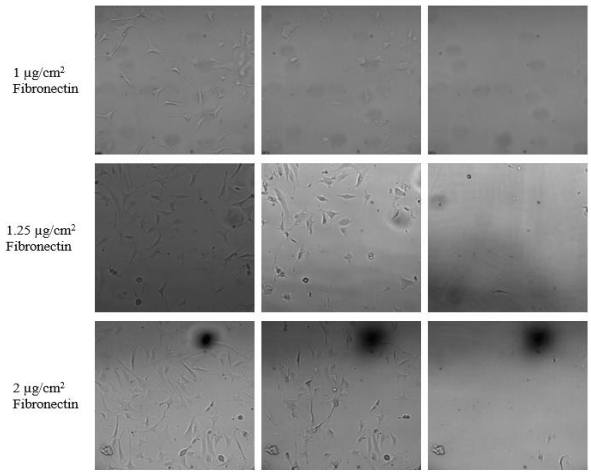


Figure 3: Results of serial stress application using imaging parallel plate chamber. A: Number of cells that remain attached after each application of shear stress is shown, using different concentrations of fibronectin to coat glass slides. Conditions with lower fibronectin concentrations allowed for cells to detach at lower shear stresses. B: Images taken during three different points: before shear stress application, when approximately 50% of cells remain bound to the glass slide, when all cells have detached. C: PPFC data from sort tried using 20 dynes/cm^2 and 1 ug/cm^2 of fibronectin based on imaging parallel plate chamber data. Cell death remained high at these conditions.

Given that lower fibronectin concentrations led to lower shear stresses needed to detach cells and Figure 3A showed that approximately 50% of cells detach at about 20 dynes/cm ^{2}in the lowest fibronectin concentration condition, the next step was to attempt the PPFC assay using these new conditions. However, when the PPFC assay was run using a glass slide coated with 1 µg/cm^2 of fibronectin and using 20 dynes/cm^2, both the number of cells recovered from the sort and the number of cells that remained adherent were very low. Because the cells from these populations did not add up to the number of cells plated, it was clear that cell death remained high.

In order to further optimize the sorting conditions and reduce cell death, the next iteration was performed with a glass slide coated with 0.1 μg/cm^2 of fibronectin. This concentration was chosen based on the trend that lower fibronectin concentrations allowed for the usage of lower shear stresses to detach cells. According to the lower fibronectin concentration, a lower range of shear stress values ranging from 4 - 9 dynes/cm^2 were tested. From these trials, the shear stress value of 8 dynes/cm^2 was chosen for all experiments going forward. This shear stress value was the lowest value at which enough cells were viably detached in order to be reseeded. Therefore, optimization of the PPFC assay to sort wtEGFR astrocytes led to new sorting conditions of using 0.1 μg/cm^2 of fibronectin to coat the glass slides onto which cells were seeded and 8 dynes/cm^2 of shear stress to sort cells into distinct populations.

II. Characterizing Sorted Populations of wtEGFR Astrocytes

Once the appropriate conditions were established to use the PPFC to sort wtEGFR astrocytes, various downstream assays were performed to characterize differences between the sorted populations.

