**BME Senior Capstone Project:**

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

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December 9, 2022

All Updates in Yellow

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

**Abstract:** *Maia*

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 (Waks & Winer, 2019). TNBC is prevalent in 20% of all breast cancer diagnoses; it is known to be an aggressive, metastatic subtype with high mortality rates (Deepak, 2020). 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 (Marisa Weiss, 2021). Understanding the mechanisms driving metastasis is crucial for the development of more effective therapies for TNBC. One avenue for exploration is the role of neuronal genes in 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(22976542)(20579400). Our goal in this project is to further investigate how these neuronal genes play a role in chemotherapy sensitivity of TNBC cells. 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 triple negative breast cancer cells. In addition, we plan to investigate the effects on sensitivity to chemotherapy agents. The focus of this project's design work is optimizing the platform to perform gene knockdown that is needed to study these effects. 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 that gene knockdown will lead to 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 knocked down in triple-negative breast cancer cells, which is why both methods need to be evaluated to determine which is more efficacious in gene knockdown.

We will then evaluate the effects 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 morphology, and cell proliferation. Furthermore, to analyze our results, we will mathematically determine the differences in cell proliferation, movement, morphology, and viability 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 at least 70% knockdown of the gene is achieved, as this is the standard used in literature. A study from 2015 has shown that 70% knockdown was sufficient to produce significant changes in breast cancer cells (Fan, 2015). If the gene is not sufficiently knocked down, it introduces confounding variables to the study. We will be assessing the efficiencies of knockdown with the use of immunohistochemistry with TUBB3 and qPCR for MAPT. These methods were chosen given that TUBB3 has a very common antibody used for staining while MAPT does not. A limitation of ours is what is available in the lab; we will only be able to gauge gene expression of MAPT via qPCR. We will have to use immunohistochemistry, which assesses protein expression instead of gene expression, to quantify knockdown of TUBB3.

We will use a one-way ANOVA t-test between two groups to assess the significance of the effect of knockdown on migration, proliferation, and morphology. 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 migratory behavior and proliferation rate 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 for significant differences in 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. Lastly, we will assess whether chemotherapy induces morphological changes in cancer cells after knockdown. CellProlifer will be used to analyze cell morphology metrics.

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. (Waks & Winer, 2019). 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). (Hudis et al. 2011). 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 (Waks & Winer, 2019). The disease has a 5 year survival rate of 40%. (Yavuz et al. 2022).

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. (Yao et al. 2019). 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. (Lippman & Brown, 1999). 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 allow 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(10535944). 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(15615645). 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(10535944). MAPT and TUBB3 therefore have opposite roles on microtubule dynamics but at the same time induce similar effects in cell morphology and migration (Le, 2022). 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 (26998111,<https://breast-cancer-research.biomedcentral.com/articles/10.1186/bcr2598> ). 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. 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. (O’Reily et al, 2019). Cancer cells spread throughout the body via the lymphatic system, the bloodstream, or through perineural invasion. Metastatic triple negative breast cancer is fatal (Harbeck & Gnant, 2017).

