Brown University

Behaviour of Ovarian Cancer Cells in 2D and 3D Under Adherent and Non-Adherent Conditions

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Table of Contents

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Abstract

High Grade Serous Carcinoma (HGSC) is the deadliest form of ovarian cancer among women and causes the most deaths of gynecological cancers overall (Tudrej, Patrycja et al., 2018). One reason for this is that most cases of HGSC's are diagnosed once the disease has reached an advanced stage, rendering frontline therapies ineffective. The goal of my project was to identify genetic variations between 4 mice ovarian cancer cell lines; p53 control, p53 knockout (KO), BRCA2 control, and BRCA2/P53 double knockout (DKO). For the remainder of this project I will refer to the cell lines as F3-C, F3, BRCA2-C, and BRCA2, respectively. In the beginning of the project, I attempted to grow all 4 cell lines under high and low attachment conditions to obtain comparative data on their survival in each. I aimed to do this by obtaining RNA sequences of each cell line under both conditions to see if there were any genetic differences. However, the cell lines exhibited extremely poor survival on the low attachment plates, and I was unable to collect enough cells for RNA sequencing. Immunofluorescent staining was then carried out to determine if any of the cell lines were exhibiting characteristics of cancer stem cells, but the results were inconclusive. Finally, I used qPCR to determine expression levels of several genes previously found to be elevated in ovarian cancer. A significant increase in expression of TAF4A and ER-A was found in in the BRCA2 cell line (normalized to 18s), while a significant decrease in expression of ER-A was found in the F3 cell line. There was no significant correlation found between TAF4B and ER-A expression, but the results still pose an interesting question about potential estrogen responsiveness of these cell lines. The work done in this project could be followed up by another undergraduate to determine the importance of TAF4B regulation by estrogen.

Background

High Grade Serous Ovarian Cancer (HGSOC)

Ovarian cancer accounts for 2.5% of all female cancer cases, but 5% of cancer deaths because of the extremely poor survival rates of the disease. One of the main reasons for the poor survival rates is that the majority of cases are diagnosed once the cancer has spread to major organs in the peritoneal cavity. The three major types of ovarian cancer are epithelial, stromal, and sex-cord stromal. Epithelial ovarian cancers constitute 90% of cases, 52% of which fall in to the category of serous cancers. Of serous tumors, the majority are characterized as high grade serous ovarian tumors/ carcinomas[1]. The location of origin of this cancer type has long eluded researchers, a fact that likely contributes to the ineffectiveness of frontline chemotherapeutic agents in combating the disease. Though it has been hypothesized that the cellular origin of HGSOC's is either the epithelium of the ovaries or the fallopian tubes, there is a significant amount of research supporting the fallopian tube as the point of origin[2]. Another question about this disease that lacks a clear answer is how the cancer cells are able to use the peritoneal fluid like the blood stream to metastasize. In order to even begin to generate a sufficient chemotherapeutic agent for High Grade Serous Ovarian Carcinomas, both questions of origin and dissemination from the point of origin need to be better understood. If researchers are able to accurately pin point the origin of these tumors and identify genetic factors involved with their metastasis in the peritoneum, it could be possible for current therapies to adapt to this new information and create new therapies that target the identified genes.

Mutations in TP53 and BRCA1/2 in HGSOC

The majority of cancers are directly associated with mutations in genes associated with regulation and repair in the cell cycle, sometimes referred to as check point genes. The purpose of these genes is to prevent cells from undergoing uncontrolled proliferation that can eventually lead to the development of cancer. The TP53 gene provides a template for the creation of tumor protein 53, or p53, which acts as a tumor suppressor by controlling cell proliferation and is responsible for initiating apoptosis in cells with damaged DNA[3]. The BRCA1 and BRCA2 genes are another set of genes that each play a role in DNA repair, and it is believed that a germline mutation in either gene predisposes patients to be at a higher risk for certain cancers, namely breast and ovarian[4]. Similar to most human cancer cases, somatic TP53 mutations are reported in the majority of ovarian cancer cases, while mutations in BRCA1 or BRCA2 are reported in about 15% of cases[5].

CRISPR Modified ID8-F3 Cells for Murine Model of HGSOC

In order to investigate the intricate interactions and genetic mutations present HGSOC, it is critical to be able to recreate these mutations in a model that is both ethical and informative. Using CRISPR/Cas9 gene editing, novel ID8 derivatives with single ($TP53^{-/-}$) knockouts and double ($TP53^{-/-}$ / $BRCA2^{-/-}$) knockouts have been generated[6]. The ID8 cell line is the only murine ovarian cancer cell line that can be successfully transplanted into syngeneic mice with intact immune systems. The double knockout (DKO) cell line generated with this methodology allows researchers access to a cell line representative of HGSOC, while also allowing for further research to be conducted on the effect of these genetic alterations on cancer cell behavior, spread, and survival. The table below shows the ID8 cell line with the abbreviation used in the remainder of this paper and its corresponding genotype.

ID8 Cell Line	Abbreviation Used	Genotype
P53 – Control	F3-C	Wild type p53, wild type BRCA2
P53 -/-	F3	P53 null, wild type BRCA2
BRCA2-Control	BRCA2-C	P53 null, wild type BRCA2
P53 -/- BRCA2-/- (DKO)	BRCA2 /DKO	P53 null, BRCA2 null

Project Goal

This project is an extension of a former Freiman lab undergraduate researcher, Amy Wang, and is meant to elucidate on her findings on the differential behaviour of 4 novel ID8 F3 CRISPR derived cell lines under low attachment conditions. Originally, the goal of this project was to identify genetic differences between the four cell lines (F3-C, F3, BRCA2-C, and BRCA2) when grown under high attachment conditions and low attachment conditions. The purpose of the low attachment condition was to try and get a better understanding of how ovarian cancer cells are able to use the peritoneum like the bloodstream to proliferate and spread to other parts of the body. However, after attempting to grow the cells on Ultra-Low Attachment (ULA) plates, it was evident that this would not be a sufficient method to contrast the genetic inequalities under the different attachment conditions because the cell lines were not showing adequate survival, which prompted alternate examination of the cell lines. The alternate methods we chose to use were immunofluorescent staining and qPCR. The goal of the immunofluorescent staining was to determine if any of the cell lines exhibited characteristics of cancer stem cells (CSC's). The primary antibodies we examined were OCT4A, ALDH1A1, and SOX2. These were used in combination with fluorescent secondary antibodies to aid in visualization. The results of the immunofluorescent staining were inconclusive, so we decided that it would be worthwhile to carry out qPCR to analyze expression levels of several genes the Freiman Lab is interested in identifying a connection with ovarian cancer. The genes we hoped to obtain expression levels for with qPCR were OCT4A, TAF4A, TAF4B, ER-A and ER-B. Unfortunately, OCT4A and ER-B came up as undefined in our qPCR, so we were only able to look at expression levels of TAF4A, TAF4B, and ER-A. Based on the expression levels of these 3 target genes, we were interested in determining if a correlation existed between ER-A expression and TAF4B expression. We were unable to find a statistically significant correlation, but are now interested in looking in to the relationship between estrogen responsiveness and TAF4B in these cell lines. Though I will be unable to expand on this topic in the remainder of my time at Brown, I believe the question of the relationship between estrogen responsiveness and TAF4B is the most noteworthy outcome of my work.

