Evaluation of Triple Negative Breast Cancer Tumor Matrix

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by

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Undergraduate honors thesis under the direction of

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Abstract –

Despite recent advancements in therapy, breast cancers still fail to respond to therapy or re-emerges after a period of dormancy. Most breast cancers have targeted therapy for endocrine receptors (ER & PR) and or growth factor receptors, HER2. Thus, there is a molecular target that can help inhibit cancer growth. Triple Negative Breast Cancer (TNBC) however, is negative to all these receptors resulting in no treatment from hormone therapy and leading patients to receiving chemotherapy which is known for its co-morbidities and can result in patient mortality. Only 20% of patients respond to any therapy. As a result, there is a critical need to understand what mechanisms result in TNBC drug resistance. Prior research done in our group showed that TNBC has a unique micro tumor environment unlike the other breast cancer subtypes both in its architecture and composition. Thus two different studies were done to observe what these unique characteristics are. Prior studies have shown that breast tumors have fibers that are randomly aligned. Scanning electron microscope (SEM) was used to analyze multiple breast tumors to magnify the topography of the tumor. Through computational analysis of the SEM images, the data demonstrated fiber alignments trends and suggests that a more accurate representations for breast cancer 3D models are random fiber orientations. Another study was done to further understand the composition of the tumor’s microenvironment. This was done by researching Microfibril-associated protein 2 (MFAP2), an extracellular matrix protein that interacts with fibrillin to moderate the function of microfibrils. Prior studies show that MFAP2 protein expression is upregulated in different cancers and correlates to poor patient outcomes. Despite this, the underlying role of MFAP2 in breast cancer has remained unclear. The current study investigates the expression pattern of MFAP2 in triple-negative breast cancer cells and evaluates the effect of MFAP2 overexpression in triple-negative breast cancer (TNBC). Results demonstrated that MFAP2 was overexpressed in the MDA-MB-231-MFAP2 overexpressed line
compared to the breast cancer cells with the control vector (pCMV). Following confirmation of MFAP2 overexpression, proliferation studies were performed to observe how MFAP2 overexpression alters cellular proliferation and Survival studies with chemotherapies (taxol and doxorubicin) were performed to test the effects of MFAP2 on survival.
Acknowledgements

I would like to acknowledge Dr. Martin and my lab for the immense support. Meggie Lam (Research Technician) and Jonathan Savoie (PhD graduate student) have been important to me during my research as they have guided me and helped me throughout experiments and helping retrieve papers. I would also like to thank the LSU MARC and McNair program for all the support and advice they have provided me. They have been a pivotal part of my LSU experience and have helped me love research even more. I am thankful for my boyfriend and friends as they have been with me throughout my college experience. Finally, I would like to thank my family for always believing in me and shaping me to become the woman I am today.
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Figure 10. **MFAP2 does not alter markers for cell senescence.** qRT-PCR for Cell Senescence markers expression in MDA-MB-231 cell line. Housekeeping gene was beta-actin and normalization was to MDA-MB-231-pCMV-vector transfected cells.

Figure 11. **MFAP2 does not alter markers for Cancer Stem Cells (CSC).** qRT-PCR for CSC markers expression in MDA-MB-231 cell line. Housekeeping gene was beta-actin and normalization was to MDA-MB-231-pCMV-vector transfected cells.

