Characterization of human colorectal cancer stem cells and their role in chemoresistance

by

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ABSTRACT

Characterization of human colorectal cancer stem cells and their role in chemoresistance

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University of Guelph, 2015

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Treatment of advanced colorectal cancer (CRC) is impacted by tumour heterogeneity and the development of drug resistance. Cancer stem cells (CSCs) are a subpopulation of tumour cells with the ability to self-renew and evade current treatments and therefore are implicated in tumour recurrence. Our laboratory used serial colonosphere culture to enrich for CSCs from two human CRC cell lines, HCT116 and SW480. The expression of CSC markers was assessed via flow cytometry and RT-qPCR. The effect of chemotherapy on self-renewal and drug resistance-related gene expression was also examined. The sphere formation assay enriched for cells with higher levels of CSC markers. Chemotherapy treatment significantly reduced sphere and colony formation in parental cell lines. Treatment also altered the expression of the drug-resistance related genes ABCB1, ABCG2 and ALDH3A1. These data provide evidence that chemotherapy reduces the ability to self-renew and alters the expression of multi-drug resistance genes influencing the CSC phenotype.
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Nathan Farias performed the initial enrichment for the CSC enriched cell lines. Along with his guidance for colonosphere culture, this gave me a considerable head start with my project.

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DECLARATION OF WORK PERFORMED

I declare that all work in this thesis was performed by me, with the exception of the items listed below.

Nathan Farias performed the initial enrichment of the cancer stem cell lines using the sphere formation assay.

Under my supervision an undergraduate student, Lisa Richardson, contributed the clonogenic survival assays and the limiting dilution analysis following chemotherapy treatment. She also assisted with the generation of the drug-resistant cell lines.

Andrew Dalrymple assisted with the design of Figure 1.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>AB</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABC</td>
<td>Adenosine tri-phosphate-binding cassette</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ATP-binding cassette, sub-family B, number 1</td>
</tr>
<tr>
<td>ABCC1</td>
<td>ATP-binding cassette, sub-family C, number 1</td>
</tr>
<tr>
<td>ABCG2</td>
<td>ATP-binding cassette, sub-family G, number 2</td>
</tr>
<tr>
<td>ALCAM</td>
<td>Activated leukocyte cell adhesion molecule</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ALDH1A1</td>
<td>Aldehyde dehydrogenase 1 family member A1</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD44</td>
<td>Cluster of differentiation 44</td>
</tr>
<tr>
<td>CD133</td>
<td>Cluster of differentiation 133</td>
</tr>
<tr>
<td>CD166</td>
<td>Cluster of differentiation 166</td>
</tr>
<tr>
<td>CK20</td>
<td>Cytokeratin 20</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>DAAA</td>
<td>Dansyl amino-acetylaldehyde</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ESA</td>
<td>Epithelial surface antigen</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Hes-1</td>
<td>Hairy and enhancer of split 1</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory concentration for 50% of cells</td>
</tr>
<tr>
<td>ISC</td>
<td>Intestinal stem cell</td>
</tr>
<tr>
<td>Lgr5</td>
<td>Leucine-rich repeat containing G-protein coupled receptor 5</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multi-drug resistance protein 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRPI</td>
<td>Multi-drug resistance associated protein 1</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>P-gp</td>
<td>Permeability glycoprotein</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient derived xenograft</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SCM</td>
<td>Stem cell media</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SP</td>
<td>Side population</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-base, acetic acid and EDTA buffer</td>
</tr>
<tr>
<td>ULA</td>
<td>Ultra-low adhesion</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless related integration site</td>
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INTRODUCTION

Tumour heterogeneity and drug resistance are important factors impacting the treatment of advanced colorectal cancer (CRC). Current treatment methods include surgical resection, chemotherapy and/or radiation therapy. Although aggressive treatment is often effective, approximately 30 - 50% of patients with advanced CRC will develop recurrent tumours [1], [2]. The high recurrence rate and tendency for the cancer to metastasize has a negative impact on patient survival. In fact, CRC is the second and third leading cause of cancer-related deaths, in Canadian men and women respectively [3].

Cancer stem cells (CSCs) are a subpopulation of tumour cells with the capacity to self-renew and divide asymmetrically. They were first identified in acute myeloid leukemia [4] using fluorescent activated cell sorting. Since then, CSCs have also been identified in a number of solid tumours including but not limited to: brain [5], breast [6], prostate [7], and colorectal [8], [9]. The identification of CSCs is typically achieved by enriching for a subpopulation of cells that highly express markers shared in common with normal stem cells. These subpopulations have consistently formed tumours in immunodeficient mice using fewer cells than unsorted populations. The tumours formed by CSCs recapitulate the heterogeneity of the tumour they were originally isolated from [5]–[7], [10], [11]. The in vitro sphere formation assay is also used to infer tumourigenicity and enrich for CSCs [12], [13] as it relies on the ability to self-renew.

The CSC population is proposed to be highly resistant to chemotherapy [14]–[16]. If CSCs survive treatment they can undergo asymmetrical cell division and repopulate the tumour. Thus CSCs are believed to play an important role in disease recurrence. In
general, cancer cells can utilize a number of mechanisms to evade chemotherapy. The CSC subpopulation is shown to have heightened protection in comparison to the bulk of tumour cells [17]–[19]. ATP-binding cassette (ABC) transporters are a family of drug efflux pumps that actively rid the cell of both endogenous compounds and xenobiotics [20]. Several chemotherapy agents, and their metabolites, are known ligands for ABC transporters [20], [21]. ABC transporter expression and activity is a common resistance mechanism for many cancer cells. Functional assays can be used to identify a population of cells with high ABC transporter expression and this population has properties consistent with CSCs [19], [22].

The intracellular enzyme, aldehyde dehydrogenase (ALDH), is another CSC marker with a potential functional role in drug resistance. ALDH detoxifies reactive aldehydes, which are formed by excessive oxidative stress, or chemotherapy metabolism [23]. ALDH1A has been used to identify CSCs from a number of cancer types including: breast [24], [25], bladder [26], pancreatic [27] and colorectal [10], [28].

Our laboratory previously enriched for CSCs from two human colorectal cancer cell lines, HCT116 and SW480, using the sphere formation assay [29]. This thesis aims to characterize the parental and CSC enriched cell lines through examining the expression of CSC markers, determining their sphere formation ability, and evaluating the cellular responses to chemotherapy. The commonly employed chemotherapeutics; 5-fluorouracil, cisplatin and epirubicin were included in this study. This thesis provides evidence that chemotherapy affects colorectal CSC properties including the ability to self-renew and the expression of multi-drug resistance genes. Through examining the relationship
between properties of CSCs and responses to chemotherapy, this thesis aimed to illustrate correlations between drug resistance and CSCs.
LITERATURE REVIEW

The Healthy Colon

Hierarchical organization of intestinal crypts

The intestinal epithelium consists of multiple cell types with varying degrees of differentiation. A cellular hierarchy exists from the intestinal stem cell (ISC) population residing at the base of the crypts to the mature differentiated cells located at the lumen [30], [31]. The ISC population is capable of self-renewal and dividing asymmetrically to give rise to the transit amplifying cells lining the lower half of the crypts. These cells proliferate and migrate towards the lumen where they are eventually sloughed off allowing the colonic epithelium to be renewed every 3-5 days [32]. As cells move up the crypts towards the lumen they differentiate into the more specialized cell types of the colon, and lose their capacity to proliferate [30]. A number of important signaling pathways are responsible for maintaining the cellular hierarchy and normal colon homeostasis. Dysregulation of cell signaling pathways, along with other disruptions in normal cellular processes, leads to uncontrolled proliferation and ultimately the development of colorectal cancer (CRC) [33].