Materials and Methods

ID8 F3 Cell Culture

The four CRISPR derivative cell lines used in this project were ID8 F3 (p53 -/-), ID8 F3 Control (p53C BRCA2 C), ID8 F3 BRCA2 (p53 -/-; BRCA2 -/-), and ID8 F3 BRCA2 C (p53 -/- BRCA2C). To simplify these names, the cell lines are referred to as F3- C, F3, BRCA2-C, and BRCA2, respectively. Each cell line was recovered from the Nitrogen gas freezer and thawed from -120 °C to 37.5 °C in a hot water bath before being transferred to 10 cm^2 plates with 9 ml of DMEM media (Gibco, 21969-035). The DMEM media was prepared by removing 35 ml from the bottle and then adding 20 ml of heat inactivated FBS, 5 ml of L-Glutamine (100X) (Gibco, 25030-024), 5ml Pen/Strep (Gibco, 15140-122), and 5 ml ITS (100X solution) (Gibco, 41400-045). The cells were visualized under a light microscope to ensure viability and then stored in a 37.0 °C incubator (5% CO2). Cells were washed and fed new media as needed, using the color of the media in each dish as a guideline (less pink color/ more orange color and less vibrant meant they needed to be washed and fed). Cells were split by removing DMEM media from each plate with aspirating pipet, washed with 2-3 ml of PBS 2-3 times each, trypsinized with 1 ml of trypsin in 37.0 °C incubator for 5 minutes, diluted with 5 ml of DMEM media, washed >5 times each, and then 1 ml of the trypsinized cells in 5 ml of media was transferred to a new plate with 9 ml of media. Each cell line was visualized under a light microscope and split 1:10 accordingly to ensure the plates did not reach or surpass 100% confluency. Cell counts were used to determine the volume of cells that needed to be transferred to each new dish to maintain a cellular concentration of $9x10^4$ cells in each 10 cm^3 plate. These were performed by combining 10 μl of Trypan blue stain and 10 µl of each cell line on a separate C-Chip and then visualizing each under a light microscope. Each C- Chip had two ports with four quadrants each, so each cell line was counted on an individual port and the average value from the four quadrants was used to find the volume required for the split.

RNA Extraction

RNA was extracted following the RNA extraction protocol modified from the TRIzol protocol provided by Life Technologies. RNA extraction was only carried out for the 4 cell lines grown under high attachment conditions because the cells did not demonstrate sufficient survival under low attachment conditions. Percent recovery and duration data was collected on the most poorly surviving line (F3) to find ideal seeding density and collection time for low attachment conditions, but this did not lead us to believe it was worthwhile to carry out RNA extraction under these conditions. RNA extraction was carried out using Zymo Research's Direct-zol RNA mini prep kit. After extraction was complete, the RNA obtained from each cell line was brought to the Genomics core facility and placed in the Nanodrop to be sequenced and determine purity

and concentration before being stored at -80.0 °C. This RNA was used to prepare cDNA for each cell line using Bio-Rad's iScript Reverse Transcription Supermix for RT-qPCR kit.

Immunofluorescence Protocol

Immunofluorescent staining was carried out following the Protocol for Immunofluorescence Culture adapted from Brown Patho-Bio Histology Lab Protocol. After being in culture for ~2 days on Corningstar's tissue culture treated 6 well cell culture plates, cells were fixed in 4% Paraformaldehyde for 10-15 minutes at room temperature, washed 2x with PBS and stored at 4°C. To prepare for application of the primary antibody, the cells were washed 2x with PBS and placed on rocker for 3 minutes between each wash. Cells were then permeabilized with 0.1% Triton/ 0.1% Citrate for 5 minutes and washed with 0.1% PBST. 3-4 drops of blocking solution, 2.5% goat serum, were applied to each well and then cells were stored in 37°C for 30 minutes or 4°C overnight. The primary antibodies that we opted to use were Oct4A, ALDH1A1, and SOX2, and each was diluted 1:100 with blocking solution. Each primary antibody was individually plated on each of the 4 cell lines and allowed to incubate at 37°C or 4°C overnight. Cells were then rinsed again with 0.1% PBST 3x and placed on rocker for 5-10 minutes before the secondary antibody was applied in the blocking solution and stored at 37°C for 1 hour with no exposure to light. Secondary antibodies were diluted 1:500 with blocking solution. The final rinse was performed 3x through with PBST again for 5-10 minutes on the rocker between washes, slips were placed right side up on to the coverslip and covered in 1 drop of DAPI mounting media. Nail polish was used around the edges of the coverslip to ensure the slips would not be disturbed, and then the coverslips were stored at 4°C until they were visualized.

Immunofluorescence Visualization Protocol - Zen Blue

The coverslips were visualized using the Zeiss Axio Imager M.1 microscope for multi-channeled fluorescent acquisition in combination with ZEN blue software. The ZEN blue software allowed me to capture still images of real time visualizations of my stained cells and the split view allowed for comparison of the DAPI blue staining in the nucleus, the secondary antibody staining alone, and a combination view of the two. To visualize the coverslips, the "active camera" was first set to black and white under the "locate" tab, "multichannel experimental default" was selected under the "Acquisition" tab, "LIVE" view was selected and then the image was focused using a combination of the "ctrl" keyboard button with the mouse scroller. Once the image in view was properly focused, "auto-exposure" was selected. If the image exposure was not giving a clear image, the histogram minimum/ maximum values were skewed until exposure was ideal and kept by selecting "set exposure". The image was captured by pressing the "snap" button and visualized with the "split" view option on the left-hand side of the image.

qPCR Protocol

cDNA was prepared from the RNA obtained from RNA extraction (protocol described earlier) using Bio-Rad's iScript Reverse Transcription Supermix for RT-qPCR kit. This protocol recommends using 500-1000 ng of RNA for the cDNA reaction, but we used 1 μg. The genes we were interested in were OCT4A, TAF4A, TAF4B, ER-A, and ER-B. To quantify the expression levels of the selected genes, ThermoFisher's Scientifics Applied biosystem Power SYBR Green PCR Master Mix was used. The primer sequences utilized for each gene are listed below:

18s Forward: CCG-CGG-TTC-TAT-TTT-GTT-GG
18s Reverse: GGC-GCT-CCC-TCT-TAA-TCA-TG
OCT4A Forward: TGG-AGA-AGG-TGG-AAC-CAA-CTC-CC
OCT4A Reverse: ACA-CGG-TTC-TCA-ATG-CTA-GTT-CGC
TAF4A Forward: ATC-TCC-ACT-GTG-CAG-GCT-CC
TAF4A Reverse: GGT-CAC-CTG-CCG-TGC-AAT-A
TAF4B Forward: GAT-GTT-ACT-AAA-GGC-AGC-CAA-GAG-T
TAF4B Reverse: CTG-CTC-TGG-ATC-TTC-TTT-ATT-GGA-G
ER-A Forward: GTC-GCC-TAG-CCC-GCT-GAT-GC
ER-A Reverse: CGC-TGG-GCT-CGT-TCT-CCA-GG
ER-B Forward: AAA-CAG-AGA-GAC-CCT-GAA
ER-B Reverse: CCT-CTT-GGC-GCT-TGG-ACT-A

The first attempt to quantify expression levels of OCT4A and ER-B came up as 'undefined', so information on those genes' amplification is not included in this report.