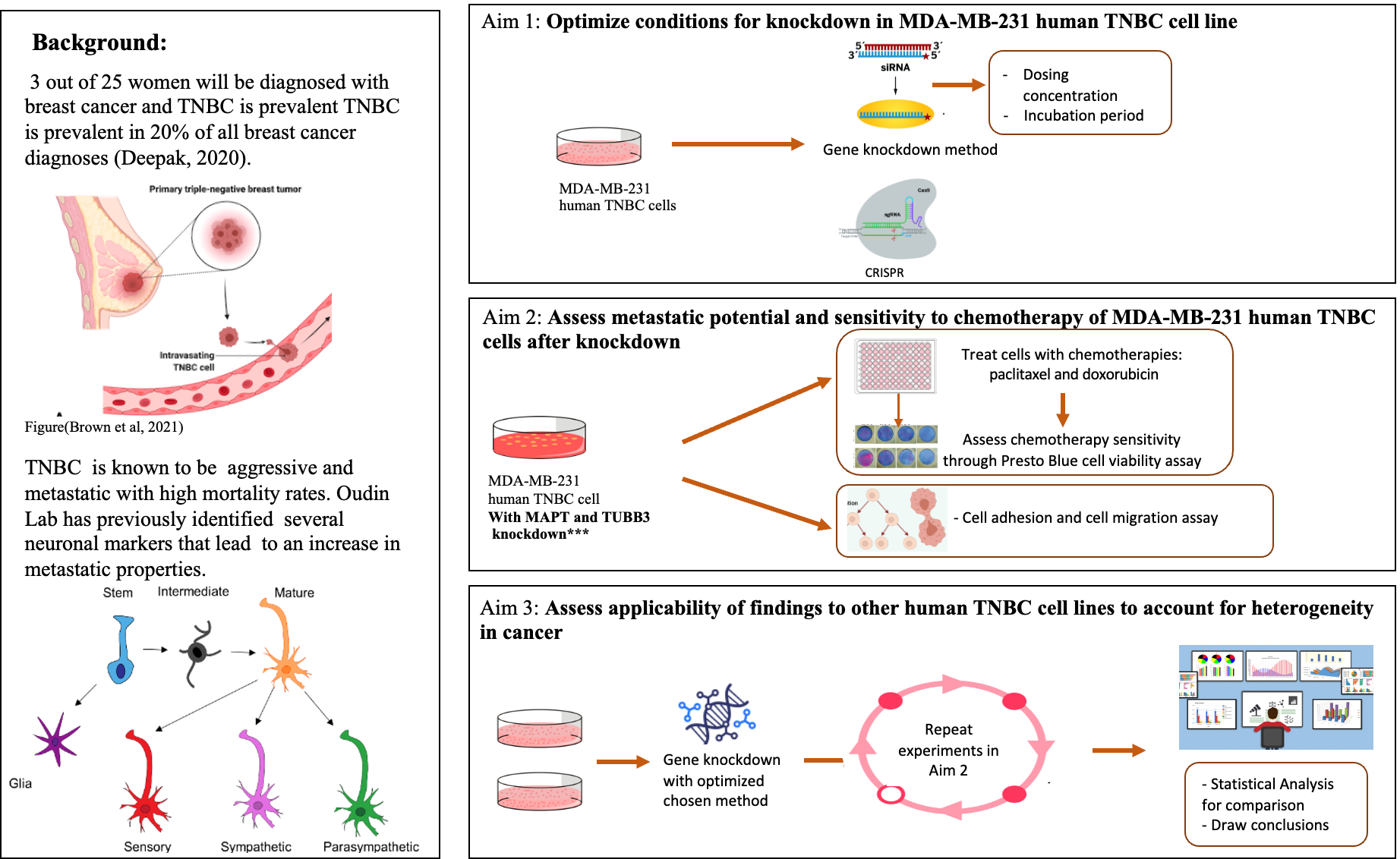
Chemotherapy remains the only FDA-approved treatment for TNBC; however, it has significant negative side effects including hair loss, appetite loss, and anemia. This treatment does not prevent metastasis, as roughly 30% of early-stage breast cancers become metastatic regardless of chemotherapy (Marisa Weiss, 2021). 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) (Rakha & Chan, 2011).

A recent article by Yang et al revealed an upregulation of neuronal genes, including TUBB3 and Tau, in SCLC. They found this upregulation affected the cell morphology and cell migration. 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 (Le, 2022). 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 (cite: <https://pubmed.ncbi.nlm.nih.gov/23379899/> and <https://pubmed.ncbi.nlm.nih.gov/22976542/>). This insight gives us motivation to investigate directly if knockdown of these genes will sensitize the cells to chemotherapy.

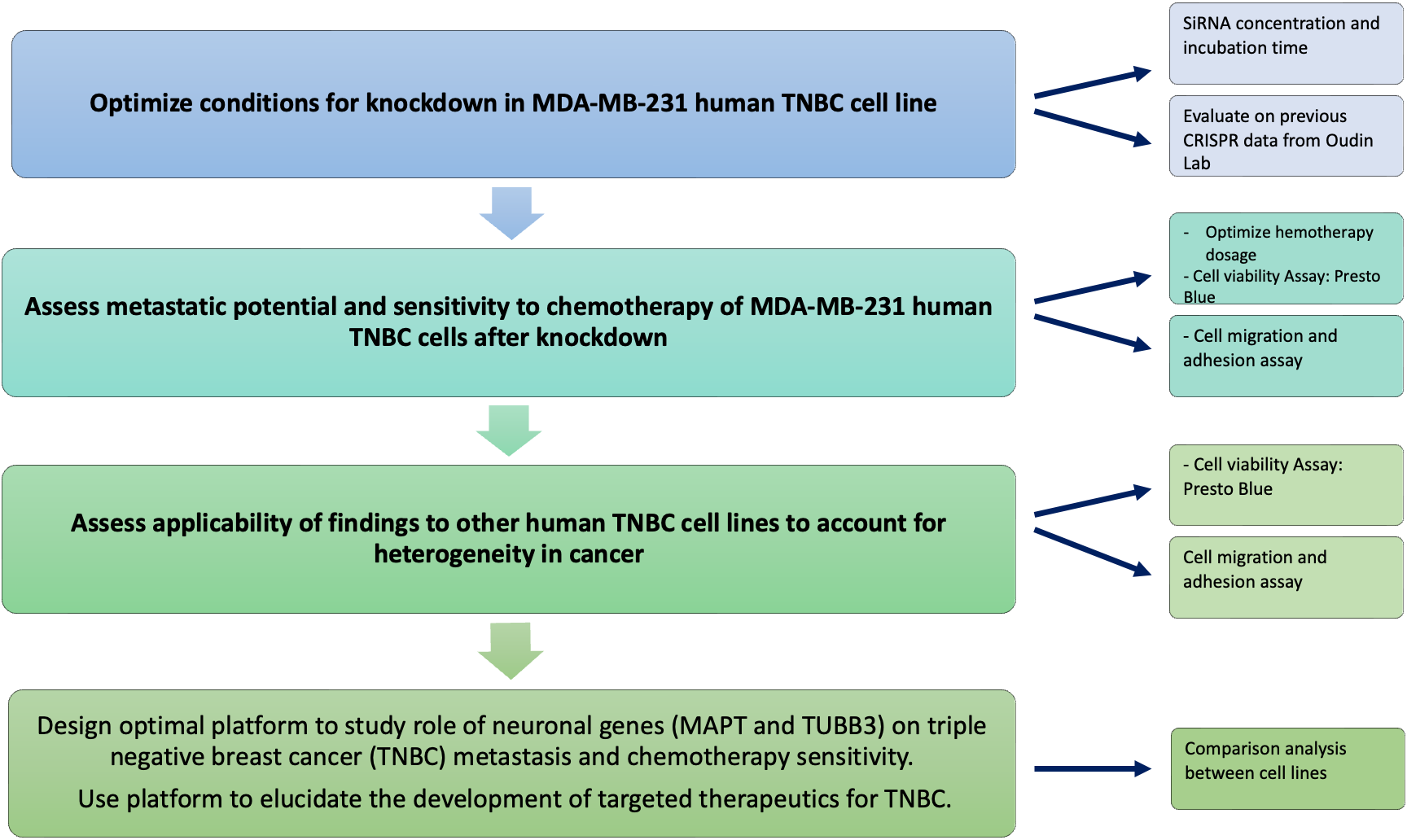
Previous research in the Oudin Lab revealed that knockdown of TUBB3 in MDA-MB-231 breast cancer cell line led to the formation of long processes (Le, 2022). In addition, knockdown led to an increase in cell speed, saltatory movements, and cell migration. MAPT was then investigated, and those cells exhibited consistent behavior with that of TUBB3 knockdown cells (Le, 2022). 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*

