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CHARACTERIZATION OF CANCER STEM CELLS IN TUMOR PATHOGENESIS

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ABSTRACT

Recent studies have shown that many cancerous tumors contain “cancer stem cells” (CSCs) that may be responsible for the self-renewing properties of the tumor. A common cancer treatment, chemotherapy, targets rapidly dividing cells, but could possibly be ignoring the more slowly dividing CSCs. The CSCs were characterized in a variety of tumors using immunofluorescence to identify stem cell markers after treatment with a common chemotherapy, Doxorubicin. The resulting data show statistically significant evidence for the presence of stem cell markers in cells left behind after chemotherapy treatment.

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BACKGROUND

Cancer Stem Cell Theory

Cell turnover is a tightly regulated process in human tissues. Each tissue in the human body is comprised of many highly specialized cells with life spans of a few days or even hours. Many cells are constantly being replenished with new, healthy, yet short-lived cells, while other special cells in the tissue (stem cells) are unusually long-lived, are less specialized, and are fewer in number than the short-lived population. One hypothesis about the derivation of the specialized cells is that the stem cells control the cell turnover process, which helps to renovate human tissues (Dalerba et al., 2007.)

Stem cells have three main properties: differentiation, self-renewal, and homeostatic control. Differentiation allows a stem cell to become a highly specialized, short-lived, tissue-specific cell. This property allows stem cells to continuously replenish tissues with short-lived specialized cell populations. Stem cells also have the ability to divide and give rise to genetically identical cells with similar levels of differentiation, self-renewal, and homeostatic control properties – essentially, they keep the stem cell population continuous. When genetic constraints and environmental stimuli are present, stem cells are able to evenly regulate the balance between self-renewal and differentiation (Dalerba et al., 2007).

Like normal tissues, tumors also contain long-lived cells similar to the stem cells found in normal tissue. These "Cancer Stem Cells" (CSCs) are thought to be diseased stem cells that give tumors their heterogeneous cell populations (Clarke & Fuller, 2006). It is also thought that tumors arise from a single target cell that undergoes multiple genetic mutations over a period of years (Fearon & Volgelstein, 1990). Since the target

mutations take time, the only cells able to accumulate these mutations would be stem cells (Dalerba et al., 2007).

According to the CSC model, tumors are comprised of a heterogeneous population of cells with different differentiation properties (Dalerba et al., 2007). CSCs, when transplanted into mice, are able to reconstitute the original tumor, including the phenotypic heterogeneity of the parent tumor (Gu et al., 2007). This was demonstrated by the induction of glioblastoma tumors *in vivo* using chemotherapeutic drug-resistant cancer stem-like cells isolated from brain cancer tissue (Kang & Kang, 2003).

In order to define the existence of CSCs, Cho, Clarke and Dalerba explain three observations seen in CSC populations. First, stem cells isolated from a tumor regenerate when transplanted into mice. Only a small subpopulation of cells from the tumor has this potential. Second, a specific collection of surface markers (cell determinants, CD) classify tumorigenic cancer cells. Third, CSCs produce tumors of a mixed population of cells that are a complete regeneration of the parent tumor (Dalerba, et al., 2007).

Evidence for the Theory

The first experimental proof that showed cancer derived cells mimicking a normal stem cell hierarchy was seen in human neoplastic disease. NOD-SCID mice injected with a CD34+/CD38^{neg} subpopulation of human leukemia tumor displayed the original tumor (Dalerba, et al., 2007).

In addition, in human colon cancer cells it has been shown that CD133^{+} (a possible CSC marker) cells - unlike CD133^{-} cells - are able to initiate tumor growth when transplanted into immunodeficient NOD/SCID mice (Ricci-Vitiani et al, 1998; O'Brien, et al., 2007). These human colon cancer-initiating cells (CC-ICs) were discovered to

reestablish tumor heterogeneity by differentiating and also regenerating themselves upon several serial transplantations (O'Brien et al., 2007).

In another study at Vanderbilt University, a fully reconstituted human prostate cancer tumor was recovered through serial transplantation experiments *in vivo*. The cell lines recovered in the experiment positively expressed CD44 and Nestin (early progenitor markers) (Gu et al., 2007).

In breast tissue, it has been shown that a subpopulation of most human breast cancer tumor clones, defined as CD44+/CD24$^{-/low}$, can sustain tumor growth in NOD/SCID mice. These cells represent only 11-35% of the total cancer cells, yet can completely regenerate the phenotypic heterogeneity of the parent tumor (Raouf et al., 2005).

Chemoresistance

Several studies have also demonstrated that cancer stem cells have high levels of drug resistance, thus they can survive after a tumor has been treated with chemotherapy. These findings have raised concerns about current drug treatments for tumors, since the drug resistant properties of CSCs could allow them to be selected for during chemotherapy treatment. If CSCs in fact are drug resistant, then chemotherapy treatments essentially are selecting for tumorigenic stem cells that will eventually give rise to new tumors. A study by Levina et al. (2008) examined lung cancer stem cells selected with various chemotherapy drugs. The study found that the cells surviving after treatment with drugs such as doxorubicin, cisplatin, or etoposide expressed the stem cell markers CD133, CD117, SSEA-3, TRA1-81, Oct-4, and β-catenin. The surviving cells also exhibited the loss of differentiation markers such as cytokeratins 8/18. In addition,

the drug-surviving cells were able to create tumor spheres *in vitro* and created metastatic tumors when implanted into SCID mice (Levina et al., 2008). All of the results of this particular experiment provide strong evidence that cells that survive chemotherapy treatments are in fact stem cells, and that these stem cells indeed have the capability of forming new malignant tumors.

Doxorubicin

Chemotherapy is a widely used cancer treatment that involves the use of a single or a combination of anti-cancer drugs. Most chemotherapy drugs target cells that are rapidly dividing (a characteristic of cancer cells); however, several normal cells of the body, such as hair cells and blood cells, also divide rapidly. Since all chemotherapy drugs affect both cancer and normal cells, the goal is to design drugs that affect cancer cells more severely than normal cells (Merck, p.1043-1044).

An effective chemotherapy drug will interrupt a key function of a cancer cell, thus killing or severely impairing the survival of the cell. There are several main classes of chemotherapy drugs: alkylating agents, anti-metabolites, anti-tumor antibiotics, topoisomerase inhibitors, mitotic inhibitors, and corticosteroids.

The drug used in this project was Doxorubicin, a drug classified most often as an anthracycline, but also as a topoisomerase inhibiter and alkylating agent. According to the American Cancer Society (2008), anthracyclines are a broad class of anti-tumor antibiotics that interfere with enzymes during DNA replication. More specifically, Doxorubicin interferes with topoisomerases, which are enzymes involved in the separation of DNA strands during replication. Finally, Doxorubicin can be classified as

an alkylating drug, one that directly damages DNA in a nonphase-specific manner (American Cancer Society, 2008).

As mentioned above, chemotherapy targets rapidly dividing cells, possibly leaving behind slower dividing cancer stem cells that have the ability to reconstitute the entire tumor. DOX was used in the current MQP to test the hypothesis that recurring tumors are caused by the presence of tumor stem cells left behind after chemotherapy treatments.

Discovery

The discovery of Doxorubicin was first published by scientists in Milano, in 1969 under the name Adriamycin. This new cancer biotic, as it was called at the time, was the 14-hydroxy derivative of daunomycin. Daunomycin, isolated from the bacteria *Streptomyces peucetuius*, was shown to successfully treat acute leukemia in children. In order to produce other successful cancer treatments, Arcamone et al. thought it would be worthwhile to create a derivative of daunomycin by mutagenizing the original strain *S. peucetius* (Arcamone, et al., 1969).

After treating the parent culture with the mutagen N-nitroso-N-methyl urethane, the surviving colonies were termed *S. peucetius* var. *caesius*. An isolated metabolite produced by this mutagenic strain, adriamycin, was a new compound similar to the structure of daunomycin. Initial chemotherapeutic studies of the new drug indicated that there was a "marked retardation" of cancers more favorable than the original drug daunomycin (Arcamone, et al., 1969).

Current Usages

Doxorubicin inhibits normal cell function by interfering with DNA replication in three ways: via enzyme interference, direct DNA damage, and the formation of reactive oxidative species. The drug prevents protein synthesis and DNA replication by intercalating between DNA base pairs, thus damaging the helix and preventing replication. Also during DNA replication, Doxorubicin inhibits Topoisomerase II by specifically inhibiting the ligation properties of the enzyme after the induced double-strand breakage, therefore resulting in the formation of a cleavable DNA-enzyme complex (Cutts et al., 2005). Finally, Doxorubicin produces oxygen free radicals, which result in cytoxicity (National Cancer Institute, 2009).

Since Doxorubicin is able to be used in treatment against a wide variety of tumors, it is one of the most valuable agents in clinical use (Cutts et al., 2005). The major downside of the drug is that it has been shown to induce cardiotoxicity (DeVita, et al., 2001), which has sparked major efforts to develop less cardiotoxic and more effective derivatives. The research involved in finding and using these derivatives has allowed doxorubicin to become one of the most widely used of all clinical anti-cancer drugs (Weiss, 1992).

Dangerous Effects of DOX on the Body

One of the most concerning problems with doxorubicin is that it has been seen as highly cardiotoxic. This cardiotoxicity limits the vast utility of the drug, and has fueled research into alterations/modifications that could make the drug safer for human treatment. In a review of the dangerous side effects of this popular chemotherapy treatment, Saltiel and McGuire showed that the prevalence of cardiomyopathy is

anywhere between 1.7-6.8% which can appear within a minimum of one week following the final dose of the drug. Risk factors include: age, dosing regimen, total dose, mediastinal irradiation, and others (Saltiel & McGuire, 1983).

One way doxorubicin may induce cardiomyopathy is typical of anthracycline drugs (Saltiel & McGuire, 1983): the production of superoxides caused by the initial reduction of doxorubicin to a semiquinone radical (Doroshow et al., 1979), followed by the reduction of NADPH to form the superoxide (Myers et al., 1977). The formation of these superoxide radicals cause a chain reaction (Saltiel & McGuire, 1983) which ultimately produces malondialdehyde, levels of which have been measured in rats and in human plasma (Myers et al., 1977). Saltiel and McGuire also suggest that doxorubicin degrades glutathione, which in reduced form seems to protect the heart from the effects of doxorubicin (Saltiel & McGuire, 1983).

To combat the possible lethal effects of this anthracycline, Saltiel and McGuire argue that vitamin E, when use as a pre-treatment, can "reduce the incidence of typical cardiac lesions and of cardiomyopathy (Saltiel & McGuire, 1983)." Known as an agent against free radicals in the body, vitamin E (in large doses) was shown to prolong the life of rabbits treated with doxorubicin (Van Vleet & Ferrans, 1990).

Also discussed was the inhibition of coenzyme Q10 by doxorubicin. Like the studies with vitamin E, treatment with coenzyme Q10 has been shown to reduce the interference of doxorubicin with oxidative metabolism in the heart (Saltiel & McGuire, 1983).

