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

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Abstract: The ability to identify and treat cancer early can drastically improve patient survival outcomes. While much effort is placed on anti-cancer therapeutics, a developing area of interest is the identification of cancer biomarkers for the early detection of different types of cancer. Trefoil factors (TFFs) are a family of proteins whose known functions suggest that they may be major contributors to cancer; however, these proteins are understudied in a disease state like cancer. In this work, we identify expression of TFF1 among varying cancer types. We then investigate the correlation between varying levels of TFF1 expression and the aggressiveness of the cancer. Cell culture of seven different kinds of cancers was performed. Western blots were conducted, and the blots were probed for TFF1. After establishing TFF1 levels, we aimed to analyze cancer cell function through a series of cancer assays examining proliferation, viability, and migration. We hypothesized that there would be varying levels of TFF1 expression correlated to the functional characteristics of the cancer cells, and that increased levels of TFF protein would support a more aggressive cancer. The breast cell lines tested were the only lines to express TFF1 and were less aggressive than the lines with no expression.

Keywords: *cancer biomarkers, oncogenes, trefoil, tumor suppressors*

Cancer is a detrimental disease 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[1]. Cancer is characterized by uncontrollable cell growth that gains resistance to death signals, increasing pro-survival mechanisms of the cancerous cell and its properties[2]. Although cancer treatments have caused survival rates to increase, there is a demand for viable biomarkers for the many types of different cancers. A viable biomarker is indicative to the presence of cancer within the body and helps diagnose patients promptly.

Trefoil factors (TFFs) are secreted proteins that help in healing epithelial tissue in the stomach[3], thus have the potential to be biomarkers in many types of cancers. The normal function of TFFs is to regulate cell growth and assist cells in recognizing environmental stress that leads to inflammation. While TFFs are an attractive 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. Further research on TFFs is required to understand their expression and function in cancerous cells. The need for this research is not only to better understand TFFs in cancers but hopefully to identify a new source of markers for detecting cancer in its earlier stages, and therefore mitigate aggressive and potentially intractable metastic disease.

In this study, we aimed to identify the levels of TFF1 expression in cancerous cells that have not been investigated for the 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. We also expected that increased levels of TFF1 would be a form of support for a more aggressive cancer. We found 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 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 world-wide[2]. 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. Cancer markers are substances naturally

produced by the body, and when cancer is present these substances are produced at different levels[2]. TFFs are proteins secreted by epithelial cells primarily for wound healing in mucosal membranes, which makes its investigation as a biomarker attractive[3]. The proteins are named after a trefoil motif structure composed of disulfide loops[5]. There are three TFFs: TFF1, TFF2 and TFF3. TFF1, previously known as pS2, was first found in breast cancer, and TFF2 was extracted and purified from the pancreas[6-7]. Intestinal trefoil factor, now TFF3 was later found in both intestines[8]. This family of proteins has similar characteristics but vary in expression and function throughout the body[9].

The stomach is the only organ in which all three TFFs are expressed[9]. 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[9]. Other studies have looked at particular parts of the body and cancer cells located within those parts (e.g., gastric and breast) to determine TFF expression[11-20]. Recent studies have found TFF expression may lead to detection of various cancers[21-24]. 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 world-wide, accounting for almost 1,000 of the new cancer cases in 2012[10]. There are known classic biomarkers for gastric cancer but TFFs may be novel markers for the disease[11]. TFF1 expression is essential for gastric mucosa cells to differentiate[12]. When expression of TFF1 is low, gastric adenomas can develop and a percentage of the tumors can become cancerous[12]. 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 it is overexpressed in gastric cancer[13]. 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[14]. 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[15].

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[15]. Overexpression of the two proteins suppressed cell proliferation and induced apoptosis of the cancer cells and significantly lowered cell invasion as well. These findings showed that Sp3 is needed to regulate TFF2 and together their overexpression (or their absence) could make for cancer markers[15].

Trefoil Factors in Breast Cancer

Breast cancer is the most common cancer among women and the second most common cancer world-wide[10], and it is well established that TFFs are expressed in MCF-7 breast cancer cell lines[16]. In a study that used knockdown TFF1 in MCF-7 cells, the effects of estrogen with doxorubicin treatment were tested[17]. 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[17]. Kannan et al. used MCF-7 and T47D cells transfected with TFF3 to determine TFF function and relationship with estrogen[18]. TFF3 was identified as a novel growth factor in breast

cancer. Forced expression of this gene 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[17-18].

