**BME Senior Capstone Project:**

**Investigating the Role of Neuronal Genes in Breast Cancer Metastasis and Chemotherapy Sensitivity**

Ariana Barriero, Maia Buckwald, and Kevin Lyons

Madeleine Oudin

March 3, 2023

All new edits in green

**Keywords:** Triple-negative breast cancer (TNBC), metastasis, Neuronal Markers

**Abstract:** *Maia Buckwald*

The goal of this project is to investigate the role of neuronal genes in triple-negative breast cancer metastasis and chemotherapy sensitivity. Currently, three out of every twenty five women in the U.S. will be diagnosed with breast cancer.1 TNBC is prevalent in 20% of all breast cancer diagnoses; it is known to be an aggressive, metastatic subtype with high mortality rates.2 TNBC in particular does not have a therapeutic target for treatment, making chemotherapy only a palliative measure and a way to slow the course of the disease.3  Understanding the mechanisms driving metastasis is crucial for the development of more effective therapies for TNBC. Transcriptome data has indicated that neuronal genes TUBB3 and MAPT are highly upregulated in TNBC cells. Their expression levels are especially high compared to normal breast cells. Previous research in the Oudin Lab revealed that knockdown of upregulated neuronal genes TUBB3 and MAPT in MDA-MB-231 human TNBC cells led to an increase in metastatic properties: increase in cell speed, increase in saltatory movements, increase in cell migration, and the formation of elongated cell morphology. Additionally clinical data indicates a correlation between resistance to paclitaxel (a taxane-based chemotherapy) and an overexpression of MAPT and TUBB3.4,5 Our goal in this project is to further investigate how these neuronal genes play a role in chemotherapy sensitivity of TNBC cells. Our goal in this project is to further investigate how these neuronal genes play a role in chemotherapy sensitivity. A second goal of this project will be to investigate whether these trends are consistently seen in two other human TNBC cell lines to account for heterogeneity differences in cancer.

**Elements of Engineering Design:** *Ariana, Maia and Kevin*

The objective of the project is to define the influence that neuronal gene expression has on the metastatic potential of breast cancer cells. In addition, we plan to investigate the sensitivity to chemotherapy agents. The focus of this project's design work is on optimizing a platform to study how neuronal gene expression plays a role in the malignancy of breast cancer. The project fulfills a research need on better understanding the mechanism behind metastasis. Expanding this knowledge will provide avenues for new and more effective treatments. We hypothesize the differences in cell behavior after gene knockdown will have an increase in metastatic potential.

To optimize our platform, we will first test two different gene knockdown methods: siRNA and CRISPR. It is necessary to perform both of these methods since their efficacies are dependent on factors such as cell line and the target gene. These neuronal genes, TUBB3 and MAPT, have yet to be knockdown in triple-negative breast cancer cells, which is why both methods need to be evaluated to determine which is more efficacious in gene knockdown.

To investigate this we will first optimize two knockdown methods: siRNA and CRISPR. We will then evaluate the effect of each type of knockdown. This will be evaluated with biochemical assays and experimental lab methods such as imaging and immunostaining to measure cell migration, cell adhesion, and cell proliferation.  Furthermore, to analyze our results, we will mathematically determine the differences in cell proliferation, movement, and viability across various cell lines using advanced statistical analysis. Our most notable constraint is time.

After optimizing knockdown methods and evaluating its effect in cell behavior and chemotherapy sensitivity, we will assess the applicability of our findings to two other TNBC cell lines (that will later be determined). We expect that these other cell lines will behave similarly. The same gene knockdowns are being performed; therefore, the experiments should yield the same result. The purpose of using these other cell lines is to control for the heterogeneity of cancer cells. At this point, an optimal platform of using either siRNA or CRISPR knockdown will have been determined. The preferred knockdown method will be used in these other cell lines. Within each cell line, we will have three biological replicates: Scr, MAPT knockdown, and TUBB3 knockdown.  Each of these will be tested in duplicate. We will perform a comparative analysis on the sum of data that is collected. With such a large volume of data to collect and analyze, we are limited principally by the time and maintenance constraints. It is expected that these experiments can be completed in six months and the comparative analysis will be done in the subsequent months.

Down the line, we may have alternative plans based on what we observe. We will evaluate our progress as we finish the experiments of the first cell line and decide whether to do the second and third cell lines knockdowns with CRISPR or siRNA. The first quantitative milestone will be the extent of gene knockdown. In every case, it is necessary that a 75% knockdown of the gene is achieved. If the gene is not sufficiently knocked down, it introduces confounding variables to the study. We will use a one-way ANOVA t-test between two groups to assess the significance of the effect of knockdown on saltatory movement and proliferation. Each group (MAPT and TUBB3 knockdown) will be compared to control cells (Scramble). It would be optimal if the cells have at least a 2-fold difference in both saltatory movement and proliferation compared to controls. These metrics are based on preliminary, yet incomplete, data from the Oudin Lab that investigated these cell lines. Additionally, our goal is that the control and knockdown groups will demonstrate a differential dose-dependent response to paclitaxel and doxorubicin. We will use a one-way ANOVA t-test between two groups to assess the significance of viability when exposed to chemotherapy. Each group (MAPT and TUBB3 knockdown) will be compared to control cells (Scramble). Previous members in the Oudin lab expected a 3 fold decrease in viability.  when exposed to chemotherapy. One-way ANOVA t-test was chosen as a statistical test to determine the significance in these experiments because it is used in cancer literature to determine significant differences in response to chemotherapy between groups. Each knockdown will be compared to the control (Scramble) in this test.