Combination Therapy

Generally, in the United States, cancer patients are treated with a combination of chemotherapy drugs, in order to achieve the highest success rate. In gastric cancers, a combination therapy that includes doxorubicin increases survival rate significantly (Wagner et al., 2006).

In the treatment of hepatocellular carcinoma (HCC) that retains the asialoglycoproten receptor (ASGP-R, a surface receptor), doxorubicin has been largely ineffective when administered systemically due to its high toxicity to surrounding cells. To make doxorubicin more effective and safe, the drug can be coupled with macromolecules that bind to the ASGP –R so it can be targeted toward the cancer cells and avoid other tissues surrounding the liver. In a preclinical study, when coupled with lactosaminated human albumin, which contains a clinically safe glycoprotein that is internalized by the ASGP-Rs of HCC cells, it was shown that the conjugate was effectively targeted in the HCC tumor with "increased anticancer efficacy and tolerability (Fiume et al., 2008)."

However, in some studies the combination of doxorubicin and drugs such as cyclophosphamide, mitomycin, and megestrol acetate, have increased the patients chance of cardiomyopathy (Saltiel & McGuire, 1983).

Lyle Lab Interests

Past experiments in the Lyle laboratory have focused on identifying characteristics of stem cells that could allow them to give rise to tumors. Several mutations in stem cells have been identified and are currently being studied by the Lyle lab. A past project in the Lab identified keratin-15 as an epithelial stem cell marker (Lyle

et al., 1998). The same study also identified skin stem cells as residing in the "bulge" area of the hair follicle, which give rise to several skin components such as the epidermis, hair follicle, and sebaceous glands (Lyle et al, 1998). These findings have driven further research to identify mutations and mechanisms that could cause stem cells to produce skin tumors.

LEF/TCF Mutations

Current experiments in the Lyle lab aim to understand the mutations in stem cells that can cause tumor formation. In particular, the lab is focusing on LEF/TCF transcription factors, which normally function to maintain cellular skin homeostasis. One third of human sebaceous tumors have mutations in the LEF-1 gene, a significant effect of LEF mutations in tumor incidences (Takeda et al., 2006). LEF/TCF is a part of the Wnt/β-catenin/LEF/TCF pathway, which causes differentiation of skin stem cells into epidermis, hair follicles, and sebaceous glands. Mutations in LEF/TCF may therefore cause dysregulation of these stem cells, giving rise to sebaceous and other epithelial tumors. The role of LEF/TCF signaling in both normal and abnormal skin cell differentiation is shown in **Figure 1**.

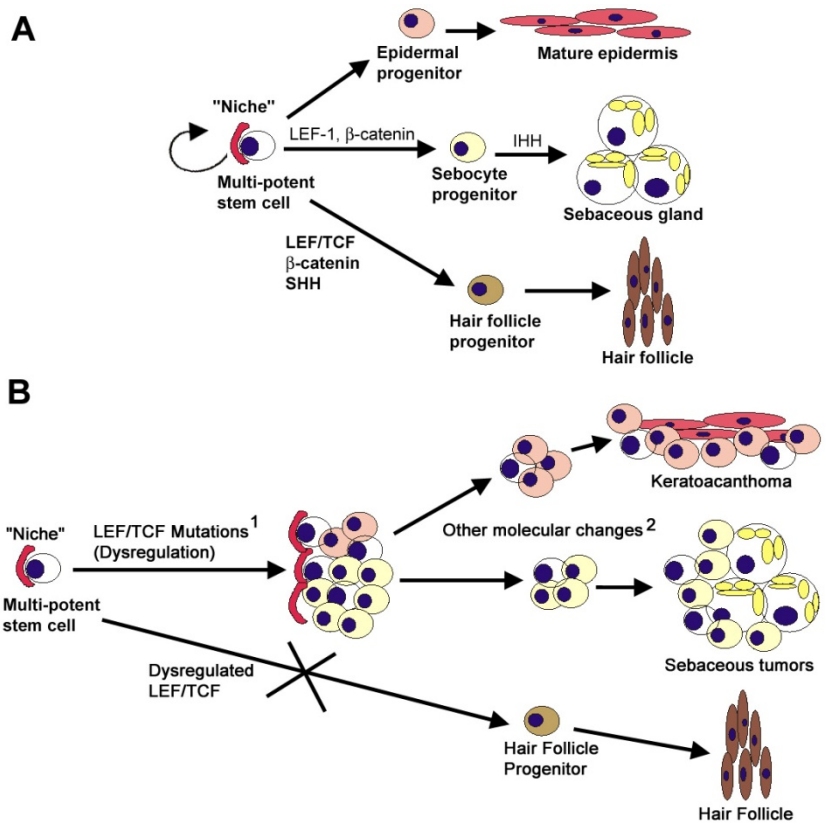


Figure 1: The Role of LEF/TCF in Skin Cell Differentiation and Tumorigenesis. (A) Normal differentiation of multi-potent stem cells into epidermis, sebaceous, and hair follicle cells under LEF/TCF signaling. (B) Hypothesized mechanism of tumorigenesis due to mutations in LEF/TCF.

Muir-Torre Syndrome

Muir-Torre syndrome is a condition in which patients have multiple skin tumors on the face such as sebaceous carcinomas, usually in conjunction with other internal cancers, especially colon cancer (Cohen et al., 1991; 1995). Most of these patients also show defects in the MLH1 and MLH2 DNA mismatch repair genes (Lyle grant, 2004). As previously mentioned, mismatch repair errors in LEF-1 occur in one third of sebaceous tumors. DNA mismatch repair has also been linked with microsatellite instability, which is thought to be a cause of TCF-4 mutations (Fukushima et al, 2001). All of these factors therefore suggest that Muir-Torre syndrome patients could possess

abnormal LEF/TCF signaling pathways, making them good candidates for research on cancer stem cells in the Lyle lab.

Midbody Retention

Previous experiments in the Lyle lab have also suggested that midbodies, or remnants of cell division, are retained in stem cells but are degraded in normally differentiated cells. Midbodies form in the cleavage furrow during telophase, to aid in the splitting of the cell during cytokinesis. When the cell divides, the midbody must be retained within one of the two daughter cells. Cells present within adult stem cell niches appear to have more than one midbody, suggesting that stem cells accumulate them during a symmetric cell division (Lyle & Doxsey labs, unpublished). When comparing normal cells to cancer cells, it was found that a high percentage of cancer cells contained greater than one midbody, while almost none of the normal cells contained multiple midbodies. Lastly, colocalization assays showed that MKLP-1 and LAMP2 (a lysosome stain) existed in the same area in normal cells (Lyle & Doxsey labs, unpublished). This information strongly suggests that normal cells have a mechanism for degrading midbodies, while stem cells retain midbodies, making them a useful stem cell marker.

Immunofluorescence and Chosen Stem Cell Markers

Immunofluorescence is a microscopy staining technique used to identify the presence of antigens in cells using antibodies to the molecules of interest, tagged with fluorescent dye. Cells containing antigens of interest are incubated with a primary antibody, and then are incubated with a secondary antibody containing a fluorescent dye. The stained cells and antigens are then observed using a fluorescence microscope. In this

MQP, the following stem cell markers were stained and observed using immuno-fluorescence.

MKLP-1

Mitotic kinase-like protein-1 (MKLP-1) is encoded by the KIF23 gene, located on chromosome 15. MKLP-1 is a motor protein involved in mitosis of mammalian cells, and is specifically involved in cytokinesis. During mitosis, MKLP-1 is found in the midbody of the cell, which forms in the cleavage furrow during telophase (Zhu, 2005). After cytokinesis, MKLP-1 is left in the midbody which is left behind in one cell's cytoplasm. As mentioned before, previous experimental data has suggested that stem cells retain their midbodies after division, while differentiated cells do not (Lyle and Doxsey, unpublished). **Figure 2** below is an example of a midbody in a primary breast tumor cell.

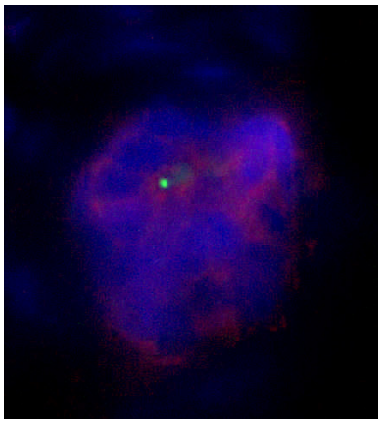


Figure 2: Example of a Midbody in Primary Breast Tumor Cell (60x). MKLP-1 is stained in green (midbody) (diagram center), and CD44 is stained in red.

Keratin-15

Keratins contribute to the structure and strength of the cytoskeleton of epithelial cells. Keratin-15 (K15) is an "acidic" keratin, and part of the type I keratin family. K15 works in conjunction with K5, its "basic" type II keratin counterpart, to create a keratin

filament network within epithelial cells (Radoja, 2004). Many basal cell carcinomas express K15, which suggests that epithelial tumors arise from stem cells (Lyle et al, 1998; Jih et al., 1999).

CD133

CD133 is a cell surface glycoprotein with five transmembrane domains. It is usually found on neuroepithelial stem cells and is generally associated with brain tumors (Miki, 2007). It has also been identified in cancer stem cells of many other tissues, including prostate cancer (Miki, 2007) and colon cancer (Chu, 2009). CD133+ colon cells have been shown to be highly proliferative and tumorigenic when injected into immunodeficient mice (Ricci-Vitiani, 2007).

CD44

CD44 is a cell adhesion molecule involved in cell signaling functions. It is usually found in the basal cells of both normal and tumoric prostate tissue. Some studies suggest that CD44+ prostate cancer cells are more tumorigenic and metastatic than CD44- cells (Patrawala, 2006). CD44 has also been indicated as a colon cancer stem cell marker in several studies (Chu, 2009).

PROJECT PURPOSE

As discussed in the background, malignant tumors often recur after treatment with chemotherapy. This is thought to be because current chemotherapy treatments target rapidly dividing cells, leaving behind the more slowly dividing cancer stem cells that could eventually give rise to an entire new tumor. The hypothesis being tested by this MQP is that cancer stem cells remain in a tumor cell population after treatment with chemotherapy. Normal and abnormal (cancer) cells from several types of human tissue will be treated with the common chemotherapy drug doxorubicin (Dox), which will kill rapidly dividing cells, as occurs in normal chemotherapy treatments. Immuno-fluorescence staining and microscopy will then be used to determine the amounts of various stem cell markers remaining in the human tumor cells, and statistical analysis will be done to compare stem cell marker presence in normal and abnormal cell lines. We hypothesize that there will be a higher percentage of cells staining positive for stem cell markers in tumor cell lines than in normal cell lines, and that the percentage of positive staining will increase after treatment with doxorubicin.

METHODOLOGY

Cell Culture

Culture Splitting

All the cell lines used in this MQP were generously given by colleagues in the Cancer Biology department at UMASS Medical School. These cell lines were: MDA231, PC3, NPrEC, SEBE5E7, Tumor 1C, Clone A, Clone J, HCT116 +/+, and HCT116 -/-.