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**Introduction**
A healthy person’s cells normally undergo a regulated cell cycle in which cells grow in a controlled manner. When this process becomes impaired, cells begin to divide and grow out of control; if left unchecked, this can lead to tumor formation. 1 in three people in the United States are affected by this disease (cancer.org). Breast cancer is a malignancy that commonly begins at the breast’s milk ducts or lobules (komen.org). In the United States, about 1/8 women will develop breast cancer over the course of her life (Breastcancer.org). In the past year (2021), 287,859 new cases of breast cancer in women have been identified with 43,250 women dying from the disease (komen.org). Patients are diagnosed on the subtype of breast cancer based on factors such as tumor morphology, tumor size, and its molecular expression of estrogen receptor (ER), progesterone receptor (PR), Erb-B2 Receptor Tyrosine Kinase 2 (HER2) protein (Fragomeni et al., 2018). Despite the common tissue of origin, breast cancer can be categorized to four molecular subtypes based on the genes expressed in breast cancer cells: Luminal A breast cancer, Luminal B breast cancer, HER2 Enriched breast cancer, and Triple-negative breast cancer (TNBC). These breast cancer subtypes are designated based on their molecular expression (Fragomeni et al., 2018) (Table 1).

<table>
<thead>
<tr>
<th>Molecular Subtype</th>
<th>Hormonal Status</th>
<th>HER2 Presence</th>
<th>Incidence Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER +, PR -/+</td>
<td>-</td>
<td>75%</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER +, PR -/+</td>
<td>-/+</td>
<td>10%</td>
</tr>
<tr>
<td>HER2+</td>
<td>ER -, PR -</td>
<td>+</td>
<td>5%</td>
</tr>
<tr>
<td>TNBC</td>
<td>ER -, PR -</td>
<td>-</td>
<td>12%</td>
</tr>
</tbody>
</table>

**Table 1. Molecular Subtype Descriptions** (Rutkovsky et al., 2019).
Luminal A and Luminal B have a better prognosis than the other breast cancer and can be treated with targeted endocrine therapy (breastcancer.org). HER2 breast cancer tend to grow faster than the luminal cancers thus, having a worse prognosis. HER2 amplified breast cancer can be treated with targeted therapy that inhibits the HER2 receptor (breastcancer.org). Because TNBC is negative for ER, PR, and HER2 expression, endocrine therapy nor HER2 treatment are successful in treating patients with TNBC. Currently, this leaves chemotherapy as the standard therapy to be administered. Chemotherapy has known co-morbidities and can result in patient mortality. TNBC not only has a more aggressive history with worse outcomes (Santonja, 2018), it also has the second highest incidence rate (Table 1) from all the other subtypes; thus, new methods for TNBC treatment are being researched.

Furthermore, breast cancer differs among different patients due to its intertumoral heterogeneity and intratumoral heterogeneity (Turashvili, 2017). The intratumor heterogeneity of breast carcinoma occurs at the morphologic, genomic, and proteomic levels that causes challenges when it is time for diagnosis and treatment (Turashvili, 2017). Recently, the tumor matrix has garnered attention on its contribution to tumor heterogeneity.