Ahmed et al. investigated TFF3 expression in normal breast cancer cells, benign breast tumors and *in situ* carcinomas[19]. In the normal breast tissues, TFF3 protected the epithelial tissue, but TFF3 expression was lost in invasive breast cancers. TFF3 expression was also restricted at certain times during a woman's menstrual cycle. TFF3 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[20]. 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[19]. 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[2], and only a few forms of cancer have been studied for TFF expression. Prostate cancer, endometrial adenocarcinoma (EAC), and lung adenocarcinoma have been investigated for TFF expression, including their possible roles as biomarkers for these specific cancers[21]. 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, as stated before estrogen or estrogen receptor (ER) have different roles in mediating TFF expression[21].

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[22]. In EAC it has been confirmed that high levels of ER lead to good prognosis for the cancer[22]. Mhawech et al. aimed to evaluate TFF3 expression and prognosis as well as look at its relationship with ER[22]. 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 in proliferation, migration and invasion of tumors and as a cancer marker[22]. Identified biomarkers for uterine cancer exist, but TFFs can be compared to the cancers 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.[9]. 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[23]. 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[23]. 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[9]. 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 are left 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 varying levels of TFF1 expression correlated to 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 that in turn may lead to the use of less invasive and detrimental forms of cancer treatment. Further investigation of these proteins as biomarkers is also 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) 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), and 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) when 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 35000ml of cells from a passage were placed into six well plates and allowed to grow over 3 days. For cell lysis, plate was put on ice and 100µl of 1X Lysis Buffer (Cell Signaling Technology, Danvers, MA), with protease inhibitors leupeptin and orthovanadate, was added to each well. After lysis and of proteins into the buffer for 10-15min the mixture was centrifuged at 14,000xg for 10min 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 a 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 for 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 1h with gentle agitation. After blocking rabbit anti-human TFF1 (1:1000 dilution factor in block buffer; Cell Signaling Technology) was added to each blot, which were incubated overnight with gentle agitation at 4^{o}C. 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 1h at ambient temperature with gentle agitation. Secondary incubation was followed by rinsing with TBS-T. The secondary antibody has a horse radish 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 and Daily Count

A scratch test was conducted to assess the migration of each cell line. Approximately 50000ml of cells was added to 10cm plate and allowed to settle and cover the plate. The cells were then scratched with a 200□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 counts for every cell line was also done to assess growth rate, by adding 20µl of cells into 6-well plates 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 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*A*) Equal loading of the protein was assured through Ponceau S staining (Fig. 1*B*). Breast MCF-7 was used as our positive control for TFF1 expression. Breast E3 and EWD8 cell lines also expressed the protein. MCF-7 on average 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 1 (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 made, MCF-7 took an average of five days to seal the closure, and EWD8 averaged six days. Cell line A549 that did not express TFF1, on average closed the scratch in two days. Daily cell count for each cell type showed that MCF-7, E3 and EWD8 cell lines 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. A) TFF expression is shown through Western blot. MCF-7 expressed the most protein overall. B) Ponceau S staining of the blots was done to ensure equal loading among each cell line.

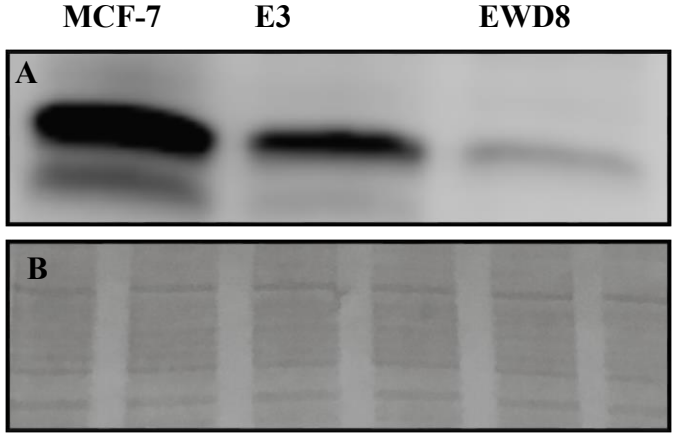


Figure 2. Pixel density was taken from the blots. The values were averaged and a ratio was taken from the average of MCF-7 expression. E3 and EWD8 expressed TFF protein.