The MDA231 cell line was purchased originally from ATCC (#HTB-26). It is a female human breast cell line that expresses the WNT7B oncogene and was isolated from an adenocarcinoma. The cells also express epidermal growth factor (EFG) and transforming growth factor alpha (TGF alpha).

The PC3 cell line was also purchased from ATCC (#CRL-1435) and is a prostate grade IV adenocarcenoma. It is a tumorigenic prostate cell line obtained from a 62-year old male. The cells express the antigens HLA, A1, and A9. Normal prostate epithelial cell line (NPrEC) was used as the normal prostate cell line. This immortalized normal human cell line was purchased from an unknown source.

The normal sebaceous cell line (SEBE6E7) was immortalized from a human sebaceous gland from an unknown source. The tumor sebaceous cell lines were genetically engineered in the Lyle Lab at UMASS Medical School. Tumor 1C was an immortalized sebaceous tumor cell line. The Clone A cell line was a selected clone from Tumor 1C with very low K15 expression levels. Clone J was also a selected clone from

Tumor 1C that was transfected by a K15 promoter regulated GFP construct. This cell line expresses high K15 levels when cultured with J2 conditioned media.

Finally, the HCT116 cell lines were used for human colon tumor lines. This line was derived from a colon adenocarcinoma from colon epithelial cells. The HCT116 -/- cell line has an isogenic deletion of the p53 loci using retrovile insertion. HCT116 +/+ is the p53 wild type.

Culture Splitting

All cell lines were split using the same procedures, in either 10cm or 6-well Cronin dishes. The cells were washed with PBS, and 1-2mL of Trypsin 1x with Versene was used to dislodge the adhered cells from the bottom of the plate. Finally, the cells were spun at 200G (1000 RPM) for 6 minutes, then re-plated at the desired dilution either on the same plate or a new plate of the correct size.

Trypsin Stock

10x stock Trypsin was diluted 1:5 with Hanks Balance Salt Solution to give 2x Trypsin. Stock Versene was mixed 1:1 with the Trypsin to give the final working solution of Trypsin 1x with Versene.

Freezing Cells

To freeze cells that were no longer needed in active culture, the split procedure (above) was used until the spin down step. After spinning, cells were re-suspended in 1mL media. Freezing Media (20% DMSO and 80% FBS) was used 1:1 with the cell suspension (1mL freezing media, 1mL cell suspension), and the mixture was placed in a 2mL vial. The vial was placed in a freezing box in the -90°C freezer.

Cell Thawing

When not in culture, cells were stored in a -90°C freezer. To thaw, cells were placed in a 37°C water bath for several seconds until fully thawed. 10-15mL of cell media was placed in a 15mL conical tube while cells were thawing. Newly thawed cells were gently added to the cell media 50µL at a time, until the vial was fully transferred. The new cell suspension was spun at 200G for 6 minutes, then plated on either a 10cm or a 6-well Cronin plate.

Culture Media

The cell lines for each tissue used different media (summarized in **Table I**) for optimal growing conditions. The procedures for making each media are explained below. All media was stored in a light-blocking box in a 4°C refrigerator, and thawed in a 37°C water bath before use.

Breast Cell Line (MDA231)

The MDA231 cell line was cultured in stock DMEM (Dulbecco's Modified Eagle Medium) with 1% Penn/Strep and 10% FBS.

Prostate Cell Lines (PC3 & NPrEC)

The prostate cell lines were cultured using Serum Free Media (SFM). A stock 500mL Defined K SFM bottle of media was used along with 1% Penn/Strep and 1 vial of supplement (Keratinocyte-SFM).

Sebaceous Cell Lines (Tumor 1C and SEBE6E7)

The Sebaceous cell lines were cultured in Kupffer cell medium + EGF Media with J2 Cell byproducts. 500 µL EGF was added to the stock 500mL bottle of KCM along with 1% Penn/Strep and 10% Fetal Bovine Serum (FBS) and J2 cell byproducts.

Colon Cell Lines (HCT116 +/+ and HCT116 -/-)

Both colon cell lines were cultured in DMEM with 1% Penn/Strep and 10% FBS.

Table 1: Summary of Culture Media Used for Each Cell Line.

Cell Line	Media Used	Additives
MDA231	DMEM	1% Penn/Strep, 10% FBS
PC3, NPrEC	Defined K SFM	1% Penn/Strep
Tumor 1C, SEBE6E7, Clone A, Clone J	KCM J2 Conditioned Media	EGF, 1% Penn/Strep, 10% FBS, J2 Cell
HCT116 +/+, HCT116 -/-	DMEM	1% Penn/Strep, 10% FBS

Treatment with Doxorubicin

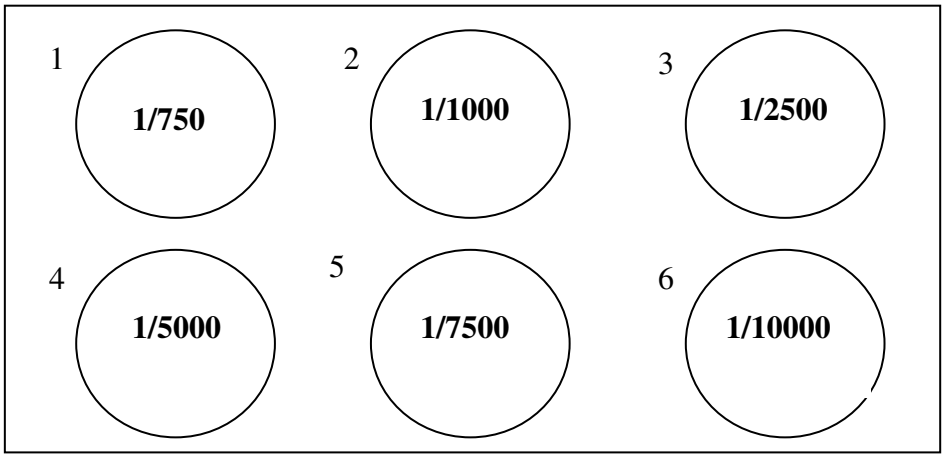
10 mg of Doxorubicin HCl was ordered from Sigma-Aldrich (catalog number D1515). The powder was solublized in DMSO to form a 10 mM stock solution and divided into 70 µL aliquots stored at -20^{o}C in a light-blocking box. The working concentration of Doxorubicin, used for the kill curves and for treatment, was a 1:10 dilution with PBS of the 10 mM stock solution.

All cell lines in this experiment were treated with Doxorubicin and controlled with PBS at specific doses. A "kill curve" was used for each cell line to determine which dose of Doxorubicin would be effective in eliminating 95% of the cell's population. The determination used a visual qualitative estimation of the percentage of cells left.
Once the appropriate dosage was determined, the cells were plated in a 6-well dish with the top 3 wells designated for the treatment with Doxorubicin, and the bottom 3 for the control with PBS. 2-3 glass cover slips were placed in 4 of the 6 wells. One well for both the treatment and the control were designated for the viability count, therefore were free of cover slips.

The cells were treated every 2-3 days until 95% of the treated cells were eliminated. An immunofluorescent (IF) stain was completed on both the treatment and the control cover slips, with antibodies (Ab) specific for each of the stem cell markers.

Kill Curves

As previously mentioned, a kill curve was used to determine the correct dosage of Doxorubicin needed to eliminate 95% of the cell's population. One stock solution of a 1:10 Doxorubicin:PBS mixture was created, then varying amounts of that dilution were used to establish the kill curve. There were 6 established concentrations used in the kill curve: 1/750, 1/1,000, 1/2,500, 1/5,000, 1/7,500, and 1/10,000. The wells were set up with the concentrations as follows:



In order to generate the concentrations in each well, varying amounts of the 1:10 diluted Doxorubin were added to each well (**Table 2**). An example calculation of how to determine the amount is shown below with the 1/750 used. 1/750 = 1/750 of the stock

10 mM Doxorubicin; Diluted 1:10 with PBS = [(1/750)/10] = 1/75; 2500 µL total volume of well, therefore: 2500/75 = 33.3µL 1:10 Doxorubicin.

Table 2: Kill Curve Concentrations.
These values were used in determining optimal concentration of 1:10 stock to use for treatements.

Well #	Concentration	1:10 Doxorubicin or PBS added (µL)
1	1/750	33
2	1/1000	25
3	1/2500	10
4	1/5000	5
5	1/7500	3
6	1/10,000	2.5

Each cell line was plated on a 6-well plate until 80% confluent. Once confluent, the cells were treated with 1:10 Doxorubicin in the specified concentrations above. Once one of the wells achieved 95% elimination of cells, that concentration was indicated as the optimum concentration for that cell line, shown in Table 3.

Table 3: Concentrations of 1:10 Doxorubicin Chosen From Kill Curves to be Used for Cell Line Treatment.

Cell Line	Concentration	Amount 1:10 Doxorubicin (µL)
MDA231	1/1000	25
PC3	1/750	33
NPrEC	1/5000	5
SEBE6E7	Between 1/750 and 1/1000	30
Tumor 1C	1/10,000	2.5
Clone A	1/10,000	2.5
Clone J	1/10,000	2.5
HCT116 +/+	1/750	33
HCT116 -/-	Between 1/1000 and 1/2500	14

Doxorubicin Treatments

Each cell line was treated with Doxorubicin until 95% of the cell population was eliminated. **Table 4** shows the amount of time each cell line was treated for. Additional

1:10 Doxorubicin was added to the treatment wells approximately every 2 days during the course of treatment.

Table 4: Treatment Duration for Each Cell Line.

Cell Line	Number of Days of Treatment	Amount 1:10 Doxorubicin Added (µL)
MDA231	5	25
NPrEC	7	5
PC3	7	33
SEBE6E7	3	30
Tumor 1C	4	2.5
Clone A	3	2.5
Clone J	3	2.5
HCT116 +/+	6	33
HCT116 -/-	3	14

For two of the cell lines (HCT116 -/- and SEBE6E7) an amount of 1:10 Doxorubicin was used that was in between the tested concentrations. This was determined because the upper and lower concentrations were too effective or too ineffective, respectively; therefore, an in between amount was used.

In regards to the number of days each cell line was treated, they were retreated with the same amount of both Doxorubicin and PBS every 2 days during the week, and not during the week. The schedule was planned in a way that treatments mostly began on Friday, and were either fixed or retreated on Monday, and every 2 days after that until they were ready to be fixed.

Fixing Coverslips

In order to fix cells to their coverslips after experimentation, all of the media was vacuumed off and the slips rinsed once with PBS. The glass coverslips were then placed in a glass dish for fixing with either acetone or methanol. All glass coverslips were fixed with acetone for 30 seconds, and plastic slips with methanol for 5 minutes. When fixing time was up the fixing chemical was removed, and the slips rinsed again with PBS.

Fixed coverslips were stored in a six well plate with the edges sealed with parafilm in the 2-8^{o} C refrigerator.

