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TESTING A LABEL-RETAINING FUNCTIONAL ASSAY FOR DETECTING CANCER STEM CELLS

A Major Qualifying Project Report

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ABSTRACT

Cancer stem cells (CSCs) are a newly hypothesized subpopulation of cancer cells that may help explain many cases of cancer resurgence. CSCs possess the ability to self-renew, similar to normal human stem cells (nHSCs), and may possess other nHSC traits, including a slow-cycling, quiescent nature, and resistance to chemotherapy drugs. An assay to select for CSCs would allow tailored research on these cells, but there is no known genetic marker for CSCs at this time. Because of this slow-cycling nature, a pulse-chase assay using CFSE fluorescent label may provide a functional method to select for CSCs, as these cells would retain the label longer than non-CSCs. My project focused on this assay, pulsing cells from the HCT116 human colorectal cancer line with CFSE before a seven-day chase period. These samples were then treated with one of several chemotherapy treatments for three days and analyzed by fluorescence-assisted cytometric sorting (FACS) to determine whether the population of label-retaining cells (LRCs) was enriched by the chemotherapy. A separate sample of HCT116 cells was examined using immunofluorescence to test for possible cancer stem cell markers. Statistical analysis of the data indicated that a label-retaining pulse-chase assay could indeed select for cancer stem cells. None of the markers tested, however, seem to possess potential for use as an HCT116 CSC marker.

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BACKGROUND

Cancer Stem Cells – General Theory

Despite advances in the treatment of cancer, such as chemotherapy and radiation therapy, resurgence remains a significant problem, one whose causes are often unknown. The recent cancer stem cell model provides a possible explanation. According to this model, cancerous growths contain a sub-population of cells that possess a stem-like ability for self-renewal and tumor maintenance, and are responsible for initiating the growths. A cancer stem cell (CSC) is defined as a cell from a cancerous tumor capable of self-renewal and production of the various cell lineages that constitute such a tumor (Clarke *et al.*, 2006).

Most chemotherapy treatments are equally toxic to both quickly-dividing cancer cells and "normal" differentiated cells. Normal human stem cells (nHSCs), however, are resistant to chemotherapy treatments, and, through proliferation, replace the differentiated cells that were lost (N. Moore, personal communication). Because of their similarity to nHSCs, these theorized cancer stem cells would also be resistant to chemotherapy treatments, increasing the potential for tumor resurgence.

The cancer stem cell theory originated with the observation that both normal tissues and tumors are composed of a heterogeneous mixture of cell types. The conventional view was that these variations were due to genetic and epigenetic changes triggered by micro-environmental influence and genomic instability. It was recently proposed, however, that these heterogeneous collections of malignant cells might indicate the continued execution of dysfunctional differentiation mechanisms, resulting in a pyramidal hierarchy of development, originating from a small population of CSCs, similar to the standard developmental hierarchy in normal tissue

(Clarke *et al.*, 2006). Recent gain-of-function experiments with mice support this theory: several fusion proteins that induce leukemia were discovered to also be capable of bestowing self-renewal abilities on myeloid progenitor cells. Other data from the tests indicate that leukemia stem cells—and, by association, CSCs in general—may, through proliferation, produce two populations: one of cells capable, and one of cells incapable, of self-renewal (Lobo *et al.*, 2007).

Possible Mechanisms of Cancer Stem Cell Genesis

There are currently two proposed mechanisms by which cancer stem cells (CSCs) may arise. A normal human stem cell may mutate in such a way as to allow uncontrolled proliferation (**Figure 1**), or a progenitor cell may, through mutation, gain the ability to self-renew (Guo *et al.*, 2006; Lobo *et al.*, 2007). Recent experiments indicate that—at least in leukemia—both of these processes may occur in humans (Fialkow, 1990: reviewed in Lobo *et al.*, 2007). Dr. Stephen Lyle, of the University of Massachusetts Medical School in Worcester, MA, has performed extensive research on CSCs and possible treatments.

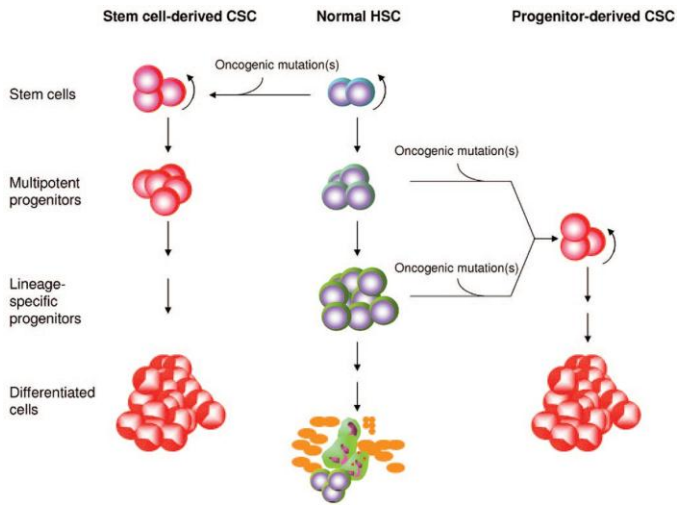


Figure 1: Diagram comparing the differentiation of nHSCs and the development of CSCs (Guo *et al.*, 2006).

Recent Lyle Lab Research

Much of Dr. Lyle's recent research has dealt with cancer and/or stem cells. In 1998, he performed a study in collaboration with Drs. Christofidou-Solomidou, Liu, Elder, Albelda, and Cotsarelis indicating that keratin 15 (K15) is a stem cell marker. Samples of human scalp skin were immunostained with one of several mouse monoclonal antibodies, including C8/144B, DK25, and 4B11 (Lyle *et al.*, 1998). The results of this test indicated that C8/144B preferentially immunostains keratinocytes in the bulge area of the hair follicle while leaving the rest of the follicle unstained. As the bulge area (**Figure 2**) was known to contain epithelial stem cells (Cotsarelis *et al.*, 1990: reviewed in Lyle *et al.*, 1998), these data suggest that the C8/144B immunoglobulin may preferentially immunostain epithelial stem cells (Lyle *et al.*, 1998). These data were further investigated by taking samples of whole, mounted hair follicles and double-labeling the samples with either C8/144B mouse or Ks 19.1 mouse (anti-cytokeratin 19) (C6930 Monoclonal Anti-Cytokeratin Peptide 19 antibody, n.d.) as a primary antibody and a mixture of Texas Red-conjugated anti-mouse and FITC-conjugated anti-β_1 as secondary antibodies. A label retention assay—similar to that performed by Cotsarelis *et al.* (1990)—was then used to identify slow-cycling cells, which were assumed to be stem cells and used to create a cDNA library by expression cloning (Lyle *et al.*, 1998). Total RNA was extracted from a sample of hair follicles and used to synthesize cDNA, which was subsequently amplified with PCR. A sample of the follicle-specific RNA was

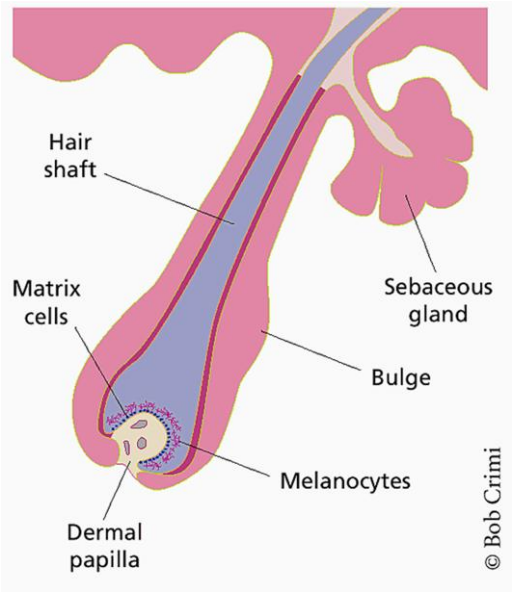


Figure 2: Hair follicle microanatomy – the bulge (Hoffman, 2000).

also used to synthesize a ^{32}P-labeled cDNA, which was used to determine the location of the K15 gene via Southern dot blot analysis of the cDNA library. After the K15 gene was located, the corresponding clone was used for *in vitro* synthesis of K15. The resulting K15 solution was immunoprecipitated using C8/144B or another isotype-matched monoclonal antibody (IgG_1) and the precipitate analyzed with SDS-PAGE and autoradiography. The results of this analysis indicated that C8/144B indeed binds to K15.

