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Trefoil Factor 1 (TFF1) Expression in E3 and EWD8 Breast Cancer Cell Lines

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Introduction

Cancer is one of the largest epidemics in our world. In 2012, an estimated 14 million new cases of cancer and 8.2 million cancer-related deaths were documented worldwide (National Cancer Institute, 2015). Cancer is also associated with aging; as life expectancy rises, so does the risk of getting cancer. Globally, an estimated one in three women and one in two men will develop cancer in their lifetimes (RT Answers, 2016). Cancer is characterized by uncontrollable cell growth that gains resistance to death signals, increasing pro-survival mechanisms of the cancerous cell and its properties (National Cancer Institute, 2015). Early detection and prevention of cancer has become just as paramount a concern as treatment, leading many to investigate new possible biomarkers for the disease. A viable biomarker is indicative of the presence of cancer within the body and helps diagnose patients promptly. Trefoil factors (TFFs) are proteins secreted by epithelial cells primarily for wound healing in mucosal membranes, which makes their investigation as biomarkers attractive (Thim & May, 2005).

TFFs are secreted proteins that help in healing epithelial tissue in the stomach and thus have the potential to be biomarkers in many types of cancers (Thim & May, 2005). The normal function of TFFs is to regulate cell growth and assist cells in recognizing environmental stress that leads to inflammation (Aihara, Engevik, & Montrose, 2017). While TFFs are an intriguing cancer biomarker, further investigation of cancer cell function in the context of TFF expression could also provide information on the plausibility of TFFs as treatment targets. Such research is required to understand their expression and function in cancerous cells and therefore mitigate aggressive and potentially intractable metastatic disease.

In this study, we aimed to identify the levels of TFF1 expression in cancerous cells that have not been investigated for this family of proteins. We then correlated the varying levels of

expression to the aggressiveness of the cancer. We hypothesized that there would be varying amounts of TFF1 expression due to the functional characteristics of the cancer cells, and it was also our expectation that increased levels of TFF1 could possibly support a more aggressive cancer. Findings concluded that the breast cell lines tested were the only lines to express the protein and were actually less aggressive than the lines with no TFF1 expression.

Literature Review

Cancer markers are substances naturally produced by the body, and when cancer is present, these substances are produced at different levels (National Cancer Institute, 2015). As proteins secreted by epithelial cells primarily for wound healing in mucosal membranes, TFFs have the potential to be viable biomarkers (Thim & May, 2005). These proteins have a trefoil motif structure composed of disulfide loops (Thim, 1989). There are three kinds of TFFs: TFF1, TFF2 and TFF3 (Aihara et al., 2017). TFF1, previously known as pS2, was first found in breast cancer, and TFF2 was extracted and purified from the pancreas (Masiakowski et al., 1982; Jørgensen, Jørgensen, Diamant, & Thim, 1982). Intestinal trefoil factor, now TFF3, was later found in both large and small intestines (Suemori, Lynch-Devaney & Podolsky, 1991). The proteins in this family are similar in structure but vary in expression and function throughout the body (Madsen, Nielsen, Tornøe, Thim, & Holmskov, 2007).

The stomach is the only organ in which all three TFFs are expressed (Madsen et al., 2007). TFF1 expression is highest in the stomach and colon, while TFF2 levels are highest in the stomach, and TFF3 levels are highest in the colon (Madsen et al., 2007). Other studies have looked at gastric and breast cancer to determine TFF expression (Lefebvre et al., 1996). TFF proteins are gaining recognition as cancer biomarkers in the field. The purpose of this literature

review is to investigate TFF expression in different cancers and identify their likelihood as biomarkers.

Trefoil Factors in Gastric Cancer

Gastric cancer is the fifth most common cancer worldwide, accounting for almost 1,000 of the new cancer cases in 2012 (World Health Organization, 2015). There are known classic biomarkers for gastric cancer, but TFFs may be novel markers for the disease (Jin, Jiang, & Wang, 2015). TFF1 expression is essential for gastric mucosa cells to differentiate (Lefebvre et al., 1996). When expression of TFF1 is low, gastric adenomas can develop, and a percentage of the tumors can become cancerous (Lefebvre et al., 1996). Low expression of TFF1 in the gastric mucosa could show signs for early detection of stomach adenoma carcinoma, but the opposite could be true for TFF3.