Results

High Attachment Conditions

All 4 cell lines demonstrated sufficient survival under the high attachment conditions, and we were able to collect a large enough cell pellet to perform RNA extraction. The Nanodrop data for concentration and absorbance under high attachment conditions for F3, F3-C, BRCA2 and BRCA2-C can be seen in tables 1-4, respectively. Each RNA isolation was performed in triplicate to ensure results were legitimate.

Table 1: ID8 F3 RNA

ng/ul	$A260/A280$	$A260/A230$
1396.3	1.96	2.13
1336.2	1.95	2.08
1816.2	1.98	2.10

***For ID8 F3 samples, 20 µl water for RNA collection**

Table 2: ID8 F3- Control RNA

ng/ul	$A260/A280$	$A260/A230$
2549.1**	2.01	2.00
2622.5**	2.02	2.28
2699.3**	2.03	2.30

***For ID8 F3-C samples, 10µl nuclease free water for RNA collection**

**** Indicates these samples need to be diluted 1:10**

Table 3: ID8 F3 BRCA2 RNA

ng/ul	$A260/A280$	$A260/A230$
1339.5	1.97	2.17
1372.6	1.98	2.24
691.0	1.96	2.07

***For ID8 F3 BRCA2 samples, 20 µl nuclease free water for RNA collection**

Table 4: ID8 F3 BRCA2 - Control RNA

ng/ul	$A260/A280$	$A260/A230$
720.6	1.89	2.32
710.1	1.88	2.26
907.0	1.89	2.22

***For ID8 F3 BRCA2- C samples, 10 µl nuclease free water for RNA collection**

In the tables above, the first column titled "ng/µl" indicates the concentration of the RNA sample. The volume of nuclease-free water each sample was diluted in is indicated below each graph. The second column, titled "A260/A280" is a ratio used to determine the purity of DNA/ RNA in the sample. An A260/A280 value of ~2.0 is generally accepted as pure RNA, while ~1.8 is generally accepted as pure DNA. The final column, titled "A260/A230", is more of a general indication of purity of nucleic acids. If the sample is pure its values should fall somewhere between 2.0-2.2, with a lower value indicating significant absorbance at 230 nm and likely indicates the presence of DNA.

Though the concentration and purity values were relatively consistent for each of the 3 samples of each respective cell line, the concentration was too high in the F3- Control cell line to perform cDNA synthesis or RNA sequencing. In order to extract cDNA to perform more qPCR with these cell lines, we diluted all samples 1:10 in RNAse free water. The data in the tables low shows the newly obtained concentrations and purities of each cell line after being diluted in 90 *ul* of RNAse free water. The dilution successfully decreased the concentration of the RNA, so we are now able to use it for future qPCR experiments to look at expression levels of TAF4A and TAF4B.

Table 1: ID8 F3 RNA

ng/ul	$A260/A280$	$A260/A230$
574.3	1.97	2.19
108.2	1.81	2.02
144.5	1.85	2.02

Table 2: ID8 F3- Control RNA

ng/ul	$A260/A280$	$A260/A230$
112.1	1.87	1.68**
557.9	1.92	2.24
228.0	1.82	2.22

Table 3: ID8 F3 BRCA2 RNA

ng/ul	$A260/A280$	$A260/A230$
200.3	1.85	2.30
94.0	1.77	2.33
129.5	1.82	2.17

Table 4: ID8 F3 BRCA2 - Control RNA

ng/ul	$A260/A280$	$A260/A230$
282.0	1.86	2.34
45.6**	1.74	2.20
104.8	1.81	2.13

**ID8 F3 BRCA2-C Sample #2 A260/A230 value indicated the presence of DNA in the sample.

Low Attachment Conditions

Due to the fact that the cells were not surviving in the ultra-low attachment (ULA) conditions, we attempted to determine ideal seeding density and collection time that would allow for greater percent recovery that would eventually allow us to perform qPCR. The F3 cell line demonstrated the worst survival of the 4 under these conditions, so this cell line was chosen to try and determine seeding density and collection time on ULA plates. F3 cells were plated on ULA 10 cm^3 plates at seeding densities of $1x10^6$, $5x10^5$, and $2.5x10^5$ grown in culture for 2 days before collection. The results for percent recovery at this collection time are shown in Graph 1 below.

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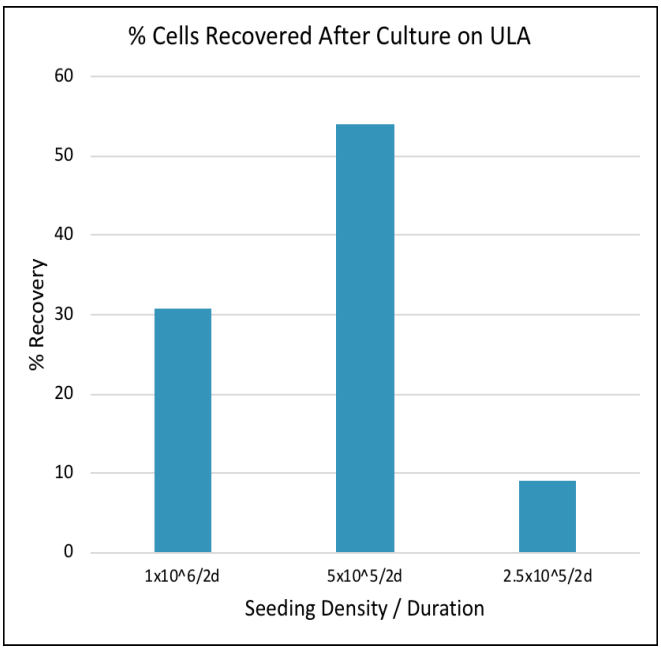
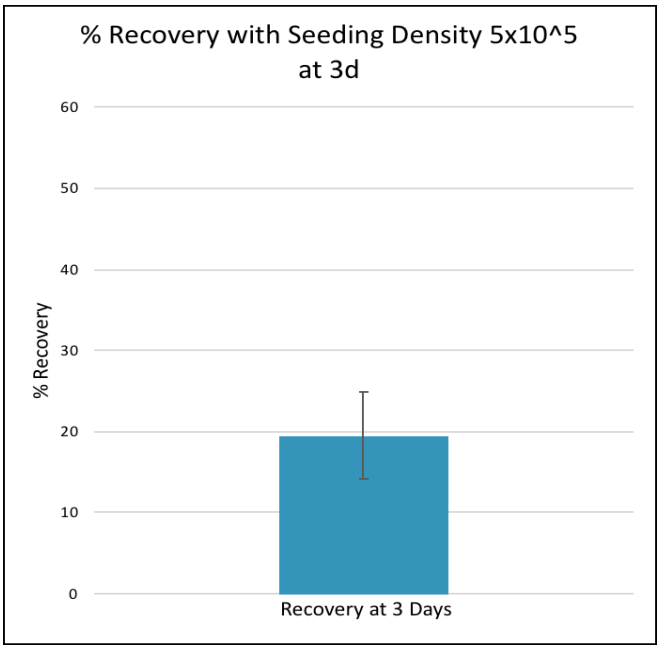
Graph 1: F3 Percent Recovery at 2 Days



