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THE EXAMINATION OF INTRATUMORAL COMPRESSION AS A MECHANOBIOLOGICAL DRIVER OF GLIOBLASTOMA PROGRESSION

By

Allison McKenzie Johnson

B.S. Biomedical Engineering, University of Alabama at Birmingham, 2022

A Thesis
Submitted to the Faculty of the
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Louisville in Partial Fulfillment of the Requirements
for the Degree of

Master of Science in Bioengineering

Department of Bioengineering
University of Louisville
Louisville, Kentucky

May 2024

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A Thesis Approved on

December 12, 2023

by the following Thesis Committee:

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Nick Hawkins

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I would like to thank my family and friends for their unwavering support and confidence in my ability to achieve the things I set my mind to. I would also like to thank my mentor, Dr. Joseph Chen, for seeing my potential and helping me grow not only as a graduate student, but as a person. Lastly, I would like to thank those who helped me and collaborated with me on various projects: Landon Teer, Bradley Mahaffey, and Zach Fowler.

ABSTRACT

THE EXAMINATION OF INTRATUMORAL COMPRESSION AS A MECHANOBIOLOGICAL DRIVER OF GLIOBLASTOMA PRGRESSION

Allison McKenzie Johnson

December 12, 2023

Glioblastoma diagnosis has one of the lowest average life expectancies at only 14 months. Recurrent tumor formation and progression despite aggressive chemotherapy and radiation significantly contributes to the poor prognosis. Recurrence is likely due to mesenchymal cells breaking off from the primary tumor, infiltrating the parenchyma to establish secondary tumors. Mesenchymal cells initially undergo an epithelial to mesenchymal transition, characterized by a loss of cell-cell adhesion, increased migration and increased proliferation. While this transition can be chemically induced, there is a gap of understanding surrounding physical activation of this pathway. The tumor microenvironment is mechanically active with the cells responding to shear stress, tensile stress, and even compressive stress. Compression largely happens when the tumor sphere expands into the surrounding tissue, experiencing normal force pushing back from all directions. This force is expectantly high in the brain as the surrounding tissue is dense, increasing the normal force against the surrounding tissue. This phenomena is largely understudied due to the lack of appropriate in-vitro testing mechanisms, but could be a predominant driver in the mesenchymal behavior

responsible for secondary tumor formation. Development of a 3D system will allow investigation into forces experienced during expansion, invasion, and secondary tumor establishment. The mechanisms behind these changes in force can be studied at a biomolecular level with the development of a 2D compression system. This thesis aims to show development of these two systems, the confirmation of viability, and initial results.

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I. INTRODUCTION

1.1 Background

Glioblastoma (GBM) is a grade four brain tumor, accounting for over 50% of all brain cancer diagnoses (Omuro & DeAngelis, 2013). While prevalent, GBM also comes with a short life expectancy of just 14 months after prognosis (Mohammed et al., 2022). The survival rate beyond 5 years is currently only 5% (Mohammed et al., 2022). While cancer therapies have progressed, increasing the life span and quality of life for patients, this cancers mortality rate lacks significant improvement. Common therapy after diagnosis is surgical recission of the primary tumor, following with chemotherapy and radiation (Davis, 2016). These treatments are precarious in the brain due to the vitality of the tissue, as resecting too much or extensive tissue death due to treatment may significantly harm the patient (Davis, 2016). Initially, these therapies show promise, slowing the cancerous progression. However, the therapy's effectiveness is not retained and soon after the patient will present with secondary tumors (Mohammed et al., 2022). The establishment of secondary tumors drastically reduces the patient's life expectancy and quality of life as the cancerous tumor destroys more areas of the brain, impacting basic functions (Davis, 2016). Understanding the underlying mechanisms of invasion and secondary tumor formation can offer up novel therapeutic targets increasing both patient survival and quality of life.

1.2 Pro-neural to Mesenchymal Transition

Similar to epithelial like cancers, neural cells can exhibit metastatic behaviors which promote invasion (Manfioletti & Fedele, 2023). A widely accepted theory behind metastasis is the epithelial-to-mesenchymal-transition in primary tumor cells, causing them to adapt behaviors that are advantageous in migration and invasion (Lamouille et al., 2014). This phenomenon is seen in metastatic epithelial cancers, beginning the process of intravasation into the vasculature and eventually to a secondary tumor site (Lamouille et al., 2014). Neural cells are hypothesized to follow a similar transition mechanism, priming them for invading into the surrounding tissue (Fedele et al., 2019). This pro-neural to mesenchymal transition (PMT) is characterized by loss of cell-cell adhesion, increased migration, and increased proliferation (Lamouille et al., 2014). Research into mesenchymal transition has highlighted specific biomolecules, secreted growth factors, and metabolites in the tumor microenvironment (TME) as robust inducers of this transition (Lamouille et al., 2014). However, inhibitors of these biomolecules often fall short in *in vivo* studies to reverse the progression of GBM (De Las Rivas et al., 2021). Thus, recent work focusing on alternative means of activation has identified the biophysical compartment of the TME.

1.3 The Biophysical tumor microenvironment

While biomolecular cues are pertinent, they are not the only axis in the TME that can drive invasion, migration, and even metastasis (Pathak & Kumar, 2012). Mechanical cues including substrate stiffness, interstitial fluid flow, tension, and compression in the TME can affect the metastatic and invasive potential (Swartz & Lund, 2012) (Fan et al., 2021).

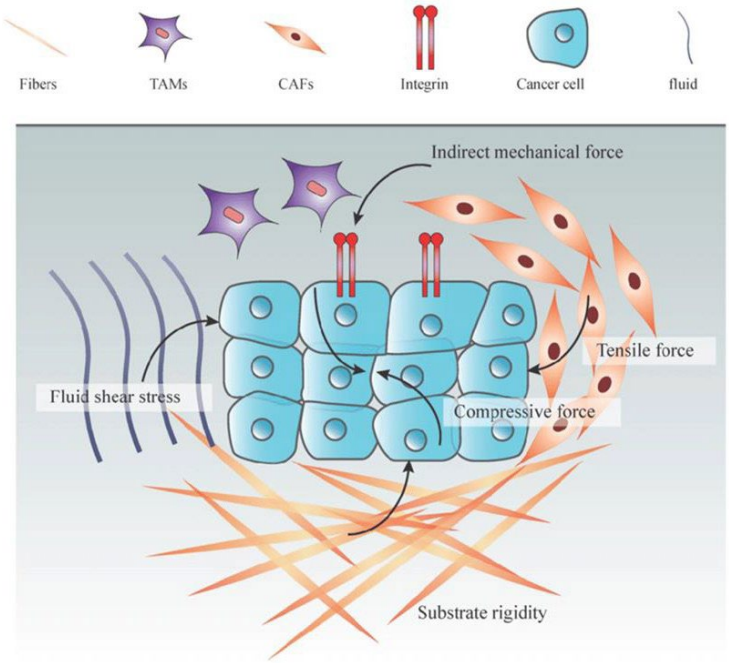

Figure 1. Mechanical Forces in the Tumor Microenvironment (Li & Wang, 2020)