TFF3 expression levels are typically low in gastric mucosa but are overexpressed in gastric cancer (Chan et al., 2005). High TFF3 expression could be an effective biomarker for gastric cancer. In one study, 90 gastric cancer patients provided blood and urine samples that were examined for TFF3 levels (Xiao, Liu, Xiao, Ren, & Guleng, 2014). TFF3 levels were significantly higher in the serum and urine of cancer patients compared to the healthy individuals. These higher levels correlated with the advancement of the stages as well as the distance of metastasis. The authors concluded that TFF3 serum could be a biomarker for gastric cancer in detecting tumor stages and in identifying metastases. Overexpression of TFF3 in gastric cancer has led researchers to consider that it has potential as a cancer marker, and the same may be true for TFF2 (Cai et al., 2016).

TFFs appear to be regulated on their own, but in some cases other proteins or substances help mediate their regulation. Sp3 protein was found to be an essential binding partner to TFF2,

which mediates the biological functions of the protein in gastric cancer cells (Cai et al., 2016). Knockdown of Sp3 protein prompted TFF2 upregulation and invasion of the gastric cancer cells. Overexpression of the two proteins suppressed cell proliferation, induced apoptosis of the cancer cells, and significantly lowered cell invasion. These findings showed that Sp3 is needed to regulate TFF2, and together, their overexpression (or their absence) could make useful cancer markers (Cai et al., 2016).

Trefoil Factors in Breast Cancer

According to the World Health Organization, breast cancer is the most common cancer among women and the second most common cancer worldwide (2015). It is well established that TFFs are expressed in MCF-7 breast cancer cell lines (Thim & May, 2005). In a study that used knockdown TFF1 in MCF-7 cells, the effects of estrogen with doxorubicin treatment were tested (Pelden, Insawang, Thuwajit, & Thuwajit, 2013). The cells that started with a low expression of TFF1 had upregulated expression after being transfected. The proteins expressed pro- or anti-apoptotic properties, which led the authors to the idea that TFF1 has a role in the apoptotic status of the cells. The authors made connections that estrogen inhibits the actions of doxorubicin and that TFF1 can play a role in allowing estrogen to do this (Pelden et al., 2013). Kannan *et al.* used MCF-7 and T47D cells transfected with TFF3 to determine TFF function and relationship with estrogen (2010). TFF3 was identified as a novel growth factor in breast cancer, and its forced expression enhanced oncogenesis. Estrogen regulates TFFs and protects MCF-7 breast cancer cells from death. Together, estrogen and TFF expression can be a viable biomarker for breast cancer (Pelden et al., 2013; Kannan et al., 2010).

Ahmed, Griffiths, Tilby, Westley, and May investigated TFF3 expression in normal breast cancer cells, benign breast tumors, and *in situ* carcinomas (2012). In the normal breast

tissues, TFF3 protected the epithelial tissue, but its expression was lost in invasive breast cancers. TFF3 expression was also restricted at certain times during a woman's menstrual cycle. It was found to have a negative relationship with tumor grade for the cancers studied but could better differentiate the type of tumor. The authors found that TFF3 stimulates angiogenesis in the cancer cells. The authors also stated that *in vitro* studies of this kind would help back up their claim that TFF3 may be a prognosis marker.

May and Westley evaluated TFF3 as a biomarker and noted the response of this protein in breast cancer cells (May & Westley, 2015). Human TFF3 was transfected into breast cancer tissues from patients that underwent endocrine response therapy. Tumors with high TFF3 levels had evenly distributed expression compared to tumors with lower TFF3 levels, which had sparse expression. This pattern could be due to the amount of estrogen present in the body or the woman's menstrual cycle, as stated previously (Ahmed et al., 2012). The authors concluded that TFF3 expression might work as an independent predictive biomarker for estrogen and be specific and sensitive as a marker in breast cancer.

Trefoil Factors in Other Cancers

There are over 100 types of known cancers, and only a few forms of cancer have been studied for TFF expression (National Cancer Institute, 2015). Prostate cancer, endometrial adenocarcinoma (EAC), and lung adenocarcinoma have been investigated for TFF expression, including their possible roles as biomarkers for these specific cancers (Bougen et al., 2013). In prostate cancer, forced expression of TFF1 was shown to decrease E-cadherin (protein) expression leading to an increase of metastases and invasion of the cancer cells, both *in vivo* and *in vitro*. *In vivo*, the cells invaded and metastasized at an increased rate. The authors concluded that overexpression of TFF1 could be a biomarker for prostate cancer and determine tumor

metastasis or tumor burden. In this study, E-cadherin transcription was mediated by TFF1; however, it is unclear exactly how the TFF expression is mediated. Some studies indicate that estrogen or estrogen receptor (ER) may play different roles in mediating TFF expression (Bougen et al., 2013).