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*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 and 3) Assess applicability of findings to other human TNBC cell lines (to be determined) to account for heterogeneity in cancer*

**Design Flow Chart:** *Ariana*

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*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 to be evaluated in Specific Aim 3.

**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.

**Specific Aim 3: Assess applicability of findings to other human TNBC cell lines (*to be determined*) to account for heterogeneity in cancer**

The purpose of this Aim is to account for heterogeneity differences that can be seen across cancer lines. It has become standard for cancer research to assess findings in more than one cell line to validate the results. Knockdown will be performed via the optimized method determined in Aims 1 and 2. This aim does not involve any further comparison of the two knockdown methods. It will simply use the protocol that was found to be most effective in earlier experiments. After, the same assays will be performed to investigate effects of knockdown on cell migration, cell morphology, and chemotherapy sensitivity. If results are consistent in other human TNBC cell lines, the data found will be further supported and validated. If results are not consistent, then TUBB3 and MAPT must play different roles in each cell line, which opens the door for new avenues of investigation. Overall, these aims will further the understanding behind the mechanisms through which these neuronal genes play a role in triple-negative breast cancer and is a step closer to developing better therapies.

**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*

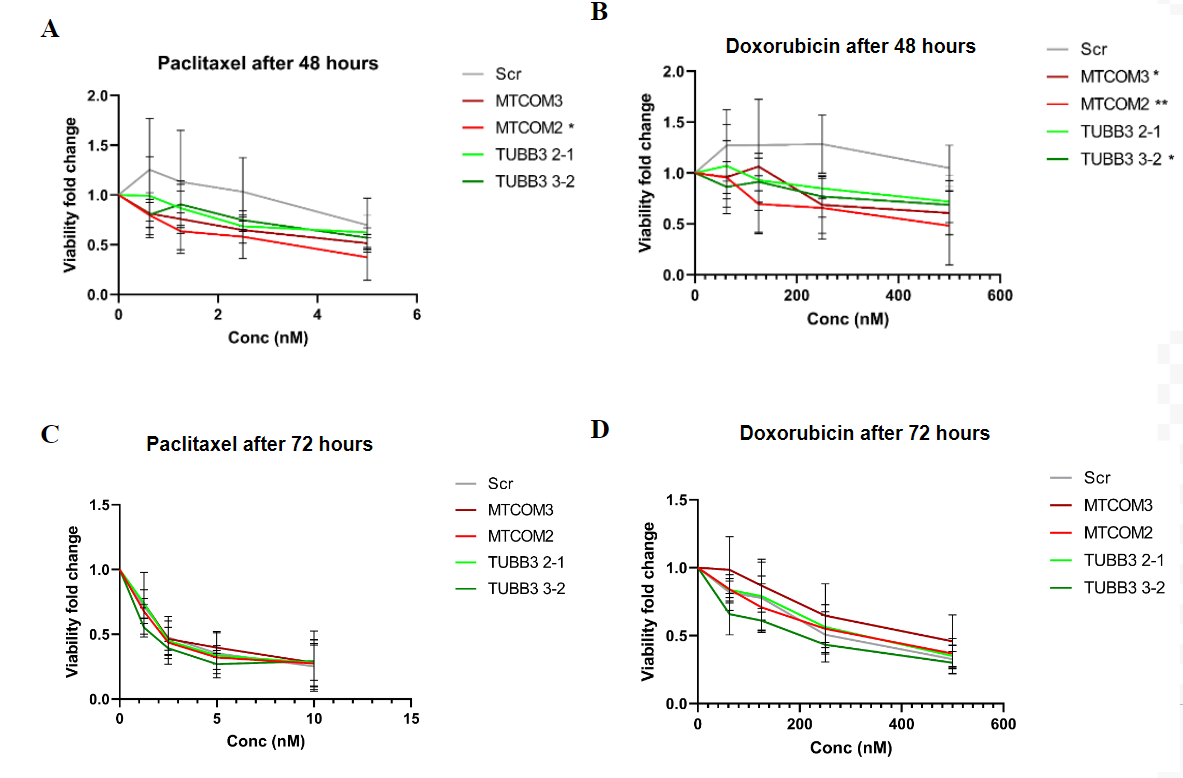
A glass-bottom 24 well plate will be used. One the first day, the plate will be coated with 0.1mg/mL Collagen I for 1 hour at 37C. After, 300 uL cells will be seeded at 12k/well in media. The plate is then incubated for 2 hours at 37C. The migration chamber will be set up using image acquisition setting, 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*