A 2006 Lyle lab publication with collaborators Drs. Bieniek, Lazar, and Photopoulos, published in the *British Journal of Dermatology*, discussed the discovery of a subpopulation of sebaceous cancer cells expressing the K15 stem cell marker. This subpopulation was present in all tumors examined—a set which included sebaceous adenomas, carcinomas, and hyperplasias (Bieniek *et al.*, 2006). As K15 is known to be a marker for multi-potent hair follicle stem cells (Lyle *et al.*, 1998), these $K15^+$ cells are likely CSCs.

In 2007, Dr. Lyle was a member of a team—Grossman *et al.*—investigating the possible use of the Shc signal transduction adaptor as an indicator of the risk of recurrence for colon cancer. Despite a significant risk of disease-related death, many stage IIA colon cancer patients are not given adjuvant chemotherapy after tumor excision (Grossman *et al.*, 2007)—adjuvant therapy is an additional treatment, such as radiation or chemotherapy, administered after a primary course of treatment (Definition of adjuvant therapy, n.d.). The Shc molecule is a recurrence risk indicator for breast cancer, and therefore may be a similar indicator for colon cancer (Grossman *et al.*, 2007).

Two cohorts of colorectal tumors were observed in the experiment: one cohort of stage IIA tumors from 130 patients that received no adjuvant therapy after tumor excision and one cohort of stage IIA tumors—none rectal—from 110 patients who also received no adjuvant

therapy post-surgery. The relapse rates for the first and second cohorts were 15% and 23.6%, respectively (based on what data could be found for the patients from the first cohort). The first cohort was prepared as 1-mm microarray cores and the second cohort as whole tissue sections. These cohorts were then treated with PY-Shc and p66 Shc antibodies as part of an immuno-histochemical assay, calibrated for nonspecific staining with a negative tissue sample. The results of this assay were then normalized to fit the two "scores" used in breast cancer recurrence risk analysis, which were then incorporated into a larger body of data—including age, sex, lymphovascular involvement, etc.—about the source patients, and the entire body of data statistically analyzed. The results of this statistical analysis indicated that measurement of p66 Shc levels in stage IIA colon tumors allows clinicians to classify patients based on risks of recurrence and disease-related death (Grossman *et al.*, 2007).

The most recent of Dr. Lyle's publications is a 2009 study investigating the effect of certain aspects of hair follicle microanatomy (**Figure 3**) on melanoma growth behaviors. 100 melanoma samples were retrieved and examined using both immunohistochemistry and immunofluorescence. Of these samples, 18 (18%) exhibited no *in situ* malignant melanoma in the follicles, 57 (57%) exhibited melanoma at the infundibulum (**Figure 3A**), 24 (24%) at the isthmus (**Figure 3A**), and one (1%) displayed melanoma encroachment as far as the bulge (**Figure 3A**). These data

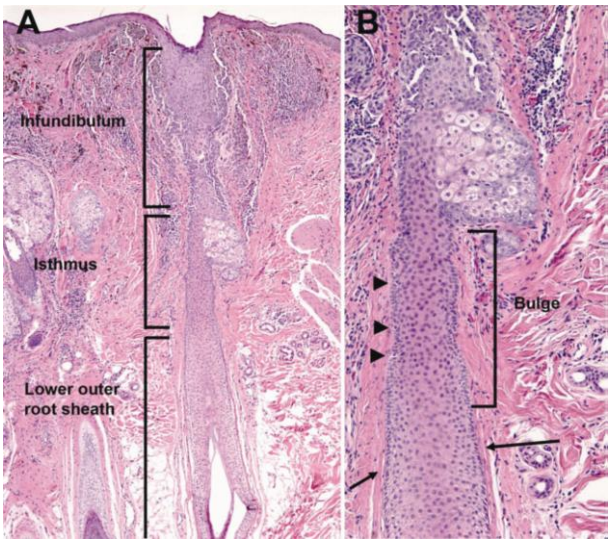


Figure 3: Hair follicle microanatomy (Pozdnyakova *et al.*, 2009)

indicate that there exists one or more currently-unknown physiological mechanisms that protect the interior of the hair follicle from harmful stimuli (Pozdnyakova *et al.*, 2009).

Detection of Slow-Cycling, Quiescent Cancer Stem Cells

Currently, one of the most effective methods for identifying stem cells involves the use of stem cell markers—proteins that are associated with stem cell properties. This method may prove valuable in selecting for CSCs that rose from normal human stem cells (nHSCs). Many cells that express some of these markers, however, are not stem cells, and these markers do not themselves bestow stem cell attributes (N. Moore, unpublished). Therefore, these markers will be of little—if any—use in selecting for CSCs that originated from progenitor cells or other, more highly differentiated cells. Until a reliable CSC marker can be found, alternative identification methods must be used to select for CSCs. One such method is a pulse-chase label retention assay (N. Moore, unpublished).

Stem cells are commonly slow-cycling—that is, they progress through the standard cell cycle at a slower and more controlled rate than differentiated cells. This slow-cycling, quiescent nature is an integral component in maintaining the proliferative capabilities and longevity of stem cells, and may potentially be used as the basis for a functional stem cell assay (N. Moore, unpublished).

Pulse-chase analysis is a method commonly used in cellular and molecular biology to examine internal cellular processes—such as protein folding, protein degradation, and so forth—in live cells. The cells to be examined are treated with a label for a short period of time—the pulse—and then the label is diluted or washed away—the chase—a process which may take a significant amount of time. The levels and/or progression of label may be observed in real time,

at several discrete points in time, or at a single point in time (Alberts *et al.*, 2002). Another application of pulse-chase analysis, and one of particular interest in stem cell research, is the identification of slow-cycling cells—stem cells—within a large, heterogeneous population, such as a tumor. Such a cell population is treated with a non-transferrable label—that is, a dye that cannot be transferred to neighboring cells through the membrane—and incubated for a few days, after which the population may be analyzed (N. Moore, unpublished). As the cells undergo division, the amount of label per cell will be halved with each generation of daughter cells. The slow-cycling cells will diffuse label more slowly—and thus remain detectable longer—than the other cells within the population being assayed. If the sample is given a proper chase period, only the slow-cycling cells will retain a detectable amount of label, and may be separated from the rest of the sample with a label-sensitive assay. Based on recent studies performed in the Lyle lab with cells from one of the MDA lines, this assay has significant potential to successfully identify CSCs (Moore, unpublished).

Carboxyfluorescein succinimidyl ester (CFSE) was chosen for this project because of its nontoxicity and its static nature (it is not transferred between cells). In its administered form, carboxyfluorescein diacetate succinimidyl ester, the molecule is highly membrane-permeable and enters the cell easily. Once inside, however, the acetate side chains are cleaved, converting the molecule into CFSE. In this form, it is much less membrane-permeable and exits the cell much more slowly. This slower exit rate allows most of the molecules to bind covalently to amine groups on various intracellular molecules, distributing itself throughout the cell. Due to this widespread binding, the CFSE label will be equally divided between daughter cells when the original cell undergoes mitosis. This functionality forms the basis of the assay being tested in this project. As the cells labeled with CFSE undergo mitosis, the CFSE label within normal cells

will be diluted at a much higher rate than the CFSE label within the slow-cycling cells. The slow-cycling cells will, therefore, remain detectable longer than the normal-cycling cells, allowing for selection of the slow-cycling cells.