The biophysical TME emerges through the aberrant action of cancer cells and often create a feedback loop that reinforces malignancy and invasion (Kharaishvili et al., 2014). Epithelial cancer types have also been shown to recruit fibroblasts, cancer associated fibroblasts (CAF), to reorganize the matrix in favor of metastatic conditions (Kharaishvili et al., 2014). The increase in interstitial fluid flow and fluid viscosity due to leaky vasculature growth is shown to drive malignant intravasation into the surrounding blood vessel network (Northcott et al., 2018). Also, the change in mechanics have been shown to regulate proliferation and migration, with a stiffer tumor microenvironment promoting metastasis and therefore induce highly migratory

cancer (Northcott et al., 2018). Biophysical forces in the TME have also shown to assist in therapy resistance and immune evasion. Solid stress in the TME and interstitial fluid flow due to leaky tumor induced angiogenesis create a barrier for T cell infiltration (Zhang et al., 2022). The reduction of adequate fluid flow also hinders the delivery of chemotherapeutic drugs (Baronzio et al., 2015). Overall, the role the TME is known to play a role in cancer survival, but the extent of which is poorly understood.

Changes in the biophysical TME in GBM is largely regulated by the cancer cells themselves instead of cancer associated fibroblasts as these cell types are not often observed within GBM (Mohiuddin & Wakimoto, 2021). This lends to the ability of the GBM cells to rearrange the extracellular matrix in tumor supportive ways. Our lab has demonstrated that the ultrastructural makeup is heterogeneous with more porous and less dense regions extending towards the invasive margin, revealing that tissue remodeling occurs to support infiltration and spread (Bradley J Mahaffey*). We and others have shown that the core is dense and has reduced porosity, which decreases the interstitial fluid flow and creates hypoxic conditions that are known to upregulate HIF1-α and promote metastatic behavior (Hockel & Vaupel, 2001).

With the growing recognition of the importance of these mechanical features, a wealth of investigations has taken place to build systems capable of recapitulating these biophysical cues. The advancements made in biomaterial science has paved the way for increasingly complex in vitro systems, including tunable hydrogels, microfluidics, and microfabricated bioengineered systems that

incorporate multiple cell types (Ananthanarayanan et al., 2011). As these platforms have begun to unravel the complex interplay between the biophysical TME and cancer cells, much of these insights have been explored in the 2D context; however, new studies begin to highlight the vast differences that exist in cell responses in 2D and 3D environments, necessitating further investigations of the biophysical cues in the 3D context (Tse et al., 2012) (Pathak & Kumar, 2012).

1.4 Intra-tumoral Compression in the Tumor Microenvironment

An emergent 3D mechanobiological driver of cancer malignancy is intra-tumoral compression. As tumors rapidly grow in the body, they inevitably will run out of space to expand, pushing against the surrounding tissue. In this 3D context, there will be a level of compressive stress generated within the tumor as the tumor continues to expand (Jain et al., 2014). Interestingly, the elevation of this compressive stress has been linked to more malignant cancer (Stylianopoulos et al., 2013). Compression on epithelial breast cancer lines showed an increase in leader cell formation, overall invasion, and rate of invasion (Tse et al., 2012). Compression is highly relevant in the brain TME as the dense tissue creates a higher normal force against the growing tumor mass than less dense tissues would. Previous studies have also shown that the core region in GBM tumors was stiffer in patients with worse prognosis, leading to the understanding the core would experience high levels of compression due to the stiff tissue (Bradley J Mahaffey*). While Tse et al. showed an increase in migration in response to compression, the paper lacked any mechanistic insight that would be applicable therapeutically (Tse et al., 2012).

Initial key findings have led to the increased investigation of mechanical forces in the tumor microenvironment. While 2D mechanical stress application is becoming more commonplace in research, there is still a lack of effective treatment. This is largely because the tumor microenvironment is a complex 3D space. The incorporation of more aspects, and therefore complexity, into the research microenvironment reduces the researchers' variable control but increases the biologic relevance. One way to maintain control while increasing biologic relevance is to utilize a 3D system. A 3D system allows researchers to observe phenomena in a setting most like the patient tumor microenvironment especially when incorporating vital ECM components and cues.

1.5 Proposed Research

The major gaps in our understanding of the emergence of intra-tumoral compression is 1) the magnitude and dynamics of compressive stress over time, and 2) the biological consequence of compressive stress on GBM cell malignancy. To address this gap, I will develop new bioengineering platforms and tools that incorporate biologically relevant physical forces that will allow for an accurate representation of the TME, enabling me to recreate an environment to observe and investigate tumor expansion *in vitro* and study the effect of compression in a controlled setting. To investigate the magnitude of compression, a 3D hyaluronic acid (HA) system composed of a matrix that mimics brain tissue in structure, chemical makeup, and mechanical properties will be used in conjunction with alginate beads embedded in a tumorsphere.

Within this setup, I will be able to monitor bead deformation over time and extract the applied stresses through finite element analysis. To study, the biological consequence of compression, a 2.5D system incorporating transwells and 3D printed compression application tools will be used to mimic the compressive strain seen in patients and allow for mechanistic exploration of the biological consequence of compression and the identification of novel therapeutic targets for GBM therapy. In summary, my thesis aims to develop *in-vitro* systems that are biologically relevant to allow for the phenomenological and mechanistic study of compression driven malignant nature of GBM cells.

Cell lines were chosen to represent both an epithelial and mesenchymal nature. U87s (sourced) proliferate rapidly and are highly migratory, exhibiting more mesenchymal behaviors, while U251s (sourced) are slower to proliferate and migrate, exhibiting more epithelial like behaviors. The incorporation of both cell lines allows for evaluation of the effect of compression on cells primed and not primed for migratory behavior and compare the degree to which compression effects mesenchymal behavior in both.