**Figure 1:** Dose dependent response of cell viability to chemotherapy agents **A+C)** 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 1**. 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.

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

TUBB3 has been previously considered to promote microtubule dynamics and MAPT to have stabilizing effect in microtubules.(10535944)(15615645)(10535944). MAPT and TUBB3 therefore have opposite roles on microtubule dynamics but at the same time induce similar effects in cell morphology and migration (Le, 2022).

As 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(22976542)(20579400). If this resistance is caused by overexpression of these genes, then knockdown cells should be more sensitive to paclitaxel. In **Figures 1A** and **1C,** 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 1B** and **1D** 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(22976542)(20579400), 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.

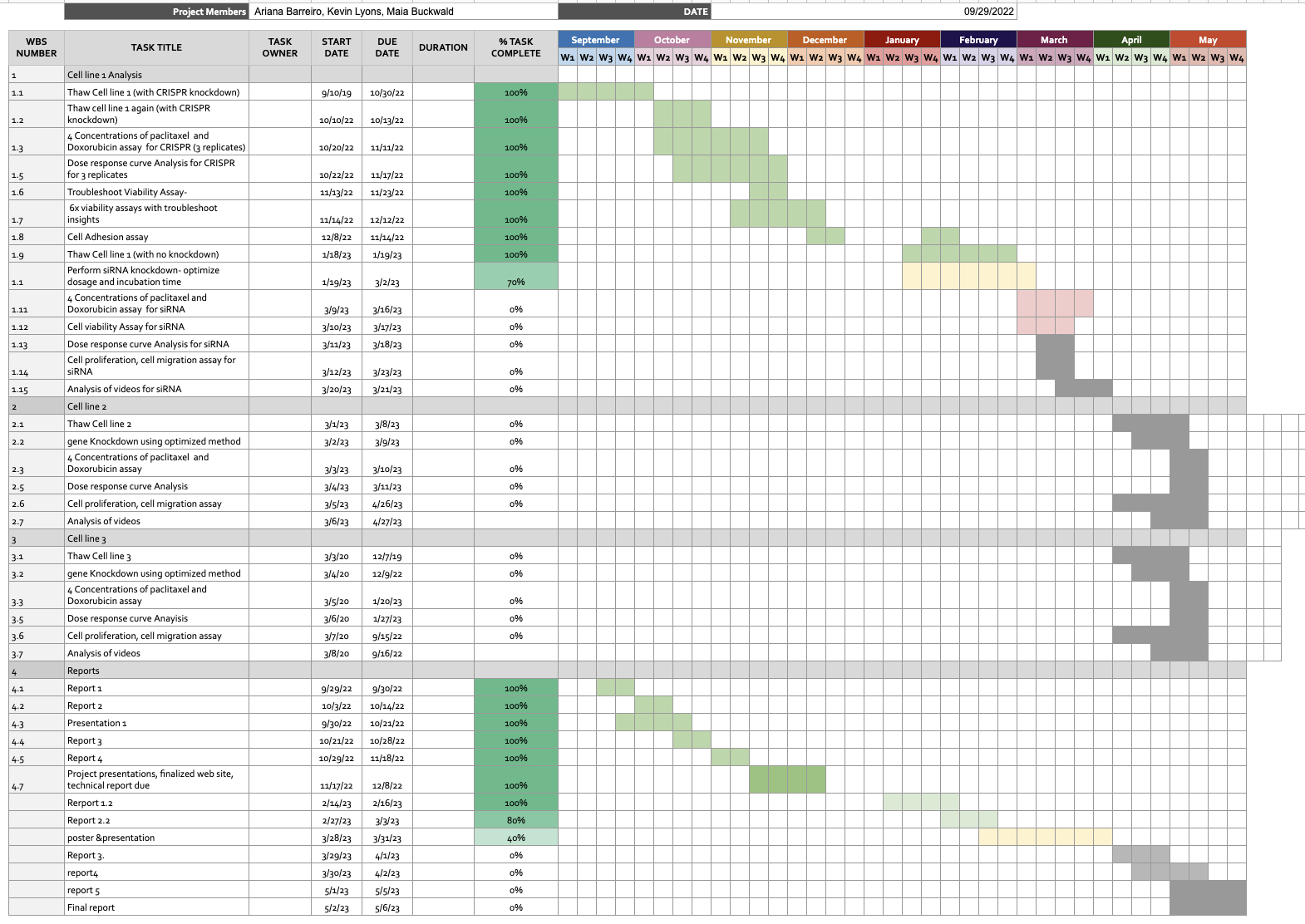
**FUTURE WORK** – what should students do next year to pick up on your project from this year, were it to continue. Or let us know it should not continue and why, etc.