Cancer Stem Cell Markers

Many types of markers can be used to identify stem cells or specific types of cancers. Examples of cancer markers include the cell-surface glycoprotein CD44, two particular mutations of which are associated with the progression of head and neck squamous cell carcinoma (Assimakopoulos *et al.*, 2002; Wang *et al.*, 2009). Examples of stem cell markers include the glycoprotein CD133, which is expressed by human hematopoietic stem cells (Shmelkov *et al.*, 2005). While recent research has found potential markers for CSCs in specific cancer types—such as CD44, which may serve as a CSC marker in prostate and breast cancers (Li *et al.*, 2007)—no markers have been found for identifying CSCs regardless of cancer type.

The genes CD24, CD44, Cyclin E (hereafter abbreviated as "CycE"), p16(MTS-1/CDKN2/INK4a) (hereafter abbreviated to "p16"), and $p21^{WAF1}$ (hereafter abbreviated as "p21") are used as cancer cell markers. The surface protein CD24 may be used to predict prostate adenocarcinoma relapse (Kristiansen *et al.*, 2004), while CD44—another surface protein—functions with CD24 as a marker for tumorigenic breast cancer cells (Al-Haji *et al.*, 2003). CycE, as a member of the cyclins, has been shown to be at least a potential prognostic marker for breast and gastric cancer (Keyomarsi *et al.*, 1994; Jang *et al.*, 1999). The cyclin-dependent kinase inhibitors (CDKIs) p16 and p21 are both involved in the modulation of the mammalian cell cycle—specifically, as tumor suppressors—and are used as markers to identify

and track multiple cancer types (Stone *et al.*, 1995; Chilosi *et al.*, 1996; Jang *et al.*, 1999; Rocco and Sidransky, 2001).

Some stem cell markers are also of interest in the field of cancer biology. Though CD133 is primarily used to isolate neuronal stem cells (Uchida *et al.*, 2000), as well as hematopoietic and endothelial progenitor cells (Shmelkov *et al.*, 2005), it has also been shown to function as a CSC marker in brain tumors (Li *et al.*, 2007). Keratin 15, specific to hair stem cells, has also been expressed by cancer cells—in this case, cells from certain pilar tumors of the skin (Kanitakis *et al.*, 1999).

PROJECT PURPOSE

As discussed above, cancer stem cells (CSCs) cannot currently be identified by genetic markers, except in certain cases where the marker is specific to the cancer type. This project therefore investigated other mechanisms that might select for CSCs. The primary focus of the project was the CFSE pulse-chase label-retaining assay. As CSCs are assumed to possess increased resistance to chemotherapy treatments, samples of HCT116 cells labeled with CFSE then chased to display label-retaining cells (LRCs), were treated with either DMSO vehicle or one of the following chemotherapy drugs: doxorubicin hydrochloride, oxaliplatin, 5-fluorouracil, or a combination of 5-fluorouracil and oxaliplatin—the cytotoxic components of the chemotherapy treatment FOLFOX (Moore, personal discussion). The cells were then analyzed by FACS to determine the percent LRCs, and whether treatment with chemotherapy enriches for LRCs. A secondary focus was the testing of certain cancer and stem cell markers for potential use as CSC markers. CFSE-labeled cells were chased and prepared for immunofluorescence with primary antibodies against CD24, CD44, CD133, Cyclin E (CycE), Keratin 15, p16, or p21.

METHODS

General Methods

HCT116 Cell Culture

HCT116 cells were obtained from cryogenically preserved samples of the HCT116 cell line. When passaging HCT116 cells, the medium was suctioned off, the plate rinsed with 1X PBS, and 1 mL of either 1X trypsin/versene or 2X trypsin was applied. The plate was incubated in the 37°C incubator for a very short time (often no longer than thirty seconds), after which more medium was added to inactivate the trypsin, then the entire mixture transferred to a 15-mL conical tube. The mixture was spun down for five minutes at 200 rcf (hereafter assumed to be the speed and duration for the term "centrifuged" unless otherwise specified) and resuspended in 2-3 mL PBS. If specific data on the cell suspension was necessary, a trypan blue cell count (see below) was performed, otherwise the culture was replated at a simple dilution ranging from 1:5 (1-2 days before next passage) to 1:40 (5-7 days before next passage).

CFSE Labelling of HCT116 Cells

HCT116 cells were only labeled after passaging a culture. Once the trypsin solution was inactivated and the concentration (in $^{cells}/_{mL}$) determined by a trypan blue cell count (see below), a sample of sufficient size—as determined by the experiment to be performed—was taken and re-suspended at a concentration of $1.0\times10^{6\ cells}/_{mL}$. A 5 mM solution of CFSE (from the Invitrogen CellTrace™ kit) was then added to the cell suspension at a rate of 2 μL of CFSE solution per 1.0×10^{6} cells. The resulting mixture was then incubated in the 37°C water bath for ten minutes, after which it could be used as desired.

Single-Cell Suspension

When forming a single-cell suspension from a suspension of cells (often when sampling a culture grown in sphere medium), the mixture in question was transferred to a tube and centrifuged as described above. The resulting pellet was resuspended in PBS (2-3 PBS unless otherwise specified) to wash away any residual medium, spun down again, resuspended in 1 mL of 1X trypsin/versene or 2X trypsin solution, and incubated at 37°C for several minutes. After incubation, the sample was vigorously resuspended with a 200 μL micropipettor, spun down again, resuspended in medium to inactivate the trypsin, and spun down again before being resuspended in PBS.

Trypan Blue Cell Counting

When specific data on a cell suspension was necessary, a cell count was performed using the dye trypan blue and a phase-contrast microscope. Trypan blue is actively expelled by live cells, and therefore only stains dead cells blue. 10 μL samples were taken of the cell suspension under examination and placed in 500 μL or smaller microfuge tubes. A 0.4% solution of trypan blue was then added to stain and dilute the cells, and 10 μL of the resulting mixture placed in one or more wells of a hemocytometer. The wells were then examined at 100x magnification and the cells in two or more of the corner sections of the grid were counted.

Optimal Chase Period

To determine the optimal chase period—one resulting in an approximately 5% CFSE-positive culture—HCT116 samples were labeled and plated at a concentration of $2.00\times10^{4\ \mathrm{cells}}/_{\mathrm{mL}}$ in 10-cm shallow petri dishes with sphere medium (DMEM/F12 medium with amphotericin B, vitamin B27 supplement, EGF, and FGF) and incubated at 37°C. Four, five, six, seven, eight,

and nine days after plating, a sample was taken from half of the plates (alternating plates sampled every day) and prepared for immunofluorescence microscopy.

Each sample was prepared for immunofluorescence microscopy as follows: a 1 mL sample of cell suspension was transferred to a 5 mL round-bottomed tube (hereafter referred to as a "FACS tube") and diluted in 2 mL PBS. The diluted sample was spun down at default settings and gently resuspended in 200 μL PBS. This sample was then placed in a CytoSpin™ funnel and spun onto a CytoSpin™ prepared slide (3 minutes at 300 rpm in a CytoSpin™ centrifuge). The slide was fixed with a 4% paraformaldehyde solution (enough to cover the sample area) for five minutes, after which it was mounted with VectaShield® Mounting Medium with DapI™, covered with a cover slip, and stored at 4 °C in the dark.