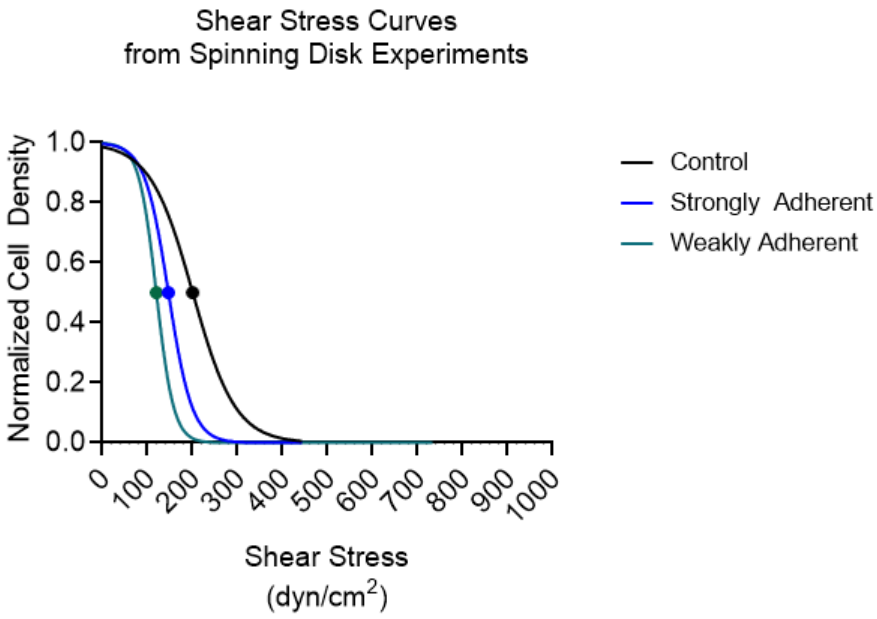
A. Spinning Disk Shear Assay and Immunofluorescence Analysis

In order to perform the first set of downstream analyses, weakly adherent cells and strongly adherent cells that were retrieved through trypsinization were seeded onto fibronectin coated glass coverslips and incubated for 48 hours to allow for cell attachment and proliferation.

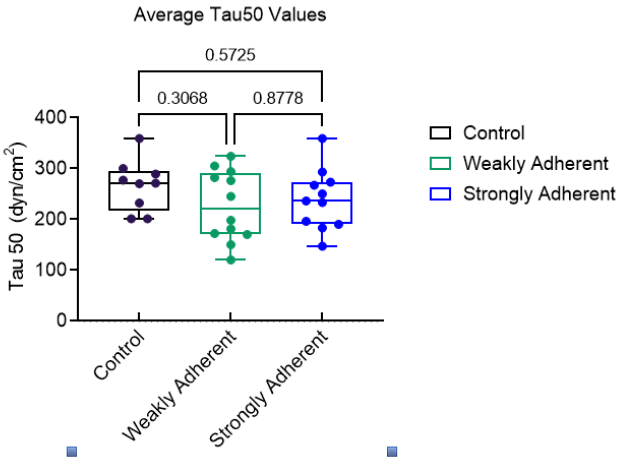
In the spinning disk assay, cells experience a radially increasing shear stress profile. As a result, cells detach from the coverslip and fall into a PBS dextrose bath, after which the number of cells remaining at each radial position on the coverslip can be determined. Since the shear stress experienced by a given cell depends on its radial position, a shear stress curve based on the percentage of cells that remain at each shear stress value can be generated. A value called the Tau50 is calculated, which identifies the shear stress at which 50% of cells have detached from the coverslip. Therefore, the Tau50 value can be used to compare adhesion strength between different cell populations. A cell population with a lower Tau50 value can be classified as having weaker adhesion strength than a population with a higher Tau50 value. The spinning disk shear assay was performed on “weakly” and “strongly” adherent populations retrieved from the PPFC assay as well as unsorted control populations to confirm that these populations truly had adhesion strength differences between them. Shear stress curves were obtained for nine sets of sorted populations. Even though some of these trials showed that the supposedly weakly adherent population from the sort had a lower Tau50 value than the supposedly strongly adherent population and the unsorted control population, this trend only applied to about 50% of the data. Further, when the average Tau50 value across multiple replicates was calculated for each population of cells, there were no statistically significant differences ($p > 0.05$) in adhesion strength between any of the groups. These results point to inherent variability within each population, meaning that each time the PPFC assay was performed using wtEGFR astrocytes, the “weakly” and “strongly” adherent sorted populations couldn’t be replicated and likely had differences that were leading to inter-sort variability in adhesion strength.