Immunofluorescence

Staining

Immunofluorescence staining was performed so that treated cells could be examined for the presence of stem cell markers using fluorescence microscopy. In preparation for staining, all coverslips were rinsed once with PBST (0.1% Triton-X 100) and then blocked with a 5% goat serum diluted in PBST for one hour. The slips were then washed once more with PBST before application of primary antibody. Slips were incubated with primary antibody either for 2 hours at room temperature, or overnight in the 4^{o} C refrigerator. After incubation with primary antibody, the slips were washed 4-5 times with PBS for 3 minutes per wash. Slips were then incubated with secondary antibody for 1-2 hours in the dark at room temperature, and rinsed again 4-5 times with PBS. The coverslips were finally blotted dry and carefully mounted with Vectashield containing DAPI stain, the edges of the slide sealed, and stored in a light blocking paper slide folder in the 2-8^{o} C refrigerator. **Table 5** shows the different stains performed for each antibody and the dilutions used for the primary and secondary antibodies.

Table 5: Immunofluorescent Stains Used for Each Cell Line Including Antibody Dilutions

Cell Line	Primary Antibody	Primary Antibody Dilution	Secondary Antibody	Secondary Antibody Dilution
MDA231	Mouse CD44	1:100	Mouse Rhodamine	1:100
	Rabbit MKLP-1	1:1000	Rabbit FITC	1:100
PC3 and NPrEC (CD133/MKLP-1)	Rabbit CD133	1:25	Rabbit Texas Red	1:300
	Goat RACGAP1	1:1000	Goat FITC	1:100
PC3 and NPrEC (K15/MKLP-1)	Mouse K15	1:100	Mouse Rhodamine	1:100
	Rabbit MKLP-1	1:500	Rabbit FITC	1:100
PC3 and NPrEC (CD44/MKLP-1)	Mouse CD44	1:100	Mouse Rhodamine	1:100
	Rabbit MKLP-1	1:1000	Rabbit FITC	1:100
SebE6E7, Tumor 1C, Clone A, Clone J	Mouse K15	1:100	Mouse Rhodamine	1:100
	Rabbit FITC	1:500	Rabbit FITC	1:100
HCT116+/+, HCT116-/-	Rabbit CD133	1:25	Rabbit Texas Red	1:300
	Goat RACGAP1	1:1000	Goat FITC	1:100

Microscopy

Once staining was complete, the stem cell marker staining for each cell line was examined using the fluorescence microscope. For each slide, photos were taken of at least six randomized fields of view on the slide to get a representative depiction of the slide's cell population. Magnification was used at either 20x or 40x, depending the size of the cells in the field (for ease of counting). The photos of each cell line and treatment

type were then merged using Adobe Photoshop to create a composite image of the DAPI stain and the red or green stains performed.

Statistical Analysis

Counting Cells

After the images were merged in Photoshop, the positive staining of each cell type was quantified. First the total number of cells for each slide was counted by looking at the DAPI nuclear staining in each image. Then, the number of cells staining positive for the variety of stem cell markers were counted. For the MKLP-1 stain, both the number of cells staining positive for MKLP-1 with one midbody, and the number of cells with more than one midbody were counted.

Statistics

A standard t-test using a 5% significance level and a one-tailed P-value was used for statistical analysis in these experiments. Microsoft Excel was used to analyze all of the data collected from counting. Percentages of positive staining out of the total number of cells for each field photo were calculated for each cell line. The resulting percentages were then used to perform the t-test for the cells treated with Doxorubicin and the control cells for each cell line. Statistical analysis was done in two ways; first, the difference in stem cell marker levels in treated and untreated cells of a cell line were analyzed and second, the difference between stem cell marker levels in normal tissue were compared to tumoric tissue.

RESULTS

As mentioned above, the purpose of this MQP was to determine whether there is a population of cancer stem cells leftover following chemotherapy treatment that could be responsible for the genesis of a new tumor. Immunofluorescence was used to look for known stem cell markers in the different cell lines tested. Cells treated with Dox and untreated control cells were fluorescently stained and counted. Statistical analysis was done to examine the differences in staining for stem cell markers between Dox treated and untreated cells, as well as between normal and tumor tissue.

Immunofluorescence

In this MQP, immunofluorescence was used to quantify the amount of positive CSC markers present with or without treatment with Doxorubicin in each of the cell lines. The data shown below supports the hypothesis that there was an increased number of CSC markers present in cells after treatment with Doxorubicin. All quantitative data can be found in the Appendix.

The data below are grouped by cell line – MDA231, PC3, NPrEC, SEBE6E7, Tumor 1C, Clone J, and HCT116-/-. Each cell line used one or more of the following stem cell markers: K15, MKLP-1, CD44, or CD133. Each stain for each line was photographed separately at 20x magnification then merged to show all stains together. Photos of each cell line were taken in six randomly selected fields in order to capture the appropriate collection of stained cells.

To quantify the number of cells that tested positive for CSC markers, the cells in each photo were counted (using Adobe Photoshop) for evidence to support or not support the hypothesis. Tables were generated to show the quantitative data collected.

MDA231 cells were treated with Doxorubicin and PBS (as a control) and the IF was performed using the appropriate antibodies for MKLP-1 and CD44, known stem cell markers (**Figure 3**). Of the cells that were photographed, 18% of the treated cells and about 3% of untreated cells stained positive for MKLP-1 (one midbody). None of the MDA231 cells stained positive for more than on midbody. 95% of the treated cells and 4.4% untreated cells stained positive for CD44.

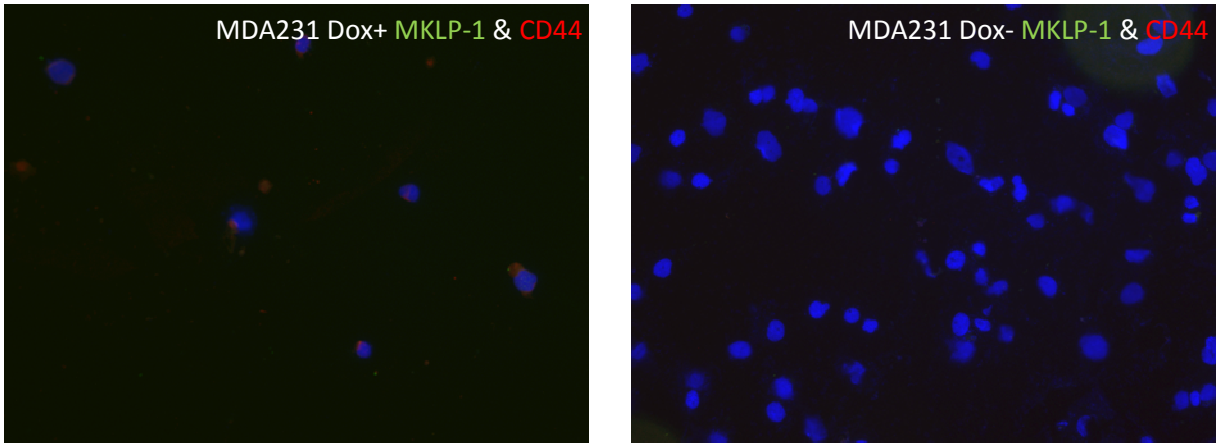


Figure 3: Photos of MDA231 Cells. Cells were treated (left panel) or untreated (right panel) with doxorubicin and stained for stem cell markers MKLP-1 and CD44. Shown at 20x.

Six random fields were photographed for PC3 co-stained with either MKLP-1 & CD44 (**Figure-4**) as well as MKLP-1 & K15 (**Figure-5**). Of the cells photographed with the MKLP-1 & CD44 co-stain, 16% of the treated cells and 2.7% of the untreated cells stained positive for CD44. For one midbody, 2.6% of the treated cells and 1.8% of the untreated cells stained positive. Only 0.3% of the treated cells and about 0.05% of the untreated cells stained positive for greater than on midbody.

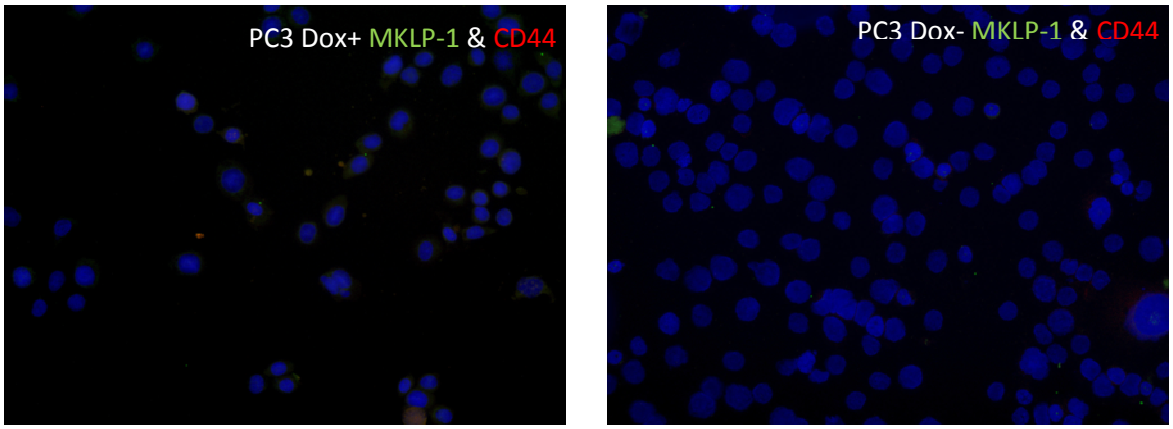


Figure 4: Photos of PC3 Cells. Cells were either treated (left panel) or untreated (right panel) with Dox, then stained for MKLP-1 & CD44. Shown at 20x.

For the MKLP-1 & K15 co-stain, close to 12% of the treated cells and about 2.8% of the untreated cells stained positive for K15. For the midbody staining, 2% of the treated cells stained positive for one midbody, and none stained positive for more than one midbody. For the untreated cells, however, about 4% were positive for one midbody, and 0.2% for more than one midbody.

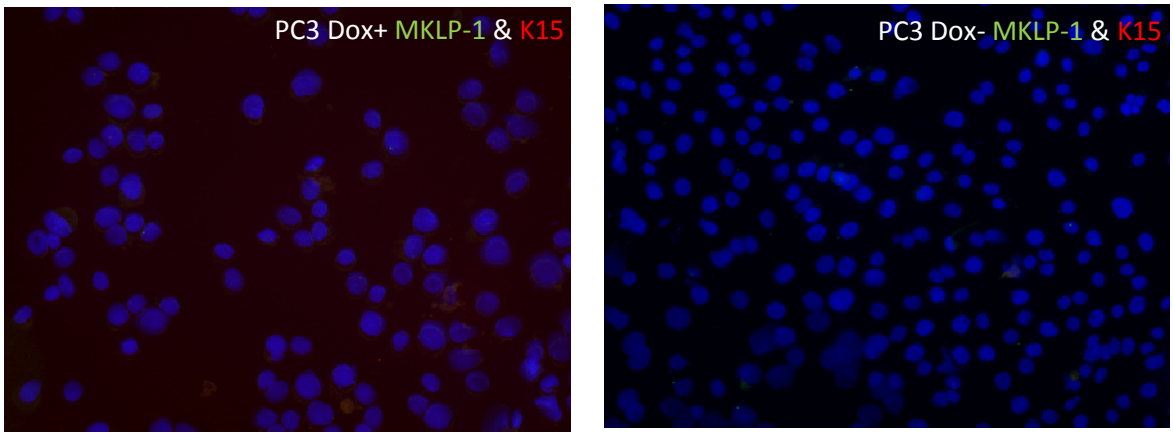


Figure 5: Photos of PC3 Cells. Cells were either treated (left panel) or untreated (right panel) with Dox, then stained for MKLP-1 & K15. Shown at 20x.