**PARTICIPATION** – list individual contributions of each group member to the project

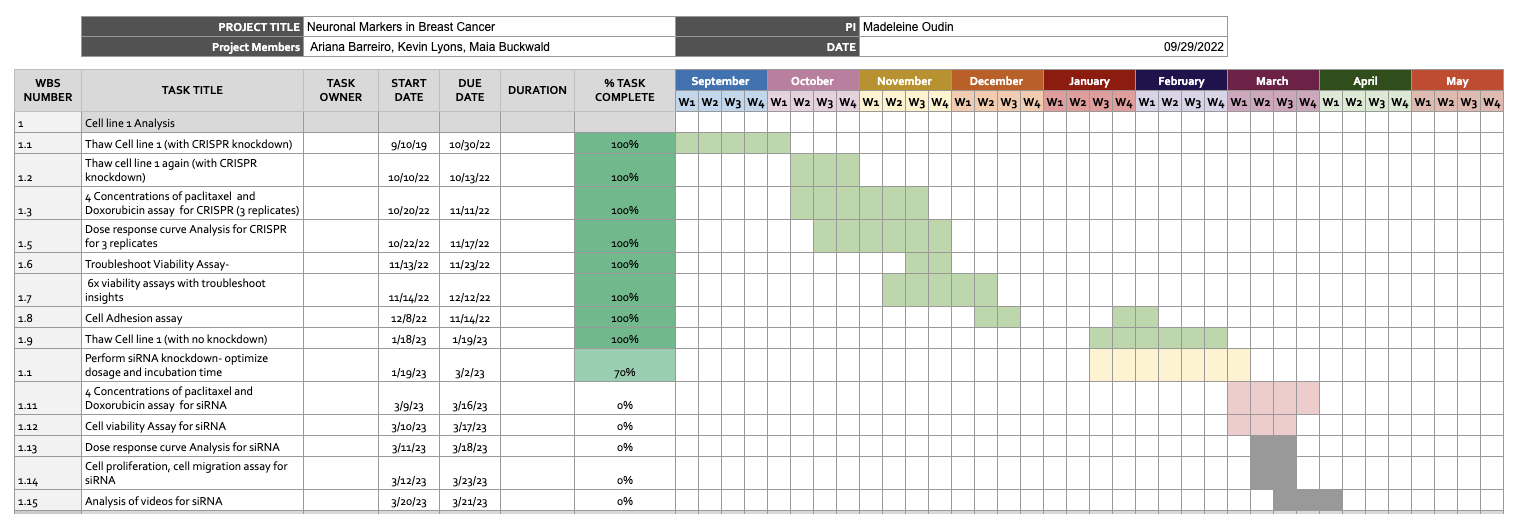
*Lab work has been distributed evenly between Ariana, Kevin, and Maia.*

**GANTT CHART: TRACKING PROJECT PROGRESS (UPDATED AS OF 12/09/22) *-*** *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).

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**Zoom in for relevant dates:**