Figure 4: Results of downstream assays used to compare sorted astrocyte populations from PPFC. A: Example of shear stress curve generated from spinning disk shear assay in which the weakly adherent population from the sort demonstrates a lower adhesion strength than the strongly adherent and unsorted control populations. B: Average Tau50 values of weakly adherent, strongly adherent, and unsorted control populations. Statistically significant differences in adhesion strength were not shown between any of the populations. C: Tau50 values of weakly and strongly adherent populations across nine replicates normalized to those of unsorted control population. D/E: Cell area and major:minor axis ratio from ellipse fit of sorted populations and unsorted control populations across 3 biological replicates. Statistically significant differences were not shown across either measurement.

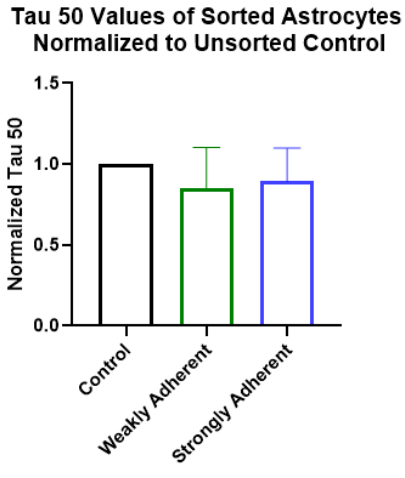
A


Shear Stress Curves
from Spinning Disk Experiments
Normalized Cell Density
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
700
800
900
1000
Shear Stress
(dyn/cm2)
Control
Strongly Adherent
Weakly Adherent

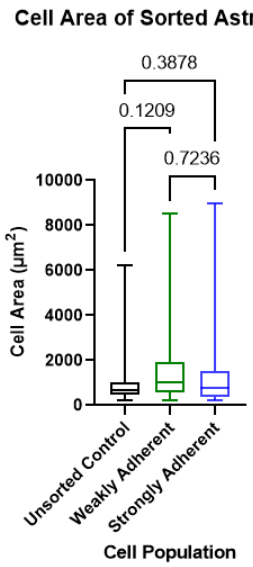
B


Average Tau50 Values
0.5725
0.3068
0.8778
Tau 50 (dyn/cm2)
400
300
200
100
0
Control
Weakly Adherent
Strongly Adherent
Control
Weakly Adherent
Strongly Adherent

C


Tau 50 Values of Sorted Astrocytes
Normalized to Unsorted Control
Normalized Tau 50
1.5
1.0
0.5
0.0
Control
Weakly Adherent
Strongly Adherent

D



E

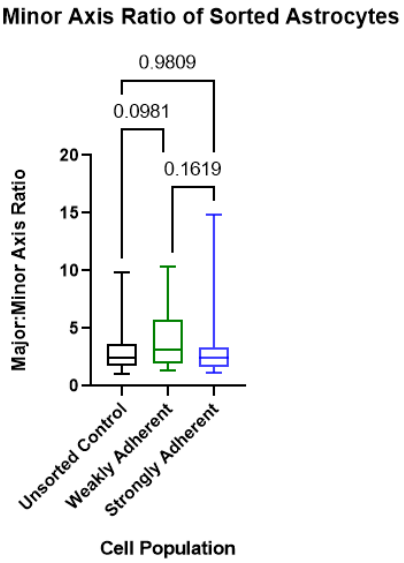


Figure 4: Results of downstream assays used to compare sorted astrocyte populations from PPFC. A: Example of shear stress curve generated from spinning disk shear assay in which the weakly adherent population from the sort demonstrates a lower adhesion strength than the strongly adherent and unsorted control populations. B: Average Tau50 values of weakly adherent, strongly adherent, and unsorted control populations. Statistically significant differences in adhesion strength were not shown between any of the populations. C: Tau50 values of weakly and strongly adherent populations across nine replicates normalized to those of unsorted control population. D/E: Cell area and major:minor axis ratio from ellipse fit of sorted populations and unsorted control populations across 3 biological replicates. Statistically significant differences were not shown across either measurement, Continued.