Doxorubicin Kill Curve

To determine the amount of doxorubicin necessary to kill approximately 85-90% of an HCT116 culture, CFSE-labeled HCT116 cells were plated at $2.00\times10^{4\ cells}/_{mL}$ in 10-cm plates with sphere medium and incubated at 37°C for 7 days. Each plate was then transferred to a separate 15 mL conical tube, trypsinized into single-cell suspensions with 1 mL 2X trypsin for 1 minute (see above), and plated in 6-cm plates with sphere medium. Each plate was then treated with either 150 μL or 200 μL of 1 mM doxorubicin hydrochloride, and incubated for an additional 3 days at 37°C, after which they were transferred to separate FACS tubes and trypsinized into single-cell suspensions with 1 mL 2X trypsin for 1 minute. These samples were then subjected to a trypan blue cell count to determine the percent survival of the cells.

Label-Retaining Cell Assay – Chemotherapy Enrichment

To test the label-retaining assay for positive identification of cancer stem cells, the HCT116 cells treated in the CFSE pulse-chase assay were treated with one of several chemotherapy agents or the dimethyl sulfoxide (DMSO) vehicle, and incubated for 3 days at 37°C, after which the cultures were analyzed by fluorescence-assisted cytometric sorting (FACS) to determine whether the label-retaining cell (LRC) populations had increased in cultures treated with chemotherapy.

Five plates of CFSE-labeled HCT116 cells and one plate of non-labeled ("dark") cells were incubated at 37 °C for 7 days. Each plate was then transferred to 15 mL conical tubes, spun down, and resuspended in 2-6 mL PBS (6 mL for Dark sample, 2 mL for all others). The labeled cells were combined and all samples were trypsinized into single-cell suspensions with 1 mL 2X trypsin. The concentrations were ascertained by trypan blue cell count and the cells plated into 12 plates of sphere medium—2 dark, 10 labeled—at $2.00\times104\ ^{cells}/_{mL}$. Two of the labeled plates were treated with 6.5 μL each of DMSO vehicle, two with 500 μL each of doxorubicin (1 mM), two with 3.0 μL each of oxaliplatin (12.5 mM), two with 6.5 μL each of 5-fluorouracil (5-FU; 384 mM), and two with 3.0 μL each of oxaliplatin (12.5 mM) & 6.5 μL each of 5-FU (384 mM)—the two toxic components of the common chemotherapy treatment FOLFOX (Moore, unpublished). The plates were then incubated for 3 days and prepared for FACS analysis.

FACS Analysis

To prepare the plates for FACS analysis, each pair of treated plates—Dark, DMSO, Doxo, Oxali, 5-FU, FOLFOX—was consolidated within a 50 mL conical tube and trypsinized into a single-cell suspension—washing with 4 mL each of PBS, incubating with 2X trypsin for 1 minute. The samples were then resuspended in 10 mL each of PBS, filtered through 40 MM

nylon, spun down, and resuspended in 2 mL each of PBS. The samples were then split between 18 FACS tubes at 500 μL each: 2 tubes from the Dark sample ("No Stain," "Live/Dead Blue"), 4 from the DMSO sample ("CFSE," "DMSO 1," "DMSO 2," "DMSO 3"), and 3 each from the other treatment samples ("Doxo 1–3", "Oxali 1–3," "5-FU 1–3," "FOLFOX 1–3"). A 1.5 μL sample of Live/Dead Blue—a fluorescent label that functions similarly to trypan blue, as it is only taken up and retained by dead cells—was added to each of the tubes except "No Stain" and "CFSE," after which the tubes were mixed by flicking and incubated on ice in the dark for 15 minutes. All tubes were diluted with 1 mL each of PBS, spun down, resuspended in 200 μL each of BD Cytofix/Cytoperm™, and incubated on ice in the dark for 15 minutes. The tubes were once again diluted with 1 mL each of PBS and spun down, after which the pellets were resuspended in 300 μL each of FACS buffer—PBS 1X with sodium azide (1 mM) and BSA (0.05 $^{g}/_{mL}$)—and stored at 4°C in the dark until FACS analysis. This entire experiment was performed in triplicate.

Cancer Stem Cell Marker Test

As more sophisticated methods of marker analysis—such as serial genetic expression analysis (Velculescu *et al.*, 1995)—were unavailable, immunofluorescence staining and FACS analysis were used to test the label-retaining assay for enrichment of each of the following potential markers: CD24, CD44, CD133, CycE, keratin 15 (K15), p16, or p21. Two plates of HCT116 cells were prepared. One was pulsed with HCT116, and both were chased for 7 days, after which the plates were split into 12 samples at 1 mL each—3 from the unlabeled ("dark") cells, 9 from the labeled cells—using PBS as a diluent. The samples were spun down and resuspended in 100 μL each of FACS wash buffer, after which they were treated with the

primary antibody (see **Table 1** below) and incubated for 15 minutes on ice in the dark. The samples were then diluted in 1 mL each of PBS, spun down, and resuspended in 100 μL each of secondary antibody solution (see **Table 1** below) in FACS buffer to incubate for 15 minutes on ice in the dark. After the secondary incubation, the samples were washed again with 1 mL each of PBS and spun down. Each sample was then resuspended in 400 μL FACS buffer and stored at 4 °C in the dark until FACS analysis.

Table 1: Marker immunfluorescence stain – antibodies used

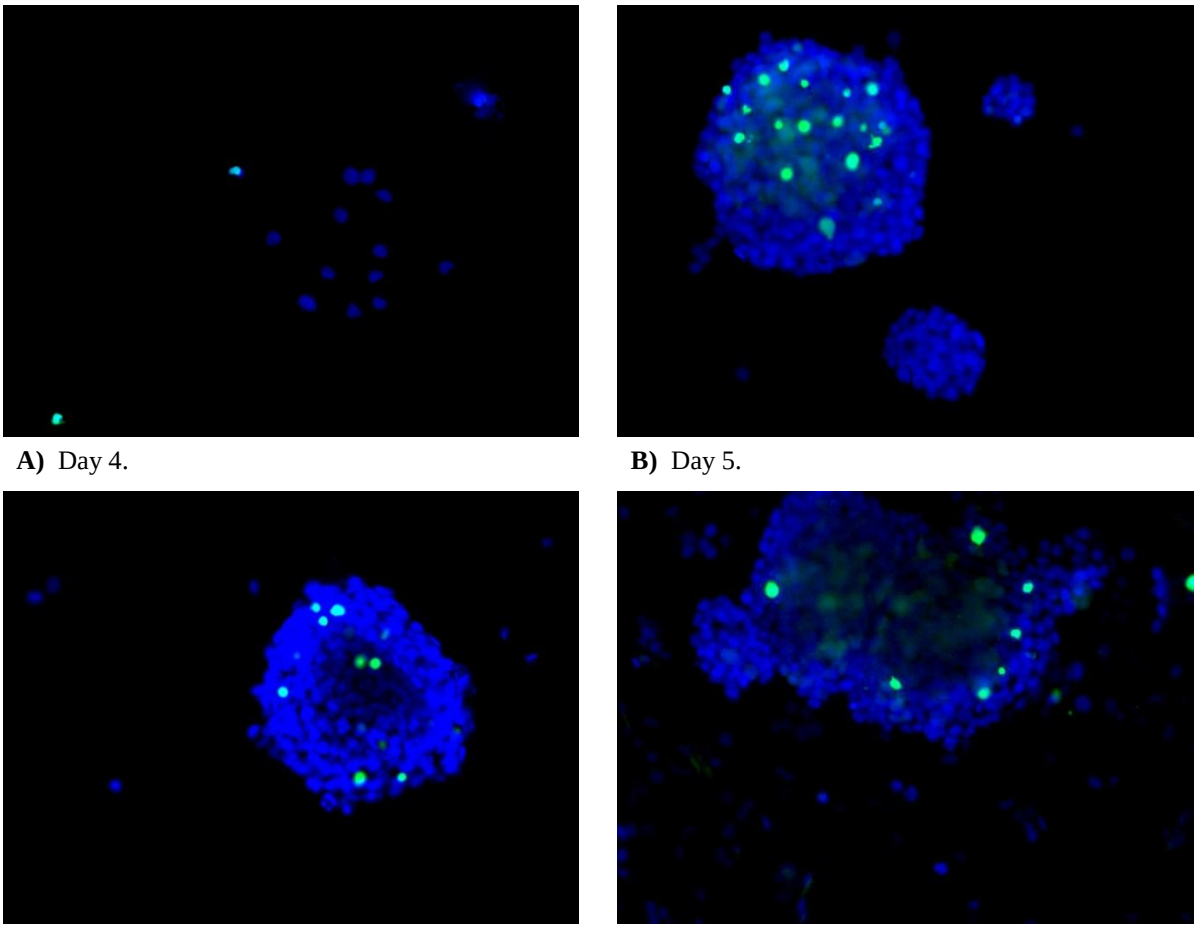
#	Tube	Primary	Secondary
1)	Dark Control	--	--
2)	Live/Dead Blue Control	--	--
3)	CFSE Control	--	--
4)	Marker Control (CD44)	Mouse α-CD44	α-Mouse
5)	CFSE + Live/Dead Blue	--	--
6)	CD44	Mouse α-CD44	α-Mouse
7)	p21	Rabbit α-p21	α-Rabbit
8)	p16	Mouse α-p16	α-Mouse
9)	CD24	Mouse α-CD24	α-Mouse
10)	CycE	Mouse α-CycE	α-Mouse
11)	CD133	Rabbit α-CD133	α-Rabbit
12)	K15	Mouse α-K15	α-Mouse