Intestinal stem cells and signaling pathways

Stem cells are defined by their ability to self-renew and undergo asymmetrical cell division to generate progenitor cells that can further differentiate [34], [35]. ISCs are multipotent and therefore can only generate the cell types in the intestinal epithelial crypts [36], [37]. Generally stem cells are believed to exist in a highly quiescent state and are long-lived [36], [37], allowing them to provide progenitor cells when required.
Dye retention can be used to trace the stem cell lineage and highlights the slow turnover rate [38]. However, the ISC population has an average cycling time of one day [31] which is much shorter than the average cycling every 57 days for hematopoietic stem cells [39]. The quicker cycle is likely due to the homeostatic role of ISCs in replenishing the intestinal crypts.

A number of techniques can be used to identify the ISC population. Often the expression of cell surface markers, specific to a slow cycling population, are used to identify ISCs. Stem cell-related transcription factors can also be used. A number of ISC markers have been proposed, with the most widely regarded being LGR5 (leucine-rich repeat containing G-protein coupled receptor 5) for both mouse [31], [40], [41] and human [42] ISCs. Other markers that have been used to identify the ISC population include Musashi-1 [43], Hes-1 [44], Ephrin type B (EphB) receptor [30], [45], Bmi-1 [41], CD166/ALCAM [46], and aldehyde dehydrogenase (ALDH) 1 [28], [47], [48]. All of these markers are recognized for their high expression in ISCs although there are discrepancies in their ability to uniformly identify the ISC population. For example, it has been shown that Bmi-1 and LGR5 are expressed by two distinct populations with different functional characteristics [41]. LGR5 appears to mark a more rapidly proliferating population than the quiescent cells expressing Bmi-1 [41], [49]. Bmi-1+ cells can acquire an active phenotype and give rise to LGR5+ cells [41]. However, LGR5+ cells can also give rise to Bmi-1+ cells [50] and a single LGR5+ cell can form crypt structures that consist of differentiated cell types when grown in organoid culture [40]. It is evident that both Bmi-1+ and LGR5+ cells can be used to identify ISCs.
although it is unclear as to which is a more ISC putative marker. This also highlights the plasticity among the ISC population [51]–[53].

The location of ISCs is critical for their homeostatic functions. ISCs are located in close proximity to stromal cells which secrete factors stimulating signaling pathways required to maintain the stem cell state [31], [54]. Thus the microenvironment is of critical importance to ISC function and maintenance. Many of the proposed ISC markers are targets of stem cell signaling pathways. For example, EphB receptors [30] and LGR5 [37] are targets of Wnt/β-catenin signaling.

The Wnt/β-catenin signaling pathway is crucial in maintaining the cellular hierarchy of the colonic epithelium. Wnt pathway activation is observed at the base of the intestinal crypts where the ISC population resides [31] in close proximity to the stroma containing abundant Wnt factors [55]. Overexpression of β-catenin induces the expression of Wnt target genes and increases the proportion of stem cells [34]. As cells migrate upwards toward the lumen of the intestine, they lose their proximity to Wnt factors. This decrease in Wnt signaling is believed to drive the differentiation in the intestinal epithelium [32].

Homeostatic renewal of the intestinal epithelium involves the crosstalk between Wnt signaling and a number of other developmental signaling pathways. Therefore, Wnt signaling is not the only pathway responsible for maintaining the stem cell state. Bone morphogenetic protein (BMP) signaling suppresses the Wnt pathway, which effectively inhibits self-renewal [56]. Stromal cells in the colonic crypts express BMP antagonists, such as gremlin 1, gremlin 2 and chordin-like 1 [57] that activate Wnt signaling in normal intestinal epithelial cells and also inhibit the differentiation of the CRC cell line Caco-2 in
vitro [57]. Along with Wnt and BMP signaling, the Notch [44], [58] and transforming growth factor-β (TGF-β) signaling pathways also act to maintain the ISC state [48]. The ISC marker Hes-1 is a target of Notch signaling [44] and the deletion of Hes-1 led to excessive goblet, enteroendocrine and paneth cells in the intestinal epithelium [59], highlighting its role in maintaining the stem cell state.

Collectively these signaling pathways actively maintain the ISC state. However, their dysregulation is often involved in the development and progression of CRC [60]. Uncontrolled cell growth resulting from aberrant cell signaling is considered a hallmark and early event in the development and progression of many cancers [61]. Specifically for CRC, disruption in Wnt signaling often occurs early [62]. The progression of CRC follows a series of alterations and is referred to as the adenoma to carcinoma sequence [33]. The progression from normal colonic epithelia to dysplasia, to adenoma and eventually carcinoma results from mutations in tumour suppressor or oncogenes including k-ras, p53, and Smad4 [33], [47], [63]–[65]. Ongoing genomic instability and uncontrolled proliferation ultimately results in CRC [33].

**Colorectal Cancer**

CRC is the second and third most commonly diagnosed cancer in Canadian men and women respectively [3]. It is a highly prevalent disease with approximately 25,000 Canadians expected to be diagnosed in 2015 [3]. Despite aggressive treatment options including surgery, radiation and chemotherapy, approximately 30 - 50% of patients will develop recurrent tumours [1], [2]. Recurrent disease has poor survival rates and is often more aggressive than the primary tumour [2]. Detection of CRC in the more advanced
stages, as well as the high recurrence rate and tendency to metastasize, has a negative impact on patient survival. In Canadian men and women, CRC is the second and third leading cause of cancer-related deaths respectively [3].

The development of CRC is influenced by a number of contributing lifestyle factors including obesity, physical inactivity, dietary influences and smoking [66], in addition to hereditary or disease-related aspects. Early detection generally results in a good prognosis and current detection methods include the fecal occult blood test or fecal immunochemical blood test and colonoscopy. New detection strategies are focused on less invasive techniques with equal sensitivity. The stool test for CRC has received FDA approval in the United States for average risk individuals that are over 50 years old. The stool test has been demonstrated to have equal sensitivity to a colonoscopy [67]. The development of these less invasive and easily compliant techniques will greatly impact the prognosis of CRC as the outcome is more favourable if detected in the early stages [3]

**Treatment of colorectal cancer**

Currently CRC is treated with surgical resection when feasible, followed by or in conjunction with radiation and/or chemotherapy. The first-line chemotherapeutic for CRC is 5-fluorouracil (5-FU), or its pro-drug form capecitabine (Xeloda). 5-FU is a uracil analogue that can be incorporated into DNA or RNA, interfering with DNA replication [68]. 5-FU also inhibits thymidylate synthase to prevent nucleotide synthesis [68], [69].

Often a combination approach is used to treat CRC. Other chemotherapeutic compounds that offer synergistic effects are used in conjunction with 5-FU. Folinic acid
(leucovorin) stabilizes the inhibition of thymidylate synthase and increases the efficacy of 5-FU [70]. Another commonly used agent to treat CRC is irinotecan. Irinotecan inhibits topoisomerase I, acting at a separate site from 5-FU to induce DNA damage [68]. The combination of 5-FU, folinic acid and irinotecan, is referred to as FOLFIRI therapy.

Oxaliplatin, another approved agent to treat CRC, is an analogue to the commonly used chemotherapy drugs cisplatin and carboplatin. These compounds induce DNA damage and apoptosis through intercalating into DNA and causing intra-strand or inter-strand crosslinks [71], [72]. Carboplatin forms the same types of DNA adducts as cisplatin but is less nephrotoxic or neurotoxic [72]. The combination of 5-FU, oxaliplatin and folinic acid is also often used to treat CRC and is referred to as FOLFOX therapy. Any other combination of 5-FU, or an analogous compound, and the other agents mentioned above, could also be used to treat CRC [73].