Estrogen levels have been found to correlate with TFF levels in many breast cancer studies. Like in breast cancer, the combination of ER and TFF can make for a specific marker in uterine cancer (Mhaweck-Fauceglia et al., 2013). In EAC, it has been confirmed that high levels of ER lead to good prognosis for the cancer (Mhaweck-Fauceglia et al., 2013). Mhaweck-Fauceglia *et al.* aimed to evaluate TFF3 expression and prognosis as well as look at its relationship with ER (2013). High levels of TFF3 corresponded with those of ER, providing evidence that TFF3 and ER lead to better tumor outcomes. TFF3 could possibly stand alone as a biomarker for EAC patients, but additional investigation is needed to support this. The authors suggested further research of knockdown TFF3 in EAC to determine its role as a cancer marker and in proliferation, migration, and invasion of tumors (Mhaweck-Fauceglia et al., 2013). Identified biomarkers for uterine cancer exist, but TFFs can be compared to these to analyze their effectiveness and expression.

Lung cancer is the deadliest and most common cancer worldwide, which compels the need for biomarkers for early onset detection (Madsen et al., 2007). Wang *et al.* studied the expression of established biomarkers TTF-1, CK7, P63, and CK5/6 in lung adenocarcinoma and squamous cell carcinoma and compared their expression patterns to those of TFF3 (2015). In comparison to the established markers, TFF3 had a 90% expression pattern in lung adenocarcinoma. The authors found higher levels of TFF3 in the cancer cells, which varied in the amount of expression between the cell lines, and TFF3 played a role in differentiating the cells.

Lastly, the authors concluded that TFF3 is all around “more sensitive” as a biomarker for all organs, not just specific organs compared to the established biomarkers (Wang et al., 2015). This study showed that TFF3 has the potential of being a biomarker when compared to established markers.

Conclusion

Cancer treatment has progressed, allowing researchers and physicians to get the five-year survival rate up to 66%, yet there is more that can be done to raise this percentage (Madsen et al., 2007). Earlier detection of the disease can lead to better prognosis and lower the amount of cancer-related deaths. TFF proteins have the potential to be biomarkers in many types of cancers. The expression levels of these proteins could identify various cases of early onset cancer or the amplitude of metastases. Many cancers still need to be investigated for TFF expression. We looked at breast cell lines MCF-7, E-3, EWD-8, skin A375, lung A549, colon COLO205, and mast cell lines P815 for TFF1 expression. We expected that varying levels of TFF1 expression would correlate with the functional characteristics of the cancer cells and that increased levels of TFF protein would support a more aggressive cancer. TFFs may be viable biomarkers that can aid in the early prognosis of cancer, which in turn may lead to the use of less invasive and detrimental forms of cancer treatment, and further investigation of these proteins as biomarkers is certainly necessary.

Methods

Cell Culture

Breast (MCF-7, E3, and EWD8), skin (A549), lung (A375), colon (COLO205), and mast (P815) cells were cultured in 10cm diameter dishes and T75 cell culture flasks (VWR, Radnor, PA) in Dulbecco’s Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA).

The culture medium was supplemented with (% volume) 10% equine and fetal bovine serum mixture (FBE from VWR), 2mM L-glutamine, 1mM sodium pyruvate, 0.01M HEPES, and 1 unit/mL penicillin/streptomycin mixture (all from Thermo Fisher Scientific). Cells were enzymatically passaged with trypsin-EDTA (Thermo Fisher Scientific) once the bottom of the plate was fully covered by the cancerous cells. Since trypsin passaging can epigenetically influence cells over many passages, low passage number samples were used from liquid nitrogen storage.

Protein Isolation and Concentration Measurements

Approximately 3.5×10^4 cells/ml from a passage were placed into each six-well plate and allowed to grow for 3 days. For cell lysis, the plate was put on ice and 100 μ l of 1X Lysis Buffer (Cell Signaling Technology, Danvers, MA) with protease inhibitors leupeptin and orthovanadate was added to each well. After lysis, the protein extract was centrifuged at 14,000xg for 10 minutes to separate leftover cell/organelle membrane fractions from solubilized proteins. The supernatant, which contains intracellular proteins like TFFs, was kept to measure protein concentration. A Nanodrop 2000 device was used to measure protein concentrations from the samples.