Similar research in the field of lung cancer is currently being done. Research has focused on small cell lung cancer (SCLC) exploring the neuroendocrine and neuronal gene characteristics of SCLC cells. Recent findings have shown that cancer cells become more neuronal and lose some neuroendocrine characteristics as they gain the ability to metastasize. The research in this field could advance and further explore the effect of silencing genes in chemotherapy treatment. Given that some neuronal characteristics are similar this could decrease the novelty of our experimental findings.

**Introduction:** *Kevin and Maia*

Breast cancer is the most frequently diagnosed type of cancer and the leading cause of cancer-related deaths among women.1 About 20% of breast cancers are classified as triple negative, meaning they test negative for the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2).6 The lack of molecular markers in TNBC makes it particularly difficult to treat, as these typically serve as therapeutic targets. The disease has a poor prognosis, and those with TNBC have a shorter median life expectancy than patients with other forms of breast cancer.1 The disease has a 5 year survival rate of 40%.7

Metastasis is the process by which cancer cells leave the original tumor site, disseminate to a foreign tissue, and colonize in a new site to form a new tumor. Metastasis drastically decreases the overall survival rate and results in greater systemic effects of the disease.8 One way to reduce metastasis is through the use of targeted therapies. These were first introduced with the approval of the breast cancer drug tamoxifen in 1998.9 Targeted therapies work by silencing or inhibiting malignant signaling pathways that are associated with the expression of the cancer phenotype. The uncovering of more and more targeted therapies allows for a wider range of cancer mutations to be mitigated. This provides incremental progress towards a definitive cure for the disease. However, this progress is inherently reliant on the detailed understanding of the effects of gene expression on cell behavior. These systems must be investigated thoroughly in order to elucidate an avenue for effective therapeutic intervention.

Our project is specifically investigating the role of neuronal gene expression of TUBB3 and MAPT in TNBC cells. TUBB3 has been previously considered to promote microtubule dynamics. A study in A549 lung cancer cells showed that knocking down TUBB3 resulted in more stable microtubules when quantifying growth/shrink rate.10 Other studies have shown that expressing mutant TUBB3 in mouse models resulted in a higher level of de-tyrosinated α-tubulin, which is correlated with stabilized microtubules.11 On the other hand, MAPT has been considered to have stabilizing effect in microtubules as Tau protein attaches to tubulin subunits and suppresses the microtubule shrink rate.10 MAPT and TUBB3 therefore have opposite roles on microtubule dynamics but at the same time induce similar effects in cell morphology and migration.12 These genes are also upregulated in triple-negative breast tumors and have been associated with chemotherapy resistance, suggesting these genes may be giving the cancer cells an advantage.13 Data-mining also reveals that these genes are correlated with poor survival of patients. This correlation suggests that these genes play a significant role in the progression of TNBC, which is why we plan on investigating the effects of gene knockdown on cellular metastatic potential. 13

We plan to first create a knockdown protocol. CRISPR and siRNA are the two most common ways to silence a gene in-vitro. Each of these requires its own set of optimization and quality assurance procedures. Additionally, they are better suited for different scenarios, which is best determined experimentally. The knockdown method will allow us to genetically engineer a platform that will allow us to study the gene’s effect on the malignancy of breast cancer. We plan to investigate how the genes affect cancer cells’ ability to metastasize by looking at differences in proliferation rate, migratory behavior, and cell morphology. We also plan to look at how chemotherapy affects the viability and morphology of cancer cells after knockdown of these genes. Lastly, we plan to validate and generalize these findings by repeating the experiments in multiple cell lines.

**Background:** *Kevin and Maia*

Breast cancer is caused by genetic mutations in epithelial cells of the breast. These mutations dysregulate the proliferative or apoptotic signaling pathways which create aggregates of harmful nonfunctional cells called tumors. The tumors disrupt normal cell function and can be fatal if left untreated. Classification of breast cancers is based on the surface receptors that the cell line expresses. Surface receptors tend to make the cancer more aggressive, but they can also serve as therapeutic targets. There are four molecular subtypes of breast cancer: luminal A, luminal B, HER2+, and triple negative. This study specifically uses triple negative breast cancer cells, which do not express any surface receptors.

Metastasis takes place when cancer cells form new tumors distant from the original cancer site, typically in the brain or soft organs of the body.14 Cancer cells spread throughout the body via the lymphatic system, the bloodstream, or through perineural invasion. Metastatic triple negative breast cancer is fatal.15

Chemotherapy remains the only FDA-approved treatment for TNBC; however, it has significant negative side effects including hair loss, appetite loss, and anemia. This treatment does not prevent metastasis, as roughly 30% of early-stage breast cancers become metastatic regardless of chemotherapy.3 Therefore, chemotherapy is mainly utilized as a palliative measure and to slow the course of the disease. Research is currently being performed to seek out potential targets for new therapies for TNBC. Examples of these targets include rapamycin (regulator of an impaired pathway), EGFR (plays a role in inducing metastatic behavior like cell proliferation), and VEGF (a factor that is associated with more aggressive cancer).16