RESULTS

The primary goal of this project was to determine whether a CFSE label-retaining functional assay would be effective in selecting for cancer stem cells (CSCs) from a HCT116 colorectal cancer cell line. This was tested by labeling HCT116 cells with CFSE, then chasing and treating the cells with one of several chemotherapy drugs or with the DMSO vehicle, and the samples analyzed via fluorescence-assisted cytometric sorting (FACS) to determine whether the percentage of CFSE-positive cells had increased. Testing for potential CSC markers via immunofluorescence and FACS was later added to the experiment as a secondary goal.

The label-retaining experiment was divided into three components, the first of which was determining the optimal chase period. HCT116 cells were labeled with CFSE, plated in two separate plates with sphere medium, and incubated at 37°C for a period of 9 days. Starting at day 4, one of the plates was sampled daily—alternating plates every day to reduce stress on the cells—and prepared for fluorescence microscopy using immunofluorescence staining. This experiment was performed twice, after which the slides were examined, micrographs were taken, and the percent CFSE-positive cells estimated for each slide. Before running the experiment, the target CFSE-positive population was determined to be 5% of the total population. See Figure 4 below for sample micrographs, Figure 5Figure 4: Pulse-chase micrographs to help define the optimal chase period for the CFSE label. Blue indicates a regular cell and green indicates a label-retaining cell.

below for a graph of the data gathered, and **Table 2** in the **Appendix** for the raw data from the experiment. Based on the data and on schedule factors, a 7-day chase period was selected for the main experiment.



A) Day 4. **B)** Day 5.

C) Day 7. **D)** Day 9.

Figure 4: Pulse-chase micrographs to help define the optimal chase period for the CFSE label. Blue indicates a regular cell and green indicates a label-retaining cell.

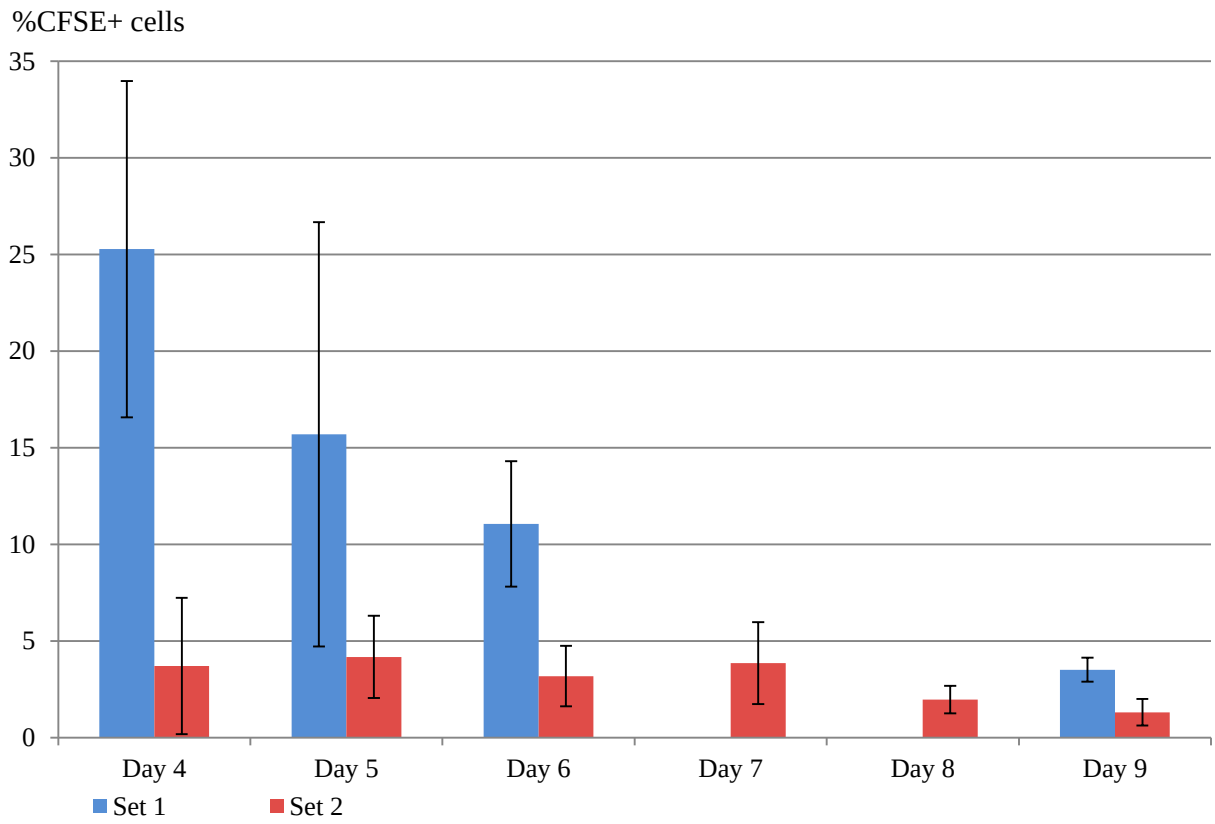


Figure 5: CFSE-positive cell counts from optimal chase period experiment. Error bars denote standard deviation. The black bars indicate the error as determined by the standard deviation.

The second component of the main project was the generation of a kill curve for the chemotherapy drug doxorubicin hydrochloride to determine its optimal concentration—determined to be the concentration with a 15-20% survival rate—for use in the assay. HCT116 cells were grown in 6-cm plates with sphere medium for 7 days, after which each plate was treated with either 150 µL or 200 µL of 1 mM doxorubicin solution in DMSO and PBS. The plates were incubated for an additional 3 days, after which they were sampled, the cells digested into a single-cell suspension, and counted with trypan blue to calculate the percent survival. The data points were then plotted to form a kill curve, with % Survival on the *y*-axis and µL Doxo represented on the *x*-axis (**Figure 6** below). Taking the kill curve into account, a final concentration of 50 µL doxorubicin per mL of culture medium was determined to be ideal.