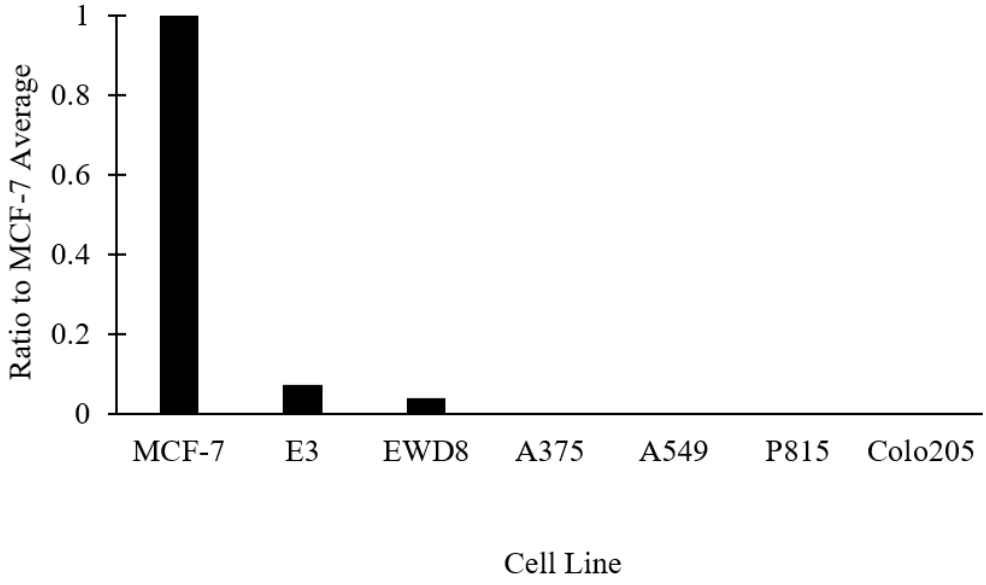


Figure 3. Cell migration over time is shown after scratch tests were performed. Three scratches were made in 10ml petri dishes after the cells had completely covered the surface for each cell line expect P815 because of their floating properties. ImageJ was used to obtain the surface area of the scratches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

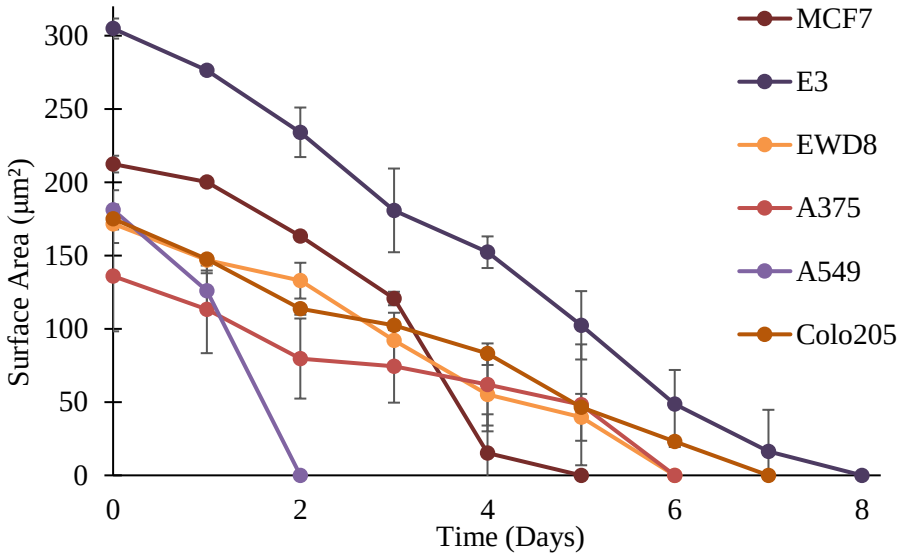
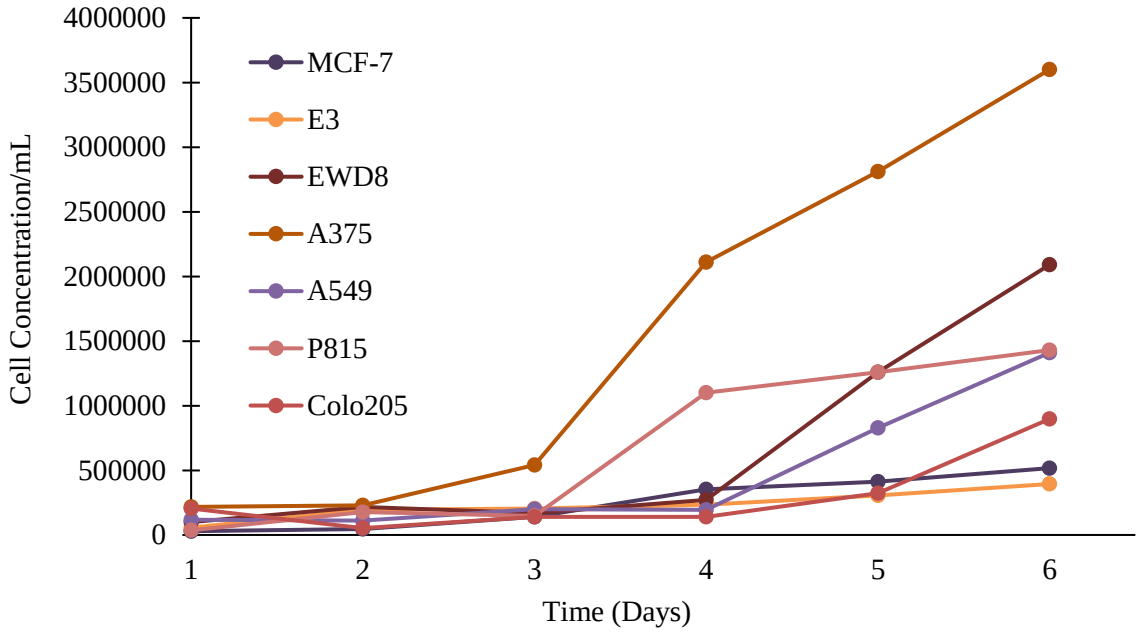