II. TUNABLE 3D HYALURONIC ACID-METHACRYLATE PLATFORM

2.1 Background

The brain ECM largely consists of the glycosaminoglycan (GAG) hyaluronic acid (HA), chondroitin sulfate proteoglycans, heparan sulfate proteoglycans, and glycoproteins (Gupta et al., 2019). HA has vital bio-physical and bio-chemical properties for brain ECM including high viscoelastic properties and high moisture retention (Yasin et al., 2022). HA can bind to both ECM molecules and cell surface receptors, making it a key factor in matrix and cell process regulation (Jensen et al., 2020). Specifically, brain ECM comprises of high levels of high molecular weight HA, which allows for high moisture and protein retention (Yasin et al., 2022). HA is synthesized by hyaluronan synthases at the cell membrane and broken down by hyaluronidases (Wolf & Kumar, 2019). HA can be modified chemically with functional groups (hydroxyl, carbide, and amine) increasing its capacity to bind to crosslinkers and substrate proteins (Spearman et al., 2020).

The biophysical properties are influenced by HA molecular weight, percent HA, and incorporation of additional matrix factors like link proteins or proteoglycans (Nishimura et al., 1998). The molecular weight of HA differs per tissue type and function. Low molecular weight is prominent in synovial fluid and acts as a lubricant in joints (Wolf & Kumar, 2019). High molecular HA is largely produced by HAS-2, high levels of which have been shown to correlate with

worse prognosis, and has anti-inflammatory and homeostatic properties (Wolf & Kumar, 2019) (Valkonen et al., 2018). Increase in HA density in the ECM increases the potential binding sites, therefore increasing the overall stiffness of the substrate (Ananthanarayanan et al., 2011). In the brain, link proteins and chondroitin sulfate proteoglycans bind together with tenascins to form an HA matrix (Nishimura et al., 1998). High amounts of tenascins in gliomas are correlated with a worse prognosis and shorter life expectancy (Cai et al., 2018). Artificial methods of HA binding, i.e., methacrylate and a crosslinker, are commonplace in research instead of tenascin/proteoglycans due to the tunability of both chemically (Ananthanarayanan et al., 2011).

HA is biologically active in the TME through binding via certain receptors. HA binds to cells via the CD44 and RHAMM complexes, linking the ECM with the cellular cytoskeleton, inducing a mechanotransducive response (Cooper & Giancotti, 2019). CD44 specifically is highly overexpressed in cancerous and highly metastatic cells, increasing the binding capacity with the extracellular matrix and contributing to the increased motility also seen in these cells (He et al., 2018). CD44/HA binding can promote the MEK/MAPK cascade, promoting rapid proliferation. HA binding can also promote integrin signaling (Chen et al., 2018). Integrin binding to substrate activates the FAK pathway, increasing intracellular binding to the actin cytoskeleton (Chen et al., 2018). This mechanotransductive pathway is shown to influence cytoskeletal reorganization, shown through distinct phenotypic changes in cells based on changes in

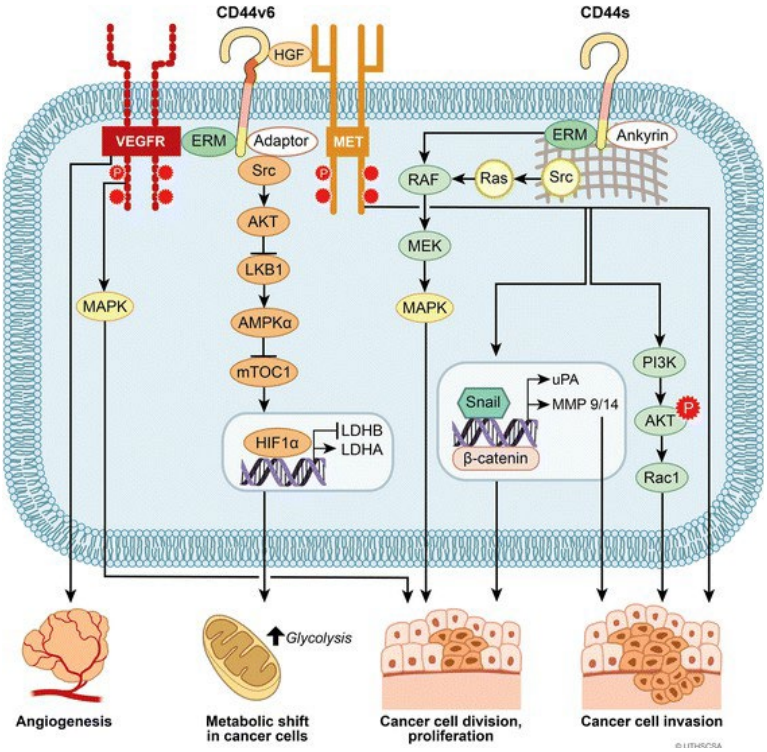

Figure 2. CD44 protein cascade and inducible effects (Chen et al., 2018).

substrate stiffness (Rens & Merks, 2020). An increase in cell receptors and therefore mechanotransduction can also increase the HAS, hyaluronidase, and subsequent matrix remodeling (Wolf & Kumar, 2019). Matrix remodeling may contribute to the overall heterogeneity in the tumor sphere and promote conditions that drive PMT, and eventually invasion.

2.2 Alginate Bead Force Probe

Additionally, the HA-Me-RGD hydrogel system can be used to quantify forces in 3D tumor expansion and invasion. Alginate beads of 10-20 μm diameter

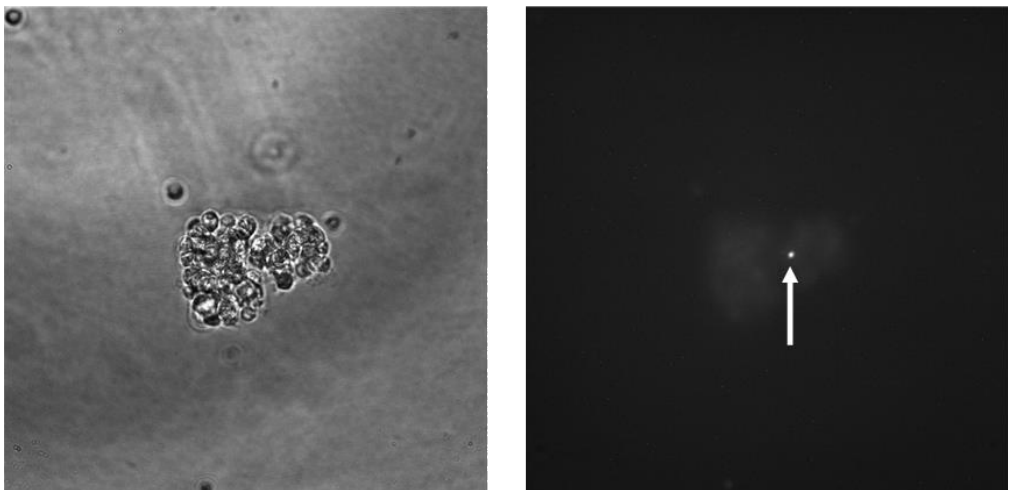

Figure 3. Glioblastoma stem cell spheroid (left) and the alginate bead encased in the spheroid (right).

were made using a microfluidic device designed by Zach Fowler (Fowler, 2023). The beads were in culture with glioblastoma stem cells for 5 days prior to incorporation into the HA-Me-RGD hydrogel of a 1 kPa stifness. The cell masses

with incorporated beads were imaged at various timepoints in brightfield and AlexaFlour 488. Z stacks every 1 μm for 20 slices were taken of each bead and a corresponding brightfield image of the cell mass. Images of both the cell mass and the bead central to the cell mass are shown via Figure 3.