The extracellular matrix (ECM) is an important regulator in breast cancer that includes various changes in its composition and organization compared to healthy tissue. It is a complex, dynamic and critical component of all tissues and functions as a scaffold for tissue morphogenesis (Kleinman, 2003). Because the ECM is composed of many extracellular molecules that provide structural and biochemical support to nearby cells (Malandrino et al., 2018), the ECM provides different roles in a cell and cues for cell proliferation, cell differentiation, and enhances repair after injury. Different types of collagens, growth factors, and cytokines are present in the extracellular matrices (Kleinman, 2003). Cancer cells are capable to
influence the ECM’s composition and thus, influence the progression of the cancer’s invasion. Due to the complexity of the tumor matrix, others have shown that many components of the matrix can contribute to cancer progression, included in this are matrix stiffness, fiber orientation, and matrix protein composition. Because TNBC lacks all receptors and there is a need to identify novel therapies to treat TNBC, accurate 3D models should be constructed. As stated above, TNBC is a heterogeneous disease, creating a novel 3D model that resembles natural breast tissue and its heterogeneous architecture will allow insight to how chemotherapies affect different regions of the tumor. The interaction between the surrounding ECM and cancer cells significantly influences the progression in cancer (Gurrala et al., 2021). According to Saha et. Al, breast cancer cells cultured from fibrous scaffolds presented long spindle morphology if it was aligned or flat and stellar morphology if it was random fibers after 3-5 days. These cells therefore present different topography that may have an essential role in tumor progression. Because these cells have restructured their cytoskeleton by ECM contact guidance, they create new biochemical signals within the cells that alter the gene and protein expression. The alteration caused by the ECM restructuring, can alter the alignment of the collagen architecture. Aligned collagen architecture is known as a characteristic feature to the tumor’s ECM (Taufalele, 2019), but our previous research has shown that TNBC can have a random fiber alignment. Further our group and others (Tang et al., 2019) (Byrne et al., 2021) have shown that the matrix protein composition is different between TNBC and ER+. Specifically, ER+ are rich in collagen in 1 & 3 and TNBC has no defined microenvironment matrix. Thus, the relationship between fiber alignment and tumor progression should be examined. Furthermore, the composition of the extracellular matrix should be studied with identification of key proteins elevated specifically in TNBC.
Prior research from our lab has shown that Microfibril-associated protein 2 (MFAP 2) is enhanced (figure 1) in TNBC. Microfibril-associated protein 2 (MFAP2) is an extracellular matrix protein that interacts with fibrillin to moderate the function of microfibrils (Wang et al., 2018). The relationship between MFAP2 overexpression and breast cancer progression is therefore a promising area of research. MFAP2, is associated with cancer cell proliferation and poor patient prognosis in hepatocellular carcinoma (Zhu et al., 2020), gastric cancer (Dong et al., 2020), and papillary thyroid cancer (Dong et al., 2020); thus, a study was conducted to see how this relationship is observed in triple negative breast cancer.

Chapter 1. Architectural Heterogeneity
Introduction
It has been extensively shown that tumor progression is often accompanied by architectural restructuring of the ECM within the tumor as well as the adjacent tissue surrounding it (Provenzano 2006). These significant changes in matrix architecture include modifications of stromal collagen alignment in addition to collagen structure, which we term as architectural heterogeneity. Tumor expansion can lead to increased tissue compression and interstitial pressure which leads to an increase in cell and tissue tension. This in result, leads to tumor progression due to the release, concentration and activation of many growth factors (Yu, 2011). Moreover, the onset of increased compressional forces and interstitial pressure induces the restructuring of matrix collagen fibers which can greatly affect ECM rigidity and topography, while also
encouraging EMT-like phenotypes critical for cancer cell progression and metastasis (Saha, 2012).

While matrix collagen realignment has been thoroughly established during tumor progression, more research is needed to understand how 1) the organization of collagen differs from tumor and normal mammary tissue and 2) how matrix proteins contribute to fiber organization in the breast cancer subtypes. Provenzano et al. was the first to establish changes in matrix fiber alignment through the identification of tumor-associated collagen alignment signatures (TACS) (Provenzano 2006). Alignment of collagen fibers along the perimeter of the tumor is associated with tumor cell invasion along with three-tumor-associated collagen signatures (TAC-3) (Gole, 2020). TACS are markers from alterations in structural collagen fiber alignment. Distinct types of TACS, signify unique stromal phenotypes and can be correlated with different stages of progressive breast cancer (Malik, 2015). Normal tissue are indicated by curly collagen fibers (TACS-1), pre-invasive tissue are indicated by TACS-2 and are straight, parallel collagen fibers around the tumor. The metastatic stage is indicated by three-tumor-associated collagen signatures (TACS-3) are thick bundles of straight, aligned fibers that are perpendicular to the tumor boundary (Malik, 2015). It is positively correlated to extracellular matrix proteins such as collagen and are a prognostic tumor indicator similar to estrogen and progesterone receptors. Poor survival in patients with TACs (+) have allowed for proposals to quantify collagen alignment (Conklin, 2011). Prior studies focus on area between tumor and stroma. It has been found that there is significance in breast tissue due to tumorigenesis and invasion being promoted by the misregulation of the stromal-epithelial interactions (Provenzano et al., 2006). However, these prior studies focus on area between tumor and stroma and not necessary on the tumor core.
To fully understand tumor matrix structure in depth, analysis of the inner tumor matrix structure should be further researched.