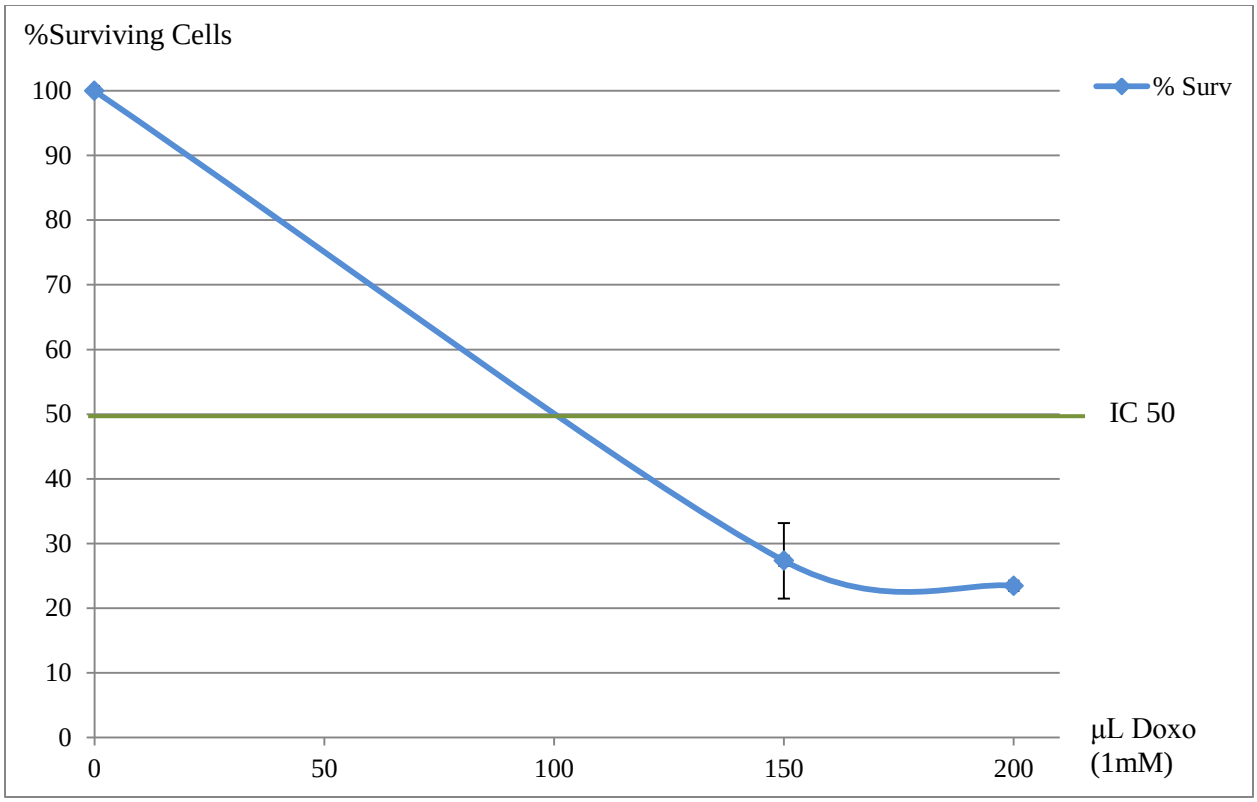


Figure 6: Doxorubicin Kill Curve. The black error bars indicate one standard deviation.

Once the optimal chase period and doxorubicin concentrations had been determined, the third and final component of the main project—the label-retaining cell (LRC) enrichment—was performed. Several plates of HCT116 cells were pulsed with CFSE and, along with an unlabeled control plate, chased for 7 days, after which the cells were re-plated and the labeled cells were treated either with one of several chemotherapy treatments—doxorubicin, oxaliplatin, 5-fluorouracil, or FOLFOX (oxaliplatin + 5-fluorouracil)—or with DMSO vehicle, then incubated for an additional 3 days before being analyzed via FACS (**Figure 7** & **Figure 8** below, **Table 3** in **Appendix**). The FACS analysis showed a nearly two-fold increase in percent CFSE-positive cells in both the doxorubicin- and FOLFOX-treated samples (histobars 2 and 5)

(compared to the DMSO-treated sample). Further analysis with a student's T-test indicated that the increases were indeed significant ($p < 0.05$).

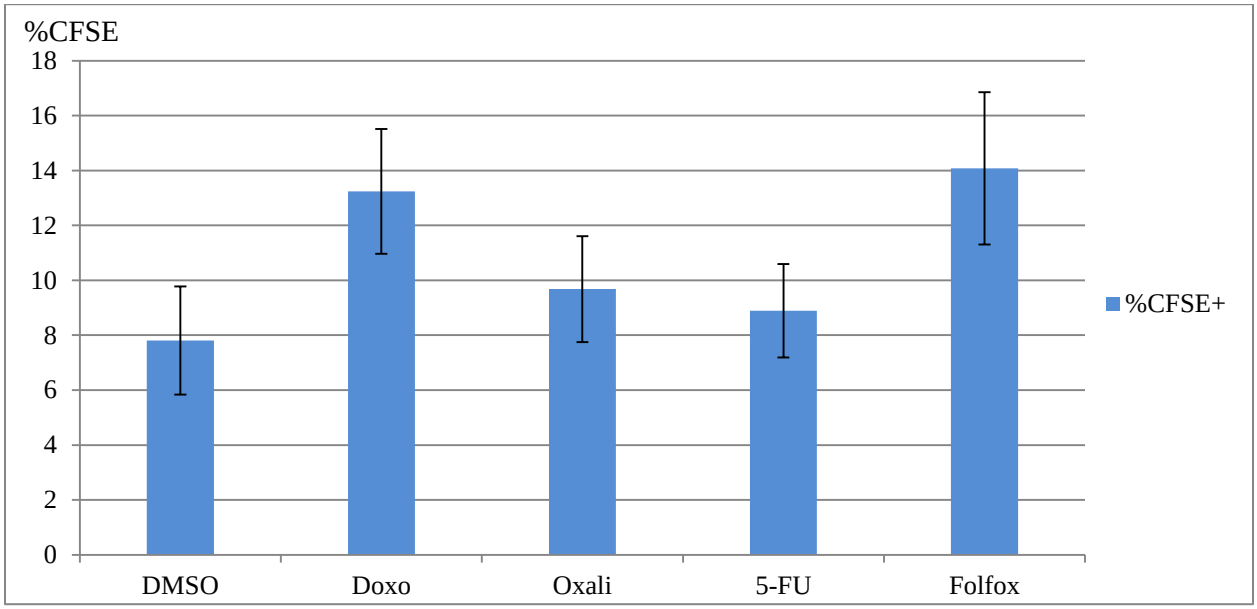


Figure 7: Percent CFSE-positive cells following various chemotherapy treatments. A star (*) indicates a sample whose change relative to the DMSO sample has $p < 0.05$.