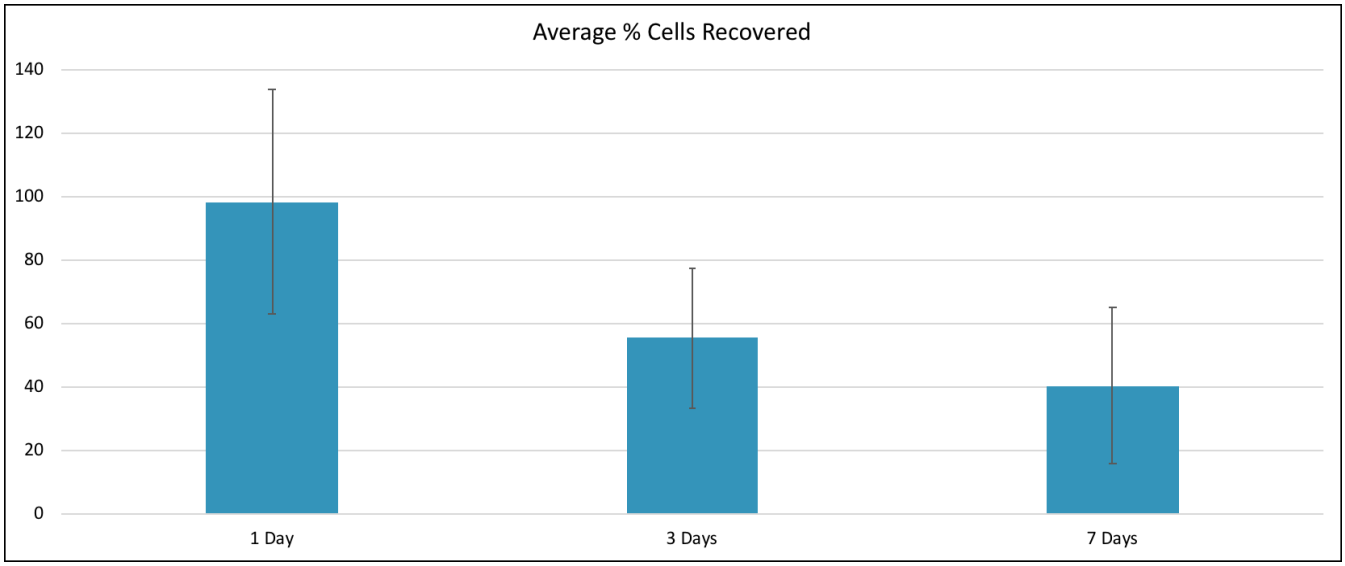
Graph 2: Percent Recovery at 3 Days



The results from the first seeding density experiment, summarized in Graph 1, revealed that the F3 cells had the highest percent recovery when collected after 2 days in culture with a seeding density of $5x10^5$ cells. The seeding density results prompted us to wonder how the percent recovery would differ if the cells were grown in culture with this seeding density and collected at a later time point. We hypothesized that the surviving cells may require more time in culture to recover the cell population. To test this hypothesis, the cells were grown in triplicate on ULA 10 cm^3 plates at a seeding density of $5x10^5$ cells and left in culture for 3 days. The collection at 3 days yielded a percent recovery of just under 20%, which was just under a 30% drop from the day 2 collection percent recovery seen in Graph 1 at the same seeding density. The value for percent recovery in Graph 2 represents an average of the 3 plates grown at $5x10^5$ cells.

The results from our 3-day collection experiment were not what we had hoped for, so we carried out a third collection experiment but allowed the cells to grow in culture for an extended time period in hopes that the surviving cells required more than 3 days to recover the population. Graph 3 summarizes the results from this prolonged collection experiment, in which we grew the cells again in triplicate on the ULA plates at the same seeding density of $5x10^5$ cells, only this time we performed collections at day 1, 3, and 7.

Graph 3: F3 Percent Recovery at 1, 3, and 7 Days



Graph 3 demonstrates that our collection at day 7 did not indicate an increased survival of this cell line when collected at a later time point. However, because the drop-in percent recovery from day 3 to day 7 is smaller in magnitude than the drop from day 1 to day 3, it is possible that the experiment needs to be carried out to a more extended time point for the cell population to recover. It is also possible the media the cells are being cultured in is not providing the cells the nutrients they require to survive under low attachment conditions. Due to time constraints, these collection time experiments were delayed until further analysis could be performed on the cell lines.

Immunofluorescent Staining Results

As mentioned earlier, the purpose of the immunofluorescent staining was to determine if any of the cell lines demonstrated characteristics of cancer stem cells (CSC's). The cells were immunoassayed using 4 specific primary antibodies and visualized using the secondary Alexa-594 fluorescently labeled antibody (red). Nuclear staining was visualized using DAPI staining (blue). The primary antibodies that we stained for were OCT4A, ALDH1A1, and SOX2, all of which are known cancer stem cell markers and have been previously documented as being expressed in ovarian cancer cells[8,9,10]. The BRCA2 and F3 cell line were stained for OCT4A, while BRCA2 and BRCA2-C cell line were stained for ALDH1A1 and SOX2. Finally, the F3, F3-C, BRCA2, and BRCA2-C cell lines were all stained for TAF4B after we obtained our qPCR results.

OCT4A Staining

In 2016, Samardzija et al. published a paper, *Coalition of Oct4A and β1 integrins in facilitating metastasis in ovarian cancer,* that looked at the association between cancer stem cells, namely OCT4A, and B-integrins in ovarian cancer metastasis. Their findings indicated an increased expression of OCT4A in high grade-serous ovarian tumors compared to normal ovarian tissue and a decreased ability of the same cancer cells to metastasize and form spheroids upon removal of OCT4A. Their indicated significant nuclear expression of OCT4A in high grade serous tumor cells, and heightened protein levels were confirmed by western blot analysis using the same tissue samples. Shown below is Figure 1 from Samardzija et al.'s paper, with a red rectangle surrounding their immunofluorescent staining results. This immunoassay demonstrates that the high-grade serous tumor cells demonstrated nuclear specificity for OCT4A while the normal ovarian epithelium cells did not. The staining results shown below were what we were hoping to find when we analyzed the same cancer stem cell factor in the 4 cell lines we examined.