In the NPrEC cell experiment stained for MKLP-1 & K15 (**Figure-6**), about 19.2% of the treated cells stained positive for K15, whereas only 3.8% of the untreated cells were positive for K15. For the midbody staining, the treated cells showed about 6% positive for one midbody and

0.8% for more than one midbody. For the untreated cells, 16% stained positive for one midbody, and 4% for more than one midbody.

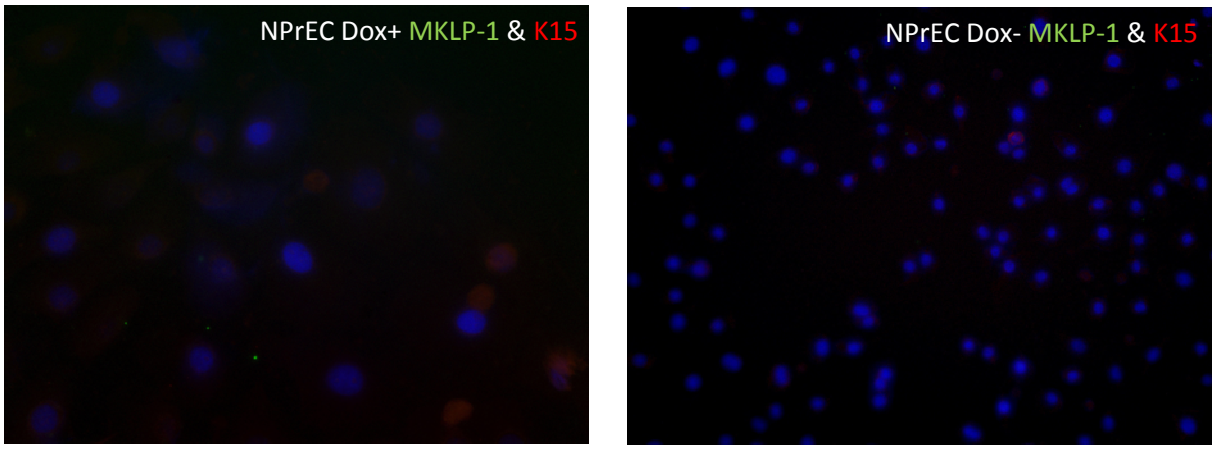


Figure 6: Photos of NPrEC Cells. Cells were treated (left panel) or untreated (right panel) with Dox, then stained for MKLP-1 & K15. Shown at 20X magnification.

For the NPrEC cells stained for MKLP-1 and CD44 (**Figure-7**), about 1.8% of the untreated and about 53% of the treated cells positively stained for CD44. For the MKLP-1 staining, 11.7% of the untreated cells and about 5% of the treated cells showed one midbody. For more than one midbody, 6.3% of the untreated cells, and 0.14% of the treated cells tested positive.

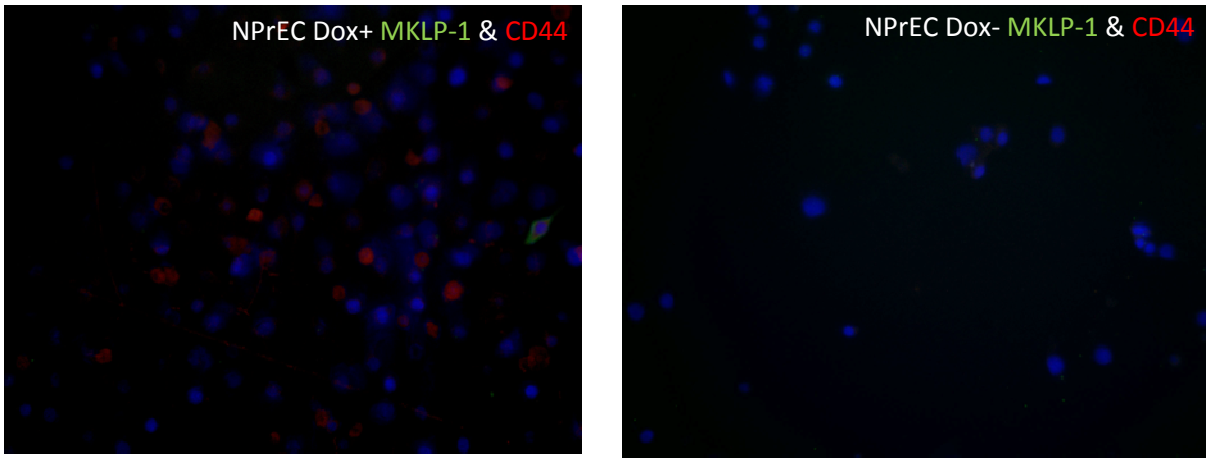


Figure 7: Photos of NPrEC Cells. Cells were treated (left panel) or untreated (right panel) with Dox, then stained for MKLP-1 & CD44. Shown at 20x.

The SEBE6E7 stains (**Figure-8**) were photographed separately (shown below). For the K15 staining (lower row), 98.96% of the treated cells and 13% of the untreated cells stained positive for the CSC marker. The MKLP-1 midbody stain (upper row) results are as follows: 0.76% treated and 3.17% untreated cells positive for one midbody, 1.7% and 0% treated and untreated, respectively, positive for more than one midbody.

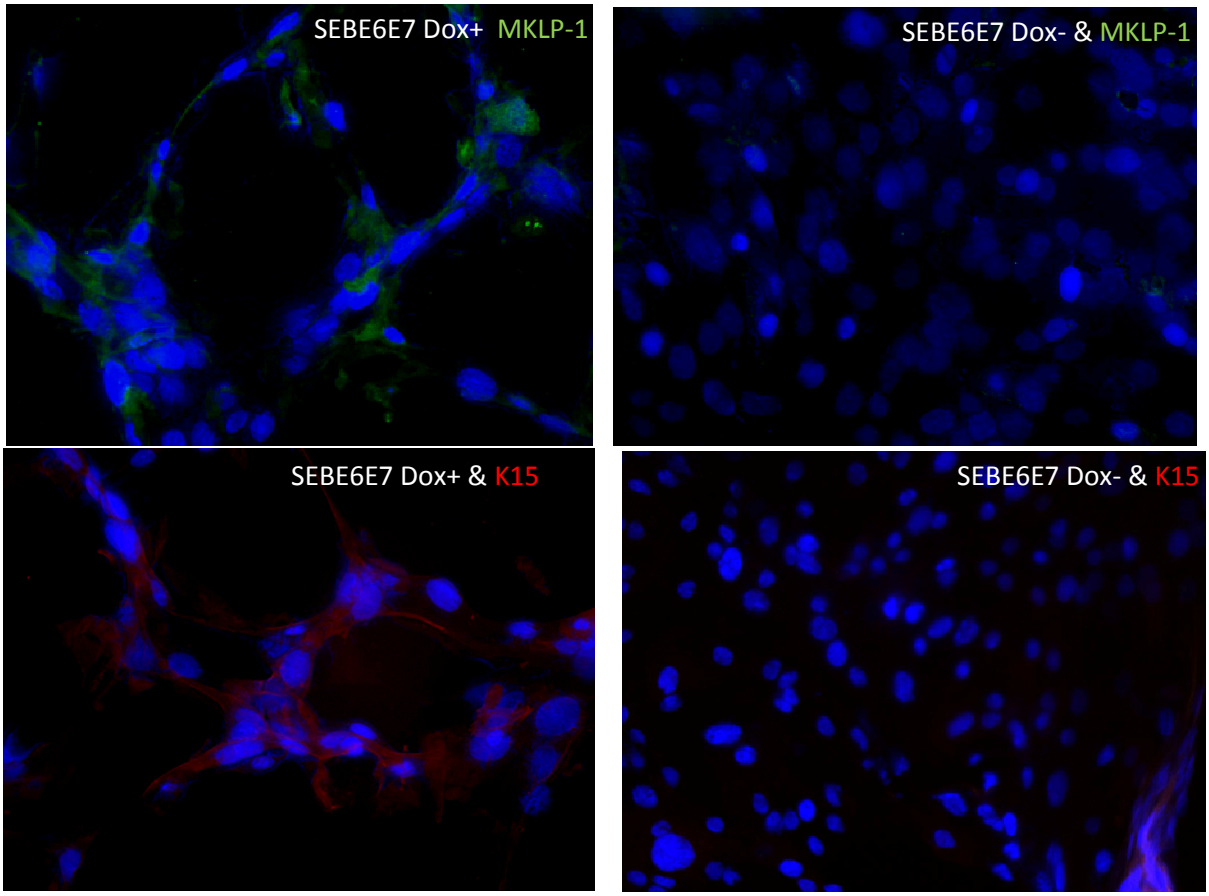


Figure 8: Photos of SEBE6E7 treated and untreated cells with stem cell markers K15 and MKLP-1.

Tumor 1C cells (**Figure 9**) were stained with MKLP-1 & K15. About 1.15% of the untreated cells and 10% of the Dox treated cells stained positive for K15. As for MKLP-1, 10% of the untreated and 15.4% of the Dox treated cells stained positive for one midbody; 1.16% of the untreated and 0.71% of the Dox treated cells stained positive for more than one midbody.

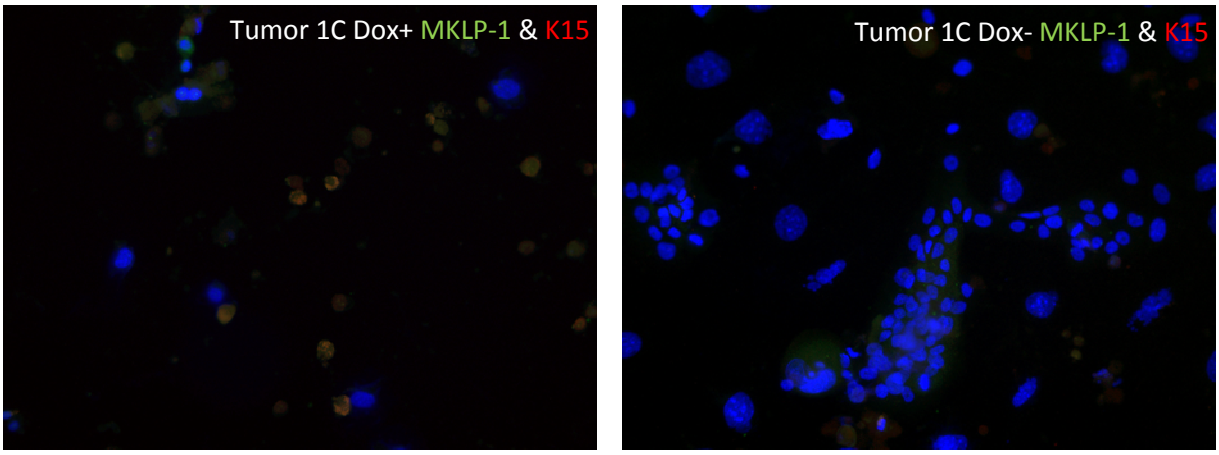


Figure 9: Photos of Tumor 1C Cells. Cells were treated (left panel) or untreated (right panel) with Dox, then stained for MKLP-1 and K15.