Although chemotherapy is often initially useful, cells can become refractory to treatment rendering chemotherapy ineffective. There are a number of ways that cancer cells can resist chemotherapy and this resistance can be either intrinsic or acquired through exposure [71]. Cells can increase levels of anti-apoptotic proteins such as Bcl-2 and decrease pro-apoptotic proteins such as bax to confer resistance to 5-FU [74]. The p53 status of tumour cells influences their sensitivity to chemotherapy, with cells with low levels of p53 are more sensitive to 5-FU treatment [70]. The chemotherapeutic target can also be increased to confer resistance as cells with high levels of thymidylate synthase are more resistant to 5-FU [75]. A number of other genes involved in RNA processing, cell-cycle regulation, apoptosis and DNA replication can be up-regulated to confer resistance to 5-FU [76]. Combination therapy that acts at separate sites to induce
cell death reduces the development of drug resistance and increases efficacy [68].

Generally, agents from different drug classes do not have overlapping resistance profiles as is the case for oxaliplatin and irinotecan [77]. Often even agents in the same drug class will have different resistance profiles. For instance, cancer cells resistant to cisplatin are often sensitive to its analogue oxaliplatin [78]. Cancer cells can also achieve resistance through drug efflux, particularly through the P-glycoprotein (P-gp) transporter. Inhibition of P-gp can overcome resistance to the commonly used anthracycline agents, epirubicin or doxorubicin [79], [80]. Many chemotherapeutic agents, such as epirubicin, also induce the formation of reactive oxygen species (ROS) and therefore up-regulation of antioxidant enzymes can also confer chemoresistance [81], [82].

Tumours are comprised of a heterogeneous neoplastic cell population [83], [84] which extends to drug resistance. Within a tumour, a subpopulation of cells can survive chemotherapy while others are sensitive and effectively eradicated. A small subpopulation of cancer cells, referred to as cancer stem cells (CSCs), are believed to have heightened protective mechanisms allowing them to survive chemotherapy treatment.

**Cancer stem cells**

*History and identification methods*

There are two separate models to describe the origin of CSCs, referred to as the stochastic and the hierarchal model. The stochastic model proposes all tumour cells are equivalent and their behaviour is influenced by both intrinsic signaling pathways and extrinsic factors, such as the tumour microenvironment [84]. This model states that
tumour-initiating activity cannot be enriched for, as all cells are equally capable given the right conditions. On the other hand, the hierarchal model states that tumours are composed of distinct subpopulations. According to the hierarchal model, a subpopulation of cells with tumour-initiating ability can be identified using functional and phenotypic assays that exploit properties shared with normal stem cells [84]. Evidence has been provided to support both models. An overwhelming number of papers have successfully enriched for a small subpopulation of cancer cells with the ability to initiate tumours from both established cell lines [11], [85] and primary patient samples [6], [9]. In addition, tumours arising from the enriched subpopulation have been shown to recapitulate the heterogeneity of the tumour they were originally isolated from [5]–[7], [10], [11] supporting the hierarchal model.

CSCs have been implicated in tumour formation, metastases and disease recurrence [84]. The origin of CSCs is still actively debated. CSCs could arise from neoplastic transformation in the normal stem cells [86], [87] or in the immature transit-amplifying cells [87]. Normal ISCs are long-lived and therefore have a greater chance of acquiring mutations that could be passed on to their progeny [47], [65]. For example, APC mutations have been shown to expand the crypt basal cell population where ISCs reside [88]. Mutations that activate self-renewal pathways could also arise in the transit amplifying cells, allowing for uncontrolled proliferation [84], [87]. However, the transit amplifying cells are quickly lost due to the constant renewal of the colonic epithelium and therefore it is less likely that the cells will persist long enough to progress to CRC [53].
CSCs were first identified roughly 20 years ago in acute myeloid leukemia [4]. Researchers used fluorescent activated cell sorting (FACS) to separate leukemia cells based on their expression of CD34 (a surface marker that is normally expressed on progenitor and pluripotent bone marrow cells) and CD38 (a surface marker expressed on differentiated immune cells). Lapidot et al determined that a very small subpopulation of the leukemia cells were CD34⁺/CD38⁻, an immature phenotype. When subsequent xenograft experiments were performed with the sorted cell populations using NOD/SCID mice, it was found that only the CD34⁺/CD38⁻ population was able to establish a human leukemia. The other populations failed to produce a new cancer in the mice [4]. This CD34⁺/CD38⁻ subpopulation of leukemia cells was denoted cancer initiating cells, or cancer stem cells (CSCs). A number of studies have since performed similar experiments and identified CSCs in several solid tumours types including: medulloblastoma and glioblastoma [5], breast [6], pancreatic [89], prostate [7] and colorectal [8], [9], among many others.

Sorting cancer cells based on their expression of surface markers shared in common with normal stem cells, remains the most frequently used method to identify CSCs. Performing xenograft experiments to determine the tumourigenic ability is the gold-standard experiment to confirm a successful enrichment for CSCs. Enrichment for tumourigenic ability is best highlighted by the requirement of a large number of cells to be implanted from unsorted or negative populations, when compared to the isolated CSC subpopulation. Results from xenograft experiments show CSCs can form heterogeneous tumours that are representative of the tumour they were originally isolated from [5]–[7], [10], [11]. For example, breast cancer cells isolated from a xenograft tumour derived
from CD44+/CD24− cells were shown to have the same percentage of cells expressing these markers as the original patient tumour. In addition, the CD44+/CD24− population maintained their tumourigenicity while the other populations that arose from these cells were non-tumourigenic [6]. The ability of CSC subpopulations to form heterogeneous tumours that consist of both tumourigenic and non-tumourigenic populations has led to the implication of CSCs in tumour formation as well as metastases, and supports the hierarchal model of tumour heterogeneity.

Currently, identification of the CSC population relies heavily on the expression of CSC surface markers. Robust CSC markers have been identified for leukemic (CD34+/CD38−) [4], breast (CD44+/CD24−) [6] and brain (CD133) [5] CSCs, however uniform CSC markers for other cancer types are still under investigation. Many colorectal CSC markers have been proposed and their ability to robustly identify the colorectal CSC population is discussed below.

In addition to surface marker expression, CSCs can also be identified using an in vitro sphere formation assay. This consists of plating a small number of cancer cells in anchorage-independent, serum-free, conditions. A media containing fibroblast growth factor (FGF) and epidermal growth factor (EGF), factors which are needed to maintain stem cells, is used [7], [8], [90]. Only cells with stem cell properties will be able to survive and proliferate under these conditions [90] and they will give rise to spheres. This method has been used successfully to enrich for cancer cells with a CSC phenotype from prostate [12], [13], breast [91], glioblastoma [92] and colorectal cancers [17], [93] among others.
**Colorectal CSC Markers**

A number of cell surface markers have been proposed for colorectal CSCs including: CD133 [8], [9], CD44 [10], [94], EpCAM [35], [93], CD166 [95], LGR5 [45], [96] and aldehyde dehydrogenase 1 (ALDH) [28]. The identification of a CSC marker is based on the observation that positive populations initiate tumours in xenograft experiments while negative populations are unable to form tumours. The CSC marker is deemed questionable when the negative population can also initiate tumours, as is seen more often in established cell lines [97]. However, the negative population often requires more cells to initiate tumour formation and many studies support the enrichment of CSCs from established cell lines [11], [19], [27], [85]. Although primary patient samples and patient derived xenografts (PDX) are the optimal samples to study CSCs, established cell lines are also a useful tool as they have been well characterized and may elucidate links between mutational status and CSCs [98].

A major issue affecting the identification of colorectal CSCs is the significant tumour heterogeneity among patient samples. Not all tumours express all of the proposed colorectal CSC markers and they are often expressed in varying amounts. Tumour heterogeneity stresses the requirement for use of multiple markers when identifying CSCs. Colorectal CSC markers need to be robust, and if possible, ubiquitously expressed, to be able to be applied across patient samples.