SDS-PAGE and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting was conducted on cell lysates and replicated to detect TFF expression in the cells. Equal quantities of total protein from each sample were loaded based on Nanodrop concentration data. The gel tank was set up using a 1X Tris-Glycine Running Buffer system. A size-standardized ladder and samples were loaded into 4%-20% polyacrylamide, Tris-Glycine gels and resolved according to manufacturer's instructions (BIO-RAD, Hercules, CA). Once the

proteins separated, the gel was transferred to a stable nitrocellulose blot using BIO-RAD's Transblot Electrode apparatus according to manufacturer's protocol. Using Ponceau S, we observed equal loading in each blot. Next, a standard primary-blocking buffer of Tris-Buffered Saline, 0.1% (v/v) Tween-20 (TBS-T), 5% (v/v) bovine serum albumin was used to block non-specific protein binding by incubating with the blots at ambient temperature for 1 hour with gentle agitation. After blocking rabbit anti-human TFF1 (1:1000 dilution factor in block buffer; Cell Signaling Technology) was added to each blot, they were incubated overnight with gentle agitation at 4°C. On the following day, an anti-rabbit secondary antibody (1:5000 dilution; LI-COR Biosciences, Lincoln, NE) solution of TBS-T, 5% (v/v) non-fat dry milk was added to the blot after removing the primary and rinsing with TBS-T. Blots were incubated with the secondary antibody solution for 1 hour at ambient temperature with gentle agitation. Secondary incubation was followed by rinsing with TBS-T. The secondary antibody has a horseradish peroxidase (HRP) enzyme attached to it; therefore, the blot was developed using a chemiluminescent HRP substrate detection reagent (WesternSure, LI-COR) and scanned with the LI-COR cDigit device.

Migration Assay

A scratch test was conducted to assess the migration of each cell line. Approximately 5×10^4 /ml of cells was added to a 10cm plate and allowed to settle and cover the plate. The cells were then scratched with a 200 μ L pipette tip, and pictures were taken over time. Images were taken daily until the scratch completely closed. ImageJ (NIH, Bethesda, MD) was used to obtain the surface area of the scratch from each image.

Daily Count

Daily counts for every cell line were also done to assess growth rate by adding 20 μ l of cells into each well of a six-well plate and counting one well per day. Trypsin was added to lift the cells and then counted with Life Technologies Countess II (Carlsbad, CA).

Statistical Analysis

A qualitative approach was taken to observe for the presence of bands indicating the presence of TFF1 among each cell line. Descriptive statistics were used to compare TFF1 expression from cell type to cell type by taking the pixel density average of MCF-7 from the total of blots run (7) and using it to take a ratio for the other cell lines average expression. Using ImageJ, the surface area was averaged from each scratch for each cell line.

Results

After running Westerns blots, we found that only three of the seven cell lines tested expressed TFF1 (Fig. 1) Equal loading of the protein was assured through Ponceau S staining (Fig. 1). Breast MCF-7 was used as our positive control for TFF1 expression. Breast E3 and EWD8 cell lines also expressed the protein. On average, MCF-7 expressed the highest amount of TFF. Cell line E3 expressed the second highest amount of TFF, and EWD8 expressed the lowest amount, yet the ratio was much smaller than MCF-7 for these cell lines (Fig 2). From the scratch tests, MCF-7, E3, and EWD8 appear to migrate the slowest (Fig. 3). E3 on average took eight days to close the scratch; MCF-7 took an average of five days to seal the closure, and EWD8 averaged six days. Cell line A549, which did not express TFF1, on average closed the scratch in two days. Daily cell count for each cell type showed that MCF-7 and E3 had lower proliferation rates compared to the cell lines that did not express TFF1. At the end of the six-day count, cell

concentration was 51,700 cells/mL for MCF-7, 39,400 for E3 cells/mL and 2,090,000 cells/mL for EWD8 (Fig. 4).

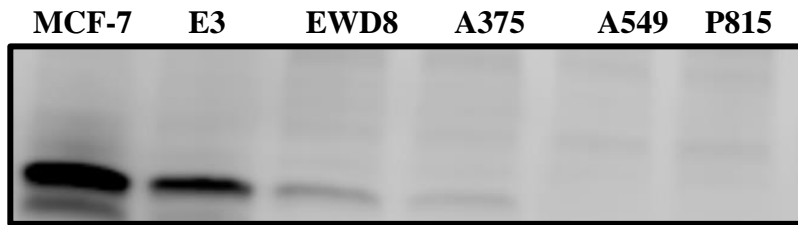


Figure 1. TFF expression is shown through Western blot. MCF-7 expressed the most protein overall. Ponceau S staining of the blots was done to ensure equal loading among each cell line.