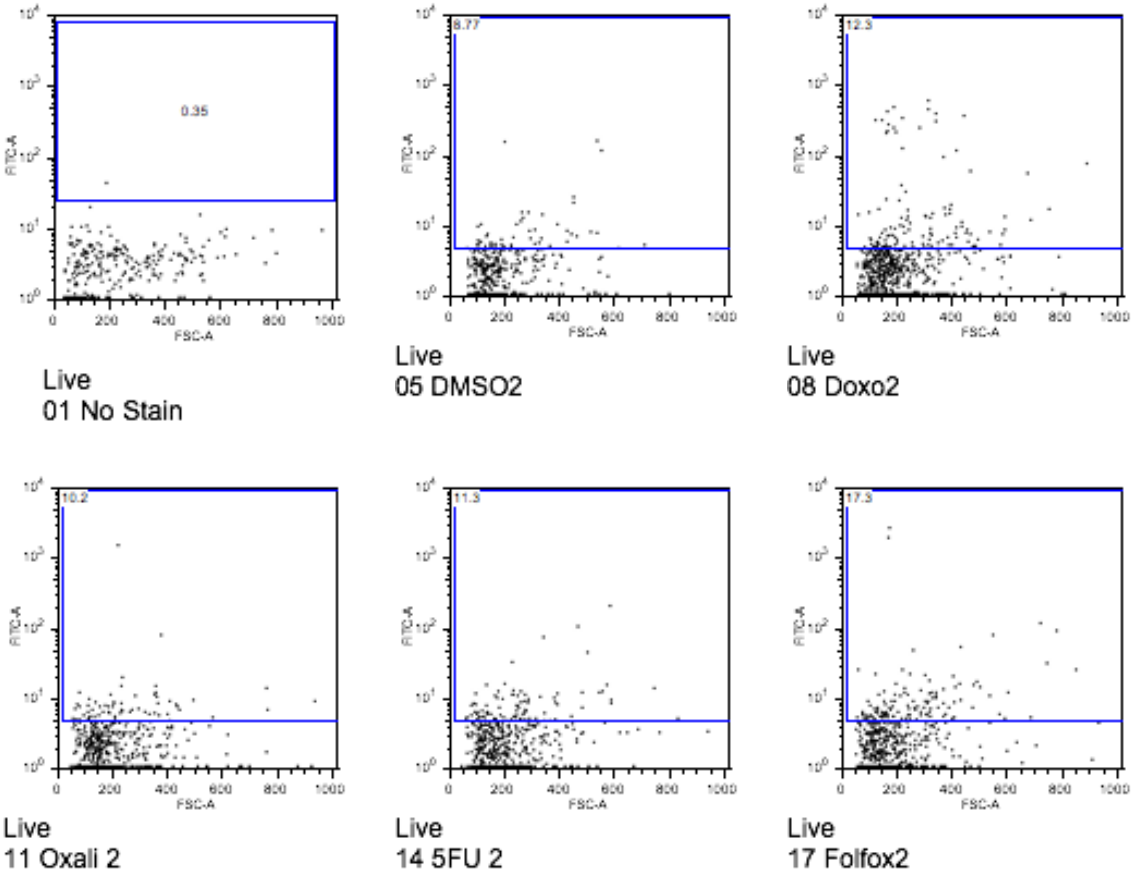


Figure 8: Example FACS plots from chemotherapy enrichment test. FITC fluorescence is plotted on the vertical axis, and forward scatter on the horizontal axis.

As a side project, several proteins—CD24, CD44, CD133, CycE, K15, p16, and p21—were tested as potential CSC markers. This was done by taking pulse-chased HCT116 cells, preparing immuno-stained samples—one for each potential marker—and analyzing the samples with FACS (**Figure 9** & **Figure 10** below, **Table 4** in **Appendix**). Based on the data, none of the markers seems to make up a significantly larger percentage of label-retaining cells than of the "bulk" (non-label-retaining) population, or vice versa. None of these proteins, therefore, seems a likely marker for the HCT116 line. These conclusions are tentative at best, however, as there was only sufficient time for a single run of the experiment.

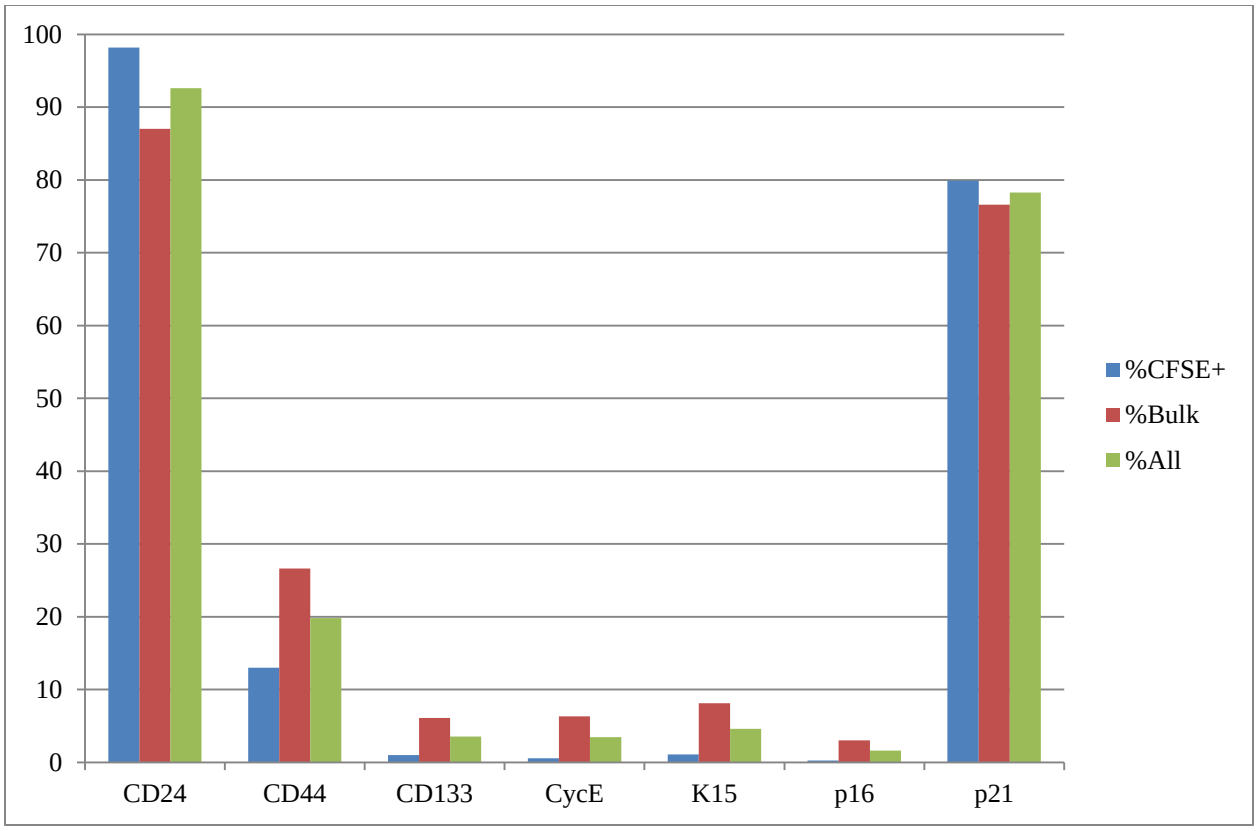


Figure 9: % CFSE-positive (%CFSE+), CFSE-negative (%Bulk), or all (%All) cells also positive for indicated marker. The %All was calculated for each sample by averaging the corresponding %CFSE and %Bulk values.