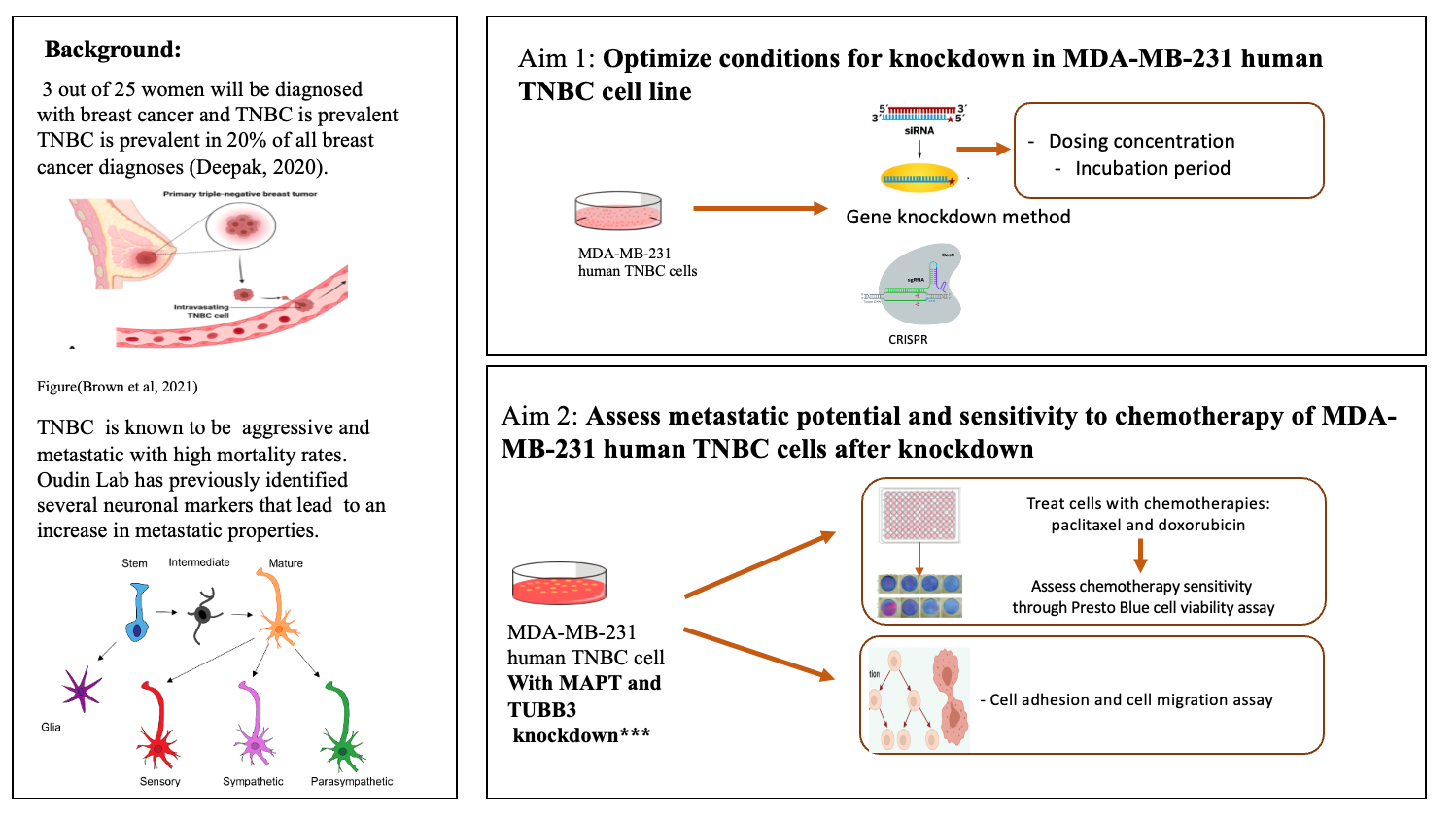
A recent article by Yang et al demonstrated an upregulation of neuronal genes, including TUBB3 and Tau, in SCLC.17 The cells became more elongated and moved in a saltatory fashion, which increases the potential for metastasis to occur. Publicly available transcriptome data of breast cancer cells has shown that these cells are also marked by an upregulation in neuronal genes. More specifically, neuronal genes are associated with the most aggressive subtype of breast cancer: TNBC. Two neuronal genes were identified to be highly expressed in breast cancer and to be biomarkers for poor prognosis are β3-tubulin (TUBB3) and microtubule-associated protein Tau (MAPT). The mechanism through which these genes play a role in TNBC is not well characterized. Further research into these gene’s roles may elucidate potential therapeutic targets or biomarkers.

TUBB3 and MAPT are two genes with slightly different purposes. TUBB3 plays a role in promoting microtubule dynamics, while MAPT plays a role in stabilizing microtubules.12 Microtubule dynamics influence cell migration, cell morphology, and cell mitosis; so, MAPT and TUBB3 are genes that affect these parameters. Some chemotherapies are taxane-based, meaning they interfere with the microtubules in order to prevent mitosis. Literature has indicated that overexpression of TUBB3 and MAPT are correlated with taxane-based chemotherapy resistance, most likely due to the genes being able to block the mechanism of action of the therapy.18 This insight gives us motivation to investigate directly if knockdown of these genes will sensitize the cells to chemotherapy.

Previous research in the Oudin Lab revealed that knockdown of TUBB3 in MDA-MB-231 breast cancer cell line led to the formation of long processes. In addition, knockdown led to increase in cell speed, saltatory movements, and cell migration. MAPT was then investigated, and those cells exhibited consistent behavior with that of TUBB3 knockdown cells.12