Figure 4. Daily count of each cell was done. Counting for every cell line was done by adding 20µl of cells into 6-well plates and counting one well per day using the trypan blue exclusion method and a Life Technologies Countess II device. Cells that did not express TFF proliferated more than those who expressed it such as MCF-7, E3 and EWD8.



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[3], and on average MCF-7 expressed the highest amount of TFF1. E3 expressed the second highest amount of TFF1, and EWD8 expressed the lowest amount. As previously stated estrogen appears to mediate TFF expression[17-20]. MCF-7 and cell lines E3 express estrogen receptor while EWD8 cell lines are estrogen-withdrawn cell lines[27]. This may explain why MCF-7 and E3 expressed more TFF1 than the EWD8s. EWD8 may be regenerating estrogen or have estrogen strands left over that would cause the low but present TFF1 expression. Treating the cells with estrogen and progesterone may have significant changes on the cell's TFF expression and is worth considering.

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 slow 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 on average 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[10-13]. Some have correlated high TFF expression with aggressive cancers

across different phenotypes, thus indicating their promise as a cancer biomarker. There is also the possibility that TFF are a side effect from the cancer, rather than direct oncogenes, yet further research is required.

A limitation to this study was only probing for one of the three TFF proteins. We plan to adjust our protocol in order to probe for the TFF2 and TFF3 antibodies. Our data suggested that the more TFF present the less aggressive the cancer suggesting it can be used as a possible target treatment for aggressive cancers. Next steps would be to treat one of the more aggressive cell lines, like lung A549 with recombinant TFF and observe for any changes in the cancers 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. Thus, we plan to observe what occurs in regards 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 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 what we would expect to see less aggressive breast cancers express higher levels of TFFs than more aggressive cancers. TFFs continue to be debated on whether they are tumor suppressors or oncogenes as well as varying levels working as biomarkers in many cancers, yet the proteins seem to have the potential to be viable cancer biomarkers in less aggressive cancer settings from our findings.

Identification of cancer biomarkers is essential for the early detection of cancer to improve patient survival outcomes. Treatment and therapy is crucial in the fight against cancer, yet it comes with many discomforts. Biomarkers would allow for the early detection of the cancer that could possibly lead to less invasive and aggressive treatments. Further investigation would allow for the categorization of TFF1 in these cancers as oncogenic or tumor suppressors. TFF could also possibly work as a treatment to suppress cancers.

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