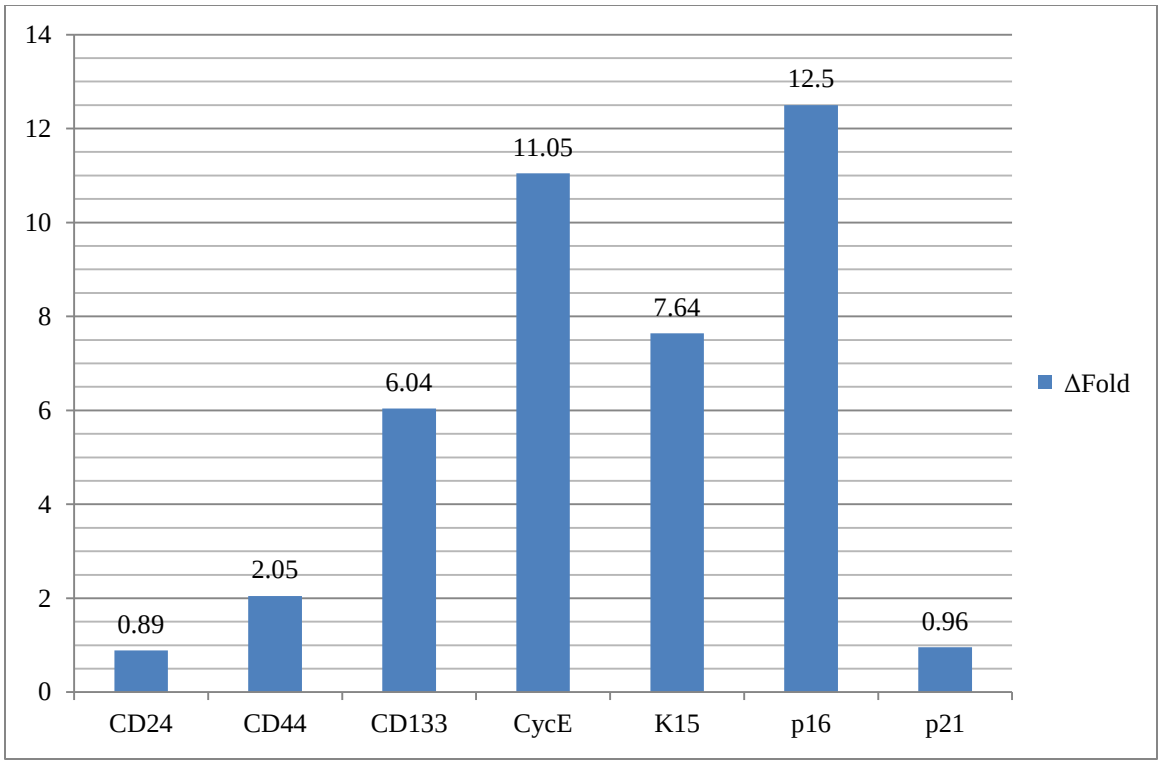


Figure 10: Fold change in % marker-positive from Bulk population to CFSE-positive population. The specific values are listed above the bars.

DISCUSSION

Once the CFSE pulse-chase assay had been optimized and its results analyzed, it was determined that the doxorubicin and FOLFOX treatments had significantly enriched the percentage of label-retaining cells (LRCs). This, in turn, may indicate that the assay was successful in selecting for HCT116 cancer stem cells (CSCs). If doxorubicin was the only treatment to produce a significant change, however, the results would likely have been a false positive, as doxorubicin fluoresces red brightly enough to bleed through into other wavelengths, potentially causing CFSE-negative cells to appear CFSE-positive (Moore, unpublished).

Conversely, the marker test did not present any indication that any of the markers tested have potential as an HCT116 CSC marker. This data is tentative at best because there was insufficient time for more than a single run of the experiment. Further experiments in this area would be best performed using more advanced techniques, such as serial genetic expression analysis (Velculescu *et al.*, 1995), as those techniques would allow for a speedier comprehensive analysis.

While the data indicate that the label-retaining assay was successful in selecting for CSCs, I feel further perfection of the technique is necessary. Due to problems with the centrifugation and re-suspension of cells, the concentrations of cells varied between experimental runs and sometimes between plates within the same experiment run. Furthermore, the concentrations of the chemotherapy treatments were estimated—no kill curve was obtained for oxaliplatin, 5-fluoruracil, or FOLFOX—and the doxorubicin kill curve was fraught with contamination problems such that only two of the concentrations tested yielded any significant data. Due to a communications error, the optimal chase period value was an estimate at best.

When grown in sphere medium, HCT116 cells form spheres, with each original cell forming its own sphere (Moore, personal communication). These were to have been digested before performing the immunofluorescence staining, but due to a miscommunication, were not. Because of time constraints, we opted to use the data currently available in an estimate of optimal chase time, rather than perform another run of the experiment. Subsequent experiments should, therefore, at least include elements that test and account for these discrepancies.

Another CSC trait is the ability to initiate tumorigenesis when cultured *in vivo* (Moore, unpublished). Due to time constraints, such a test could not be used, but in subsequent examinations of the HCT116 line, it may prove valuable, as it provides a more definitive result for CSC potential (Moore, unpublished). Therefore, when the assay is perfected, I suggest that an *in vivo* model be used for at least part of the tests, to ensure that the assay indeed selects for CSCs.

APPENDIX

Table 2: Raw pulse-chase data.

Sample	DapI+	CFSE+	%CFSE+
Day 4.1_1	14	3	21.43%
Day 4.1_2	11	4	36.36%
Day 4.1_3	15	2	13.33%
Day 4.1_4	10	3	30.00%
Day 4.2_1	115	3	2.61%
Day 4.2_2	21	2	9.52%
Day 4.2_3	95	0	0.00%
Day 4.2_4	75	2	2.67%
Day 5.1_1	23	2	8.70%
Day 5.1_2	36	3	8.33%
Day 5.1_3	26	9	34.62%
Day 5.1_4	54	6	11.11%
Day 5.2_1	193	9	4.66%
Day 5.2_2	322	24	7.45%
Day 5.2_3	159	4	2.52%
Day 5.2_4	97	2	2.06%
Day 6.1_1	41	5	12.20%
Day 6.1_2	51	3	5.88%
Day 6.1_3	27	4	14.81%
Day 6.1_4	44	5	11.36%
Day 6.2_1	182	5	2.75%
Day 6.2_2	162	5	3.09%
Day 6.2_3	107	6	5.61%
Day 6.2_4	79	1	1.27%
Day 7.2_1	470	19	4.04%
Day 7.2_2	402	26	6.47%
Day 7.2_3	206	9	4.37%
Day 7.2_4	180	1	0.56%
Day 8.2_1	350	8	2.29%
Day 8.2_2	222	6	2.70%
Day 8.2_3	191	3	1.57%
Day 8.2_4	133	1	0.75%
Day 8.2_5	363	9	2.48%
Day 9.1_1	235	10	4.26%
Day 9.1_2	126	5	3.97%
Day 9.1_3	136	4	2.94%
Day 9.1_4	70	2	2.86%
Day 9.2_1	238	2	0.84%
Day 9.2_2	208	3	1.44%
Day 9.2_3	168	4	2.38%
Day 9.2_4	340	2	0.59%

Table 3: Raw chemotherapy enrichment data – FACS analysis. NOTE: The 2/27/11 FACS data (marked in yellow) was determined to be an outlier, and as such was removed from consideration when calculating the mean and standard deviation for each treatment set.

Sample	2/27/11 FACS	3/05/11 FACS	3/15/11 FACS	Mean	Std. Dev.
DMSO 1	5.77%	6.34%	7.28%	7.81%	±1.97%
DMSO 2	71.00%	9.47%	8.77%		
DMSO 3	71.00%	4.58%	10.40%		
Doxo 1	63.20%	13.40%	15.90%	13.24%	±2.27%
Doxo 2	54.80%	11.90%	12.30%		
Doxo 3	64.60%	16.20%	9.73%		
Oxali 1	54.70%	9.24%	8.59%	9.68%	±1.93%
Oxali 2	67.40%	7.08%	10.20%		
Oxali 3	62.80%	13.40%	9.57%		
5-FU 1	60.20%	7.34%	9.43%	8.89%	±1.70%
5-FU 2	52.80%	6.10%	11.30%		
5-FU 3	55.80%	9.31%	9.85%		
FOLFOX 1	53.00%	14.40%	11.80%	14.08%	±2.77%
FOLFOX 2	62.00%	11.80%	17.30%		
FOLFOX 3	53.20%	18.10%	11.10%		

Table 4: Raw marker FACS data.

Marker	% CFSE-positive	% CFSE-negative	Fold Difference
CD24	0.57%	6.30%	11.05
CD44	98.20%	87.00%	0.89
CD133	0.24%	3.00%	12.50
CycE	1.06%	8.10%	7.64
K15	79.90%	76.60%	0.96
p16	1.01%	6.10%	6.04
p21	13.00%	26.60%	2.05

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