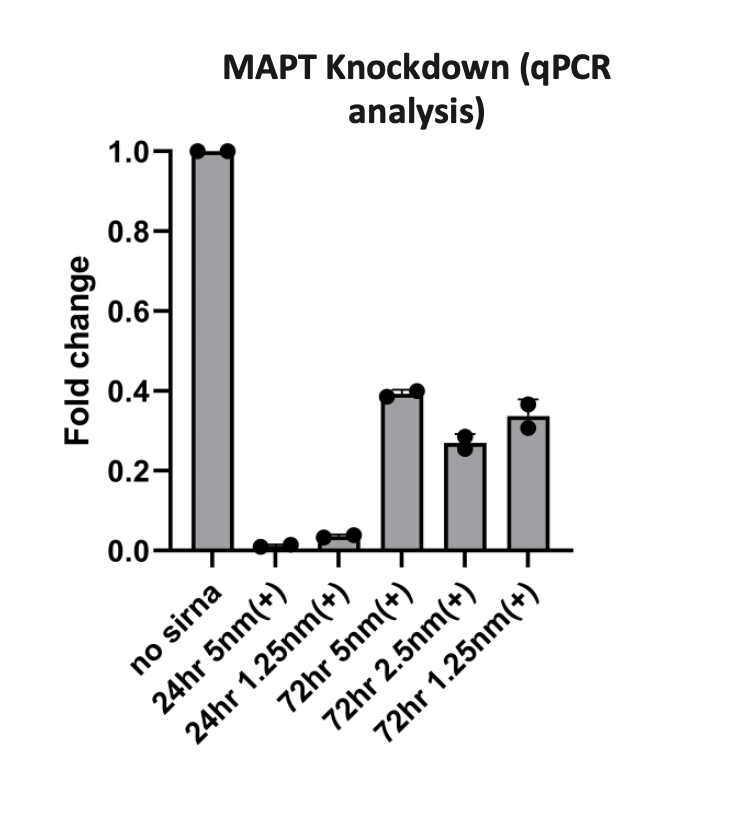
Progress Update: 3/3

This past couple of weeks, we have been continuing to try to optimize our knockdown with siRNA. This involves performing multiple trials with different concentrations of siRNA and time points in order to see which produces the most effective knockdown. In order to quantify the knockdown with β3-tubulin, we are using immunostaining since there is an effective antibody that conjugates well with this protein.We initially had many challenges in both the qPCR (for MAPT) and immunostaining (for TUBB3) protocols and were not able to obtain any data.

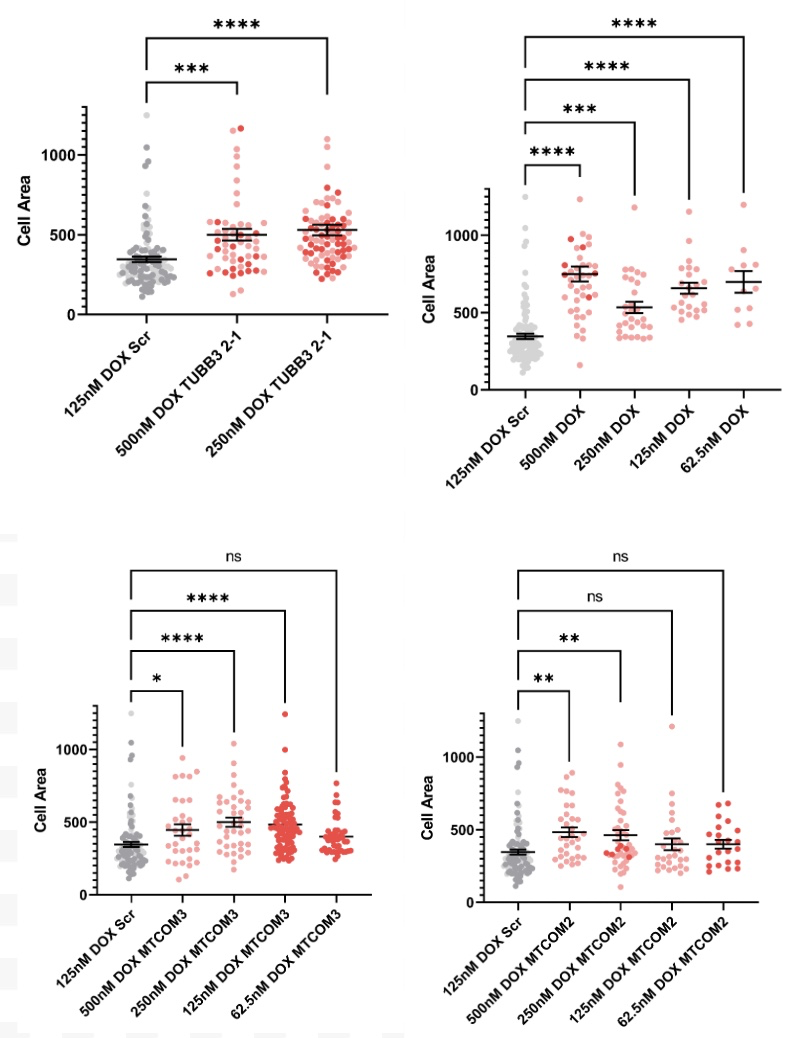
After troubleshooting and performing siRNA transfection followed by immunostaining 5 times, we finally realized we failed to initially fix the cells, which was why we were unable to obtain results.

We are performing immunostaining again this week and hope that by fixing the cells properly before adding the proper antibodies, we can finally quantify knockdown for TUBB3.