No data was acquired for the Clone J cell line (**Figure-10**) due to the aforementioned problems we encountered with the cell line. One photograph of the cell line was taken, however, and it is shown below.

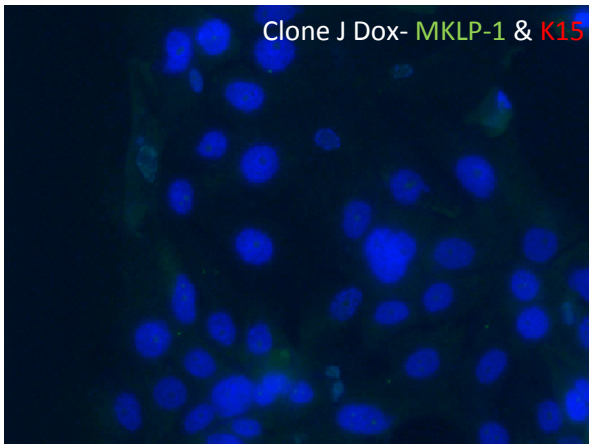


Figure 10: Photo of Clone J Cells. Untreated cells were stained for MKLP-1 and K15. Shown at 20x.

As with the Clone J cell line, no quantitative data was collected for the HCT116-/- cell line (**Figure-11**). The photos taken of the cell line are shown below.

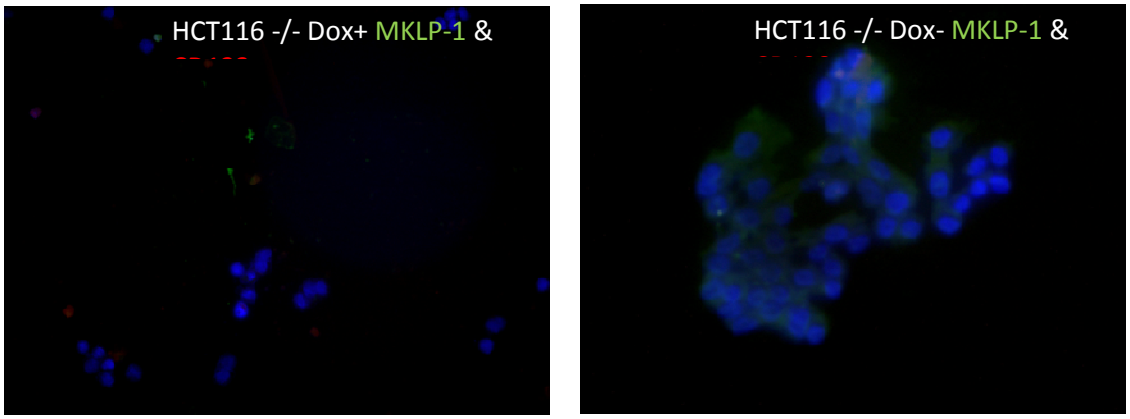


Figure 11: Photos of HCT116 -/- Cells. Treated (left panel) and untreated (right panel) cells were stained for MKLP-1 and CD133. Cells shown at 20x.

Statistical Analysis

In order to analyze the results obtained from the counting of the merged images of each stained slide, a student t-test was used with a 5% significance level. A one tailed P-value was used to determine statistical significance because it was determined that the data was one directional, in that the positive staining for stem cell markers could only increase above zero, and not go below zero.

First, the staining of stem cell markers was compared between the doxorubicin treated and the untreated control cells. For each cell line, it was found that at least one stem cell marker was significantly different between treated and untreated cells of the same type. The results of the t-test for treated vs. untreated cells are shown in **Table 7** below. The mean values of percent positive staining for each stem cell marker in each cell line are also shown in **Table 8**.

Cell Line	Stem Cell Marker	P-Values (One Tailed T-Test)	Significance (α=.05)
MDA231	CD44	0.000	Y
	MKLP-1 (1 midbody)	0.018	Y
	MKLP-1 (>1 midbody)	No P-Value	Inconclusive
PC3 (CD44 + MKLP1 costain)	CD44	0.142	N
	MKLP1 (1 midbody)	0.297	N
	MKLP1 (>1 midbody)	0.218	N
PC3 (K15 + MKLP1 costain)	K15	0.042	Y
	MKLP1(1 midbody)	0.091	N
	MKLP1 (>1 midbody)	0.089	N
NPrEC (CD44 + MKLP1 costain)	CD44	0.003	Y
	MKLP1 (1 midbody)	0.030	Y
	MKLP1 (>1 midbody)	0.012	Y
NPrEC (K15 + MKLP1 costain)	K15	0.053	N
	MKLP1(1 midbody)	0.044	Y
	MKLP1 (>1 midbody)	0.060	N
SebE6E7	K15	0.000	Y
	MKLP1 (1 midbody)	0.016	Y
	MKLP1 (>1 midbody)	0.120	N
Tumor 1C	K15	0.005	Y
	MKLP1 (1 midbody)	0.195	N
	MKLP1 (>1 midbody)	0.297	N

Table 7: Statistical Significance for Cell Line Staining. Table shows comparison of Dox treated cells to untreated control cells (Y=significant, N=not significant).

		Mean % Positive	
Cell Line	**Stem Cell Marker**	**Treated**	**Untreated**
MDA231	CD44	95.238	4.445
	MKLP-1 (1 midbody)	18.254	2.930
	MKLP-1 (>1 midbody)	0.000	0.000
PC3 (CD44 + MKLP1 costain)	CD44	16.065	2.693
	MKLP1 (1 midbody)	2.607	1.790
	MKLP1 (>1 midbody)	0.333	0.048
PC3 (K15 + MKLP1 costain)	K15	11.976	2.785
	MKLP1(1 midbody)	2.189	3.910
	MKLP1 (>1 midbody)	0.000	0.203
NPrEC (CD44 + MKLP1 costain)	CD44	1.790	52.873
	MKLP1 (1 midbody)	4.954	11.727
	MKLP1 (>1 midbody)	0.142	6.342
NPrEC (K15 + MKLP1 costain)	K15	19.287	3.800
	MKLP1(1 midbody)	5.904	16.162
	MKLP1 (>1 midbody)	0.794	4.128
SebE6E7	K15	98.965	13.001
	MKLP1 (1 midbody)	0.760	3.170
	MKLP1 (>1 midbody)	1.732	0.000
Tumor 1C	K15	10.004	0.147
	MKLP1 (1 midbody)	15.437	10.027
	MKLP1 (>1 midbody)	0.714	1.166

Table 8: Mean Percentages of Cells Staining Positive for a Particular Stem Cell Marker.

Figures 12 and **13** contain the mean amounts of positive staining for the observed stem cell markers in graphical form. Figure 12 shows percent positive staining for cells treated with Doxorubicin, while Figure 13 shows positive staining of untreated cells. It is clear from the graphs that there was significantly more staining for most of the stem cell markers in almost all cell lines treated with doxorubicin compared to the control cells.

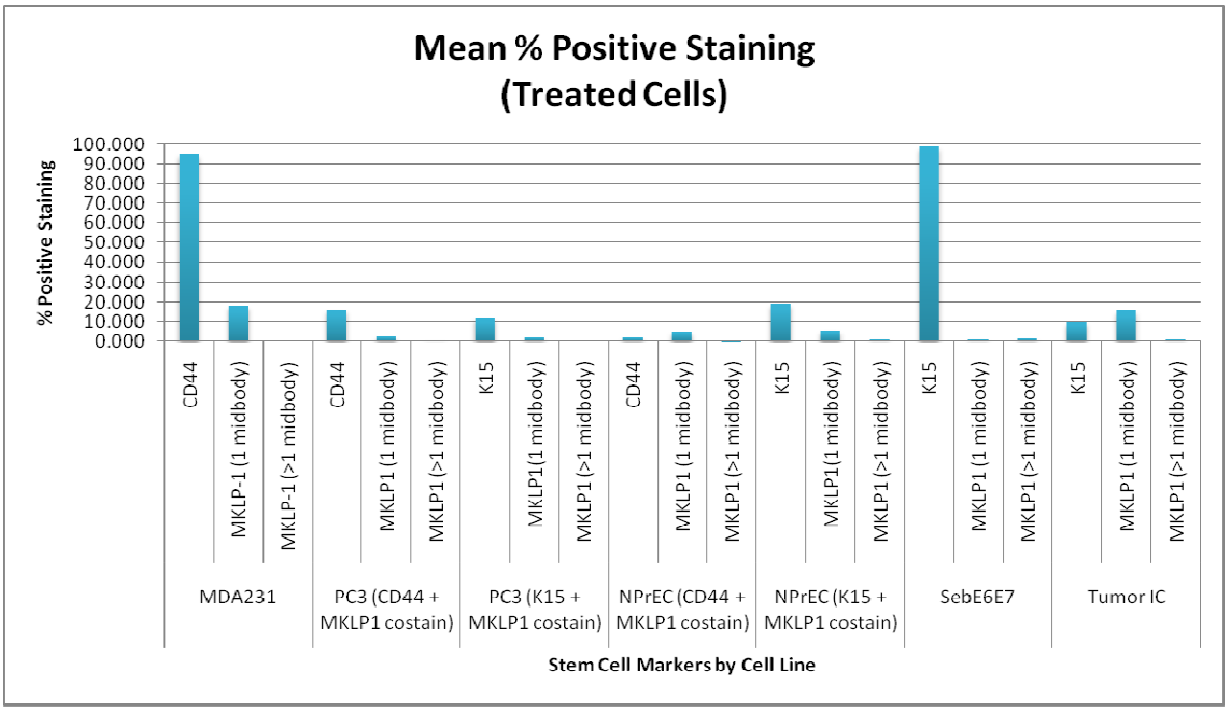


Figure 12: Bar Graph Analysis of the Mean % Positive Staining for Particular Stem Cell Markers in Cell Lines Treated with Doxorubicin.