CD44 expression is used to identify CSCs from a number of different cancer types including breast [6], [99], prostate [7], gastric [16] and colorectal [94], [95]. CD44 is a receptor for hyaluronic acid and has a role in normal cell-cell adhesion [100]. CD44 also has a role in proliferation, motility, adhesion and cell survival in cancer [101], [102] and
is expressed at higher amounts in colorectal cancerous tissue compared to adjacent normal tissue [103]. CD44\(^+\) cells isolated from primary colorectal tumours have been shown to have increased tumourigenic ability [94], [95]. As few as 100 CD44\(^+\) cells isolated from a primary colorectal tumour initiated tumour formation in mice [10], [94] and a single CD44\(^+\) cell could form a sphere \textit{in vitro} [94]. CD44\(^+\) cells also have enhanced colony formation [10], [94], [104] and can be serially passaged \textit{in vivo} [10], [94] demonstrating their ability to self-renew. In addition, CD44\(^+\) cells form heterogeneous tumours \textit{in vivo} that contain CD44\(^-\) cells [10]. This finding supports the ability of CD44\(^+\) cells to produce other cancer cell types, a property of CSCs. In breast CSCs, knockdown of CD44 resulted in the acquisition of a non-CSC phenotype, with decreased tumourigenic ability and lower levels of CSC related gene expression [99]. This finding is supported in CRC, where CD44 knockdown in primary samples inhibited colony formation and tumourigenicity [94].

Another commonly employed CSC marker is CD133, also known as prominin-1 or AC133. CD133 was first identified as a CSC marker in medulloblastomas and glioblastomas [5] and has since been used to identify CSCs in prostate [7], [105], pancreatic [106] and colorectal [8], [9] cancers. Unlike CD44, the functional role of CD133 remains unknown [100]. CD133 is also expressed in normal colon tissue [107] but with fewer cells expressing CD133 than in tumour tissue [9]. CRC cells expressing CD133 have been shown to be negative for the differentiation marker cytokeratin 20 (CK20) [108]. CD133 was the first CSC marker used to identify colorectal CSCs, where as few as 1000-3000 CD133\(^+\) cells were capable of initiating tumours in NOD/SCID mice [8], [9]. However, more recent studies have questioned the suitability of CD133 to
identify colorectal CSCs as they have been unable to enrich for tumourigenic ability using only CD133 [10]. It has also been determined that the CD133− population can also establish tumours in NOD/SCID mice [107], [109]. In addition, knockdown of CD133 did not affect colony formation or tumourigenic ability [94]. CD133 expression is highly heterogeneous in both established CRC cell lines [104], [110] and in primary patient samples [95]. In the SW620 CRC cell line, the CD44+/CD133− subpopulation correlated with most of the features of CSCs including a more invasive and migratory phenotype, increased colony formation, and the ability to resist cell death induced by the topoisomerase inhibitor camptothecin [104]. The CD133 population generally composes a larger subset of cells than other CSC marker populations such as ALDH1 [28] and there is a lack of CD133 and CD44 co-localization in patient samples [28], [94]. Therefore, CD133 does not appear to be as robust a CSC marker for CRC as was initially proposed.

CD166/ALCAM has also been identified as a colorectal CSC marker [95]. CD166 is highly expressed by normal ISCs and co-localizes with other ISC markers LGR5 and Musashi-1 [46]. CD166 is heterogeneously expressed in CRC and increased levels are associated with poor outcome [111]. CD166 is not as well studied as the other proposed colorectal CSC markers, although it has been shown that CD44+/CD166+ CRC cells had increased tumourigenic ability [95]. CD166 was also expressed on single-cell derived colonospheres along with CD133, CD44 and LGR5 among other stem cell markers [112]. Currently, no studies have single stained for CD166 to isolate a subpopulation and evaluate tumourigenic ability through xenotransplantation therefore its functional role as a colorectal CSC marker remains to be discovered.
Epithelial cell adhesion molecule (EpCAM), also known as epithelial surface antigen (ESA), has been shown to be expressed at high levels in colorectal [113] and other epithelial cancers including breast [114], ovarian [113] and gastric cancers [113], [115]. EpCAM is involved in organization of the intestinal epithelium [116] and regulates cell cycle progression through cyclin D1 [117]. Overexpression in primary human mammary epithelial cells led to increased proliferation in vitro [118]. Blocking EpCAM overexpression in gastric cancer cells inhibits proliferation [115]. EpCAM is an epithelial marker, but has been shown to be overexpressed in the CSC population [93]. In addition, overexpression of EpCAM is associated with poor survival in breast cancer [114]. Human CRC HCT116 cells grown as spheres had higher protein expression of EpCAM, along with higher expression of other colorectal CSC markers including CD44, Musashi-1 and LGR5 [93].

Most of the studies that have reported increased tumourigenic ability in EpCAM\(^\text{high}\) cells also co-sorted with a second CSC marker that is likely more indicative of tumourigenic ability [10], [95], [119]. In established CRC cell lines, including HCT116 and SW480, virtually all cells express EpCAM [104]. Immunohistochemical analysis of over 2000 primary tumour samples revealed that EpCAM is highly expressed in most gastrointestinal tumours, but absent in most hepatocellular carcinomas [113]. Therefore, EpCAM may be a more suitable marker for CSCs in hepatocellular carcinoma cells [120] or other cancers where EpCAM expression is low or absent in normal tissue.

Aldehyde dehydrogenase 1 (ALDH1) is another commonly used marker to identify CSCs from numerous cancer types including but not limited to, ovarian [121], bladder [26] and colorectal [28]. ALDH1 is also a marker for normal ISCs [28]. The
utilization of ALDH to identify colorectal CSCs is discussed in detail later in this review as ALDH may also have a functional role in drug resistance.

The normal ISC marker LGR5, has also been identified as a colorectal CSC marker [45], [93], [96], [122]. LGR5 expression is higher in CRC cell lines when grown as spheres compared to monolayer culture, and silencing LGR5 diminished sphere formation [96]. LGR5 expression is correlated with CRC stage and metastasis, contributing to the development of CRC [123]. The other normal ISC marker, Bmi-1 has also been used to identify colorectal CSCs [124].

In addition to the markers discussed above, gene expression profiles can also be used to identify the colorectal CSC population. The expression of transcription factors associated with normal stem cells including OCT4, Nanog, or SOX2 is enriched in the CSC population [12], [15], [45], [122]. The heterogeneous expression of markers, and conflicting results regarding the tumourigenic ability of isolated subpopulations, interferes with the identification of true colorectal CSCs. In addition, few studies have looked at the functional role of these markers in CSCs.

**Issues with CSCs: Drug resistance**

CSCs have been implicated in disease recurrence following chemotherapy treatment due to their heightened protective mechanisms and slower division rate compared to the bulk of tumour cells. The agents commonly used to treat advanced CRC (5-FU and oxaliplatin) belong to a category of traditional chemotherapeutics that rely on the hallmark that cancer cells are rapidly proliferating [61]. However, cell cycle analysis has revealed that CSCs are slow cycling compared to the bulk of tumour cells [125] and
resting in the G0/G1 phase [126]. Many traditional chemotherapeutics induce damage in the growth phase of the cell cycle, disrupting DNA synthesis or replication [68], [71]. Therefore, the dormant CSC population may not be targeted by these therapies. Chemotherapy has proven effective at reducing the bulk of the tumour [1], [68]. However, this reduction could be insufficient since xenograft studies have shown that a very small number of colorectal CSCs, as few as 100 CD44+ cells from a patient’s tumour sample, can establish a new tumour in mice [94]. Thus, if the majority of tumour cells are effectively abolished by chemotherapy, and the resistant CSC population remains, the tumour could recur [35], [127].