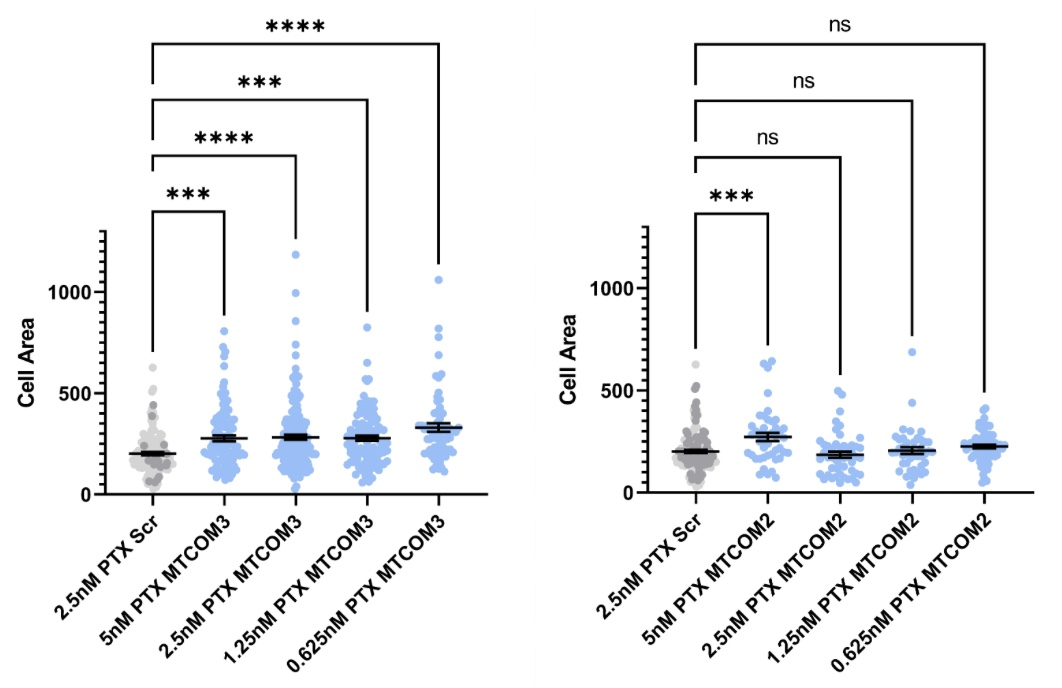
After having conducted troubleshooting procedures over the past two weeks for the optimization of MAPT knockdown through qPCR analysis, we have succeeded in obtaining amplification curve data. We failed to get data initially because we most likely did not load enough cDNA into the thermal cycler, and/or we used the wrong settings on the thermal cycler. Provided below is the preliminary data we have gathered for the purpose of quantifying MAPT knockdown. The data illustrated represents two separate qPCR experiments utilizing identical cDNA concentrations. It is evident from the graph that the 24-hour time point yields a higher degree of knockdown for MAPT (approximately 100%). Furthermore, the data collected at 72 hours suggests that knockdown was successful, as evidenced by a 0.7-fold decrease in MAPT concentration when compared to the control at a 2.5nM siRNA concentration. Unfortunately, proper readings were unattainable for the 24-hour 25nm concentration samples, prompting us to re-run the qPCR with new dilutions. Additionally, we intend to conduct further replicates to ensure the reproducibility of the data, and subsequently, we plan to perform statistical analysis.



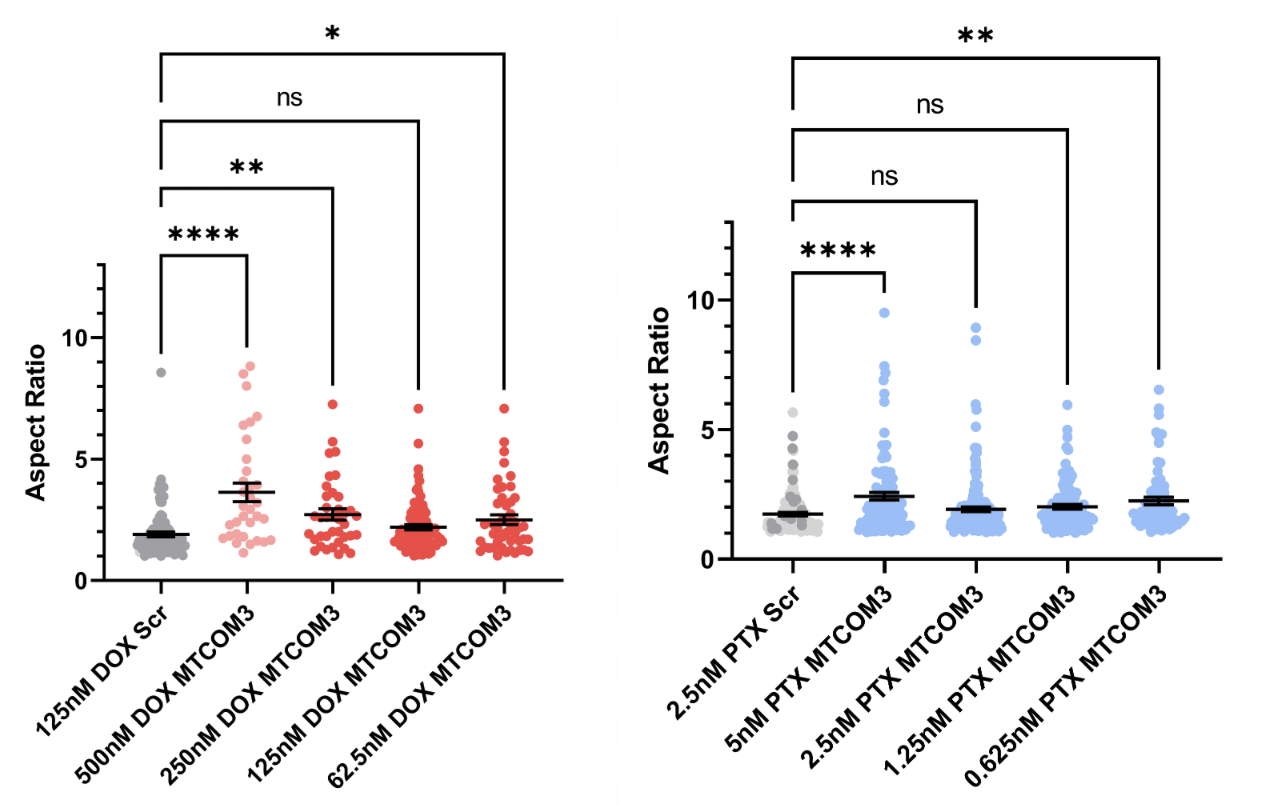
Our project is currently behind schedule because the subsequent parts of our experimental plan are entirely dependent on an effective knockdown protocol. Before running additional viability, adhesion, and migration assays, we need to determine the optimal siRNA concentration and incubation time for a knockdown. The delays we have experienced due to failed immunostaining and qPCR protocols have put our project behind schedule. Nevertheless, as we are beginning to see success in the qPCR and immunostaining protocols, we believe that we will be able to begin the viability assays soon.