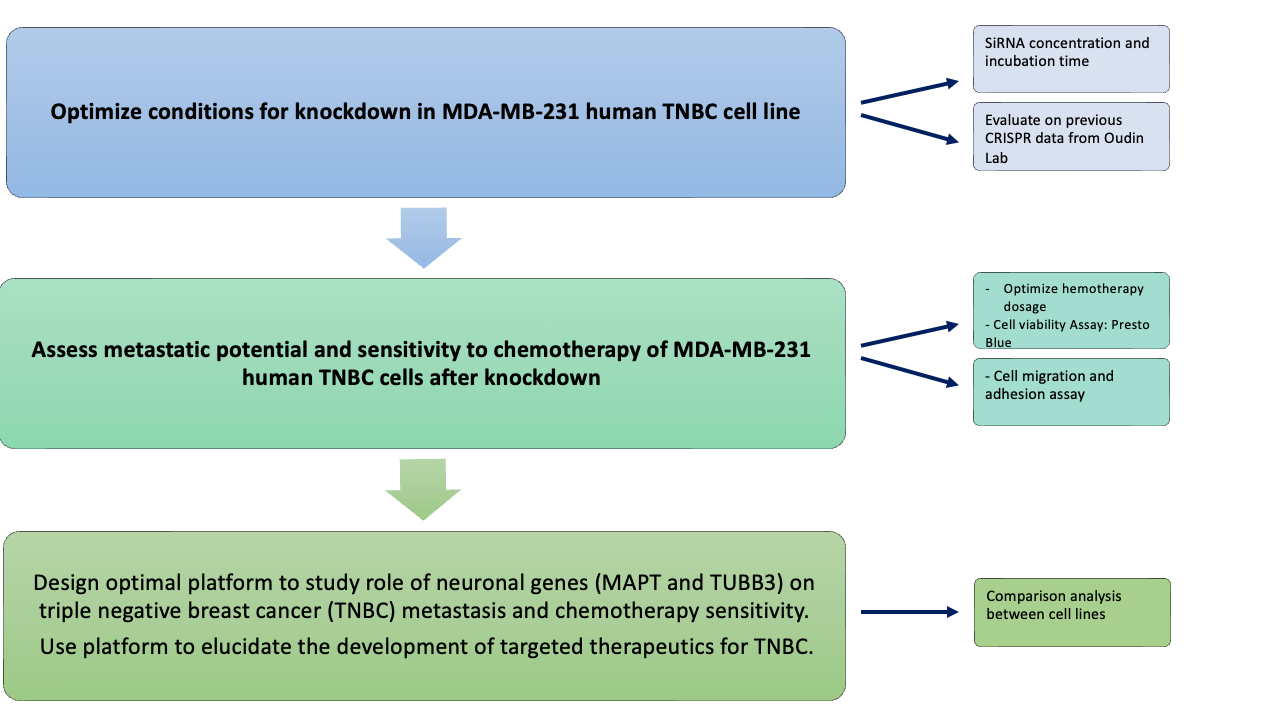
Our project will further investigate the effects of gene knockdown on triple negative breast cancer cells. Our novel findings may elucidate new therapeutic avenues that interfere with the genes in a way to exploit higher chemotherapy sensitivity while limiting the potential of metastasis. In addition, these genes may serve as biomarkers to better characterize breast cancer.

**Unifying Figure:** *Ariana*

****

*The figure illustrates the background and individual aims for our project. We provide a graphical outline of the experimental methods and deliverables. Our final project is broken into 3 main aims: 1) Optimize conditions for knockdown in MDA-MB-231 human TNBC cell line 2)Assess metastatic potential and sensitivity to chemotherapy of MDA-MB-231 human TNBC cells after knockdown*

**Design Flow Chart:** *Ariana*

****

*The design flow chart of our project illustrates our three main objectives and our ultimate goal. On the right hand side we have arrows pointing towards the methods and parameters to accomplish each corresponding goal.*

**Specific Aims:** *Maia*

**Specific Aim 1: Optimize conditions for knockdown in MDA-MB-231 human TNBC cell line**

The goal of this aim is to compare the efficacies of knockdown of TUBB3 and MAPT using two different strategies: using siRNA and using CRISPRi. These knockdowns will be performed in the MDA-MB-231 human TNBC cell line. This cell line will be used because of its availability in the Oudin Lab, but the findings will have to be investigated in multiple cell lines in order to draw any definitive conclusions. It is not feasible to perform this Aim in more than one cell line due to time constraints of the project. It is already known that both methods offer different advantages. Knockdown using siRNA is more time efficient as it can significantly alter the gene expression in just 24 hours. However, it is only able to temporarily reduce gene function. On the other hand, CRISPRi is able to edit the genetic code and completely knockout a gene of interest. The efficacy of the two methods depends on many factors such as the target gene and the cell type used. Since the experiment only involves TNBC cells with identical gene knockdowns, the optimal knockdown method should be the same across all three cell lines. Besides the assessment of method of knockdown, optimization of dosing and timing parameters while using siRNA is also required. From these experiments, we will understand the conditions and efficacy of knockdown using each method.  From there, we will be better equipped to assess the best method of knockdown in other TNBC cell lines in future directions of this project.

**Specific Aim 2: Assess metastatic potential and sensitivity to chemotherapy of MDA-MB-231 human TNBC cells after knockdown**

The goal of this aim was to take the cells from Aim 1 and assess the effects of knockdown of TUBB3 and MAPT. The mechanism through which these genes play a role in TNBC is not well understood. Metastatic potential will be assessed through migration and adhesion assays. Migration assays involve seeding cells on Collagen I, then imaging them in a migration chamber overnight to track their movements. Cell speed, cell proliferation, and persistence are parameters that will be analyzed after. Adhesion assays are performed to assess cell morphology, which is indicative of invasive capacity. Cells will be seeded onto Collagen I, fixed, stained, then imaged. CellProfiler will be used to perform morphological analysis to assess aspect ratios and cell area. Previous work in the Oudin Lab has assessed metastatic potential of knockdown using CRISPR. This work will be continued by further assessing effects of knockdown on chemotherapy sensitivity through cell viability assays. This involves seeding the cells and staining them with Presto Blue to get initial reading in a plate reader. Chemotherapies will then be added to cells except for control cells. Paclitaxel is a taxane-based chemotherapy. Doxorubicin is another chemotherapy that will be tested. The cells may also be sensitized to doxorubicin since it affects the microtubules indirectly, through preventing mitosis via damaging the DNA. Four concentrations of paclitaxel will be assessed using a two-fold dilution series: 5nM-0.625nM.  Four concentrations of doxorubicin will also be assessed using a two-fold dilution series: 500nM-62.5nM. These concentration ranges were previously optimized in the Oudin Lab. An adhesion assay will also be performed. This will show chemotherapy-induced effects on cell morphology, which allows us to assess invasive capacity of cells. In addition, characterizing metastatic potential and chemotherapy sensitivity after knockdown using siRNA will be determined to look at consistency across knockdown methods. After carrying out this Aim, we will have an understanding of the effects of TUBB3 and MAPT knockdown in TNBC cells. We also will investigate consistencies/differences seen across knockdown methods to further characterize the best method for knockdown.