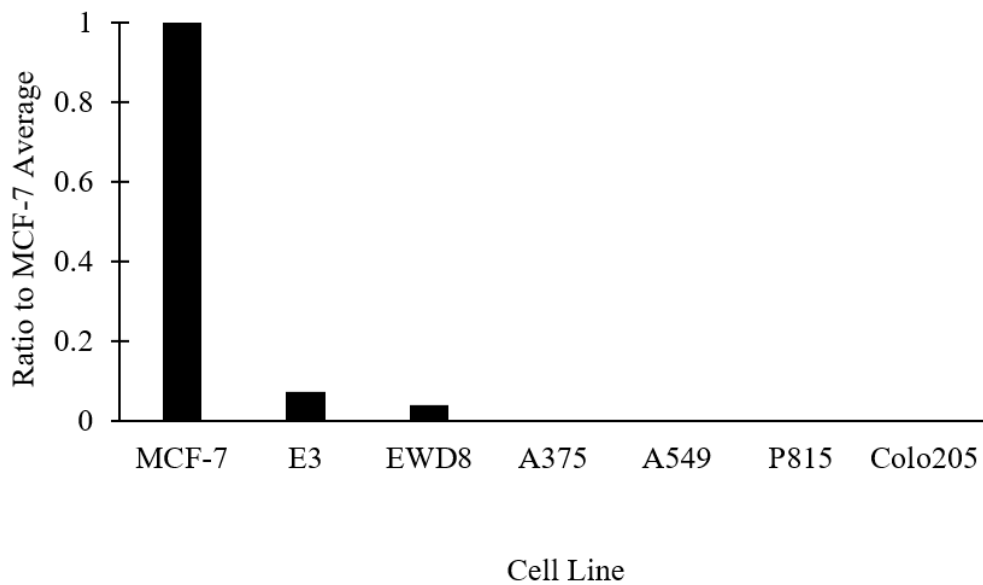


Figure 2. Pixel density was taken from the blots. The values were averaged and a ratio was taken from the average of MCF-7 expression. E3 and EWD8 expressed TFF protein.

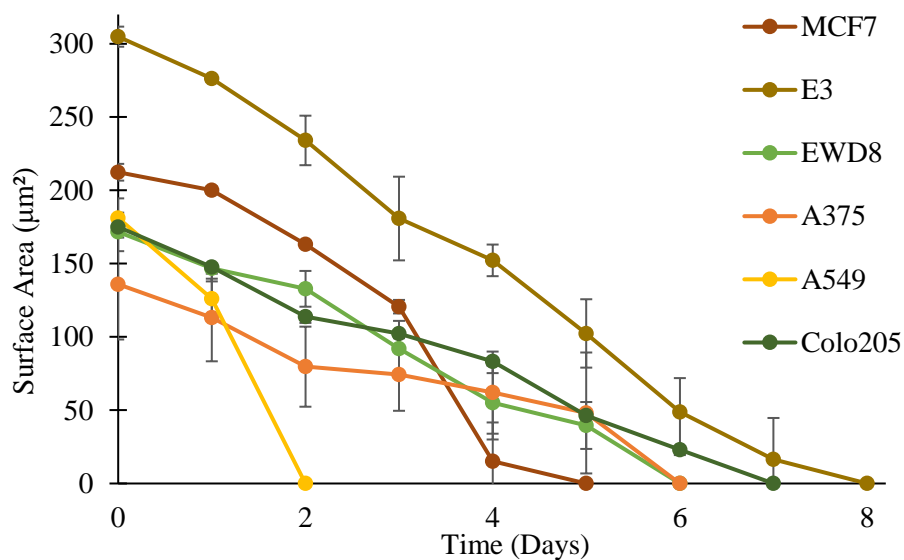


Figure 3. Cell migration over time is shown after scratch tests were performed. Three scratches were made in 10ml petri dishes after the cells had completely covered the surface for each cell line, except P815 because of their floating properties. ImageJ was used to obtain the remaining surface area of the scratches from each image. MCF-7, E3, and EWD8 appear to have migrated the slowest.