2.3 Hyaluronic Acid Methacrylate

To create a biomimetic system, a hyaluronic acid gel base with additional macromolecules embedded to promote cell attachment and invasion potential was needed. Hyaluronic Acid Methacrylate of 100 kDa was chosen. The methacrylate group binds to the hydroxyl group of the hyaluronic acid, creating a double bonded carbon, a site for crosslinker binding (Tsanaktsidou et al., 2020). Each thiol group on the cross linker, Dithioerythritol (DTT), binds to the carbon group through a Michaels Addition Reaction (Lopez-Ruiz et al., 2019). Due to the dual thiol of DTT, this happens at both ends, tethering chains of HA-Me together to form a matrix (Lopez-Ruiz et al., 2019).

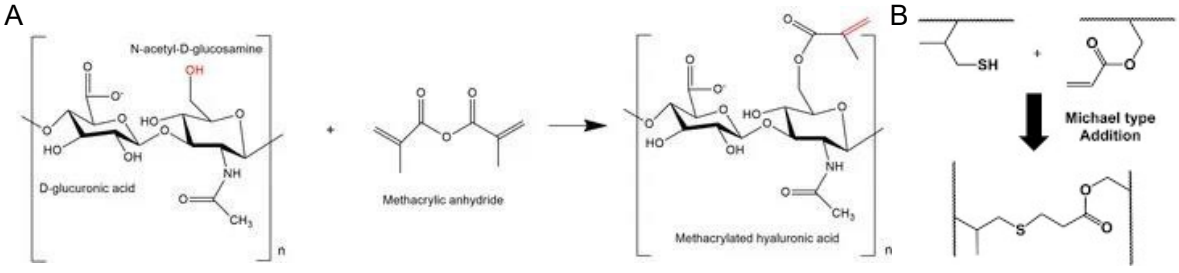

Figure 4. The methacrylate anhydride addition to hyaluronic acid (A) and the Micheals addition reaction by which DTT binds HA (B) (Tsanaktsidou et al., 2020) (Bustamante-Torres et al., 2021).

The density of HA-Me and thiol ratio can both be increased or decreased to control the amount of binding, and therefore the stiffness. This gel also incorporated Arginylglycylaspartic acid (RGD), a common adhesive peptide (Bellis, 2011). The RGD promotes cell adhesion but has no apparent effect on the mechanical aspects of the gel.

2.4 Evaluation of Principle

2.4.1 Accurate Tunability

To accurately mimic the stiffness of tissue as seen from patient data, the system must be tunable. The thiol ratio and percent HA can be changed to vary the stiffness. The thiol ratio increases the instance of bond between HA molecules, so increasing the thiol ratio, the percent HA or both will increase the stiffness of the hydrogel. Increasing the HA-Me density will also increase the instance of bond and create a more viscous gel. Previous studies have shown expected stiffnesses based off the density of HA-Me-RGD and the thiol ratio. These values were confirmed via atomic force microscopy to a previously established HA-Me-RGD hydrogel system (Ananthanarayanan et al., 2011).

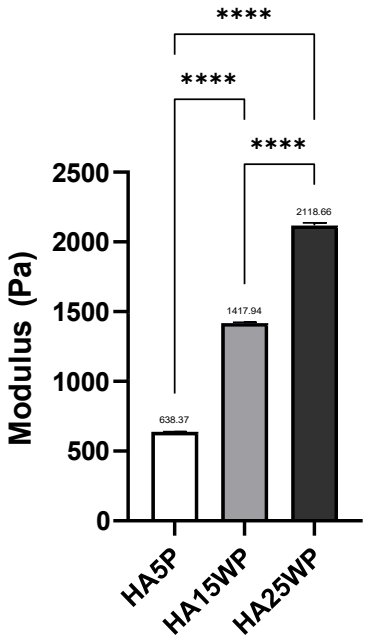

Figure 5. Modulus in Pascals of HA-Me-RGD hydrogels of varying HA weight percents.

2.4.2 Increased Biologic Relevance

This data shows the increased cell motility through velocity and persistence. U87s were seeded on a polyacrylamide gel with a stiffness of 500 Pascals, a tissue culture treated plastic plate with soluble low molecular weight HA, a tissue culture treated plastic plate with soluble high molecular weight HA, and a high molecular weight HA-Me-RGD gel. The cells were imaged over 24 hours at 15-minute intervals.