**Methods:** *Ariana and Maia*

*Cell Culture*

*Preparation of Media*

Preparation of media was needed in order to be able to grow our TNBC cells and perform the corresponding experiments.

DMEM/F12 hiNSCs complete media was the type of media primary used for this experiment. This media is made by adding 2 main components: 50mL aliquot of FBS, and 10mL of PSG. For preparing the media, both components need to be thawed. Once both components are thawed the serum is filtered in a 0.2um filter with a 50mL syringe and put into a media bottle together with the PSG. The bottle is then labeled with name, data and supplements added and stored in the refrigerator.

*Thawing and Seeding Protocol*

The cells used for this experiment were: MDA-MB-231 TNBC cell lines. The cells were previously frozen by members of the Oudin lab with CRISPR knockdowns of TUBB3 and MAPT. These were the first cells thawed in order to analyze the effect of CRISPR on cell behavior. We thaw MDA-MB-231 TNBC cells without CRISPR gene knockdown to be able to analyze siRNA as a method of knockdown in the following weeks. Media was first thawed for approximately 20 minutes in the water bath. The frozen vials of cells were removed from the -80 freezer and put in the 37C water bath to quickly thaw it. The vials were then sprayed with 70% ethanol and the solution was then pipetted and transferred to a 15 ml tube along with 10 mL of pre-warmed media. The tube was then centrifuged at 1000 rpm for 3 minutes to pellet the cells so the toxic DMSO solution could be aspirated. After centrifugation, cells were gently reconstituted in 10 ml of complete growth medium. Cells were then transferred to a labelled flask at high density and incubated overnight at 37C/5% CO2. Cell density was checked the next day to either change media or passage the cells accordingly.

*Subculturing Procedure*

 Every week, cell density was evaluated, and cells were passaged accordingly. To passage cells, the culture medium was first removed by aspiration. The flask was then rinsed with 5mL of DPBS for 1 minute by transferring it to the bottom of the flask and then aspirating again. 1mL of 0.25% trypsin-EDTA, was added to detach the cells and the flask was then incubated at 37°C for approximately 1 min. After the incubation, 9 ml of media was added to stop the reaction. A serological pipet is used to rinse the dish 4 times. Cells were then seeded at desired ratio in new flask containing free media.

*Knockdown of Neuronal Genes*

*CRISPR Protocol:*

Cells used for the analysis of the first cell line (MDA-MB-231 human TNBC cells) were thawed from a previous graduate student at the Oudin lab who, along with one of us (Maia), had performed the CRISPR gene knockdown.

*Drug resistance experiment and analysis (Cell Viability Assay)*

In order to test the effect of gene knockdowns of MAPT and TUBB3 in chemotherapy sensitivity, drug resistance experiments and analyses were performed accordingly:

5000 cancer cells per well were seeded in 96 well plate and left incubating for 24 hours. Then, media was replaced with media with chemotherapy drugs at varying concentration. Cell viability is measured on day 1 and day 4 with Presto Blue reagent (P50200) according to manufacturer recommendations. Readouts were normalized with blank, then with day 1 control to calculate the cell viability fold change, and finally with day 4 control to display viability as fractions of no drug control.

*siRNA Infection*

 For the initial viability evaluation of MAPT and TUBB3 knockouts, siRNA-TUBB3 and siRNA-MAPT Silencer will be used from provider ThermoFisher with code 4392420 and Assay ID: s21740. The protocol for infection will be followed as instructed by the provider. SiRNA will be diluted in 11.33 uL of water. 3 experimental groups will be tested infection to evaluate the best siRNA concentration for knockouts 1.33 µl of siRNA was pipetted into each 150mM condition. Then, 1.00 µl of siRNA was added for every 100nM condition. Finally, 1.33uL of H2O was added to each control.  siRNA was delivered in the media before pipetting it to the cells.

*Migration Assay*

A glass-bottom 24 well plate will be used. One the first day, the plate will be coated with 0.1mg/mL Collagen I for 1 hour at 37C. After, 300 uL cells will be seeded at 12k/well in media. The plate is then incubated for 2 hours at 37C. The migration chamber will be set up using image acquisition setting, and the cells will be placed inside after their incubation. Brightfield and TXRed will be used when imaging the cells.  Multipoint setting will be used to pick 4-5 POVs for each well. Other settings that will be set on the migration chamber will be 10 minutes between image acquisitions for a total of 97 times. There will be an initial delay of 30 minutes before the first image to allow time for the cells to settle.

*Morphological analysis via cell adhesion assay*

24h after co-culture was established, cells were fixed and stained with DAPI. Images were taken at 20x with approximately 30 field of views per condition. CellProfiler v3.1.8164 was used to identify cell shape: DAPI was used to identify individual cell then mCherry was  used  to  determine  cell shape parameters.

*Statistical analysis*

GraphPad Prism v8.4.3 was used for generation of graphs and statistical analysis. To compare between  two  groups,  unpaired  two-tailed  Student’s  t-test  was  used  and  a  p-value  of  ≤  0.05  is considered  significant. To  compare  between  multiple  groups,  one-way  ANOVA  with  Tukey’s multiple testing correction was used with a corrected p-value of ≤ 0.05 is considered significant. For  RNA-seq,  adequately  expressed  genes  passing  a  fold  change  threshold  of 1.2  and  with  p value ≤ 0.05 in edgeR analysis were considered differentially expressed. Pathways with p value ≤ 0.05 and FDR ≤ 0.01 were considered differentially regulated.