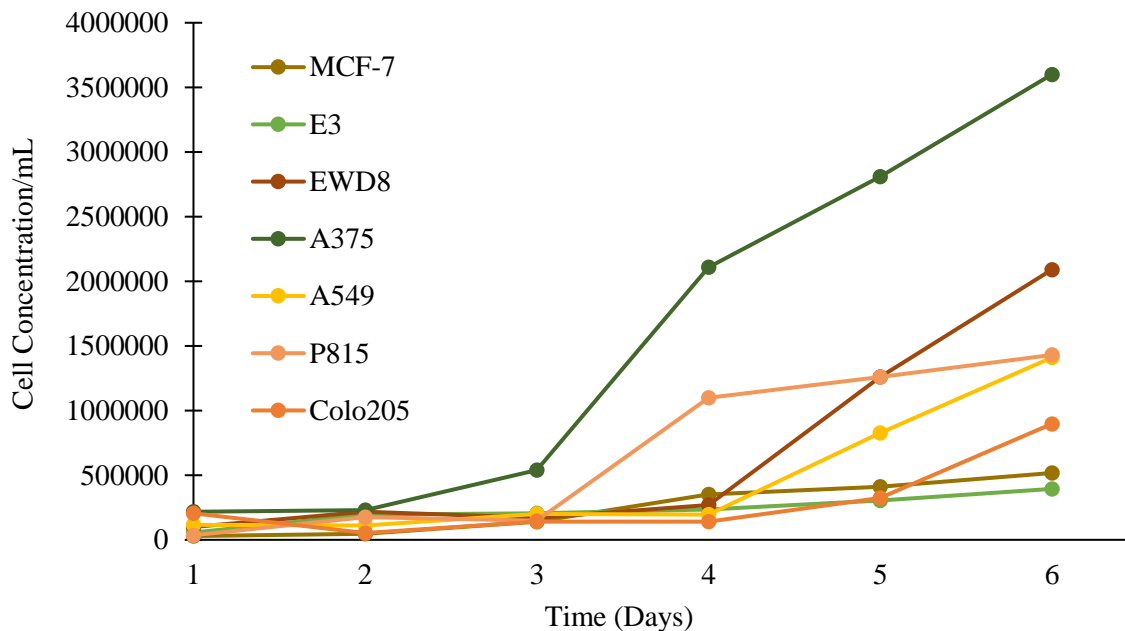


Figure 4. Daily count of each cell line was done by adding 20 μ l of cells into six-well plates and counting one well per day using the trypan blue exclusion method and a Life Technologies Countess II device. Cells that did not express TFF proliferated more than those that expressed it, such as MCF-7 and E3.

Discussion

Our results show that only three of the seven total cell lines tested had TFF1 protein expression, including the positive control. We predicted that there would be varying levels of TFF1 among the cell lines because of their morphological characteristics. All three of our breast cell lines (MCF-7, E3, and EWD8) expressed TFF1 protein; this may be due to their luminal breast cancer characteristics. TFFs are well-known for being expressed in MCF-7 breast cancer cell lines, and in general, MCF-7 expressed the highest amount of TFF1 (Thim & May, 2005). E3 expressed the second highest amount of TFF1, and EWD8 expressed the lowest amount. As previously stated, estrogen appears to mediate TFF expression (Mhaweche-Fauceglia et al., 2013). MCF-7 and E3 express estrogen receptors, while EWD8 cell lines are estrogen-withdrawn cell

lines (Haughian et al., 2012). This may explain why MCF-7 and E3 expressed more TFF1 than the EWD8. EWD8 may be regenerating estrogen or have estrogen strands left over that would cause the low but present TFF1 expression (Haughian et al., 2012). Treating the cells with estrogen and progesterone is worth considering because it may significantly change the cell's TFF expression.

Previous research has found that overexpression of TFFs can be used as a possible biomarker for certain cancers. We also hypothesized that the cancer would be more aggressive if more TFF1 was present (i.e. faster growth rate, faster scratch closure), but our current observations showed the opposite pattern. Further assays suggested that these cells grow as slowly as the positive control (MCF-7) compared to the other cell lines, among which we did not detect any TFF1 expression. The cell lines expressing TFF1 generally took a longer time to close the scratch test. Daily counting provided more evidence that there were lower rates of proliferation on average in the breast cell lines. TFFs have been found to be biomarkers and to have oncogenic or tumor suppressing effects among many cancers. Some studies have correlated high TFF expression with aggressive cancers across different phenotypes, thus indicating promise as a cancer biomarker. There is also the possibility that TFFs are a side effect of the cancer, rather than direct oncogenes, yet further research is required.