**Methods**

<table>
<thead>
<tr>
<th>ER+ Breast Cancer Xenograph</th>
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<tbody>
<tr>
<td>Decellularization</td>
</tr>
<tr>
<td>SEM Prep</td>
</tr>
<tr>
<td>SEM Imaging</td>
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<tr>
<td>FIJI-ImageJ</td>
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<td>Quantiy</td>
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**Figure 2. Overall Methods.** Schematic of Methods in the ECM Alignment Studies.

To observe changes in tumor fiber alignment, samples were decellularized in order to isolate the ECM of the tissue from the cells. Once the scaffold was made, it was prepped for Scanning Electron Microcopy (SEM) imaging. Fiber alignment was analyzed through the identification and classification of the fiber orientation within each SEM image’s region of interest. Each image sample (N=10) was analyzed and then all were averaged together. ImageJ has a FIJI adaptation feature that is used for analysis in combination with an in-house Python script written by collaborators at LSU Science Center (Gurrala et al., 2021). The magnification level of the SEM images was no lower than 5000x in order to identify the individual fibers and not cluster them together. The images chosen had minimal debris and fiber clustering. After the SEM image’s contrast was adjusted, an ImageJ plugin called Weka Trainable Segmentatin (WTS) was used to identify and classify pixels based on a machine learning algorithm. This allows the image to identify its parts as fibrous or non-fibrous. After classifying and segmenting the image, FIJI plugins “OrientationJ” were used to generate and calculate data on the fiber distribution’s
orientation. The method evaluates each pixel’s tensor, as the largest eigen vector’s direction corresponds to the pixel’s orientation (Rezakhanliah et al., 2011). Probability maps for alignment were then generated as the distribution of orientation vs the orientation in degrees. The frequency amount of fibers at each angle is given and then normalized. Because the fibers are at random directions, the histograms are arbitrary. To normalize the fiber orientation to a 90 degree axis, the most prevalent angle was set to zero. The mean of the distribution is rounded since the histogram is binary. The distribution is then shifted by adjusting the angle to be subtracted by the mean frequency. After this, all the angles are adjusted to be between -90 and 90 degrees only. This was done by adding or subtracting 180 degrees to any values outside that range. The individual peaks seen on the following images represents the fiber orientation.

Results
This study evaluated 3 sets of tumor tissues. Tumors were derived from 1) tumors from representative TNBC and were compared to distal and adjacent adipose tissue and 2) TNBC from serial passaged tumors in mice, and 3) representative ER + tumors grown in the presence of estrogen (E2).

E2 was chosen as it is required for ER+ breast cancer growth in xenograft and is a known mediator of matrix remodeling and tumor progression in ER+ cancers. Three tumors were analyzed to see the correlation of fiber alignment and over expression of matrix protein collagen III. Collagen III is previously shown to be elevated in more aggressive ER+ tumors, and to determine if collagen expression altered fiber orientation, collagen III was over expressed in the MCF-7 breast cancer cell line and injected in mice for 30 days (through collaboration with Tulane University). At end point tumors were transferred to LSU and analyzed for alterations in fiber alignment. The results show that vector samples were aligned as seen in blue yet the
aggressive phenotype with collagen III has a more random alignment as observed through the absence of a single peak. Thus, compared to control, collagen III has increased random fiber orientations. As seen on figures 3, the PIRES vector samples (figure 3) had a straight, aligned structure compared to the mcf7col3a1+e2 samples (figure 3). This suggests that the tumor’s microenvironment is changing when there is an expression of collagen III (Col3). Which may provide insight on what makes more aggressive tumors on ER+ breast cancer.