**RESULTS:** *Kevin, Ariana, and Maia*

**Optimal concentration and incubation time for siRNA knockdown found**

To create a platform to study the effects of these genes, siRNA knockdown will be optimized and compared to the previous method of CRISPR knockdown. To optimize this method of knockdown, three concentrations of siRNA will be tested: 5uM, 2.5uM, and 1.25uM. These concentrations were chosen based on the recommendations from the company from which the siRNA came from. Incubation times of 24 hours and 72 hours were tested for MAPT knockdown, and 24 hours and 48 hours were tested for TUBB3 knockdown. 48 hours was assessed for TUBB3 knockdown since the cells appeared to be too confluent to wait to be assessed at the 72 hour time point. As stated before, the metric for successful knockdown is at least 70% knockdown.

Chart

Description automatically generated

**Figure 1:** MAPT and TUBB3 siRNA Knockdown quantification. A)qPCR quantification of MAPT gene expression after siRNA exposure testing 3 different concentrations (5um, 2.5um, 1.25um) at two timepoints (24hr and 72 hr). B)immunostaining protein quantification of TUBB3. Results indicate a successful knockdown at 24hrs but partial reversal at 48hrs. 3 replicates were analyzed and averaged for analysis.

MAPT siRNA knockdown results show that there is almost 100% knockdown after incubation with siRNA for 24 hours at all concentrations. 2.4uM data could not be obtained due to a reading error by the thermal cycler. At the 72 hour time-point, there is about 60-70% MAPT knockdown across all concentrations. TUBB3 siRNA knockdown results show about 80-95% knockdown after incubation with siRNA for 24 hours across the different concentrations. After incubation with the siRNA for 48 hours, there was about 25% knockdown at 5uM siRNA. There was no knockdown of TUBB3 at the two lower concentrations of siRNA.

Chart

Description automatically generated**Figure 2:** *Migration assay results for siRNA kncodkwon of TUBB3 and MAPT cells 24hours after transfection. Results show comparable cell speed and persistence between control and siRNA knockdown cells, suggesting no significant impact of siRNA knockdown on cell migration.*

Given that we were unable to optimize the knockdown of TUBB3 for longer than 24 hours this prevented us from evaluating chemotherapy resistance after siRNA knockdown, which requires cell incubation for at least 48 hours. For this reason we decide to begin by conducting migration assays after siRNA knockdown to confirm the reliability of this approach compared to CRISPR data. However, the results shown in **Figure 2** did not show any differences in cell speed and persistence between control and knockdown cells, which contradicts the data previously obtained by the Oudin lab on CRISPR data**(Add figure or see if we should put as appendix)**. These findings suggest that siRNA may not be a dependable platform for studying these genes.

To test the effect of siRNA knockdown in the MDA-MB-231 cell line, we additionally performed an adhesion assay to assess the effects of siRNA knockdown on cell morphology. However, we encountered challenges in accurately analyzing the cells due to excessive confluency, as seen in **Appendix 1** . Consequently, we were unable to perform the adhesion assay analysis for this cells. We hypothesize that the observed proliferation induced by the siRNA knockdown, coupled with the longer incubation periods required for efficient knockdown, may have led to the high cell density at the time of imaging. These findings support our belief that CRISPR may be a more effective method for analyzing neuronal genes in TNBC. This study emphasizes the importance of carefully considering appropriate experimental methods to ensure accurate and meaningful results.

**Gene knockdown leads to susceptibility to chemotherapy at 48 hour timepoint**

In order to assess the effectiveness of chemotherapy after gene knockdown, we took into account two time points: 48 and 72 hours after chemotherapy exposure. These timepoints were selected after preliminary tests proved no effects were seen until at least 48 hours after exposure to chemotherapy. Presto blue viability assays were performed to assess viability of scr control cells, MAPT knockdown cells, and TUBB3 knockdown cells at these to timepoints after paclitaxel and doxorubicin exposure.

Diagram

Description automatically generated

***Figure 3: (****A+C) Dose-dependent response of cell viability to chemotherapy agents 48 and 72 hours after exposure to Paclitaxel. Lower dosages were used in the 48 hour condition because the 10 nM condition was determined to be well below the IC50 point in the 72 hour trial. B+D) 48 and 72 hours after exposure to Doxorubicin. One-way ANOVA t-test between two groups was used to assess significance. Each group (MAPT and TUBB3 knockdown) was compared to control cells (Scramble-Scr)*

Previously in the Oudin Lab, knockdown of MAPT and TUBB3 was performed on MBA-MD-231 cells. The knockdown was determined to be successful with >70% knockdown assessed by qPCR (MAPT) and Western Blot (TUBB3). These same cells were used to perform a viability assay with paclitaxel and doxorubicin; the results are shown in **Figure 3**. Upon exposure to paclitaxel, only MTCOM2 demonstrated a statistically significant decrease in viability compared to the control after 48 hours. Upon exposure to doxorubicin, MTCOM2, MTCOM3, and TUBB3 3-2 demonstrated a statistically significant decrease in viability compared to the control after 48 hours. No statistical significance was found at the 72 hour time point.

**Gene knockdown leads to morphological changes due to chemotherapy exposure**

Besides effects of chemotherapy on viability, effects on morphology should be analyzed as well. Morphology indicates the metastatic potential of the cell. To assess this, the cells were incubated with pacitaxel and doxorubicin and adhesion assays were performed.