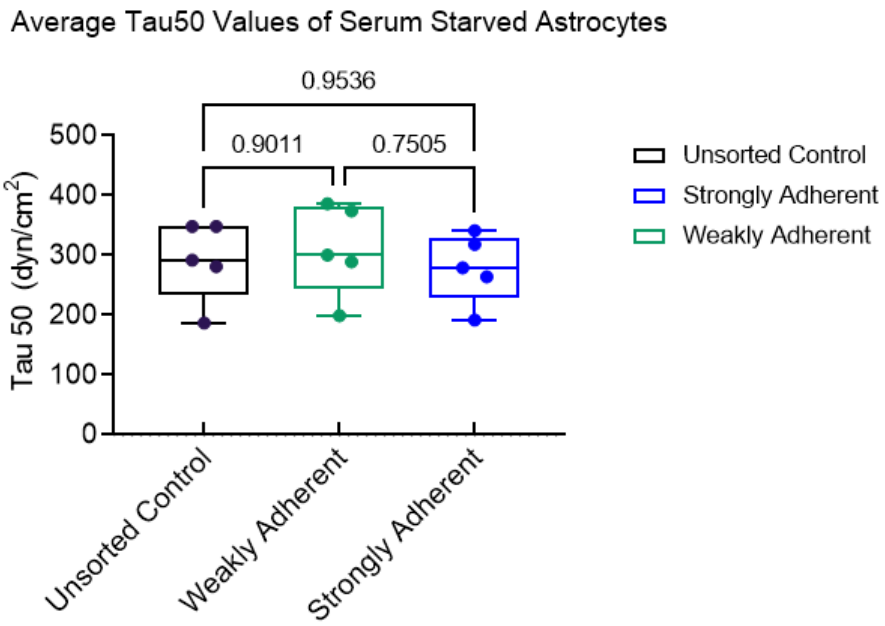
In order to identify differences between the sorted populations using another method, immunofluorescence staining was performed. By using a rhodamine phalloidin stain to stain for actin fibers in the cell, the cell area could be measured and an ellipse could be fit to the cell body in order to obtain a major:minor axis ratio measurement. Though the weakly adherent population appeared to have a larger cell area and higher major:minor axis ratio compared to the strongly adherent population and unsorted control population, the high degree of variability led to a lack of statistically significant differences ($p > 0.05$) in morphology between the populations. The variability within sorted populations across replicates in terms of morphology and adhesion strength emphasized a need to pinpoint the source of variability and identify a method to reduce it in order to study the true differences between these populations. Specifically, a method was needed to constrict variability in the cell population before it was

sorted, such that the population of cells that was sorted each time would be as consistent as possible. Since cells are likely at different phases in the cell cycle at the time that they are seeded and at the time that they are sorted, this could contribute to a variable distribution in the pre-sort population and affect various cell behaviors upon sorting such as adhesion (Jones et al., 2019). Therefore, attempting to restrict cells to a given phase of the cell cycle before they are sorted may reduce variability in sorted populations.

B. Serum Starvation of Astrocytes

In order to test whether the variable phenotypes in the sorted populations were caused by the cells being in different phases of the cell cycle before they were sorted, serum starvation was used to arrest the cells in the same growth phase before they were sorted. When cells were seeded on the glass slides, they were incubated in cell culture media containing 1% fetal bovine serum (FBS) instead of 10% FBS, which would arrest them in the in the G_0 phase of the cell cycle. After they were sorted using the PPFC, they were re-plated in cell culture media containing the normal amount of serum. When the replated sorted populations were analyzed for adhesion strength using the spinning disk assay, the inherent variability in the populations between replicates still did not seem to reduce. Even with cells being subject to serum starvation, the weakly adherent population did not consistently have a lower adhesion strength than the strongly adherent and unsorted control populations. None of the cell populations had statistically significant differences in average adhesion strength across multiple replicates. Given that regulating the cell cycle phase of the pre-sort population of cells did not reduce variability in the post-sort populations, another technique to identify reproducible differences between sorted populations was required. Since the PPFC assay didn't consistently output the high number of cells that are needed for robust results from the spinning disk assay, immunofluorescence staining to characterize focal adhesions was attempted as an alternative method to analyze adhesion differences between the sorted populations.