Another update is that we have completed a substantial amount of our adhesion assay analysis. We performed this adhesion assay to look at changes of cell morphology after administering chemotherapy to our control and knockdown cells. Morphology is indicative of metastatic potential of the cancer cells. Cells that are larger and more elongated are more likely to metastasize throughout the body. To note, we are still working on how to best illustrate our data in these figures, but these are the preliminary figures we created. First, we looked at differences in cell area between Scr control cells that have been subjected to doxorubicin and knockdown cells that have been subjected to doxorubicin. The results are shown in the figure to the right. There appears to be a trend that knockdown cells are larger in cell area after doxorubicin treatment. This does not necessarily mean the chemotherapy is responsible for larger cell area, because it was previously found that knockdown cells are larger in cell area to begin with. 

We also created a figure that compares the cell area of our paclitaxel treated Scr control cells to the cell area of our paclitaxel treated MAPT knockdown cells. The images we obtained were blurry for our TUBB3 knockdown cells, which prevented us from getting data for that parameter. The results are shown in the figure below. These results also indicate that knockdown cells may be larger in cell area compared to Scr after paclitaxel treatment. To reiterate, it is important to note that knockdown cells are larger than Scr cells without treatment. So, the larger cell area may not be caused by the chemotherapy treatment.



We also looked at one more morphological parameter: aspect ratio. This ratio is found by dividing the major axis length by the minor axis length of the cells. A higher aspect ratio means the cell is more elongated. We are still in the process of forming figures, but so far we have created figures comparing MAPT to control cells (shown to the right). It appears that doxorubicin-treated and paclitaxel-treated knockdown cells are more elongated than the Scr control cells. Prior to treatment, the knockdown cells were more elongated, so chemotherapy may not be responsible for this trend.



We looked at cell morphology after chemotherapy administration to see how the morphology of the knockdown cells compared to the Scr control cells. It appears that chemotherapy induces proportional changes in cell area and aspect ratio to all experimental groups since knockdown cells were already previously larger and more elongated. This indicates that knockdown of MAPT and TUBB3 appears to not affect chemotherapy-induced morphological changes. As noted in our previous experiments, knockdown of these genes does however sensitize the cells to chemotherapy. So overall, knockdown of these genes increases sensitivity to chemotherapy, while appearing to not affect the metastatic potential of the cells (respective of the morphology).

**REFERENCES:**

1. Liebig, C., Ayala, G., Wilks, J. A., Berger, D. H., & Albo, D. (2009). Perineural invasion in cancer: areview of the literature. *Cancer, 115*(15), 3379-3391. doi:10.1002/cncr.24396 PMID: 19484787
2. Marisa Weiss, M. D., Brian Wojciechowski, M.D., Sameer Gupta, M.D., MPH,. (2021). Metastatic Breast Cancer. Retrieved from <https://www.breastcancer.org/symptoms/types/recur_metast#:~:text=Nearly%2030%25%20of%20women%20diagnosed,cancer%20will%20develop%20metastatic%20disease>.
3. McAllister, A. J. R. S. S. (2013). Breast cancer as a systemic disease: a view of metastasis. *Wiley Online Library*. Retrieved from <https://onlinelibrary.wiley.com/doi/full/10.1111/joim.12084> PMCID: PMC3711134
4. Rakha, E. A., & Chan, S. (2011). Metastatic triple-negative breast cancer. *Clin Oncol (R Coll Radiol), 23*(9), 587-600. doi:10.1016/j.clon.2011.03.013 PMID: 21524569
5. Waks, A. G., & Winer, E. P. (2019). Breast Cancer Treatment: A Review. *JAMA, 321*(3), 288-300. doi:10.1001/jama.2018.19323 PMID: 30667505
6. Le, Thanh T. (2022). The neural identity of triple-negative breast cancer. A thesis in partial fulfillment of the requirements for the degree of Ph.D. in Biomedical Engineering. *Tufts University.*
7. Fan, Y., & Guo, Y. (2015). Knockdown of eIF3D inhibits breast cancer cell proliferation and invasion through suppressing the Wnt/β-catenin signaling pathway. *International journal of clinical and experimental pathology*, 8(9), 10420–10427.