Our data suggested that the more TFF present, the less aggressive the cancer, suggesting its use as a possible target treatment for aggressive cancers. A next step would be to treat one of the more aggressive cell lines, like lung A549, with recombinant TFF and observe for any changes in the cancer's aggressiveness. Since cell lines MCF-7, E3, and EWD8 expressed TFF1, we also plan to use gene-silencing techniques, such as siRNA transfection and/or CRISPR-Cas9, to edit the cells' genomes and remove the TFF1 protein. We thus plan to observe what happens

to the aggressiveness of the cancer in the absence of the specific TFF in cell lines typically expressing the TFF. We would expect the treated lung A549 cell lines to become less aggressive and the knockdown-TFF1 cell lines MCF-7, E3, and EWD8 to become more aggressive.

We originally predicted that TFF1 expression would be associated with more aggressive cancer cells, but we found the opposite pattern. The cancer cell lines that expressed TFF1 were less aggressive than those that did not, which implies that the loss of these proteins within these cancers would lead to a cancer that is more aggressive. While our results do not demonstrate what we initially hypothesized, it is important to note that TFFs may still be biomarkers for less aggressive cancer types. High expression levels of TFFs could allow for the detection of less aggressive or early stages of aggressive cancers. Based on these data and prospective clinical data, we would expect to see less aggressive breast cancers express higher levels of TFFs than more aggressive cancers. It continues to be debated whether TFFs are tumor suppressors or oncogenes and if varying levels of TFFs work as biomarkers in many cancers, yet the proteins seem to have the potential to be viable cancer biomarkers in less aggressive cancer settings, as shown from our findings.

Identification of cancer biomarkers is essential to improving patient survival outcomes through the early detection of cancer. Treatment and therapy are crucial in the fight against cancer, but they often come with many discomforts. Biomarkers would allow for the early detection of cancer, which could possibly lead to less invasive and aggressive treatments. Further investigation would aid in the categorization of TFF1 in these cancers as oncogenic or tumor suppressors and would continue to reveal the possibility of TFF as a cancer treatment.

Future Directions

Using the 4T1 breast mouse model, we tested for TFF expression and found that the cells do express TFFs (Fig 5.). The 4T1 mouse model is metastatic and can mimic the human immune system, making it the ideal model to study the effects of knockdown TFFs (Pulaski & Ostrand-Rosenberg, 2000). We aim to manipulate the cancer cell's genome using the CRISPR-Cas9 system to remove the expressed TFF in the 4T1 cell lines. CRISPR-Cas9 is a novel genetic engineering tool that grants the ability to disrupt or mutate genes within the cell (White & Khalili, 2016).

We will attempt to target the TFF nucleotide sequence to inhibit transcription of TFF protein in the cancer cells and then observe for phenotypic changes in the cell lines once TFF has successfully been knocked down. We expect to see changes in aggression in terms of invasion and proliferation, and we plan on treating cell lines that have no TFF expression with recombinant TFFs and observing for changes in aggression (Fig. 6). Using transwell assays, we will conduct a migration assay by placing TFF or TFF-expressing cells at the bottom of the well and seeing if the cells migrate to the signal (Fig. 7). Lastly, we will move to *in vivo* assays by injecting the knockdown TFF 4T1 cells into Balb/c mice, which will induce mammary tumors (Fig 8.). We plan to study how the metastasis of the tumors may or may not be mitigated by the absence of TFF expression.

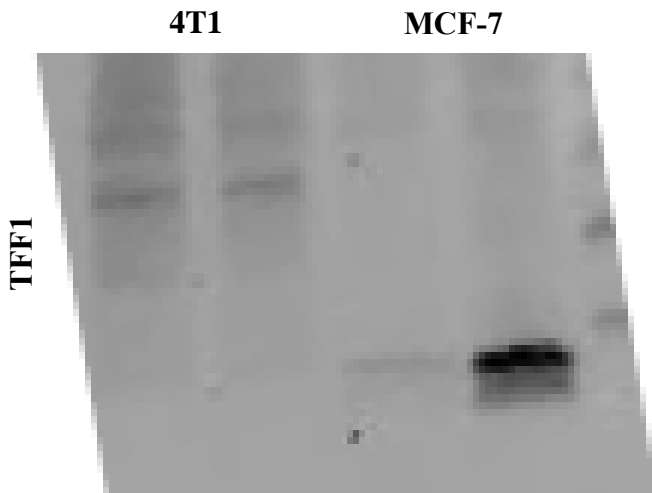


Figure 5. We have assayed 4T1 cells for their TFF expression shown in the blot. These cells are a Balb/c mouse model of metastatic mammary adenocarcinoma. We will use these cells to observe the effects of TFF expression changes *in vivo*.