If the slow dividing nature of CSCs does not allow them to evade chemotherapy they could still resist cell death through various mechanisms of drug resistance. There are a number of ways that cancer cells in general can become resistant to chemotherapy but CSCs are believed to have heightened protective mechanisms compared to the general cancer cell population. There are two important resistance mechanisms that serve as functional markers for the CSC population, over-expression of ATP-binding cassette (ABC) transporters, and high expression or activity of ALDH. Figure 1 is a schematic diagram highlighting the role of these markers in drug resistance.

**Multi-drug resistance drug efflux pumps**

ABC transporters are a family of ATP-dependent efflux pumps that have a protective role in both normal [20], [128] and cancer cells [100], [129]–[131]. The ABC transporter superfamily is organized into 7 families denoted A-G and consists of 48 genes, 16 of which are implicated in drug resistance [129], [132]. Each family member
has unique ligands and tissue dependent expression [20], [132]. ABC transporters are able to efflux endogenous compounds and xenobiotics from the cell [20], [132]. A number of ABC transporter family members are up-regulated in cancer and many chemotherapy agents are known ligands [20], [21].

The most notable family member is \textit{ABCB1} or \textit{MDR1} (multi-drug resistance protein 1), which encodes the P-glycoprotein (P-gp) pump. P-gp is up-regulated in many cancer types including colon, breast, and ovarian [129]. The anthracycline antibiotics, epirubicin and doxorubicin, as well as paclitaxel are known substrates for P-gp [20] and resistance to these compounds is associated with overexpression of P-gp [133], [134]. The chemotherapeutics gemcitabine [135], paclitaxel [135], epirubicin [134], [135] doxorubicin [133], and cyclophosphamide [134] have all been shown to induce expression of \textit{ABCB1}.

Other ABC transportor family members, including \textit{ABCC1/MRP1} (multi-drug resistance related protein 1) [20], [136] and \textit{ABCG2/BCRP} (breast cancer resistance protein) [20], [21], are also up-regulated in human cancers. Vincristine is a ligand for \textit{ABCC1} [20]. \textit{ABCG2} is encoded by the mitoxantrone resistance gene (\textit{MXR}) and overexpression in breast cancer cells is responsible for resistance to topotecan, mitoxantrone and anthracycline antibiotics [20], [21], [137]. Up-regulation of \textit{ABCG2} is most commonly found in breast cancer, however it has also been detected in lung and ovarian cancers [129]. In colorectal cancer, strong membranous \textit{ABCG2} staining significantly correlated with stage, lymph node metastasis and distant metastasis [138]. The use of ABC transporter status as a biomarker or to predict outcome to therapy has
Figure 1. ABC transporters and ALDH serve a protective role in cancer cells. Chemotherapy can induce the formation of reactive oxygen species (ROS), which at high levels are toxic to the cell inducing oxidative damage to lipids, proteins and DNA. Lipid peroxidation produces a number of reactive aldehydes that can further induce cellular damage. Aldehyde dehydrogenase (ALDH) is an enzyme responsible for the detoxification of aldehydes, catalyzing the conversion to the less reactive carboxylic acids that can subsequently be excreted. Thus ALDH serves as a protective mechanism against the indirect effects of chemotherapy. ATP-binding cassette (ABC) transporters are ATP-dependent drug efflux pumps that actively remove xenobitoics, including the chemotherapeutics epirubicin, and paclitaxel among others. ABC transporters effectively reduce intracellular drug concentrations having an important role in drug resistance.
been investigated [139]. The mRNA and protein levels of various ABC family members have been explored, but a reliable biomarker has yet to be determined [140], [141]. In addition, attempts to inhibit ABC transporters as a chemotherapeutic approach have been heavily investigated however these have failed due to widespread adverse effects in non-target tissues [129].

**Using drug efflux pumps and the side population to identify CSCs**

A functional assay to determine ABC transporter activity is the Hoechst 33342 dye exclusion assay. The ability of cells to efflux Hoescht dye is dependent on ABC transporter expression. Fluorescent Hoechst dye intercalates into DNA and affected cells will stain positive, which can be detected using flow cytometry. Cells with high expression of ABC transporters can be isolated as they remain unstained, and are referred to as the side population (SP) [128]. Hoechst dye exclusion was originally utilized to identify hematopoietic stem cells through detecting slow cycling cells as they would have little to no staining [142]. SP cells have since been isolated from a number of tissues and are shown to consist of immature cells expressing stem cell markers [128].

Due to the overlapping substrates of ABC transporters it is difficult to determine which family member is responsible for Hoechst dye efflux. Verapamil, a specific inhibitor of ABCB1 eradicated the SP in hematopoietic stem cells [142] suggesting ABCB1 is responsible for Hoechst efflux. However, more recently gene expression analysis of ABCB1, ABCC1 and ABCG2 in hematopoietic SP cells revealed that only ABCG2 was increased compared to non-SP cells [143]. Studies using an ABCG2 inhibitor, fumitremorgin C, have also shown strong inhibition of Hoechst dye efflux in
breast cancer cells [144]. Another study with human breast cancer cell lines revealed a 30-fold increase in \( ABCG2 \) gene expression in the isolated SP (which comprised only ~1% of all Cal-51 cells) [145] further supporting the role of ABCG2 in Hoechst dye efflux.

Since the SP was originally used to isolate normal stem cells, its ability to isolate CSCs has also been investigated [146]–[148]. In colorectal cancer cells, the SP has higher expression of ABCB1, ABCG2, as well as the CSC markers OCT4, Nanog, SOX2, CD44 and CD133 [148]. Isolated SP cells from human lung cancer cell lines had elevated expression of \( ABCB1, ABCC1 \) and \( ABCG2 \) and were enriched in many CSC properties including increased tumourigenic ability as well as the ability to give rise to non-SP cells [147]. The SP isolated from lung, prostate, sarcomas and colorectal cancer cells are highly tumourigenic \textit{in vivo} [19], [147]–[149]. In addition, only the SP cells can give rise to spheres \textit{in vitro} compared to non-SP cells [150] and they are also capable of generating non-SP cells [151]. In the SP isolated from SW480 CRC cells, more cells were in the G0/G1 phase of the cell cycle than non-SP cells [148]. Collectively, these data provide evidence that the SP consists of cells with CSC properties. Therefore, Hoechst dye exclusion, or determining ABC transporter mRNA or protein levels, could be used to identify CSCs. This method of identification infers that CSCs could be highly drug resistant, particularly to agents that are ligands for ABC transporters. In fact, SP cells isolated from CRC were resistant to 5-FU and cisplatin [148]. SP cells from a hepatocellular carcinoma cell line were also resistant to 5-FU, gemcitabine, and doxorubicin [151].
Aldehyde dehydrogenase: a functional CSC marker

ALDH is an NAD-dependent, intracellular enzyme that is responsible for the conversion of reactive aldehydes to carboxylic acids [152], [153]. Aldehydes can be generated by normal cellular processes through the metabolism of lipids, amino acids, carbohydrates, vitamins and steroids [154] as well as through the metabolism of xenobiotics [152]. Aldehydes are highly reactive electrophiles that can induce a variety of cellular damage [152]. A number of carcinogenic compounds, such as polyaromatic hydrocarbons, produce aldehydes that can induce DNA damage [154], [155]. In addition, chemotherapy can also produce reactive aldehydes either directly through metabolism by cytochrome P450 enzymes, or indirectly through the production of reactive oxygen species (ROS) [152]. High levels of ROS lead to lipid peroxidation that also produces many reactive aldehydes [23], [156]. Cyclophosphamide is one chemotherapeutic that has been shown to generate toxic aldehydes that are substrates for ALDH enzymes [152].