A



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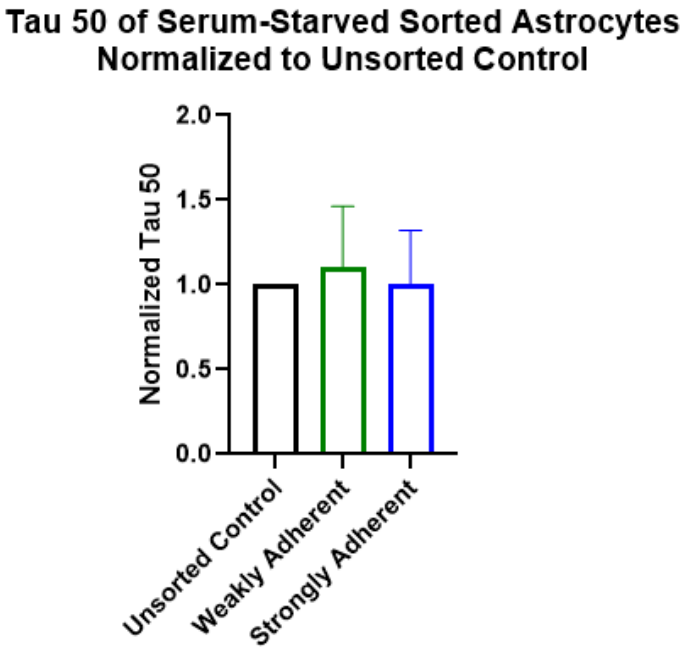
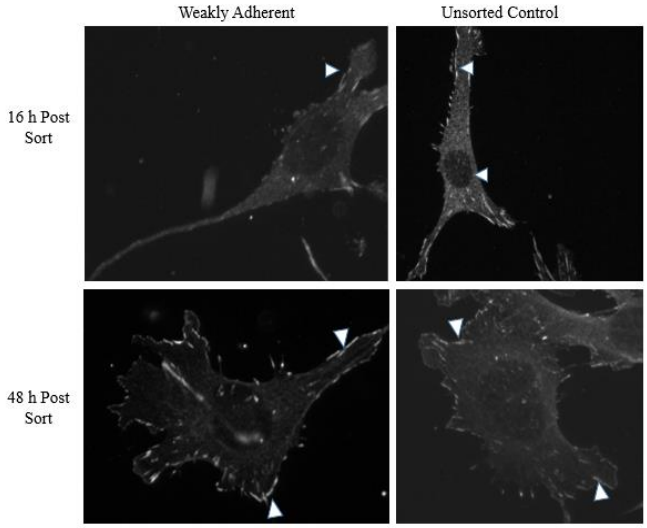


Figure 5: Results of serum starvation experiments. A: Average Tau50 values of weakly adherent, strongly adherent, and unsorted control populations that were serum starved before sorting. Statistically significant differences in adhesion strength were not shown between any of the populations. B: Tau50 values of weakly and strongly adherent populations across five replicates normalized to those of unsorted control populations.

C. Focal Adhesion Formation Analysis

As another metric to investigate the differences between sorted populations, focal adhesion formation was studied through immunofluorescence staining. Sorted cell populations and unsorted control populations were fixed and stained for paxillin, a marker for focal adhesions, either 16 hours or 48 hours after they were sorted. In addition to the 48-hour time point from previous experiments, a 16-hour time point was added in order to determine whether differences between sorted populations could be defined more clearly when cells are analyzed closer to when they are sorted. If the sorted populations truly had differences in adhesion strength, the weakly adherent population should have fewer focal adhesions per unit of cell area than both the strongly adherent and unsorted control populations. This result was seen at the 16-hour time point, in which statistically significant data showed that the weakly adherent population had fewer focal adhesions per unit of cell area compared to the strongly adherent and unsorted control populations. However, this trend was no longer seen at the 48-hour time point, since the differences in focal adhesion formation between the cell populations were no longer statistically significant.