A picture containing text, writing implement, screenshot, vector graphics

Description automatically generated

***Figure 4:*** *Effects of chemotherapy treatment on cell morphology on TUBB3 CRISPR knockdown cells. A+B) Graphs show fold change in the aspect ratio when subjected to doxorubicin and paclitaxel treatment respectively. C+D) Graphs show fold change in the form factor when subjected to doxorubicin and paclitaxel treatment respectively. E+F) Graphs show fold change in cell area when subjected to doxorubicin and paclitaxel treatment respectively.*

One replicate of TUBB3 knockdown had significantly smaller average aspect ratio when subjected to one concentration of doxorubicin (250nM) and to two concentrations of paclitaxel (2.5nM and 5nM). The results also indicate that chemotherapy leads to a larger form factor for one concentration of doxorubicin (500nM) and three concentrations of paclitaxel (0.625nM, 2.5nM, and 5nM). Lastly, both replicates of TUBB3 knockdown had larger cell areas across all concentrations of both chemotherapies. Overall, the trends seen were that TUBB3 knockdown cells had slightly smaller aspect ratios, larger form factors, and larger cell areas when subjected to chemotherapy.

A picture containing chart

Description automatically generated

***Figure 5:*** *Effects of chemotherapy treatment on cell morphology on MAPT CRISPR knockdown cells. A+B) Graphs show fold change in the aspect ratio when subjected to doxorubicin and paclitaxel treatment respectively. C+D) Graphs show fold change in the form factor when subjected to doxorubicin and paclitaxel treatment respectively. E+F) Graphs show fold change in cell area when subjected to doxorubicin and paclitaxel treatment respectively.*

Knockdown of MAPT led to larger aspect ratios of the cells for ¾ of the concentrations of doxorubicin, and for all four concentrations of paclitaxel. In addition, it leads to smaller form factors for ½ of the concentrations of doxorubicin, and for ¾ of the concentrations of paclitaxel. In terms of changes in cell area after doxorubicin treatment, one replicate of MAPT knockdown had an increase in cell area at 125nM, and it had a decrease in area at 500nM. MAPT knockdown cells had an increase in cell area after subjected to 0.625nM, 2.5nM, and 5nM of paclitaxel. However, it had a decrease in cell area when subjected to 1.25nM of paclitaxel. Overall, these results suggest that MAPT knockdown leads to larger aspect ratios and smaller form factors when exposed to chemotherapy. No conclusions can be drawn about fold changes in cell area due to contradicting data.

**DISCUSSION:** *Kevin, Ariana, and Maia*

TUBB3 has been previously considered to promote microtubule dynamics and MAPT to have stabilizing effect in microtubules.10,11 MAPT and TUBB3 therefore have opposite roles on microtubule dynamics but at the same time induce similar effects in cell morphology and migration.12As previously mentioned, TUBB3 and MAPT have shown to opposite effects on microtubule dynamics. Clinical data indicate a correlation between resistance to taxane based chemotherapies and an overexpression of MAPT and TUBB3.4,5 If this resistance is caused by overexpression of these genes, then knockdown cells should be more sensitive to paclitaxel. In **Figures 3A** and **3C,** we investigated the cell viability in response to paclitaxel, a taxane based chemotherapy agent that targets mitotic microtubules in rapidly dividing cancer cells. The TUBB3 knockdowns were not more sensitive to paclitaxel after 48 or 72 hours. One MAPT knockdown, however, did have a statistically significant decrease in viability after 48 hours. Since only one of the replicate showed this pattern, more experimentation is needed.

**Figures 3B** and **3D** show the cell viability in response to doxorubicin, which targets cancer by disrupting nucleotide formation, thereby inhibiting proliferation. So, doxorubicin induces DNA damage, however, does not affect microtubule dynamics. One replicate of TUBB3 and both replicates of MAPT had a statistically significant decrease in viability as a result of exposure to this drug.

Reasoning why we saw different data at 48 hours and 72 hours was likely due to the confounding variable of cell death due to high confluency. 72 hours may have been too long of an incubation period to keep the cells in a 96-well plate. Too long of an incubation period will cause cells to die from confluency, not solely due to chemotherapy exposure.

These preliminary results suggest that there might be a correlation between neuronal genes MAPT and TUBB3 and the effectiveness of chemotherapy. Nevertheless, more trials are needed in order to draw significant conclusions. If the differences seen in the above figures are true, these genes may serve as potential therapeutic targets for TNBC. Overall, this data may also motivate the progression of further research in the neuronal identity of TNBC.

This research may help us identify new biomarkers. The decision to start chemotherapy is difficult for physicians. The malignancy of a specific tumor is often unknown. In many cases, patients with mild tumors can receive chemotherapy unnecessarily, which subjects them to significant side effects and a decrease in quality of life. By performing genetic screening, the severity of the tumor can be determined by looking at the relative expression levels of genes like MAPT and TUBB3. Since MAPT and TUBB3 are correlated with higher malignancy,4,5 a physician may be more inclined to start a chemotherapy regimen. Conversely, if a tumor lacks malignant biomarkers, the chemotherapy treatment can be deemed unnecessary. This gives physicians more comprehensive information to improve their clinical decisions and tailor their treatment more specifically to each individual’s illness. Before this can happen, the physician’s decision has to be contextualized in a significant amount of background research on the complex effects of neuronal genes on TNBC cell behavior.