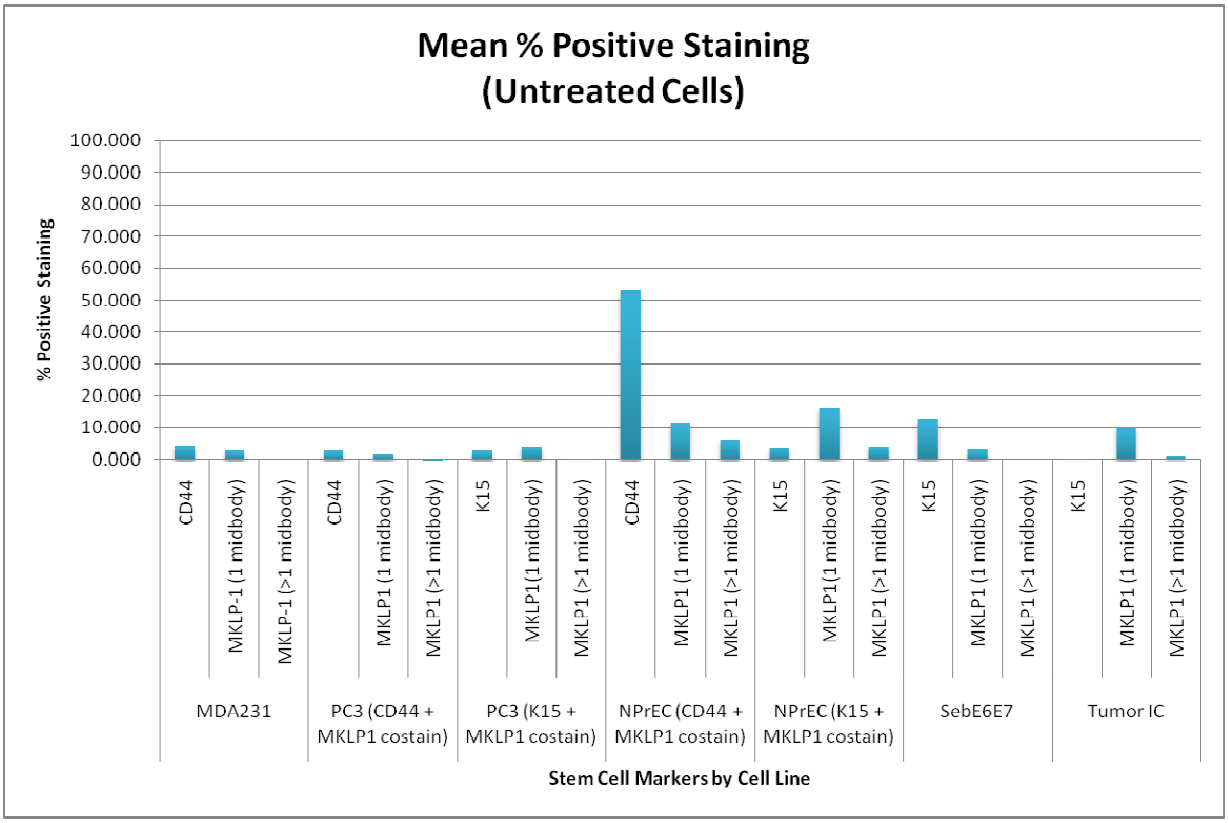


Figure 13: Bar Graph Analysis of the Mean % Positive Staining for Particular Stem Cell Markers in Control Cell Lines (untreated).

After the results of the treated and untreated cells were compared, the difference in stem cell marker staining was compared in tumor versus normal tissue. **Table 9** shows the statistical significance of the normal vs. tumoric tissue comparison for each cell line tested, both treated and untreated. There was a statistically significant difference in stem cell marker staining in normal vs. tumoric tissue in at least one stem cell marker for each tissue type compared, for both Dox treated and control cells.

		P-Values (one tailed T-test)		**Statistical Significance (α=0.05)**	
		Treated	***Untreated***	***Treated***	***Untreated***
NPrEC vs. PC3 (CD44, MKLP1 costain)	CD44	0.019	0.330	Y	N
	MKLP1 (1 midbody)	0.095	0.007	N	Y
	MKLP1 (>1 midbody)	0.307	0.011	N	Y
NPrEC vs. PC3 (K15, MKLP1 costain)	K15	0.220	0.381	N	N
	MKLP1 (1 midbody)	0.023	0.027	Y	Y
	MKLP1 (>1 midbody)	0.178	0.035	N	Y
SebE5E7 vs. Tumor 1C (K15, MKLP1)	K15	0.000	0.029	Y	Y
	MKLP1 (1 midbody)	0.014	0.011	Y	Y
	MKLP1 (>1 midbody)	0.256	0.016	N	Y

Table 9: Statistical Significance in Stem Cell Marker Staining Between Normal Cell Lines and Tumor Cell Lines (Y=significant, N=not significant).

Last, the percentage of cells containing either one or greater than one midbody was analyzed in each of the cell lines tested. The results of this analysis are shown in **Figure 14**. In Dox treated cells, midbodies were present in all cell lines tested, and multiple midbodies in all cell lines except for MDA231 cells. In untreated control cells, there were midbodies present in all cell lines, and greater than one midbody in PC3, NPrEC, and Tumor 1C cells.

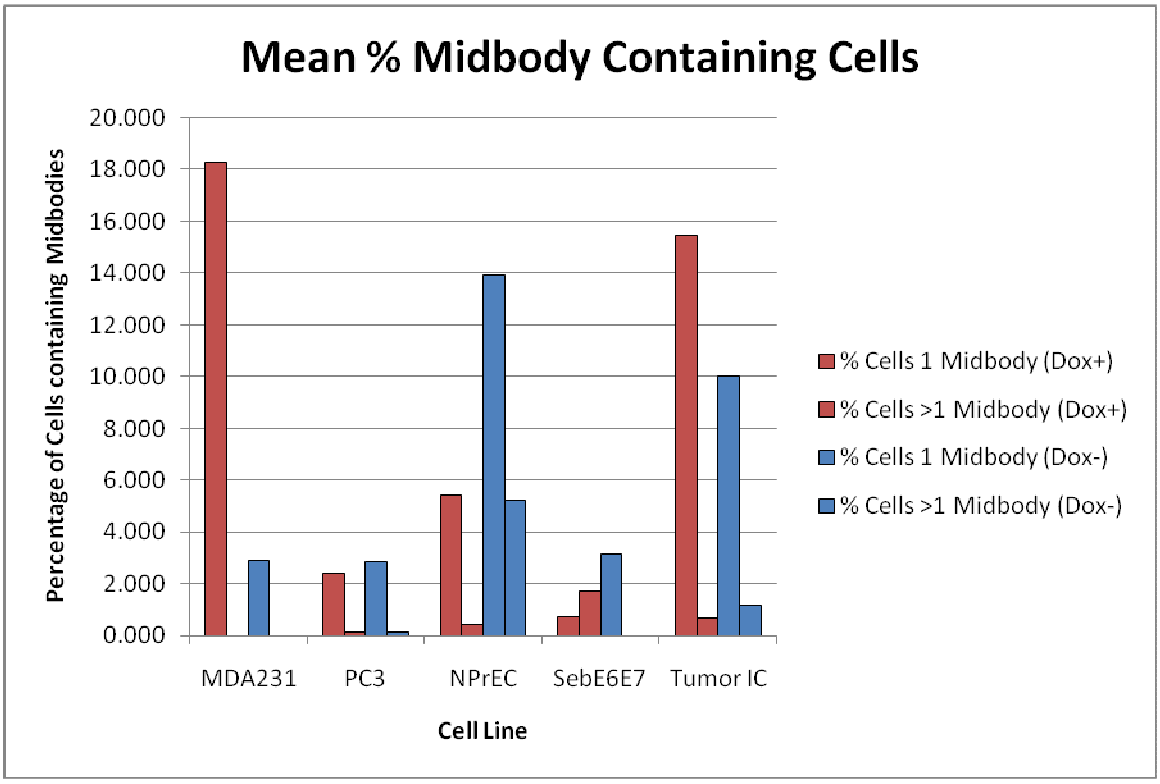


Figure 14: The Mean Percentage of Cells Containing One Midbody and Greater Than 1 Midbody for Dox+ and Dox- Cells.

DISCUSSION

Main Conclusions

The data collected from this project support the hypothesis that cancer stem cells remain in tumor cell populations after Dox chemotherapy. In the comparison between Dox treated and untreated control cells, it was shown that there was a higher mean percentage of staining in Dox treated cell lines for cell lines MDA231, PC3, NPrEC, SebE6E7, and Tumor 1C, for at least one stem cell marker tested. There was significantly different staining between Dox-treated and untreated cells for CD44 and MKLP-1 in MDA231, K15 in PC3, CD44 and MKLP-1 in NPrEC, K15 and MKLP-1 in SebE6E7, and K15 in Tumor 1C. This information supports the hypothesis that a population of stem cells remain after Dox treatment in the cell lines tested.

The presence of stem cell markers was also compared between normal tissue and tumor tissue of the same type. This analysis shows a significant difference in staining between normal and tumoric cells in all Dox-treated cell lines for at least one stem cell marker. When comparing tumoric PC3 cells to normal NPrEC cells, there was a significant difference in CD44 and MKLP-1 staining. For normal SebE6E7 and tumoric Tumor 1C cells, there was a significant difference in K15 and MKLP-1 staining. In the comparison of normal vs. tumor cell lines however, statistical significance was achieved more often in untreated than in Dox treated experiments.

MKLP-1 staining for midbodies was also examined among the cell lines tested. The percentage of cells in each experiment with one midbody and the percentage with greater than one midbody were analyzed. The results show that there is a higher

percentage of staining for one midbody in Dox treated than in untreated cells in MDA231, SebE6E7, and Tumor 1C. However, there was a decrease in midbody staining in untreated cells for the PC3 and NPrEC lines. In examining the percentages of cells containing more than one midbody, treated MDA231 and PC3 lines had more cells with multiple midbodies than untreated of the same type. In NPrEC and Tumor 1C however, there was a larger percentage of cells with multiple midbodies in untreated cells than in Dox treated. In MDA231 there were no cells with more than one midbody.

Significance

Many of the results obtained from these experiments are in accordance with the Cancer Stem Cell theory discussed in the Background section. Dalerba et al (2007) proposed that tumors are comprised of a heterogeneous mixture of cells, which was supported by our experiments with Doxorubicin. The treatment of cell lines with doxorubicin demonstrated that there was a mixture of differentiated cells, which were killed by the Dox chemotherapy drug, and cancer stem cells, which were left behind after chemotherapy and stained positive for the various stem cell markers in the immunofluorescence experiments. Cho, Clarke, and Dalerba also explained several characteristics of CSC populations; one being that tumorigenic CSCs contain surface markers such as cell determinants (CD's), and another that cells transplanted into mice regenerate a new tumor with a mixed population of cells that is the same as the parent tumor. In our experiments, both CD44 and CD133 were identified in CSC populations remaining after chemotherapy treatment.

Current research by Lyle, Doxsey, and Houghton (unpublished) indicates that midbodies may be retained in stem cells, but are degraded in differentiated cells. The data gathered from our immunofluorescence experiments indicates a significant amount of midbodies retained in the stem cell population left over after Dox treatment in several cell lines. Also several cells contained multiple midbodies, further supporting the theory that stem cells retain midbodies after cell division.