A



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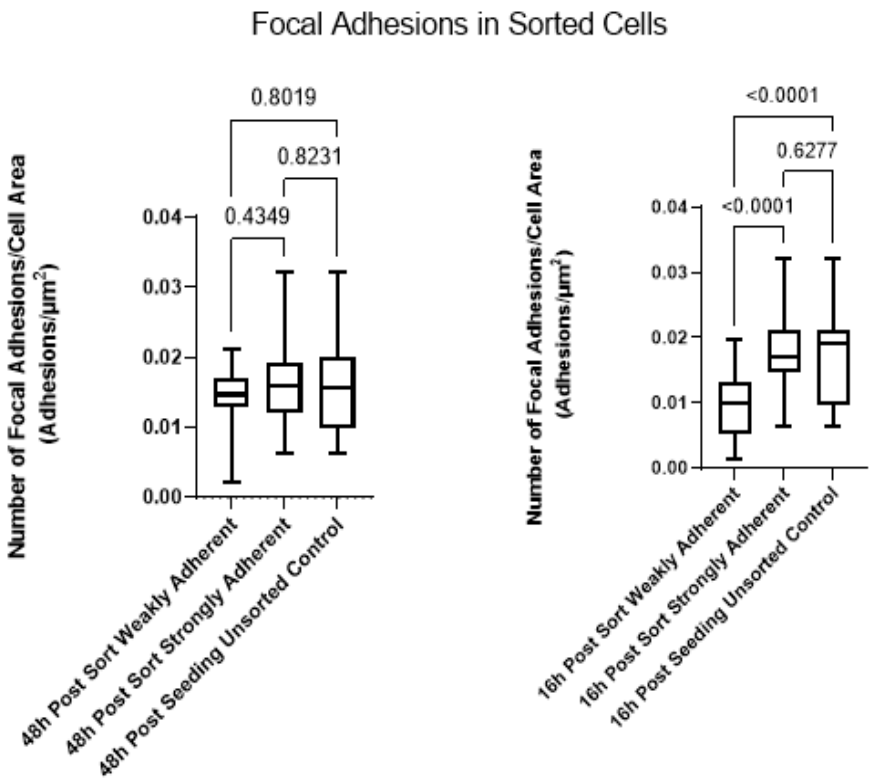

Figure 6: Analysis of focal adhesion formation in sorted cells. A: White arrows point to focal adhesions. B: The number of focal adhesions per unit of cell area is shown for each of the sorted and unsorted control populations. A statistically significant difference in focal adhesion formation between the conditions is seen at the 16-hour time point on the right, but not at the 48-hour time point on the left.

The disappearance of the statistically significant difference in adhesion strength, indicated by the number of focal adhesions per unit cell area, between the weakly adherent and strongly adherent/unsorted control populations at the 48-hour time point suggests that the phenotypic differences upon sorting are not permanent. Given that the doubling time of wtEGFR astrocytes is about 14 hours, sorted cells undergo about 1 doubling event by the 16-hour time point but almost 3.5 doubling events by the 48-hour time point. Even though there is a difference in adhesion strength between the sorted populations in the short term, this difference appears to dwindle as the cells are allowed to proliferate for longer periods of time. This observation can further be supported by performing the spinning disk shear assay at the 16-hour timepoint in order to confirm differences in adhesion strength between sorted populations at this timepoint. If differences in adhesion strength are seen, this will further prove that phenotypic differences that arise during physical selection of wtEGFR astrocytes using the PPFC are not permanent. Further studies need to be performed to determine the cause of phenotypic differences that arise during selection and the driving force behind sorted cells reverting back to their original distribution.

Discussion and Future Directions

The vast intra-tumoral heterogeneity in GBM tumors leads to aggressive phenotypes and poor prognosis in most patients. This heterogeneity often stems from the wide variety of mutations present within a single tumor. This is supported by previous studies that have shown that EGFR alterations, which are one of the most common classes of GBM mutations, can influence cell adhesion, migration, and invasion potential into healthy brain tissue. While cell lines that have various combinations of EGFR alterations and other mutations have been compared to each other in terms of adhesion strength, migration potential, and underlying causes of the aforementioned differences, it is also important to study the heterogeneity present within given cell lines in order to understand the complexity of GBM.