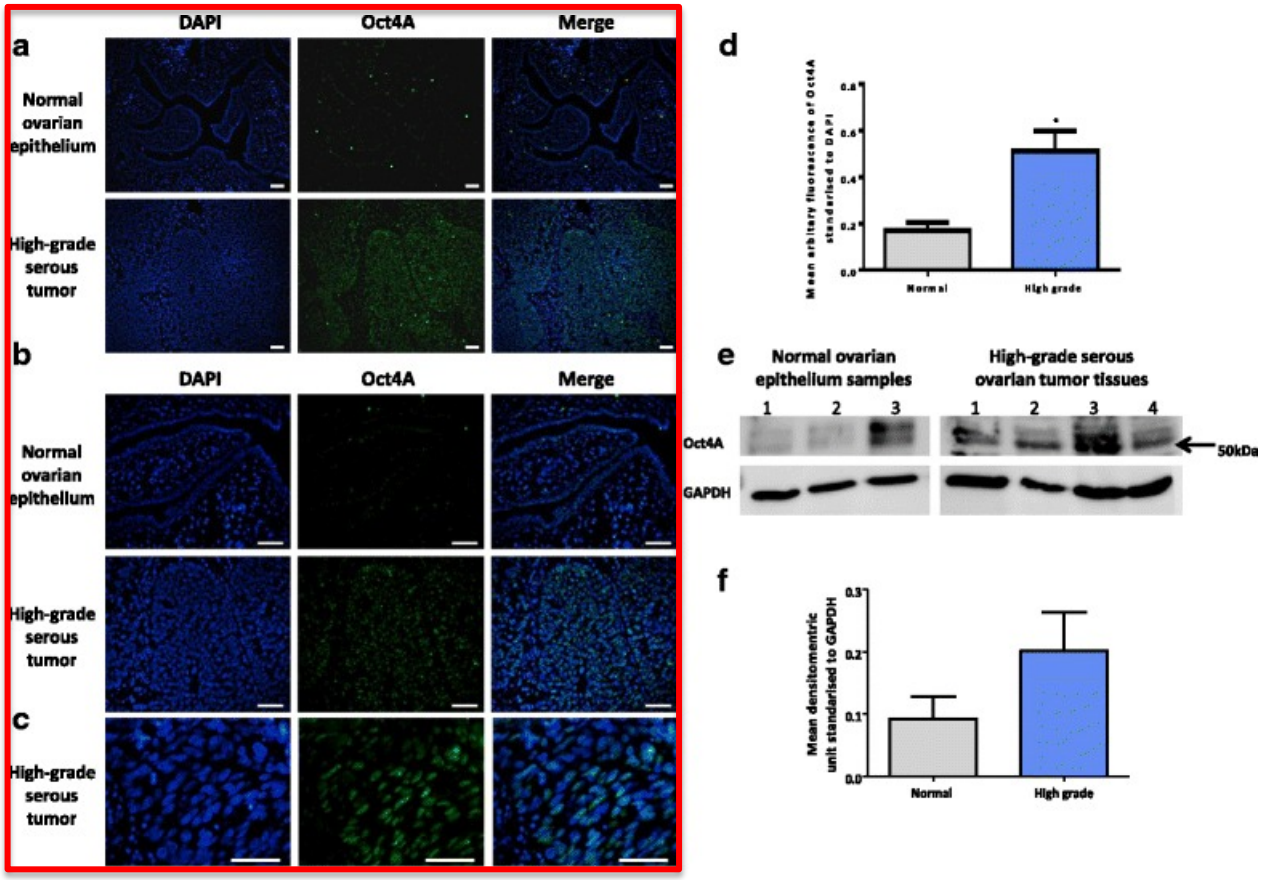


Figure 1 from Samardzija et al. (2016). a-c depicts immunofluorescent staining results showing expression and localization of OCT4A in normal epithelium and primary grade 3 serous tumor samples.

Table 5: OCT4A Staining Results in BRCA2 and F3

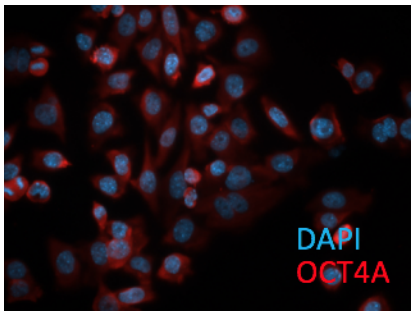
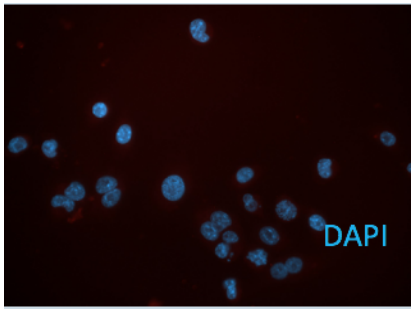
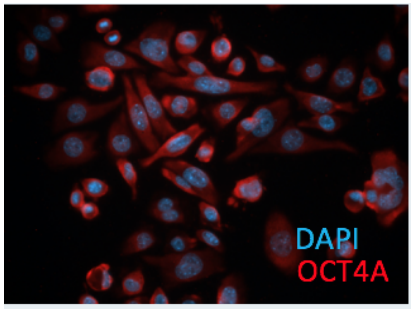
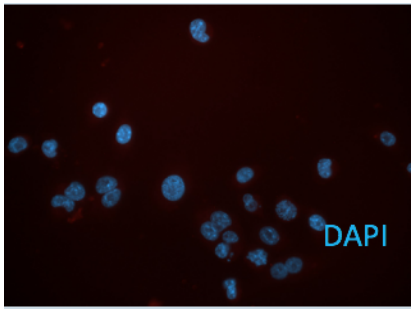
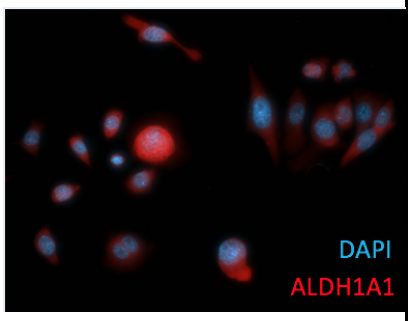
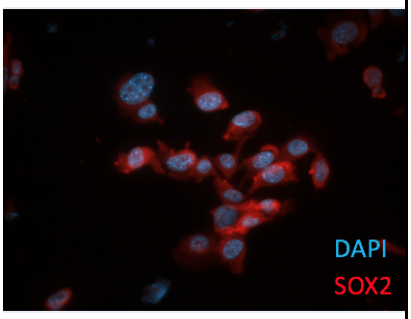
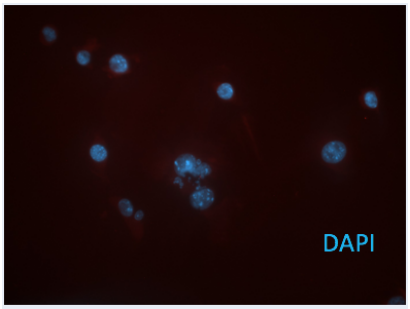
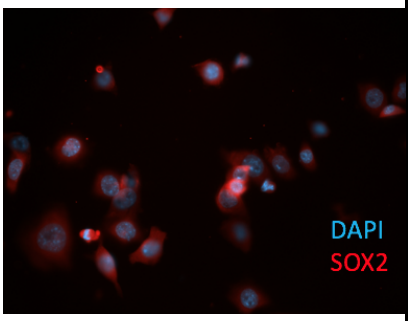
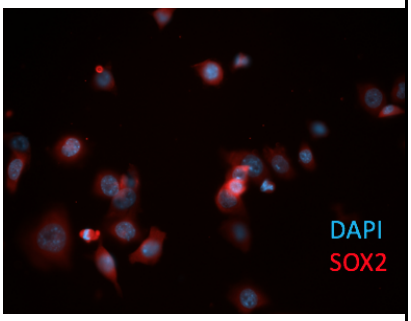
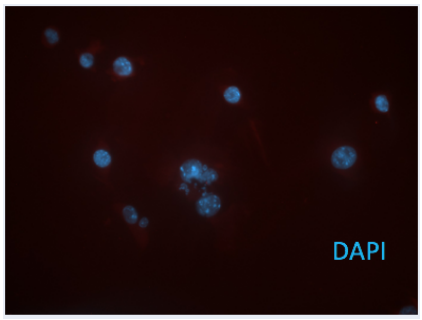
	OCT4A	SECONDARY ALONE
BRCA2		
F3		

Table 5, shown above, summarizes the results we obtained when we stained the BRCA2 and F3 cell lines for OCT4A. Unfortunately, we did not observe the same heightened expression or specificity we had hoped for, that would have been similar to Figure 1 from Samardzija et al. (2016). Though it did not appear that OCT4A expression was different between the two cell lines, it is interesting to note that the two cell lines examined were the single knockout of p53 and double knockout of p53 and BRCA2, as these are meant to simulate similar genotypes for those more at risk for cancer development.