**FUTURE WORK**:

In future work, the focus will be on assessing the applicability of the findings on other human TNBC cell lines. This will involve using the optimized knockdown method from Aims 1 and 2 to investigate the effects of knockdown on cell behavior and chemotherapy sensitivity in two other TNBC cell lines. The purpose of using other cell lines is to control for heterogeneity in cancer cells. The experiments are expected to yield the same result as the same gene knockdowns will be performed. The preferred knockdown method will be used, and three biological replicates will be tested in duplicate within each cell line. The comparative analysis of the collected data is expected to be completed in six months, and the subsequent months will be used to analyze the data. If results are consistent in other human TNBC cell lines, the findings will be further validated, while inconsistent results will open new avenues of investigation. Overall, these future aims will contribute to a better understanding of the mechanisms behind the role of neuronal genes in triple-negative breast cancer and develop better therapies

*Lab work has been distributed evenly between Ariana, Kevin, and Maia.*

**GANTT CHART: TRACKING PROJECT PROGRESS (UPDATED AS OF 04/219/23) *-*** *Ariana* [Capstone Gantt chart](https://docs.google.com/spreadsheets/d/1Z9VKEtE3O5IKDmT--ln3TSJSga9YQ2dw_hKlbJXKOek/edit?usp=sharing)

The colors in our Gantt chart represent the level of completion of the activity. Green stands for completion, yellow for “in progress”, grey for upcoming(not started), and red for delayed or canceled activity. We kept the column that indicates the percentage of the activity completed (the closer the percentage gets to 100, the darker the green becomes). Chart

Description automatically generated

Update: 4/21

Shape, square

Description automatically generatedChart

Description automatically generated

Lastly, we decided to change our viability plots into heat maps instead of line graphs. This allows us to show which specific concentrations of drug generate the significantly different viability between groups.

**APPENDIX:**

A picture containing text, tree

Description automatically generated

**Appendix 1:**  Representative image of cells after siRNA knockdown in an adhesion assay. The cells were too confluent to accurately analyze cell morphology.

**References:**

1 Waks, A. G. & Winer, E. P. Breast Cancer Treatment: A Review. *JAMA* **321**, 288-300, doi:10.1001/jama.2018.19323 (2019)

2 Deepak, K. G. K. *et al.* Tumor microenvironment: Challenges and opportunities in targeting metastasis of triple negative breast cancer. *Pharmacol Res* **153**, 104683, doi:10.1016/j.phrs.2020.104683 (2020)

3 Weiss, M. C. *et al.* A Coala-T-Cannabis Survey Study of breast cancer patients' use of cannabis before, during, and after treatment. *Cancer* **128**, 160-168, doi:10.1002/cncr.33906 (2022).PMC9413357

4 Wang, K. *et al.* Tau expression correlated with breast cancer sensitivity to taxanes-based neoadjuvant chemotherapy. *Tumour Biol* **34**, 33-38, doi:10.1007/s13277-012-0507-z (2013)

5 Ikeda, H. *et al.* The estrogen receptor influences microtubule-associated protein tau (MAPT) expression and the selective estrogen receptor inhibitor fulvestrant downregulates MAPT and increases the sensitivity to taxane in breast cancer cells. *Breast Cancer Res* **12**, R43, doi:10.1186/bcr2598 (2010).PMC2917038

6 Hudis, C. A. & Gianni, L. Triple-negative breast cancer: an unmet medical need. *Oncologist* **16 Suppl 1**, 1-11, doi:10.1634/theoncologist.2011-S1-01 (2011)

7 Cortes, J. *et al.* Pembrolizumab plus Chemotherapy in Advanced Triple-Negative Breast Cancer. *N Engl J Med* **387**, 217-226, doi:10.1056/NEJMoa2202809 (2022)

8 Yao, Y., Chu, Y., Xu, B., Hu, Q. & Song, Q. Risk factors for distant metastasis of patients with primary triple-negative breast cancer. *Biosci Rep* **39**, doi:10.1042/BSR20190288 (2019).PMC6549086

9 Lippman, S. M. & Brown, P. H. Tamoxifen prevention of breast cancer: an instance of the fingerpost. *J Natl Cancer Inst* **91**, 1809-1819, doi:10.1093/jnci/91.21.1809 (1999)

10 Panda, D., Miller, H. P. & Wilson, L. Rapid treadmilling of brain microtubules free of microtubule-associated proteins in vitro and its suppression by tau. *Proc Natl Acad Sci U S A* **96**, 12459-12464, doi:10.1073/pnas.96.22.12459 (1999).PMC22948

11 Feinstein, S. C. & Wilson, L. Inability of tau to properly regulate neuronal microtubule dynamics: a loss-of-function mechanism by which tau might mediate neuronal cell death. *Biochim Biophys Acta* **1739**, 268-279, doi:10.1016/j.bbadis.2004.07.002 (2005)

12 al., T. T. L. e. Sensory nerves enhance triple-negative breast cancer migration and metastasis via the axon guidance molecule PlexinB3. *bioRxiv* (2022)

13 Lebok, P. *et al.* High levels of class III beta-tubulin expression are associated with aggressive tumor features in breast cancer. *Oncol Lett* **11**, 1987-1994, doi:10.3892/ol.2016.4206 (2016).PMC4774425

14 O'Reilly, D., Sendi, M. A. & Kelly, C. M. Overview of recent advances in metastatic triple negative breast cancer. *World J Clin Oncol* **12**, 164-182, doi:10.5306/wjco.v12.i3.164 (2021).PMC7968109

15 Harbeck, N. & Gnant, M. Breast cancer. *Lancet* **389**, 1134-1150, doi:10.1016/S0140-6736(16)31891-8 (2017)

16 Rakha, E. A. & Chan, S. Metastatic triple-negative breast cancer. *Clin Oncol (R Coll Radiol)* **23**, 587-600, doi:10.1016/j.clon.2011.03.013 (2011)

17 Yang, D. *et al.* Axon-like protrusions promote small cell lung cancer migration and metastasis. *Elife* **8**, doi:10.7554/eLife.50616 (2019).PMC6940020

18 Karki, R. *et al.* betaIII-Tubulin: biomarker of taxane resistance or drug target? *Expert Opin Ther Targets* **17**, 461-472, doi:10.1517/14728222.2013.766170 (2013)