The ALDH superfamily is organized into 11 families denoted 1-11, and consists of 19 different genes [152], [157], [158]. ALDH family members differ in substrate specificity, function, and tissue distribution [153], [154]. During development, ALDH enzymes (particularly those of the 1A family) play a critical role in differentiation signaling pathways through catalyzing the conversion of retinaldehyde to retinoic acid. Retinoic acids are critical in development regulating the differentiation of stem cells [159]. Retinoic acids have also been used therapeutically to induce the differentiation of promyelocytic leukemia cells into neutrophils [160].

ALDH activity was first measured in live cells using dansyl ammino-acetylaldehyde (DAAA), a fluorescent aldehyde substrate [161]. When cells are
incubated with DAAA, ALDH converts DAAA to dansyl glycine, a negatively charged fluorescent compound that remains trapped within the cell, staining cells with high ALDH activity. A negative control, such as 4-(diethylamino)benzaldehyde (DEAB), a specific ALDH1 inhibitor is also included. It was later determined that DAAA was mutagenic, affecting subsequent experiments and thus the Aldefluor® assay was developed. The Aldefluor® assay relies on the same principle as the original assay but uses a non-mutagenic fluorescent aldehyde substrate, BODIPY aminoacetylaldehyde (BAAA) [162]. The Aldefluor® assay is often used to isolate stem cells from numerous tissues, based on their activity of ALDH1.

It is important to note the normal tissue distribution when considering ALDH as a potential CSC marker. It is best used as a CSC marker in tissues that normally have low expression. ALDH1 is essentially absent in normal breast and lung tissue, relatively weak in the normal colon and stomach, and high in the liver and pancreas [121]. ALDH1 in the normal colon epithelium is restricted to a small population residing at the base of the crypts, where the ISC population resides [28], [163]. Hematopoietic stem cells identified as the CD34+ population, also had high ALDH activity [161], [164] supporting ALDH activity as a normal stem cell marker.

The ALDH1A family members have been studied extensively due to their critical role in controlling differentiation in development through retinoic acid signaling. The substrate specificity of ALDH family members can overlap, as is the case for the role of ALDH1A1 and ALDH1A2 in retinoic acid synthesis. Family members can also have different substrate specificity as is evident through the role of ALDH1A1, but not ALDH1A2, in ethanol metabolism [154]. ALDH1A1 catalyzes the conversion of the
ethanol metabolite acetylaldehyde to acetate [23]. Disulfiram, a clinically proven anti-alcoholic medication, inhibits ALDH1A1 [165]. Many other known inhibitors exist that differ in their specificity for ALDH family members [165].

Due to the role of ALDH in normal stem cells, it has also been examined as a CSC marker. Sorting cancer cells based on their ALDH activity enriches for a tumour-initiating population [25]–[27], [166]. In fact, as few as 100 ALDH\textsuperscript{high} cells isolated from primary pancreatic cancers formed tumours in xenograft experiments, regardless of the expression of other pancreatic CSC markers [27].

The ALDH\textsuperscript{high} tumour cells have also been shown to be highly drug resistant in a number of cancer types [25], [167]–[171]. Drug-resistant cancer cells that express other CSC markers also have high ALDH activity or expression and are resistant to chemotherapy. [171]. The ALDH1 population has been shown to increase in breast cancer patients treated with paclitaxel and epirubicin [25]. Inhibitors of ALDH have been used effectively to reverse resistance to chemotherapy [167], [172], [173].

Chemotherapy increases ROS levels in cancer cells [25], [72], [174] which ultimately leads to the generation of reactive aldehydes. Since ALDH is a detoxification method for reactive aldehydes it is proposed that the antioxidant activity of ALDH protects against chemotherapy induced oxidative stress. Gastric cancer cells with high ROS levels also had increased expression of ALDH1A1 compared to cells with low levels of ROS [167]. Other antioxidant enzymes, including glutathione-S-transferase have been shown to protect cells against chemotherapy-induced ROS damage [82].
ALDH3A1 has also been implicated in drug resistance in both lung [175] and prostate cancer [176]. ALDH3A1 has not been well studied in CRC but studies suggest it can also confer resistance to cyclophosphamide in CRC cell lines [177].

High activity or expression of ALDH is a successful method to identify and isolate the CSC population in numerous cancer types [25]–[28]. In addition to its utility as a CSC marker, ALDH could also have a protective role contributing to chemoresistance in the CSC population.

The quiescent state of CSCs, along with their heightened protective mechanisms, warrants the need to target both the CSC population and the rapidly dividing cancer cells, to ultimately achieve disease remission.
RATIONALE

Drug resistance and disease recurrence are major obstacles that negatively affect the outcome of colorectal cancer. The CSC phenotype is suggestive of a particularly resilient subpopulation of tumour cells that may not be adequately targeted by current chemotherapeutics. CSCs are capable of establishing cancers in mice that recapitulate the heterogeneity of the tumour they were originally isolated from. This observation has led to the implication of CSCs in tumour formation, metastases and recurrence. Therefore, CSCs are a critical subpopulation of tumour cells and investigation into their chemoresistance mechanisms is warranted. Although a number of markers have been proposed to identify CSCs, reliable markers for colorectal CSCs have yet to be established. This thesis aimed to characterize two human colorectal cancer cell lines, HCT116 and SW480, and their enriched CSC populations that were previously generated in our laboratory using serial colonosphere culture. Characteristics of CSCs including self-renewal ability, expression of proposed colorectal CSC markers, and mechanisms of drug resistance were explored.

Hypothesis: Colorectal cancer stem cells are more resistant to traditional chemotherapy than their parental cell lines, and this resistance is conferred via an up-regulation of detoxification mechanisms.

Objective 1: Characterize human colorectal cancer stem cells based on proposed CSC markers and the gene expression of ABC transporters and ALDH.

Objective 2: Explore the cellular responses to chemotherapy by evaluating cell viability, self-renewal ability, and the expression of drug resistance related genes.
MATERIALS AND METHODS

(See Appendix I for a list of suppliers for all chemicals and reagents, Appendix II for detailed solution preparation, Appendix III for primer sequences and Appendix IV for details on generation of drug resistant cell lines and other supplementary data)

Monolayer cell culture

Human colorectal cancer cell lines HCT116 and SW480, referred to as the parental cell lines (purchased from the American Type Cell Culture) were maintained under standard culture conditions. Normal culture media consisted of Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% sodium pyruvate. Cells were maintained in 10 cm tissue culture plates in a 37°C humidified incubator with 5% CO₂. After forming a confluent monolayer, cells were rinsed with phosphate buffered saline (PBS) then exposed to 3 mL trypsin for ~4 minutes to detach. An equal volume of standard media was used to deactivate the trypsin and cells were centrifuged at 400 x g for 4 minutes. Cells were counted by combining 10 µL cell suspension with 10 µL Trypan Blue (Life Technologies) and using the automated cell counter Countess™ (Invitrogen) to determine cell number and viability. Cells were re-suspended in standard media and plated at the appropriate densities for subsequent experiments.

Colonosphere formation

Our laboratory previously enriched these two cell lines (HCT116 and SW480) for cancer stem cells [29]. These enriched populations, referred to as HCT116-CSC and SW480-CSC, were maintained as non-adherent colonospheres, grown either in ultra-low adhesion (ULA) plates (Grenier), or in standard uncoated 10 cm petri dishes. Stem-cell
media (SCM) consisted of DMEM/F12 (500 mL) supplemented with fibroblast growth factor (FGF) (10 ng/mL), epidermal growth factor (EGF) (20 ng/mL), and 10 mL B27 supplement (Life Technologies). Cells were plated as single cell suspensions at low cell densities and re-supplemented with SCM every three to four days. Colonospheres were allowed to form for 10 days before they were collected into 15 mL tubes and subsequently passaged. For passage, colonospheres were rinsed with PBS and mechanically dissociated by passing through a 20G syringe to achieve a single cell suspension. Cells were centrifuged at 400 x g for 4 minutes and re-suspended in SCM to achieve the appropriate cell density.