Using the parallel plate flow chamber, a given population of cells can be sorted based on their biophysical characteristics into weakly and strongly adherent populations. In this project, the input parameters for the PPFC were optimized such that wtEGFR murine astrocytes could be sorted into two distinct populations. In particular, a shear stress value that would allow weakly adherent cells to detach but minimize cell death to the greatest extent possible was chosen. In addition, a compatible fibronectin concentration for coating glass slides onto which cells of interest were seeded was determined. Once wtEGFR astrocytes could be sorted using the PPFC, various downstream assays including the spinning disk shear assay and immunofluorescence staining for various markers were performed to characterize differences between the sorted populations and unsorted control populations. The sorted cell populations exhibited a high degree of variability in terms of adhesion strength, morphology, and focal adhesion formation when they were analyzed 48 hours after they were sorted. However, when analyzed 16 hours after they were sorted, the sorted populations exhibited statistically significant differences in the number of focal adhesions per unit cell area between each other. This suggests that phenotypic differences within a cell population that present as a result of sorting that population based on adhesion strength are not permanent and do not carry across multiple doubling events.

Given that sorted cell populations exhibited differences in the focal adhesions they formed 16 hours post exposure to shear stress, the spinning disk assay can be performed on sorted cells after 16 hours instead of after 48 hours in order to determine whether the difference in focal adhesion formation translates to a quantifiable difference in adhesion strength. If the weakly adherent populations that are analyzed by spinning disk 16 hours after exposure to shear stress consistently have lower adhesion strength than strongly adherent and unsorted control populations, this finding will further support the conclusion that phenotypic differences within a cell population that arise upon physical selection pressure do not remain through multiple doubling events.

In addition to using the spinning disk assay to confirm phenotypic differences between sorted cell populations, the addition of arginine-glycine-aspartate (RGD) peptide to sorted cell populations before they have had the opportunity to spread and form focal adhesions on fibronectin-coated coverslips can help create artificially “weakly adherent” populations. Since RGD will block fibronectin-binding integrins, cells that are treated with RGD will form fewer focal adhesions/cell area, thereby making them more weakly adherent. Therefore, even if the weakly adherent population from sorting normally loses its weakly adherent phenotype 48 hours after the sort, the addition of RGD post exposure to shear stress will likely help maintain the phenotype for an extended period of time. This technique can help to confirm the ability of the parallel plate flow chamber to sort cells into distinct populations and perform downstream analyses on sorted populations that can maintain differences in adhesion.

By sorting a population of astrocytes with a given set of mutations into distinct groups based on adhesion phenotype, one facet of the innate heterogeneity within GBM tumors can be exposed. As shown in previous studies, the adhesion phenotype of cells can be correlated to their migration and invasion potential. In order to provide an accurate prognosis for patients with GBM, it is important to fully analyze the biophysical heterogeneity present within their tumor. As the parallel plate flow chamber assay was optimized in this study to sort astrocytes, it can be optimized to sort cells from patient tumor samples in order to uncover the adhesion signature of a given tumor. In order to fully predict how the adhesion strength profile of a tumor will affect patient outcomes, it is important to understand how these phenotypic differences arise in a cell population and how permanent these differences are. In the future, performing RNA sequencing on sorted astrocytes can potentially point to differences in the transcriptome that lead to phenotypic differences in adhesion. Further, serially subjecting astrocytes to selection pressure using the PPFC and analyzing their adhesion phenotype after an extended time period can further reveal whether phenotypic differences are permanent and lead to other phenotypic changes such as increased migration in the long term. Taken together, such analyses of

biophysical heterogeneity and the underlying mechanisms of this heterogeneity can lead to educated predictions of GBM patient outcomes.

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