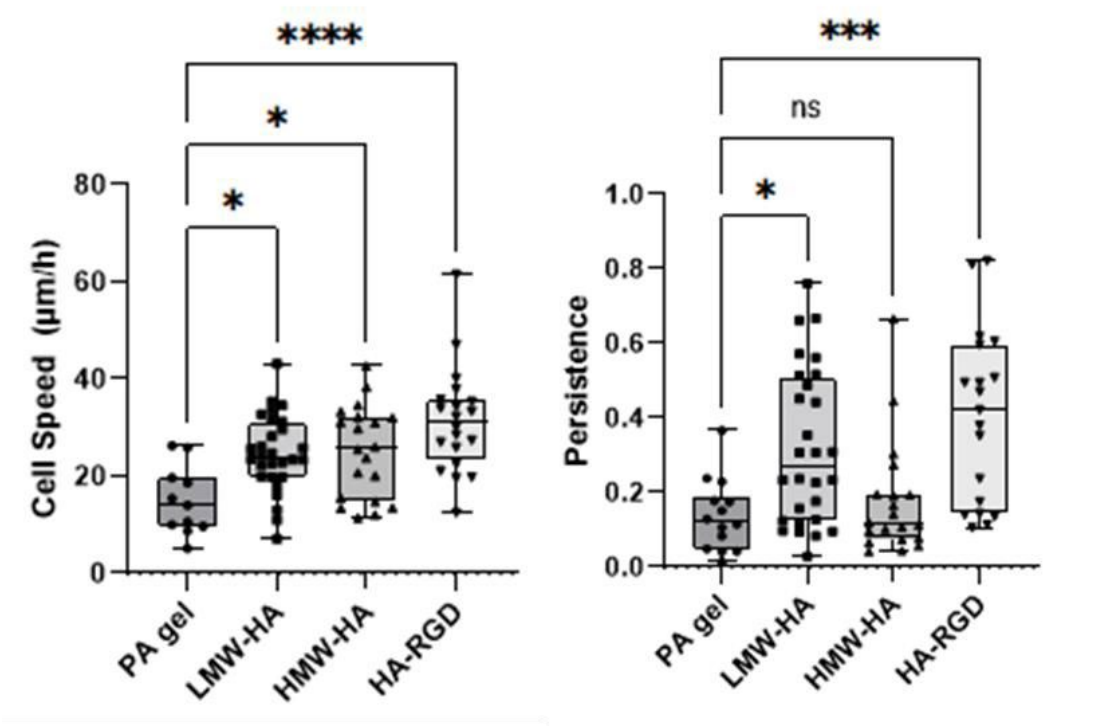

Figure 6. Cell velocity and persistence in varying HA conditions.

Velocity and persistence were quantified using the manual “cell-tracking” feature in ImageJ. Higher velocity and persistence were seen in the HA-RGD gel when compared to the PA gel of the same stiffness. This indicates the binding between the cells and the HA biomaterial is influential in migration capacity and contributes to mesenchymal nature. Higher velocity and persistence were also seen in the bound HA compared to the soluble conditions. This indicates that HA binding promotes motility, but it is most influential in a bound setting. The findings suggest that cell binding to HA plus the mechanical interaction of cell and substrate is key to promoting migratory behavior. The significant difference in cell

behavior between the HA and PA gel, and the bound and soluble HA exemplifies the importance for using biomimetic materials, as they will influence cell behavior.

2.4.3 Glioblastoma Stem Cell 3D Invasion

HA-Me-RGD gels were made at 1 kPa, representative of diseased brain tissue. Six Glioblastoma Stem Cell lines (GSC), 3 mesenchymal and 3 epithelial, were cultured for 5 days to form spheroids then imbedded in HA-Me-RGD hydrogels with a modulus of 1 kilopascal (MD Anderson). Each line was placed into 4 separate gels and 3-5 spheroids per gel were tracked. The spheroids were

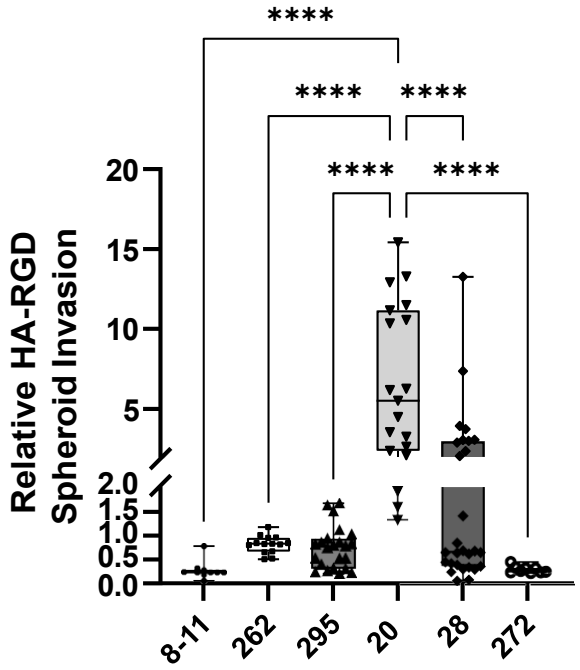

Figure 7. Relative GSC invasion into 1 kPa stiff HA-Me-RGD hydrogel.

imaged every seven days and overall expansion from day 1 to day 14 was quantified via ImageJ.

The three epithelial GSC lines showed little expansion, but the 3 mesenchymal lines showed significant invasion. The epithelial lines likely did not expand to the extent the mesenchymal lines did because they are not primed in a mesenchymal nature needed to survive, and invade, the dense hydrogel. However, this data does highlight the relationship between cell phenotype and matrix stiffness. This also confirms the cell lines ability to invade the dense gel, supporting the potentiality of quantifying the expansion and invasion forces with the use of the alginate force probes.

III. 2D COMPRESSION SYSTEM FOR MECHANISTIC TESTING

3.1. Background

Compressive stress is shown to be relevant to the tumor microenvironment, increasing migration and leader cell formation in epithelial breast cancer lines (Tse et al., 2012). The utilization of a 3D assay allows for more accurate phenomenological study but does not allow for mechanistic exploration. This approach can be followed with common molecular testing such as: Migration assays, invasion assays, qPCR, Western Blots, Immunofluorescence, etc. The creation of a 2D compression system that can recreate the force in the 3D assay, but allowing for biologic assessment, is pertinent for translating findings to a therapeutic use. This approach shows a novel system that uses weights to compress cells at a tunable pressure, while maintaining sterility and cell viability.

3.2. Design

3.2.1 Concerns & Constraints

The design is centered around maintaining cell viability and sterility. The design should be reusable and sterilizable. The design should be able to mimic both compressive stresses seen in healthy tissue and in diseased tissue, as well as the forces seen in the 3D force probe assay. The compression should be quantifiable and reproducible.

3.2.2 Design Iterations

Initial designs are as shown. The use of a hydraulic press was used in previous similar designs, but implementation is expensive and reduces the number of conditions that can be compressed at one time. A design with the agarose and compression system atop a monolayer of cells seeded on tissue culture treated plastic was not used due to the concern at the diffusion of media from the outer edges into the middle, possibly creating a necrotic core, skewing potential biomolecular assays. An additional system was considered using a transwell with a weight atop the entire six well plate, compressing a piston on to the cells. While advantageous because any manner of weight could be applied, concerns about equal weight distribution across the plate required a system redesign.