**Limiting dilution analysis**

Single cell suspensions were obtained from monolayer parental cells and 10 day-old CSC colonospheres. Cells were plated at densities of 1, 10 and 100 cells per well (in 200 µL SCM) in 96-well, round-bottom, ULA plates (Grenier). 20 wells were seeded per cell density. Cells were re-supplemented with media every 3-4 days by carefully removing 100 uL and replacing with an equal volume of fresh SCM using a multi-channel pipette. On day 10, wells were scored for the presence or absence of a colonosphere. The experiment was repeated 3 times for each cell line and the average number of positive wells was used to determine the colonosphere formation frequency. Welch’s two sample t-tests were performed using GraphPad Prism 6 software to compare colonosphere formation ability between the CSC and parental cell lines ($\alpha = 0.05$).
RNA extraction and cDNA synthesis

For untreated samples, RNA was extracted from confluent monolayers of parental cell lines and 10 day-old CSC colonospheres. RNA was also extract from confluent CSC cell lines grown in monolayer in either standard media or SCM (Appendix IV: Supplementary Data). For gene expression analysis following 24-hour chemotherapy treatment RNA was extracted from parental cells in monolayer and CSC colonospheres. A Ribozol/chloroform phase separation step was performed prior to use of the Aurum™ Total RNA Mini Kit (BioRad). RNA concentration and purity was determined using the Nanodrop ND-1000 (ThermoScientific) by measuring the absorbance at 230, 260 and 280 nm. RNA was diluted to a concentration of 250 ng/µL, aliquoted and stored at -80°C. 1 µg of RNA was converted to cDNA using the iScript™ cDNA Synthesis Kit (BioRad) according to the manufacturer’s protocol. Samples were diluted in 60 µL of RNAse free deionized water and stored at -20°C.

Gene expression analysis

The CFX96 Touch™ Real-time PCR Detection System (BioRad) was used for all reactions and results were analyzed using CFX Manager™ (BioRad) software. SsoFast™ EvaGreen® supermix (BioRad) was used to complete PCR reactions. ‘No template’ controls were included for each primer set in all experiments. All primer sequences were derived using the Harvard Primer Bank and purchased from Sigma-aldrich (see Appendix III for a complete list of primers and sequences). Specificity for all primer sequences was verified using BLAST® (NCBI).
Primers were optimized using a temperature gradient and a standard curve was generated to determine efficiency. Primer efficiencies between 90-115% were deemed acceptable. RT-PCR products were run on a 1.5% agarose gel (with 5 µL RedSafe) in 1X TAE buffer at 100V for 20 minutes and imaged using a ChemiDoc (BioRad). The size of all amplicons was confirmed using a 1kB DNA ladder (Life technologies). RT-qPCR conditions consisted of an initial denaturation step for 2 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 5 seconds at 60°C and final melt curve analysis. Gene expression was assessed relative to the expression of two housekeeper genes, β-actin and 18S ribosomal RNA, which were deemed acceptable reference genes using qBase Plus Software (BioGazzele). All samples were generated in triplicate and 3 separate RT-qPCR runs were performed for gene expression analysis for each sample. A Kruskal-Wallis test with Dunn’s multiple comparison post-test (CSC vs. parental, or control vs. treated) was performed using GraphPad Prism 6 software to compare gene expression among samples (α = 0.05).

Flow cytometric analysis of CSC markers

Parental cell monolayers were trypsinized and pelleted by centrifugation as described prior to fixation. CSC colonospheres were dissociated into single cell suspensions as described above and pelleted by centrifugation prior to fixation. Cell pellets were re-suspended in 4% paraformaldehyde and incubated at 37°C for 10 minutes then placed on ice for 1 minute prior to re-centrifugation. Paraformaldehyde was removed and cell pellets were re-suspended in PBS and 0.1% sodium azide and stored at 4°C prior to staining. Samples were diluted in PBS to achieve concentrations of 10^6.
cells/mL. Samples were centrifuged at 500 x g for 5 minutes and washed with 1 mL incubation buffer (1.5% BSA in 1X PBS). Samples were blocked in 100 µL incubation buffer for 10 minutes at room temperature prior to centrifugation and re-suspended in antibody diluted incubation buffer at the following concentrations: CD166-Alexafluor488 (1:20), EpCAM-PerCP (1:40) and CD133-PE (1:10) per 10⁶ cells. All samples were protected from light and incubated at room temperature for 10 minutes (CD133) or 30 minutes (CD166/EpCAM). Following incubation, samples were centrifuged, washed with 1 mL incubation buffer, and re-suspended in 500 µL PBS. Samples were protected from light and stored on ice until analysis. Negative unstained controls and single-stained controls were included for each cell line. Analysis was performed using a BD Accuri C6 Flow cytometer (BD Biosciences) and FlowJo software (TreeStar). A Kruskal-Wallis test with Dunn’s multiple comparison post-test was performed using GraphPad Prism 6 software to compare the percentage of positive cells between CSC and parental cell lines (α = 0.05).

**Chemotherapy treatment**

Treatment media consisted of diluting stock drug concentrations (5-fluorouracil 50 mg/mL, cisplatin 1 mg/mL, epirubicin 2 mg/mL; all stocks diluted in normal saline or 5% dextrose in water) in standard media or SCM to achieve the desired µM concentration. Treatment media was made fresh daily. Control media consisted of standard media or SCM without the addition of chemotherapy.
Monolayer dose response curves

Parental and CSC cell lines were plated at 5,000 cells/well in 100 µL standard media using standard 96-well cell culture plates (Grenier) and allowed to adhere overnight. 8 wells were plated per treatment, and control wells were included for each cell line. Blank wells (containing media only) were included in each experiment. The following day media was aspirated and replenished with 100 µL treatment media ranging in doses from 0 – 500 µM. After 72 hours of exposure to chemotherapy, 10 µL PrestoBlue cell viability reagent was added to each well and plates were incubated at 37°C for 4 hours. Absorbance was read using a spectrophotometer at 570 nm and normalized to absorbance at 600 nm. The average absorbance from the blank wells was subtracted and the resulting values were normalized to the average control value for each individual cell line. GraphPad Prism 6 software was used to generate dose-response curves and determine the IC50 value.

3D colonosphere treatment

Cells were plated in 96-well, round-bottom ULA plates at densities of 100 cells/well (parental) or 50 cells/well (CSCs) in 200 µL SCM. 8 wells were seeded per treatment group and control wells were included for each cell line. Blank wells (media only) were also included in each experiment. Media was changed on day 4 by removing 100 µL media and replacing with 100 µL SCM. Colonospheres were treated on day 7 by removing 100 µL media and replacing with 100 µL treatment media in SCM. After 24 hours of exposure to chemotherapy, 20 µL PrestoBlue cell viability reagent was added to each well and plates were incubated for 24 hours at 37°C. Absorbance was read 48, 72
and 96 hours post-treatment at 570 nm and normalized to 600 nm. The average absorbance from the blank wells was subtracted, and the resulting values were normalized to the average control value for each individual cell line. The mean value relative to control is reported. A Kruskal-Wallis test with Dunn’s multiple comparison post-test was performed using GraphPad Prism 6 software to compare cell viability between control and treated samples within each cell line (α = 0.05).