Results from the TNBC tumor matrix compared to the distal and adjacent adipose demonstrate that the tumor’s fiber orientation was distinct from that of proximal and distal adipose tissue (Figure 4). Specifically, fibers in TNBC, while still random, were slightly more aligned than that of either proximal or distal tissue, as observed by the distinct singular peak in the TNBC tumor analysis. This data provides new information on how tumors remodel the tissue environment and should be considered when designing in vitro tumor models.

Currently, patient derived xenograft provides the most relevant model for cancer studies. Tumors were evaluated from serial passage PDX samples to see if the tumor environment is maintained with tumor growth. Evaluation of patient derived xenograft tumors following serial passaged in mice, demonstrated that fiber orientation linearizes with serial passage (figure 4). This data suggests that fiber orientation in PCX models deviates from that of the original tumor and may lead to loss of clinical relevance with continual passage.
Figure 3. Comparison of fiber alignment in COL3 over expressing tumors versus vector tumors treated with estrogen. Compares frequencies of parameter degrees between control, Pires, and mcf7col3a1+e2. Data quantified after using ImageJ to create binary images that provided frequency values at different degrees. Normalization of degrees at 90° axis.
Figure 4. Comparison of fiber alignment in TNBC in different areas distal and adjacent adipose tissue. (A) Visual representation of primary TNBC across serial passages in xenograft (B) Visual representation of TN primary tumor compared to adjacent and distal adipose. (C) Compares frequencies of parameter degrees between the tumor and the distal and proximal regions of tissue. Data quantified using ImageJ to create binary images that provided frequency values at different degrees. Normalization of degrees at 90° axis.
Conclusion
In conclusion, by evaluating the alignment between tumor types, it was observed that randomly aligned fibers were found on the ER+ tumors with collagen III expression. Collagen has a crucial role in regulating tumor progression. Collagen III is a component of the tumor stroma that regulates scar formation and myofibroblast differentiation (Brisson et al., 2015). ER+ tumors that have a higher expression of collagen III present more aggressive characteristics than ER+ tumors with low collagen III expression. Overall, the tumor region along with the proximal adipose region and the distal adipose region all have random fiber alignment (figure 5), however the tumor core was more aligned than either proximal or distal adipose. Additional tumor samples will be required to make direct correlations but the study indicates that microenvironment of TNBC is different than what prior studies have presented,

Chapter 2. MFAP2 Regulation
Introduction
Microfibril-associated protein 2 (MFAP2) is an extracellular matrix protein that interacts with fibrillin to moderate the function of microfibrils. Prior studies show that MFAP2 protein expression is upregulated in gastric cancer and papillary thyroid cancer (PTC) and correlates to poor patient outcomes. Despite this, the underlying role of MFAP2 in breast cancer has remained unclear. The current study investigates the expression pattern of MFAP2 in triple-negative breast cancer cells and evaluates the effect of MFAP2 overexpression in triple-negative breast cancer (TNBC). To achieve this, the TNBC MDA-MB-231 breast cancer cell line was transfected to stably overexpress MFAP2. The effects of MFAP2 overexpression were evaluated and compared to breast cancer cells that have a control pCMV-vector. Furthermore, the expression of different genes associated with breast cancer progression (senescence phenotypic proteins, cell stressor proteins, and invasion) were evaluated. Following selection, the parent cells with the pCMV-
vector and MFAP2-overexpressing cells were collected for RNA extraction and quantitative real-time Polymerase Chain Reaction (qPCR) tests. Results demonstrated that MFAP2 was overexpressed in our MDa-MB-231-MFAP2 line compared to the breast cancer cells with the control vector. Following confirmation of MFAP2 overexpression, proliferation studies were performed to observe how MFAP2 overexpression alters cellular proliferation and survival studies with chemotherapies (taxol and doxorubicin) were performed to test the effects of MFAP2 on survival. Altogether, this research aimed to test the hypothesis that MFAP2 upregulation can lead to breast cancer progression to a more aggressive phenotype. Evaluation of MFAP2 in breast cancer may provide novel treatment options for individuals with TNBC.