3.2.3 Fabrication

Following the design constraints, a system including a transwell, a 3D printed nylon cup, and tungsten carbide discs was sourced. To satisfy the condition to retain cell viability, a transwell was used. The bottom membrane on which cells sit is approximately 10 μm thick with 0.4 μm pores, allowing for nutrient exchange between the cells and the media opposite the transwell membrane. This ensures all cells receive access to vital nutrients in the media. A 2% agarose gel is used to ensure compression around the entire top surface of the cell, not just the topmost points of the cell, and help diffuse the force across the entire cell monolayer. A 3D mold of 1 mm tall wells with a 24 mm diameter indents was made (SolidWorks) to create agarose discs. The mold was printed

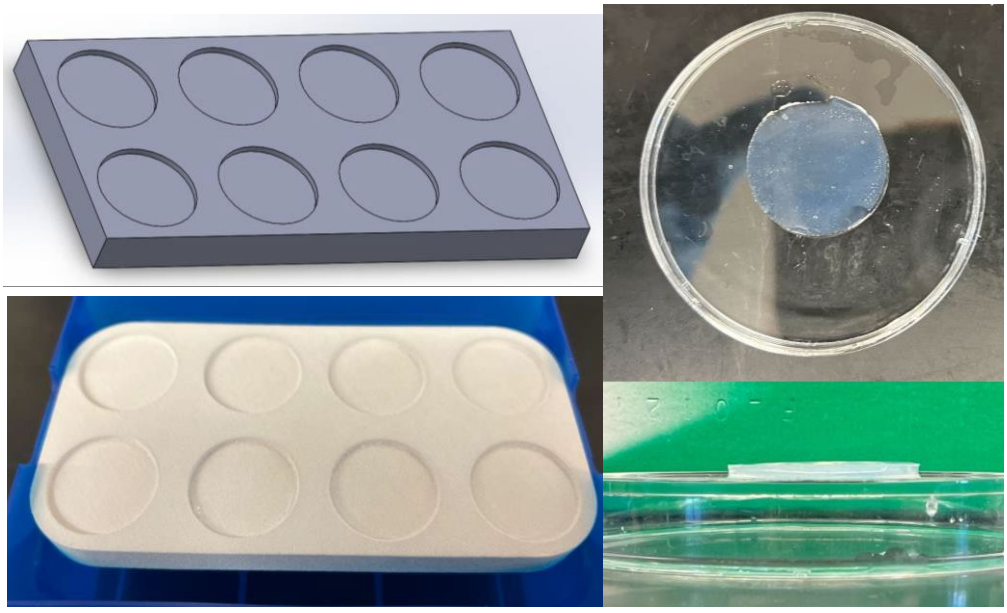

Figure 8. The rendering and print for the agarose mold and the top and side view of the resulting agarose.

by the University of Louisville Additive Manufacturing Institute of Science and Technology.

The nylon cup was designed to house the tungsten carbide, as to reduce the risk of metal leaching due to the moist environment. Nylon was chosen due to

Figure 9. Nylon cup rendering, top view of print, and side view of print atop agarose.

its resistance to erosion when sterilized using traditional methods, and its biocompatible properties (Chen et al., 2021). The size was chosen so that it was the largest that would still fit flush on the bottom of the transwell, with the height

not protruding past the well height of a standard 6 well plate. The cups were printed by the University of Louisville Additive Manufacturing Institute of Science and Technology.

Tungsten carbide was chosen due to its density. The high density allows for high amounts of weight, and subsequently pressure, to be applied given the small space, to maximize compression. The discs radius was determined based on the internal area of the housing cup. The diameter of the cup was determined to be 22 mm in diameter, allowing for only a 4.15 cm^2 of area on which force can be applied, which limited the amount of stress that could be applied. Given the need to make 1 kPa of pressure, 0.001 N/m^3, the force needed was back calculated via Eq 1. The weight needed was calculated from the required force. This presented a challenge as there was little space for a material that had to be heavy enough to cause high amounts of compression. In researching materials, tungsten carbide was chosen due to its density. The required weight was then used to calculate the volume needed per disc to elicit 200 Pascals of force (Eq. 2). The height was selected to vary as the radius of the disc was confined by the cup.

Eq. 1. $$\sigma = \frac{F}{A}$$

Eq. 2. $$Height = \frac{Weight}{(Density * \pi * r^2)}$$

Each disc is 1.5 mm tall with a diameter of 22 mm, weighing approximately 7 grams with a standard deviation of 0.02 grams. Due to this size, each disc adds approximately 200 Pascals of stress onto the cell monolayer, creating a range of 200-1600 Pascals of *in-vitro* compression. The entire system is depicted in Figure 6.

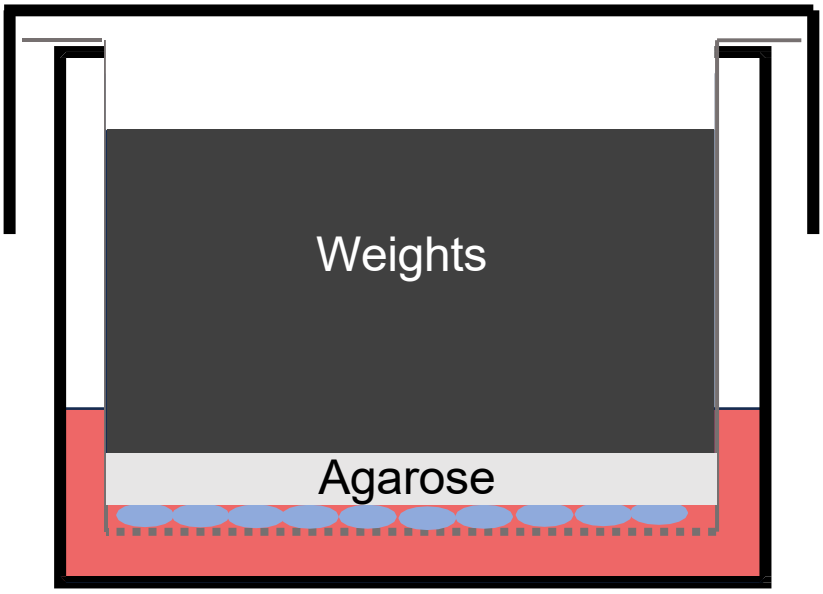

Figure 10. Schematic Representation of 2D compression system.

3.3. Results

3.3.1 Finite Element Analysis

The analysis was done via SolidWorks static simulation. The dimensions for the transwells were taken from the distributor's website. The nylon material described in the SolidWorks software was used for the transwell. The silicon described in the SolidWorks software was used in replacement for agarose due to the similar elastic mechanical properties. A singular mass of 35 grams was

used to represent the weight system. The analysis showed uniform distribution of applied pressure across the surface of the transwell, indicating the entire cell monolayer will experience the same force.