Complications with Experimentation

Due to the fact that the MQP takes place over a short period of time, most of the problems encountered during experimentation would have been solved if more time had been available. Unfortunately additional time was not a possibility, so many of the experiments were not able to be perfected. There were several complications that arose during the culturing of cell lines used for experimentation. At some times the wrong culture media were used, or different formulas of culture media needed to be created for the cells to be able to grow better. Cells sometimes failed to grow for unknown reasons. In several instances, we were not aware of the passage limit for some cell lines, and we attempted to passage them beyond their capacity. When plating cells with coverslips for treatment, plastic coverslips were mistakenly sometimes used in place of glass coverslips, which resulted in ruining the slips after fixing with acetone. In addition, drug administration sometimes did not go smoothly, in that it took longer than expected for cells to die after Dox was administered, or the wrong concentration of drug was used for the experiment.

There were also several problems that arose during immunofluorescent staining. In several instances, the wrong antibody was used to stain cells, such as using the same species of antibody to stain two different markers, so that they would not be distinguishable from one another. At times there was also overexposure of cells to dye, or the wrong concentration of antibody stain was used. In some cases, the reason that a stain failed to work was unknown. This resulted in the necessity of repeating the immunofluorescent dying process several times for some cell lines.

Lastly, there were some complications with fluorescent microscopy. At times there were not enough cells left on the slides after Dox treatment, which caused us to have to repeat some of the treatment experiments. Many of the slides that we looked at were too blurry to be used for counting; this could have been due to a dirty lens, dead cells on the coverslips, cells on both sides of the coverslip, or over-confluence of cells. In addition, it was difficult to judge how many photos should be taken of each slide to obtain a number of cells sufficient for statistical analysis. Therefore, some of the statistical data that was collected may not be sufficiently accurate because of a small sample size.

Future Experiments

Due to the time constraints of the MQP, it was not possible to perform all of the experiments that would have provided useful data for this project. Experimentation on four of the cell lines that were originally intended could not be completed: Clone A, Clone J, HCT116+/+, and HCT116-/- cells. In order to better characterize the CSCs associated with these important tumor cell lines, more chemotherapy and

immunofluorescence experiments should be done. In addition, not all of the cell lines showed data that was statistically significant for the stem cell markers tested. These experiments should be re-done to obtain more significant data.

The experiments in this MQP only tested for a small variety of stem cell markers known to exist in the tissue types that were tested. Future experiments may aim to further characterize CSCs by staining for different stem cell markers that could also be present in these tissue types. In addition, cell lines of other tissue types, such as brain tumor lines, could be tested to gain more information about a broader spectrum of diseases in which CSCs may play a role.

One last experiment that may prove to be interesting would be implantation of cancer stem cells isolated by chemotherapy treatment into mice to determine whether or not the CSCs have the ability to regenerate a tumor in vivo that is similar to the original parent tumor. Other research has demonstrated success in regenerating tumors in mice after implanting cells that remained after chemotherapy treatment (Levina et al; Gu et al., 2007; Dalerba et al., 2007; Ricci-Vitiani et al, Raouf et al., 2005; and Kang & Kang).

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APPENDIX A: Table of Cell Counts

Cell Line	Merge #	Total # Cells	# Cells Pos K15 or CD44	% Pos K15 or CD44	# Cells Pos MKLP-1 (1 midbody)	% Pos MKLP-1 (1 midbody)	# Cells Pos MKLP-1 (>1 Midbody)	% Pos MKLP-1 (>1 midbody)
MDA231 Treated (CD44/MKLP-1)	1	7	7	100	1	14.28571429	0	0
	2	7	5	71.42857143	1	14.28571429	0	0
	3	4	4	100	0	0	0	0
	4	6	6	100	2	33.33333333	0	0
	5	7	7	100	1	14.28571429	0	0
	6	6	6	100	2	33.33333333	0	0
	Total Cells	37	Mean % Pos	95.23809524	Mean % Pos	18.25396825	Mean % Pos	0
MDA231 Untreated (CD44/MKLP-1)	1	40	2	5	0	0	0	0
	2	75	2	2.666666667	0	0	0	0
	3	147	4	2.721088435	6	4.081632653	0	0
	4	89	5	5.617977528	3	3.370786517	0	0
	5	67	0	0	5	7.462686567	0	0
	6	75	8	10.66666667	2	2.666666667	0	0
	Total Cells	493	Mean % Pos	17.41578493	Mean % Pos	5.119391523	Mean % Pos	0
PC3 Treated (K15/MKLP-1)	1	59	4	6.779661017	1	1.694915254	0	0
	2	83	1	1.204819277	0	0	0	0
	3	89	28	31.46067416	4	4.494382022	0	0
	4	62	4	6.451612903	1	1.612903226	0	0
	5	101	11	10.89108911	4	3.96039604	0	0
	6	73	11	15.06849315	1	1.369863014	0	0
	Total Cells	467	Mean % Pos	12.75316208	Mean % Pos	2.607407297	Mean % Pos	0
PC3 Untreated	1	184	13	7.065217391	9	4.891304348	1	0.543478261

(K15/MKLP-1)	2	88	1	1.136363636	0	0	0	0
	3	287	4	1.393728223	18	6.271777003	0	0
	4	148	6	4.054054054	9	6.081081081	1	0.675675676
	5	215	1	0.465116279	7	3.255813953	0	0
	6	270	7	2.592592593	8	2.962962963	0	0
	Total Cells	1192	Mean % Pos	4.208604894	Mean % Pos	3.724335235	Mean % Pos	0.174164848
PC3 Treated (CD44/MKLP-1)	1	50	35	70	3	6	1	2
	2	66	4	6.060606061	0	0	0	0
	3	48	7	14.58333333	3	6.25	0	0
	4	74	3	4.054054054	0	0	0	0
	5	59	1	1.694915254	2	3.389830508	0	0
	6	63	0	0	0	0	0	0
	Total Cells	360	Mean % Pos	14.3716448	Mean % Pos	2.766309392	Mean % Pos	0.310594978
PC3 Untreated (CD44/MKLP-1)	1	91	0	0	0	0	0	0
	2	99	4	4.04040404	3	3.03030303	0	0
	3	199	1	0.502512563	0	0	0	0
	4	170	0	0	10	5.882352941	0	0
	5	192	25	13.02083333	0	0	0	0
	6	155	2	1.290322581	3	1.935483871	0	0
	7	297	0	0	5	1.683501684	1	0.336700337
	Total Cells	1203	Mean % Pos	2.693438931	Mean % Pos	1.790234504	Mean % Pos	0.048100048
NPrEC Treated (K15/MKLP-1)	1	19	2	10.52631579	2	10.52631579	0	0
	2	18	11	61.11111111	2	11.11111111	1	5.555555556
	3	22	7	31.81818182	1	4.545454545	0	0
	4	12	0	0	0	0	0	0
	5	25	1	4	1	4	0	0

	6	17	2	11.76470588	1	5.882352941	0	0
	7	19	3	15.78947368	1	5.263157895	0	0
	Total Cells	132	Mean % Pos	19.28711261	Mean % Pos	5.90405604	Mean % Pos	0.793650794
NPrEC Untreated (K15/MKLP-1)	1	37	7	18.91891892	8	21.62162162	2	5.405405405
	2	27	0	0	2	7.407407407	0	0
	3	103	2	1.941747573	14	13.59223301	4	3.883495146
	4	103	2	1.941747573	9	8.737864078	2	1.941747573
	5	113	0	0	9	7.96460177	2	1.769911504
	6	85	0	0	32	37.64705882	10	11.76470588
	Total Cells	468	Mean % Pos	6.012789525	Mean % Pos	14.69640611	Mean % Pos	3.651273758
NPrEC Treated (CD44/MKLP-1)	1	69	41	59.42028986	7	10.14492754	0	0
	2	61	38	62.29508197	3	4.918032787	0	0
	3	78	11	14.1025641	3	3.846153846	0	0
	4	65	43	66.15384615	2	3.076923077	0	0
	5	117	34	29.05982906	3	2.564102564	1	0.854700855
	6	58	50	86.20689655	3	5.172413793	0	0
	Total Cells	448	Mean % Pos	46.17875675	Mean % Pos	6.345565673	Mean % Pos	0.643710659
NPrEC Untreated (CD44/MKLP-1)	1	30	1	3.333333333	7	23.33333333	2	6.666666667
	2	27	1	3.703703704	2	7.407407407	2	7.407407407
	3	27	1	3.703703704	1	3.703703704	2	7.407407407
	4	29	0	0	4	13.79310345	0	0
	5	29	0	0	4	13.79310345	4	13.79310345
	6	36	0	0	3	8.333333333	1	2.777777778
	Total Cells	178	Mean % Pos	8.131356784	Mean % Pos	10.95850719	Mean % Pos	5.528010481
SebE6E7 Treated (K15)	1	21	20	95.23809524				
	2	39	39	100				

	3	39	39	100				
	4	34	34	100				
	5	43	43	100				
	6	69	68	98.55072464				
	Total Cells	245	Mean % Pos	85.98859667				
SebE6E7 Untreated (K15)	1	100	3	3				
	2	145	39	26.89655172				
	3	119	38	31.93277311				
	4	79	6	7.594936709				
	5	98	5	5.102040816				
	6	115	4	3.47826087				
	Total Cells	656	Mean % Pos	23.42759427				
SebE6E7 Treated (MKLP-1)	1	54		95.23809524	0	0	0	0
	2	30		4.445399883	1	0.333333333	0	0
	3	38		11.97605827	8	2.105263158	3	7.894736842
	4	8		2.693438931	0	0	0	0
	5	40			5	1.25	1	2.5
	6	46			4	0.869565217	0	0
	Total Cells	216	Mean % Pos	27.55611732	Mean % Pos	0.759693618	Mean % Pos	1.73245614
SebE6E7 Untreated (MKLP-1)	1	22			1	4.545454545	0	0
	2	58			0	0	0	0
	3	106			6	5.660377358	0	0
	4	96			4	4.166666667	0	0
	5	96			2	2.083333333	0	0
	6	78			2	2.564102564	0	0
	Total Cells	456	Mean % Pos	27.55611732	Mean % Pos	2.825661155	Mean % Pos	0.247493734

Tumor 1C Treated (K15/MKLP-1)	1	7	0	0	1	14.28571429	0	0
	2	5	1	0	3	60	0	0
	3	14	0	0	2	14.28571429	1	7.142857143
	4	11	2	0	2	18.18181818	0	0
	5	8	2	0	0	0	0	0
	6	8	1	0	2	25	0	0
	7	7	0	0	0	0	0	0
	8	12	2	0	1	8.333333333	0	0
	9	7	0	0	1	14.28571429	0	0
	10	13	1	0	0	0	0	0
	Total Cells	92	Mean % Pos	0	Mean % Pos	15.43722944	Mean % Pos	0.714285714
Tumor 1C Untreated (K15/MKLP-1)	1	31	0	0	0	0	0	0
	2	214	0	0	20	9.345794393	1	0.46728972
	3	97	1	1.030927835	9	9.278350515	3	3.092783505
	4	105	0	0	11	10.47619048	1	0.952380952
	5	177	0	0	16	9.039548023	4	2.259887006
	6	175	0	0	36	20.57142857	1	0.571428571
	7	122	0	0	14	11.47540984	1	0.819672131
	Total Cells	921	Mean % Pos	0.147275405	Mean % Pos	10.02667454	Mean % Pos	1.166205984