Methods

Figure 5. Overall Methods. MFAP2 overexpression was studied through the following tests

PCR

After extracting RNA and nanodropping, 1 ug of total RNA was used to make cDNA. To make cDNA, the RNA was mixed with 4ul of cDNA supermix (iscript kit) and water to have a total of 20ul. Once cDNA is made, 180 ul of water is further added to dilute 1:10. Three different biological replicates of pCMV vector and MFAP2 overexpressed cDNA was made. For each set of samples, three PCR tests were done. To run PCR, working primers of the chosen genes were made by adding 180 ul of DNA-RNAase Free Water, 10 ul of the reverse primer, and 10 ul of the forward primer. Then, 1ul of working primer was added to a master mix along with 10ul of
Syber Green and 6ul of DNA-RNAase free water for each well. Once the mastermix was made, 17ul of it was added to each well along with 3 ul of cDNA sample. Gene expression changes were calculated using the ΔCt method. Housekeeping gene was beta-actin.

**Proliferation Studies**

To quantitatively measure the proliferation of the TNBC cell lines, MDA-MB231 and –MFAP2 over expressing cells, crystal violet staining is done to a microwell plate. Two different well plates are done with three wells each for the vector and three wells each for the MFAP2 overexpressed cell line. These wells each had 7000 cells/well, allowing for three technical replicates. The two plates are then named Day 0 and Day 4 and placed in an incubator overnight. The next day, the well plate labeled Day 0 is stained with 100 ul of 0.3% of crystal violet into each well. The crystal violet is left to stain it for 10 minutes and then after it is aspirated and washed with water. It is then left for drying so later data can be collected based on absorbance. The same was repeated for the well plate labeled day 4, four days later. The two plates absorbance values are then compared to see if the MFAP2 overexpressed cell line proliferates more than the vector cell line

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Number of technical replicates per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV vector (control)</td>
<td>3</td>
</tr>
<tr>
<td>MFAP2 overexpressed MDA-MB231 cell line</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 2. Proliferation study set up.** This was done for day 0 and day 4 plates.
**Cell viability**

Similarly, to proliferation tests, cell viability tests following treatment with chemotherapy, use crystal violet staining in order to quantitatively measure cell number. Just one plate is used but in it two different drugs are tested in the well plate: paclitaxel (taxol) and doxorubicin, DMSO is the vehicle control. They were done for 100 nM of drug in each well. Each well was seeded with 12000 cells/well. Three trials were done for each cell line (vector & MFAP2 overexpressed MDA-MB231) at each different drug choice. Staining was followed after four days. The process is the same as the proliferation studies and then quantified.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Drug</th>
<th>Number of technical replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV Vector (control)</td>
<td>DMSO</td>
<td>3 technical replicates per drug</td>
</tr>
<tr>
<td></td>
<td>Taxol (100 nm)</td>
<td>for a total of 9 wells for</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin (100nm)</td>
<td>pCMV</td>
</tr>
<tr>
<td>MFAP2 overexpressed</td>
<td>DMSO</td>
<td>3 trials per drug for a total of</td>
</tr>
<tr>
<td>MDA-MB231 cell line</td>
<td>Taxol (100 nm)</td>
<td>9 wells for MFAP2</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin (100nm)</td>
<td>overexpressed cell line</td>
</tr>
</tbody>
</table>