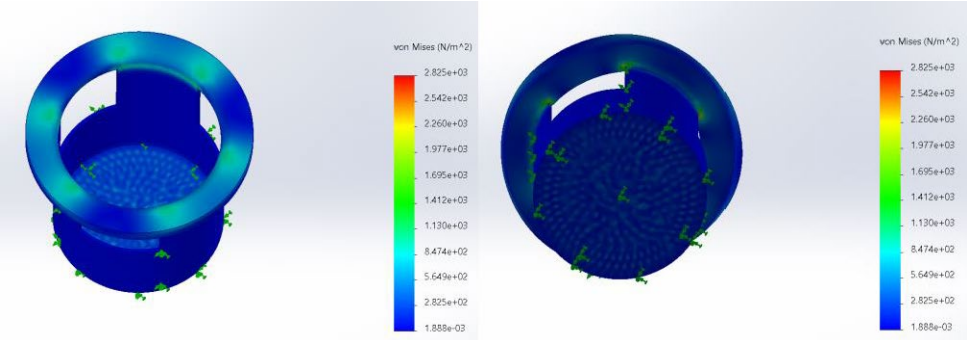

Figure 11. Finite Element Analysis of the Distribution of force on the transwell membrane.

3.3.2 Optimization

The U251s were seeded at 200,000 per well and left for 24 hours to ensure attachment to the transwell membrane. Two milliliters of media were added below the transwell, and the media atop the cells in the transwell was removed. For the non-treated conditions, an agarose gel was placed atop the cell monolayer in the transwell. For the compressed conditions, agarose, and a cup with either 3 discs or 5 discs was placed atop the cell monolayer. Each condition was compressed for 24, 48, and 72 hours. After the indicated timepoint, the cells were fixed with 10% Formalin 1% Triton. After the longest timepoint was fixed, the transwell membrane was cut from the plastic form and stained for actin

(Cytoskeleton, #PHDG1-A). The transwells were then imaged at 10X via Confocal Imaging.

The transwells were imaged at the same fluorescent intensity across each condition. Figure 7 shows a significant increase in phalloidin actin stain, indicating a larger presence of actin after 1 kPa of compression over 24 hours.

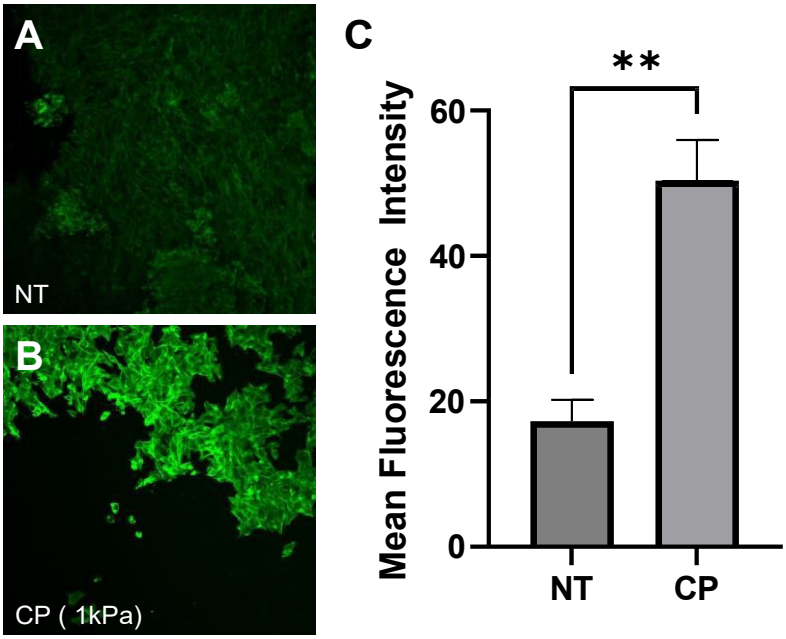

Figure 12. Fluorescent imaging of phalloidin on U251s.

The results also indicate a larger lamellipodial protrusions after compression, suggesting the cells are reacting to the compressive stress.

IV. PRELIMINARY RESULTS

4.1 2D Biomolecular Investigation

The U251s were seeded on the transwells for 24 hours prior to 2 milliliters of media being added to the plate under the transwell. After 24 hours, the media is aspirated, and appropriate equipment is placed atop the cell monolayer.

4.1.1 Motility

The cells were left for 48 hours prior to removing the equipment atop them. The cells were then lifted with a Trypsin/EDTA mixture and reseeded on a tissue culture treated plastic dish for 12 hours. The cells were then imaged at 15-minute intervals over 24 hours.

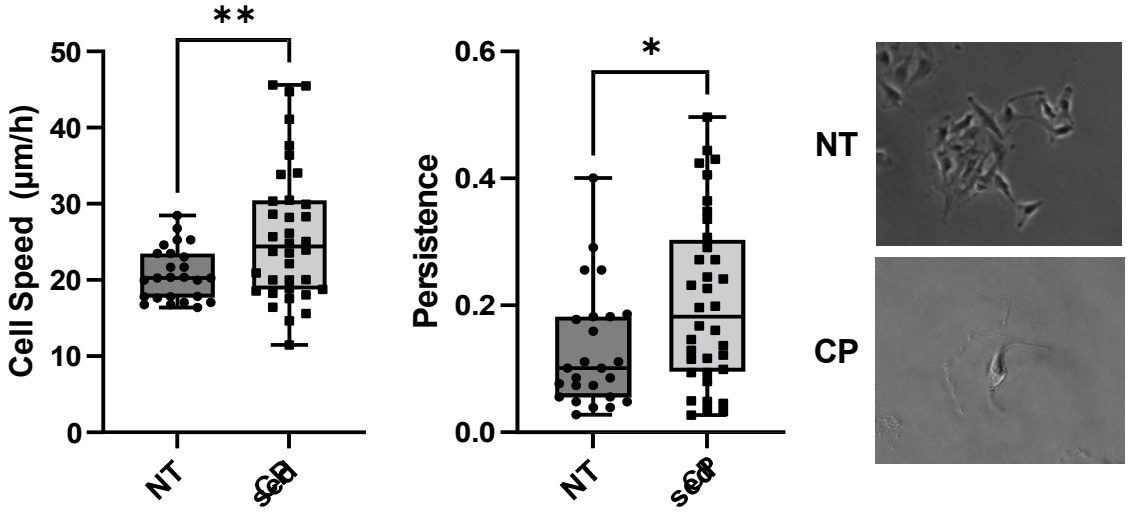

Figure 13. Cell Speed and Persistence after 48 hours of compression.