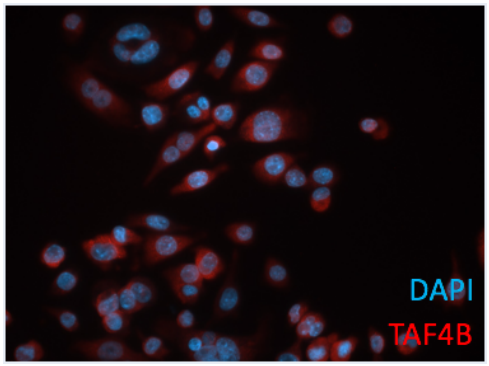
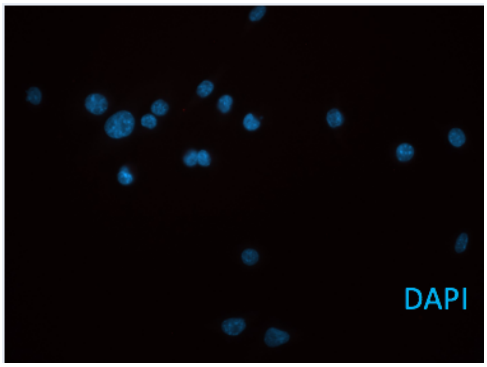
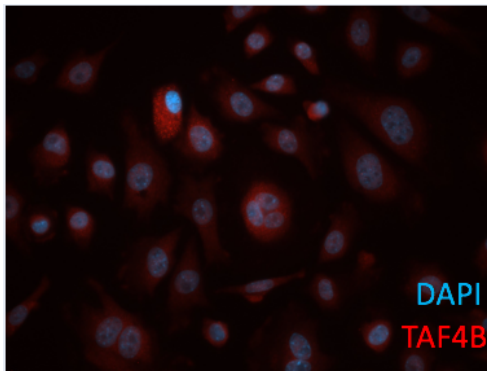
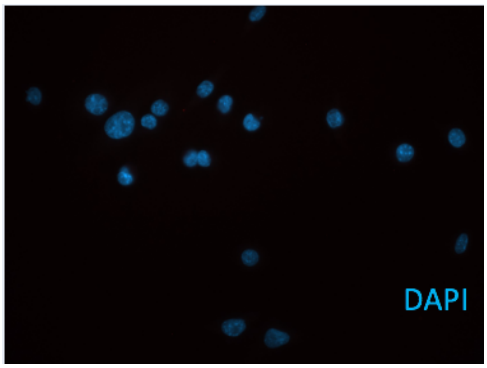
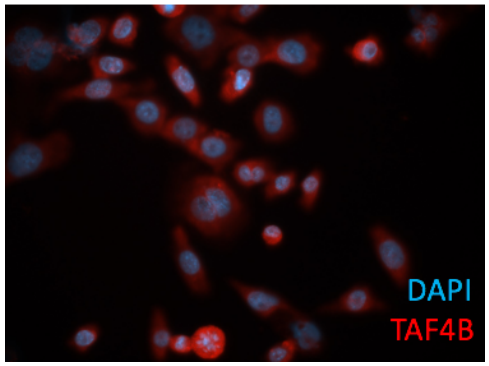
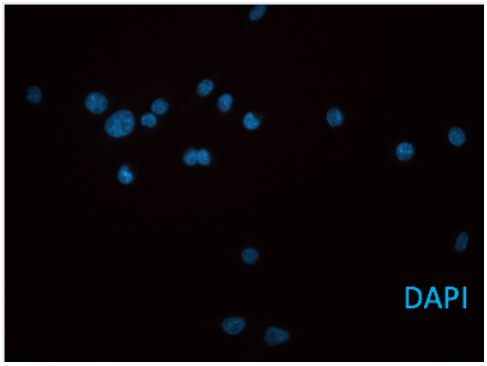
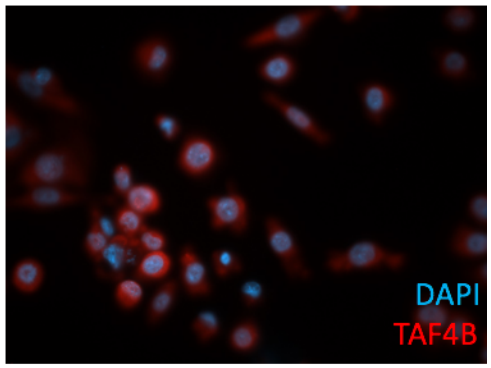
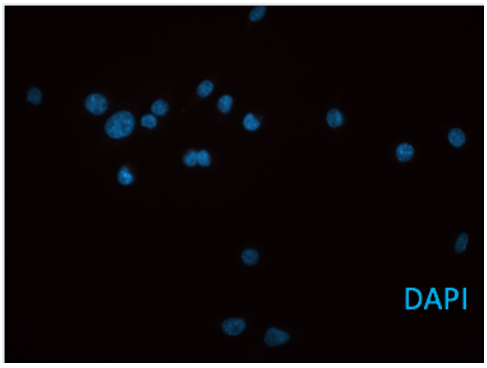
Two other cancer stem cell factors that we performed immunofluorescent staining for were ALDH1A1 and SOX2. Along with OCT4A, both of these factors have also been found to be overexpressed in ovarian cancer tumor cells, so we were interested in seeing whether or not any of our cell lines showed specificity or heightened expression of either factor. SOX2 has been found to be more highly expressed in the nucleus of ovarian cancer cells[9], while ALDH1A1 has been variably expressed in both tumor and stromal cells[10]. Table 6 summarizes the results of our immunofluorescent staining for ALDH1A1 and SOX2 in the BRCA2- C and BRCA2 cell lines. Again, we did not find either cell line demonstrated specificity or heightened expression of either cancer stem cell factor.

Table 6: ALDH1A1 and SOX2 Staining Results in BRCA2 and BRCA2-C

	ALDH1A1	SOX2	SECONDARY ALONE
BRCA2-C			
BRCA2			

Our immunofluorescent staining results were not what we had hoped for based on the literature for the cancer stem cell factors we selected. Though we opted to move on from this method in to qPCR, I performed one last staining that has been included on the following page in Table 7. After our qPCR results indicated increased levels of TAF4B mRNA in our cell lines, we decided to try and correlate this result with immunofluorescent staining. Again, the staining did not demonstrate heightened expression or specificity for TAF4B in any of the 4 cell lines.