**Table 3. Drug study test set up.** One plate done.

**Results**

To determine that MFAP2 was successfully over expressed in the MDA-MB-231 cell line, qPCR was performed for MFAP 2 expression. Visually the MFAP2 over expressing cell line was not morphologically different than the MDA-MB-231 parental cell line (figure 6). Results show that compared to the pCMV vector, the MDA-MB231 cell line with MFAP2 overexpression had an average fold change of 1000 (Figure 7). This validated the overexpression of MFAP2 in the cell line. To determine if MFAP2 altered cell growth and cell survival following drug treatment, proliferation and survival studies were performed. Results demonstrated that there was a higher level of proliferation in the MFAP2 overexpressed cell line compared to the pCMV vector (figure 8). Surprisingly, cell survival was lower when cells overexpressed MFAP2 (figure 9).
Due to the increase in cell proliferation but no increase in cell survival following drug treatment, we next evaluated differences in gene expression in genes associated with cell growth and survival. Results demonstrated no significant change in genes associated with cell senescence and cancer stem cell (figures 10 and 11). Because those genes characteristics did not present any change, the next genes that were studied were associated with inflammation and cellular migration. Results demonstrated that these genes did show a higher level of expression when there was an MFAP2 overexpression compared to the pCMV vector cell line (figures 12 & 13). Of note IL6 was significantly increased in the MFAP2 overexpressing cell line. These genes are associated with cell motility, so we next wanted to determine the clinical significance in correlation of MFAP2 expression and metastasis. For this, we next evaluated the expression correlation of MFAP2 and invasion/inflammation associated genes in patient tumors. The results demonstrated that there was a high correlation of MFAP2 with adhesion and motility genes, MMP11, MMP14, and ITGB5 (figure 14). Further, high expression of MFAP2 in TNCB was associated with poor patient metastatic free survival (figure 15).

Cell Morphology

![Cell Morphology](image)

Figure 6. Validation of MFAP2 overexpression in MDA-MB-231 cell line. Morphology of MDA-MB-231 MFAP2 overexpressing cells
Figure 7. Validation of MFAP2 overexpression in MDA-MB-231 cell line. qRT-PCR for MFAP2 expression in MDA-MB-231 cell line. Housekeeping gene was beta-actin and normalization was to MDA-MB-231-pCMV-vector transfected cells.

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Conclusion
In conclusion, MFAP 2 enhances TNBC cellular proliferation as seen from figure 9. This means that if there is an upregulation of MFAP2 expression, there seems to be an increase in the number of TNBC cells as the cells seem to be growing and dividing more than normal. There was no increase in cell survival or survival associated genes, suggesting MFAP2 does not regulate response to therapy. Furthermore, there was an increase in the expression of genes associated with metastatic characteristics such as IL6. From the results of the heat map and PCR tests, the expression of genes associated with motility and inflammation increased more when correlated with an overexpression of MFAP2. These genes include ITGB5, MMP11, and...
MMP14 that had an 80% correlation. Cells can interact with ECM by degrading them through these different types of metalloproteinases (MMPS), enzymes that degrade the extracellular matrix. Tumors then behave in this new, degraded ECM to remodel the heterogeneous profile of the extracellular matrix network. These profiles then influence the migration of the surrounding cells and the speed of the MMP to increase (Malandrino et al., 2018). Finally, from the Kaplan-Meir curves (figure 15), it was seen that MFAP2 protein overexpression was associated with poor patient survival as the time progressed in patients with TNBC. The probability for a patient with TNBC and low MFAP2 expression to survive was dropped to 70%; however, a patient with high expression of MFAP2 dropped from 100% to about 40% in month 50 to a 20% probability of survival by month 200. These drastic drops of survival rate further encourage the need to investigate MDAP2 and its effects on metastasis.

**Future Studies**

In the future, a novel 3D tumor model that represents the native TNBC tissue environment needs to be made. This will be done by electrospinning chitosan to fabricate ECM that mimics microfibers. After seeding TNBC cells to the scaffold, metastasis characteristics will be observed and quantified. These metastatic characteristics that will be observed are porosity and fiber orientation. Furthermore, the matrix composition will be further observed by performing in vitro invasion assays and xenograft models for MFAP2 metastasis. We will also be doing protein confirmation for the inflammability, invasion, and motility genes, cytokines and MMPs.
References


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