The non-treated cells proliferated steadily but were largely non-motile. The cells remained in contact and grew as previously observed. The compressed cells were highly migratory, showing a significant increase in cell speed and

persistence. Notable changes in cell behavior and shape were seen as there was little cell-cell contact and large lamellipodial protrusions. This change is indicative of an increased reliance on matrix interactions over cell-cell interactions, suggesting a more mesenchymal phenotype.

4.1.2 Circularity

The media was aspirated from atop the cells, and the non-treated condition was covered in agarose while the compressed condition was covered in agarose, the cup, and 5 discs putting the cells under an approximated 1 kPa of compression. The cells were left for a respective timepoint of 24 or 48 hours before being lysed with 10% Formalin 1% Triton. The membranes were then stained for Dapi, a nuclear stain.

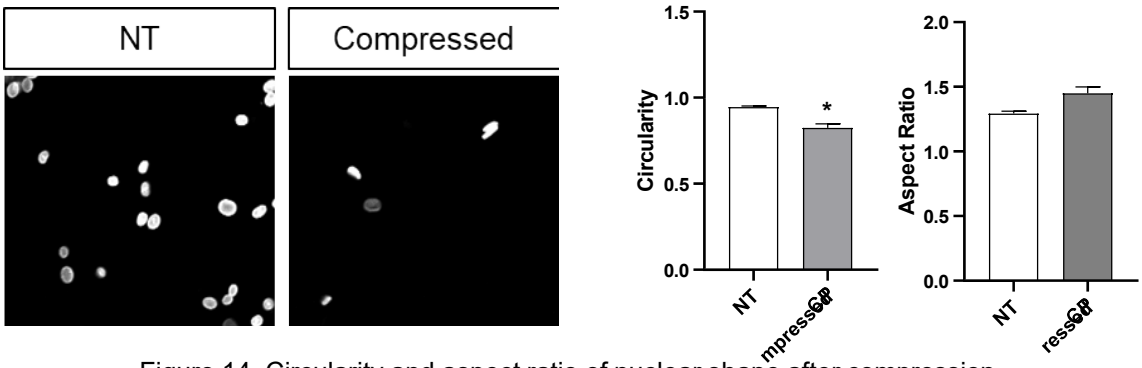

Figure 14. Circularity and aspect ratio of nuclear shape after compression.

The results showed a significant decrease in circularity after 48 hours of compression under 1 kPa of pressure. The increase in abnormal nuclear shape indicates the cells are directly experiencing the compression. The aspect ratio increase in the compressed condition suggests nuclear spreading due to the direct compressive stress.

4.1.3 Western Blot

After 72 hours of compression, the cells were lysed with RIPA and 10% Protease Inhibitor. The protein concentration was determined via BCA analysis, and the lysates were run at 20 ng per well in 10 well 12% Acrylamide gel. The blots were imaged via Olympus imager and densitometry was performed with ImageJ.

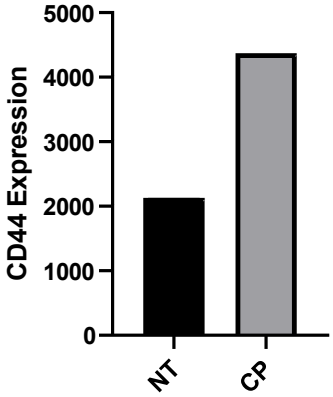

Figure 15. Relative Protein levels of CD44 after compression.

Protein analysis showed a general increase in CD44. The increase in CD44 in the absence of HA suggests the cells are reacting in a mechanoresponsive way.

4.1.4 Immunofluorescence

After 72 hours, the cells were fixed with 10% Formalin 1% Triton. The cells were then stained for Dapi and Zeb1. All conditions were imaged at the same fluorescent exposure and intensity via Confocal Microscopy. The fluorescent intensity was evaluated via ImageJ's.

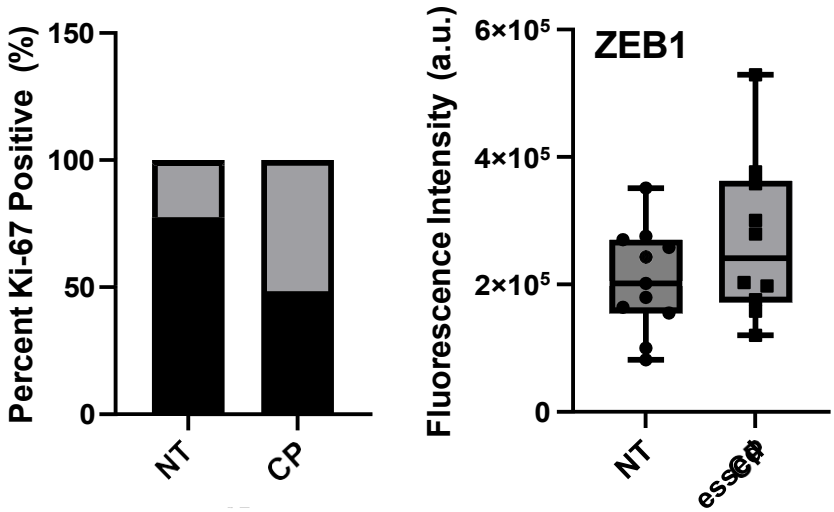

Figure 16. Fluorescent localization and FTIC of U251s after compression.

The fluorescent intensity of Zeb1 was significantly higher in the U251s that were compressed for 48 hours compared to those who were not compressed. The increase of this transcription factor typically upregulated in mesenchymal type cells indicates the early stages of PMT. The lower amounts of Ki-67 indicate reduced proliferation in the compressed condition, but in conjunction with the motility data shown, may indicate a shift in energy use towards migration.

V. CONCLUSION

The devices described offer exploration into the mechanical forces at play in the tumor microenvironment and the consequent cellular response. Each maintains cell viability and meets the design criteria described. The 3D system allows for use when evaluating specific tissue displacement and the force intratumorally during expansion and invasion. The 2D system then allows us to replicate the forces seen in 3D and explore the cellular mechanisms by which a cell survives and adapts in this environment. While beneficial, this system has some limitations. The 6 well system reduces the sample size available, only producing an “n” number of 3 per run. However, the sample size can be increased with the addition to more cups and weights as needed. This system will be used in conjunction with the alginate bead force probes to explore the forces intra- and extra-tumoral during expansion and invasion in hydrogels mimicking diseased tissue. The 2D compression system will be used to further explore the mechanism by which nuclear shape, and other potential cytoskeletal organizations, change in response to increased compression. Understanding the mechanism by which compression promotes invasive potential will allow for the development of therapeutic targets that reduce the invasive potential, reducing secondary tumor formation.

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