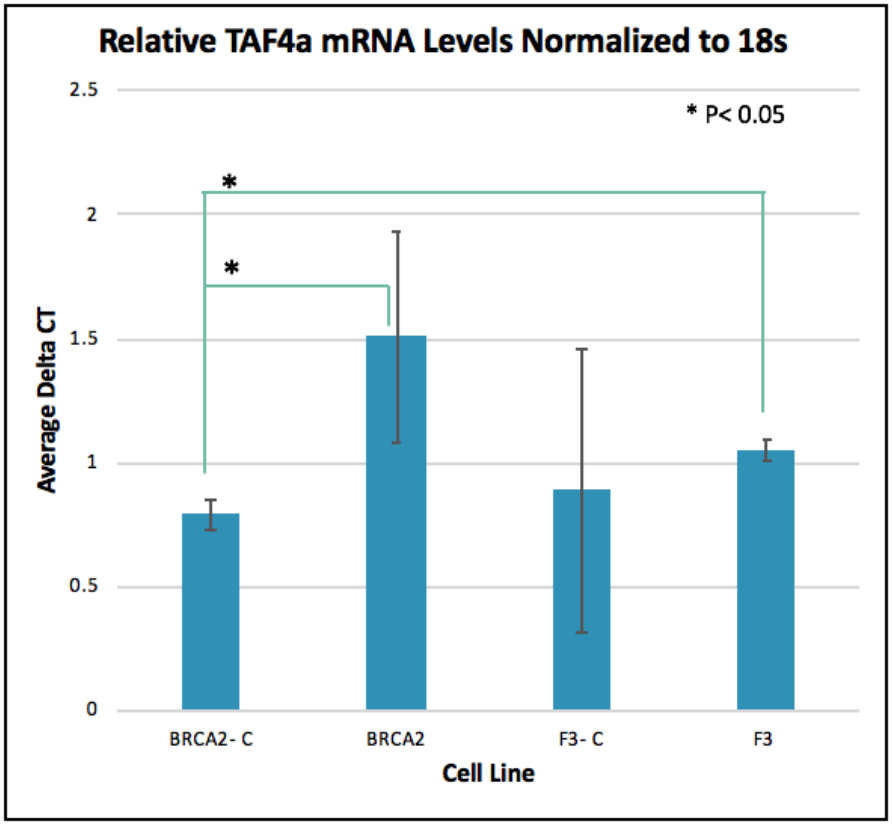
Table 7: TAF4B Staining Results in F3, F3-C, BRCA2, and BRCA2-C

	TAF4B	SECONDARY ALONE
F3		
F3-C		
BRCA2		
BRCA2-C		

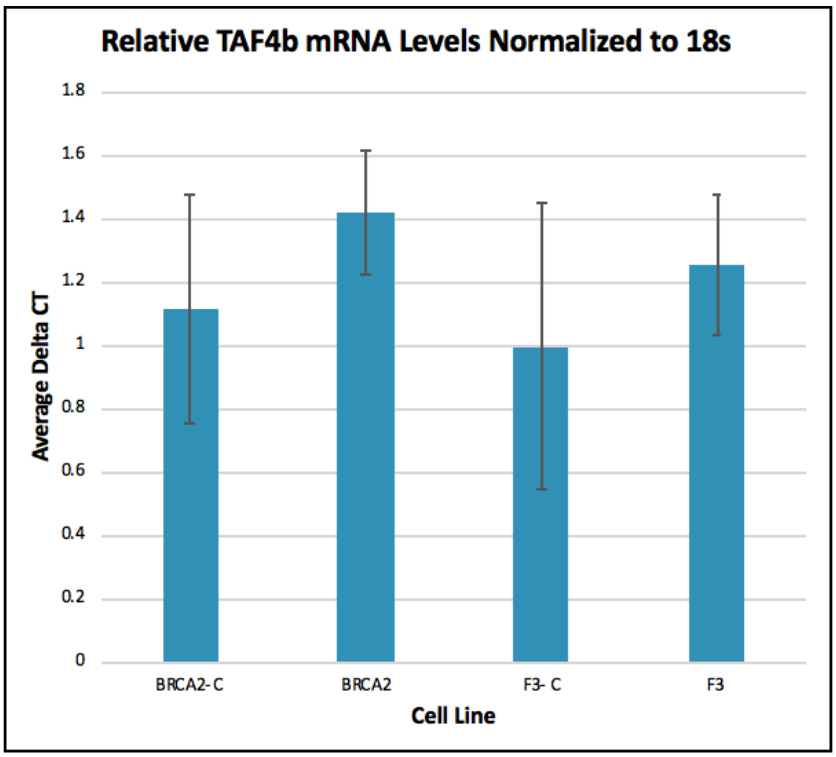
qPCR Results

Though we were unable to produce successful results from our immunofluorescent staining, we still wanted to quantify levels of mRNA in our cell lines to determine if it were possible that the primary antibodies we chose were producing illegitimate staining results. The genes we chose to look at using qPCR were OCT4A, TAF4A/ TAF4B, ER-A/ ER-B. Unfortunately, we were unable to obtain data for OCT4A and ER-B as they came up as undefined in our results, but we were able to obtain expression levels of TAF4A/TAF4B and ER-A. We chose to normalize our expression levels to 18s because it is a gene whose expression remains constant regardless of treatment and is considered one of the most stable genes despite different ovarian tissue conditions[11]. The following graphs demonstrate our qPCR results for TAF4A, TAF4B, and ER-A expression levels normalized to 18S.

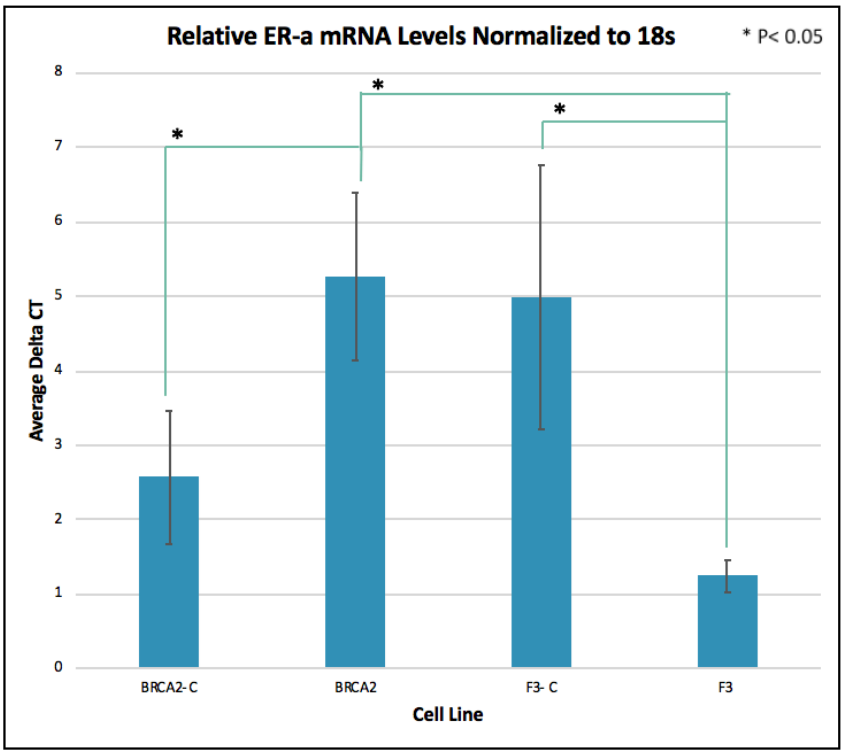
Graph 5: Relative TAF4A mRNA Levels Normalized to 18s