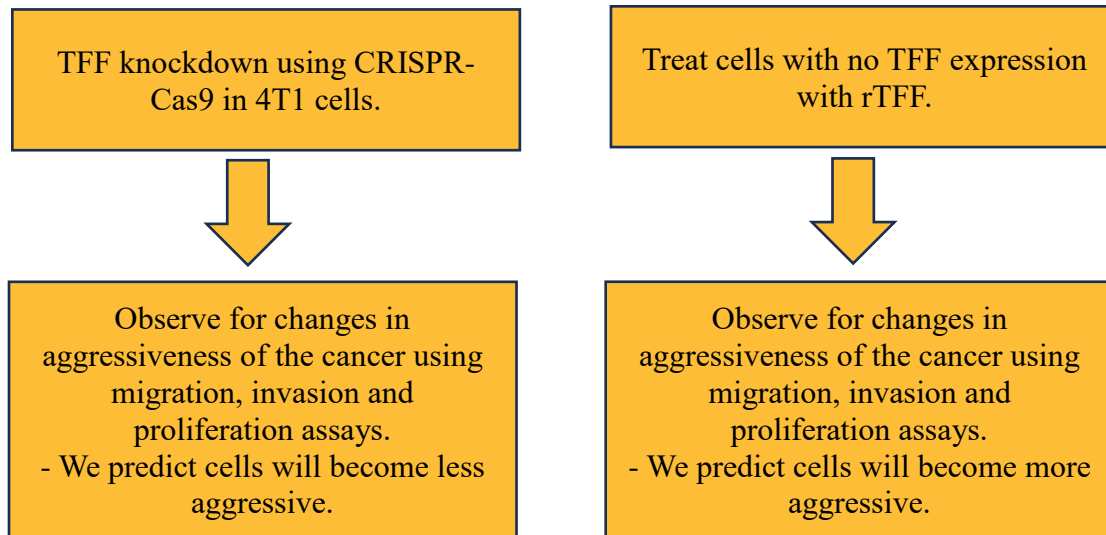


Figure 6. It is predicted that knockdown TFF will cause the cells to decrease in aggression compared to cells treated with TFF, which may become more aggressive.

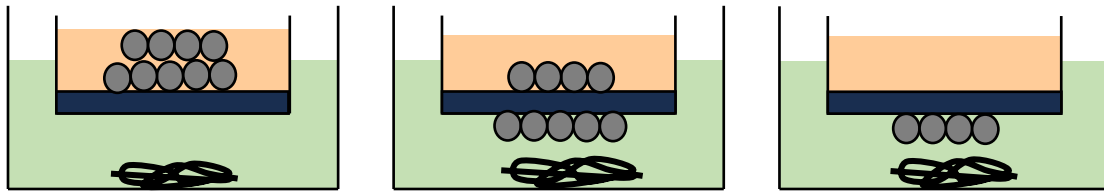


Figure 7. Transwell invasion assays will be performed to test migratory potential of the cancer cells by adding TFF or cancer cells expressing TFF to the bottom of the wells.

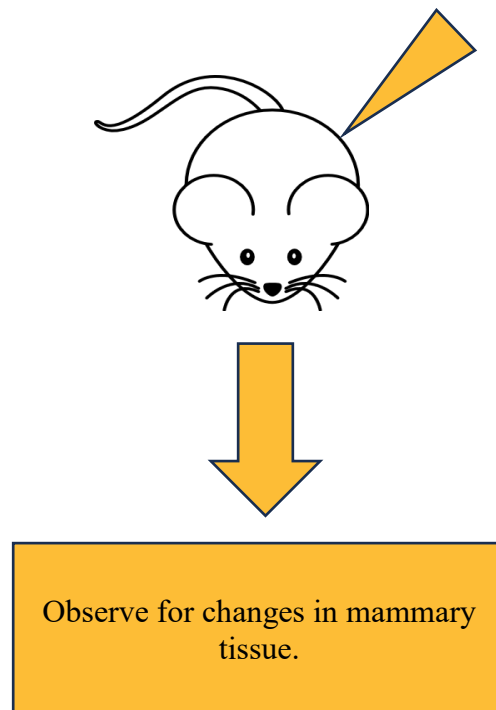


Figure 8. The 4T1 mouse model will be used to induce mammary tumors. 4T1 cell lines normally express TFFs; thus, by using knockdown cells, we will compare +/- endogenous TFFs regarding metastasis *in vivo*.

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