Graph 6: Relative TAF4B mRNA Levels Normalized to 18s



Graph 7: Relative ER-A mRNA Levels Normalized to 18s



The results from our TAF4A qPCR show that the BRCA2 and F3 cell lines have significantly increased expression of TAF4A when compared to the BRCA2-C cell line. Though none of the results from the TAF4B results were statistically significant, there was an increased level of expression in the same two cell lines as TAF4A. Though the increased expression in the single and double knockout cell lines may be noteworthy, our most interesting qPCR findings came from ER-A results. We found a significant increase in ER-A expression in the BRCA2 cell line in comparison to the BRCA2-C and F3 cell lines, and a statistically significant decrease in expression of ER-A in the F3 cell line in comparison to the F3-C and BRCA2 cell lines. Based on our findings, we wondered if there was any correlation between TAF4B and ER-A expression, as a former Freiman lab member had previously published a paper on her research regarding estrogen responsiveness of TAF4B in normal mouse ovaries and ovarian tumors. She found an increase in TAF4B mRNA in estrogen supplemented mouse ovarian tumors, highlighting the importance of TAF4B to estrogen in a normal ovary and during ovarian tumor progression in mice, suggesting basal levels of TAF4B are dependent on ER expression[12]. We attempted to find a similar relationship between TAF4B and ER-A based on our qPCR results, but were unable to find a statistically significant correlation. Perhaps a more interesting question that arises from these results is why the F3 cell line had the lowest mRNA expression of ER-A while the BRCA2 DKO cell line had the highest expression of ER-A, expressed at roughly the same level as the F3-C cell line (wild type cell line). The recovery of mRNA expression levels from the single knockout cell line (F3) to the double knockout cell line (BRCA2) is a result that should be further looked in to, as these original findings were not statistically significant.

Discussion and Future Directions

The results from the differential growth conditions in cell culture, immunofluorescent staining, and qPCR of our four CRIPSR modified ID8 cell lines did not produce any definitive answers for the original questions that I sought to answer at the beginning of this project. Though the results were not ideal, they are still informative and provide an excellent future pathway for a project that continues to look at the differential behaviour of these cell lines.

In order to try and understand the ideal growth conditions of these cell lines under low attachment conditions, I am performing the same extended collection experiment as discussed in the 'Low Attachment Conditions' section of the results. I am growing all 4 cell lines in culture with a seeding density of $5x10^5$ cells on ultra-low attachment plates and will perform collections at day 1, day 2, day 3, day 7, and hopefully day 9 or day 10. The final collection point depends on the color of the media, which will be routinely checked to ensure the cells have not used up all of the nutrients. Due to the fact that the cells are dispersed in the media itself as opposed to growing on the plate, the media cannot be changed without disrupting the cell population. This means it is crucial to use the color of the media as an indicator of cell survival under these conditions. The media is originally a bright pink color and slowly changes in to more of a dull yellow/ orange as the cells use the nutrients in it. If at any time point during the extended collection experiment the media turns to the dull yellow/ orange color, the cells will be collected and that will be the last collection of the experiment. We are anticipating this will be on day 9 or 10, which would reveal whether or not the cell population recovers with a few extra days in culture. These results will not be included in this paper but are more useful for understanding the general survival of the cells under low attachment conditions. If they demonstrate an increased percent recovery when given more time in culture, this will be critical information for the future research of these cell lines under low attachment conditions. Determining the proper seeding density and collection time point with adequate survival of these cells will allow for any of the methods discussed in this report to be carried out on the same cell lines when grown under low attachment conditions, which could be directly compared to the results already obtained for high attachment conditions. This comparison could reveal differences in cancer stem cell factors and/or gene expression that give insight in to the behaviour of ovarian cancer cells in a free-floating environment like the peritoneal fluid. If the extended collection experiment does not warrant sufficient results, it may be worthwhile to attempt to grow the cells under low attachment conditions with serum free media to determine if this variable is hindering cell growth.

The immunofluorescent staining results shown in Tables 5-7 of this paper are not indicative of what we were expecting to find in terms of expression of or specificity for the

cancer stem cell factors OCT4A, ALDH1A1, SOX2, and TAF4B. A reference immunoassay of OCT4A was shown using Figure 1 from Samardzija et al. to demonstrate the published results of similar immunostaining for that particular CSC. One reason we may not have obtained ideal results is that the primary antibodies we are using have undergone too many freeze/thaw cycles, could be too old to produce sufficient results, or the incubation period could be too short. In order to continue with any experimentation with this method, it needs to be determined in the primary antibodies are of sufficient nature. The integrity of the primary antibodies could be analyzed using western blot to determine if they have indeed undergone degradation. If this does not seem to be the case, the same experiments discussed in this paper could be repeated with a longer incubation period. If neither the western blot nor increased incubation period give improved results, it is possible new antibodies need to be used to obtain different staining results.

Due to the fact that OCT4A and ER-B both came up as 'undefined' in our qPCR results, it is worthwhile to re-run OCTA4A with a positive control to determine if the primer used is sufficient. If time permits, I will perform this experiment in the coming weeks and record the results for future reference. As briefly touched on in the 'qPCR' results section, the most interesting product of the qPCR experiments conducted was the difference in ER-A expression levels between the BRCA2 (DKO), F3 (single knockout) and F3-C (wild type) cell lines. The BRCA2 cell line showed the highest expression levels of ER-A, similar to the levels of the F3-C cell line, while the F3 cell line had a significantly decreased expression level and was the lowest of all four lines. Though the F3 and F3-C expression values were not statistically significant to each other, there still remains the question behind the heightened expression in the BRCA2 DKO compared to the F3 single knockout cell line. What could cause the BRCA2 cell line expression levels of ER-A to be closest to the expression levels of the F3-C (wild type) cell line, and why is there such a sharp drop in expression in the F3 cell line? ER-A is a component of the cell's main estrogen receptor, so it would be interesting to look at the estrogen responsiveness of these cell lines to determine if the same discrepancy is seen between the BRCA2 and F3 cell lines. If an increase in TAF4B mRNA has been previously correlated with has been previously correlated with estrogen supplemented mouse ovarian tumors[12], the relationship between TAF4B and estrogen responsiveness should be further researched to determine the importance of this subunit in estrogen receptor expression. There are several experiments that could be conducted to determine the validity of the qPCR results and further elucidate the connection between TAF4B and estrogen responsiveness. First, protein levels of ER-A should be analyzed by western blot and immunofluorescent staining. If protein levels reflect the results shown in qPCR, the next step should be to treat the cells with estrogen and analyze their growth, behaviour, and protein levels of TAF4B and ER-A with cell counts, qPCR, and western blot. Next, an ER knockdown could be generated using siRNA (small interfering RNA) to compare the cells behavior in the absence of their main estrogen receptor. This

could yield incredibly useful information on any genetic changes that take place in the cells, or between cell lines, in the absence of estrogen as it has been shown that heightened levels of estrogen significantly increase the risk for development of ovarian cancer in women[13]. Finally, the analysis of separate cell lines that are also null for p53 and BRCA2 may be useful to see if they exhibit the same ER-A expression levels as the cell lines examined in this project. Specifically, it would be interesting to obtain a cell line with the genotype wild type p53, $BRCA2^{-/-}$ to compare the behavior of a "single knockout" of BRCA2 with the F3 and BRCA2 cell lines we have used.

There are numerous studies looking at the relationship between TAF4B and estrogen responsiveness in mouse ovarian tumor cells, but there is not a significant amount of research in to response of TAFB expression levels as a result of estrogen deficiency. Combined with previous findings of the role of TAF4B in ovarian tumor formation, progression, and survival, the expression levels in ovarian tumors in the absence of estrogen could contribute to an overall increased understanding of the complexity of the interactions that allow for the disease to progress in such an aggressive manner, and ultimately aid in novel detection methods to reduce the extremely poor survival rates.

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