**Annexin V/Propidium iodide staining**

Cell viability was assessed using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Life Technologies). Parental cells grown in monolayer and CSC colonospheres were treated with chemotherapy for 48 hours as described above. Media was collected by centrifugation (800 x g for 8 minutes) combined with trypsinized cells and collected by centrifugation. Cells were washed with PBS and centrifuged prior to resuspension in 100 µL annexin-binding buffer per 10^6 cells. 5 µL Annexin V-AlexaFluor488 and 1 µL 100 µg/mL Propidium Iodide were added to each sample and incubated at room temperature, protected from light, for 15 minutes. Samples were diluted in 400 µL annexin-binding buffer and kept on ice prior to analysis using a BD Accuri C6 Flow cytometer (BD Biosciences) and FlowJo software (Tree Star). Etoposide (50 µM) was used as a positive control for apoptosis. The mean percentage of viable cells relative to control (untreated cells) is reported. A Kruskal-Wallis test with Dunn’s multiple comparison post-test was performed using GraphPad Prism 6 software to compare cell viability between control and treated samples within each cell line (α = 0.05).
Limiting Dilution Analysis post chemotherapy treatment

Parental cell lines were plated in 10 cm standard cell culture plates and allowed to adhere overnight. Media was aspirated and replaced with 8 mL treatment media. After 48 hours of exposure to chemotherapy, cells were collected and counted as described above, and single cell suspensions were achieved. Cells were plated at 1, 10 and 100 cells per well in 200 μL SCM and re-supplemented with SCM every 3-4 days as described above. After 10 days of growth, wells were scored for the presence or absence of a colonosphere. The experiment was repeated 3 times for each cell line and the average number of positive wells was used to determine the colonosphere formation frequency. A Kruskal-Wallis test with Dunn’s multiple comparison post-test was performed using GraphPad Prism 6 software to compare cell viability between control and treated samples within each cell line (α = 0.05).

Clonogenic survival assay

Parental cell lines were plated in 10 cm standard cell culture plates and allowed to adhere overnight. Cells were treated with chemotherapy for 48 hours and collected as described above. Single cell suspensions were achieved and cells were plated at limiting cell densities (10 – 10⁵ cells/well) in 6-well standard cell culture plates. Media was replenished every 3-4 days and adherent colonies were allowed to form for 2 weeks. Wells were fixed and stained using a 20% methanol, 1% crystal violet solution for 10 minutes and rinsed with deionized water. Plates were allowed to dry completely before colonies were counted using a stereomicroscope. Any group of cells with more than 50 cells was considered a colony. The colony formation ability and plating efficiency was
determined using results from the optimal cell density. A Kruskal-Wallis test with Dunn’s multiple comparison post-test was performed using GraphPad Prism 6 software to compare the survival fraction between control and treated samples within each cell line (α = 0.05).
RESULTS

CSC cell lines have superior colonosphere formation ability

The colonosphere formation ability of the CSC and parental cell lines was evaluated by limiting dilution analysis (Figure 3). Cells were seeded at 1, 10 and 100 cells/well in 96 ULA plates and each well was scored for the presence or absence of a colonosphere after 10 days. At low cell densities, CSC enriched cell lines had higher colonosphere formation compared to their respective parental cell lines (*p ≤ 0.05). Sphere formation ability was increased ~2.5 fold for HCT116 and ~13 fold for SW480 cells. When plated at 100 cells/well, colonosphere formation was 100% suggesting a large intrinsic CSC population in both of the parental cell lines (Figure 2).

CSC colonospheres have increased gene expression of CSC markers

RT-qPCR was used to analyze the gene expression of three colorectal CSC markers, CD44, CD166 and EpCAM. The amplification cycle (Ct value) was used to categorize gene expression as very high (Ct = 10 – 20), high (Ct = 20 – 25), moderate (Ct = 25 – 30) and low (Ct > 30). Both CSC and parental cell lines had high expression of CD44 and EpCAM (Table I). SW480-P cells had low expression of CD166, which was increased in SW480-CSCs but this increase was not statistically significant (p > 0.05). The fold change between CSCs and parental cells was calculated relative to the expression of two housekeeping genes β-actin and 18S ribosomal RNA (Figure 4). SW480-CSCs had a mean 3.96 ± 1.64 fold increase in CD44 gene expression compared to SW480-P cells (*p ≤ 0.05). The expression of CD166 and EpCAM was also up-
Figure 2: Images of HCT116 colonosphere formation over 10 days. Images were taken of HCT116 parental cells plated at 100 cells in 96-well ultra low adhesion plates for 10 days.
Figure 3: Limiting dilution analysis. Cells were plated at densities of 1, 10 and 100 cells/well in 96-well round bottom ULA plates. After 10 days of growth, wells were scored for colonosphere formation. The mean number of positive wells ± SEM (n=3) is shown for HCT116 (A) and SW480 (B) CSC and parental cells. There was a significant increase in the frequency of colonosphere formation for HCT116-CSCs compared to parental cells, when plated at the 1 cell/well density (*p ≤ 0.05). Colonosphere formation was also enriched for in SW480-CSCs at both the 1 and 10 cells/well densities (*p ≤ 0.05). P-values reported are from a Welch’s two sample t-test.
Table I: Gene expression of the colorectal CSC markers CD44, CD166 and EpCAM in parental and CSC enriched cell lines. RT-qPCR was used to evaluate gene expression in untreated cells. The average Ct ± SEM values (n=3) and categorical expression (low to very high) are reported for CD44, CD166, and EpCAM. CD44 was relatively highly expressed, with no major difference in the level of expression between cell lines. SW480-P cells had very low expression of CD166, which was increased in SW480-CSCs. EpCAM was highly expressed in all cell lines, with increased expression in CSCs.

<table>
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<tr>
<th></th>
<th>HCT116-P</th>
<th>HCT116-CSC</th>
<th>SW480-P</th>
<th>SW480-CSC</th>
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<tr>
<td>CD44</td>
<td>24.06 ± 1.18</td>
<td>23.13 ± 0.64</td>
<td>24.97 ± 0.56</td>
<td>24.09 ± 0.72</td>
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</tr>
<tr>
<td>CD166</td>
<td>27.30 ± 1.17</td>
<td>25.36 ± 1.22</td>
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<tr>
<td></td>
<td>Moderate</td>
<td>Moderate</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>EpCAM</td>
<td>22.49 ± 0.96</td>
<td>20.57 ± 0.82</td>
<td>21.42 ± 0.53</td>
<td>19.45 ± 1.02</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Very High</td>
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</table>
Figure 4: RT-qPCR analysis of CSC marker gene expression. Gene expression of three colorectal CSC markers, CD44 (A), CD166 (B) and EpCAM (C) was quantified in untreated cells. Target gene expression was normalized to the expression of two reference genes, β-actin and 18S ribosomal RNA. The mean fold change in gene expression ± SEM is shown for the CSC cell lines relative to their respective parental cell lines (n=3). When grown as colonospheres, expression of CD44, CD166 and EpCAM was higher in CSC cell lines than corresponding parental cell lines (p > 0.05). SW480-CSCs had significantly higher expression of CD44 compared to SW480-P cells (*p ≤ 0.05).
regulated in HCT116 and SW480 CSCs compared to the respective parental cell lines (Figure 4), although these findings were not statistically significant (p > 0.05).

**Proportion of cells expressing CSC markers**

Flow cytometry was used to determine the proportion of cells expressing CD166, EpCAM and CD133. In both CSC and parental cell lines, virtually all cells expressed EpCAM (Figure 5; Table II), confirming the high level of EpCAM gene expression detected by RT-qPCR. Over 95% of HCT116 cells expressed both CD166 and EpCAM (Figure 5). However, only a small proportion of SW480-P cells expressed CD166 (mean 7.45%), and any CD166 positive cells also expressed EpCAM (Figure 5). The proportion of cells expressing CD166 was higher in SW480-CSCs (mean 23.83%) compared to SW480-P cells (p > 0.05) (Table II). This finding corresponds with a shift from low to moderate CD166 gene expression in the CSC enriched SW480 cell line (Table II).

The expression of another CSC surface marker, CD133, was also analyzed. Fewer cells expressed CD133 in HCT116-CSCs and SW480-CSCs compared to parental cell lines (Figure 6; Table III). The proportion of cells expressing CD133, CD166 and EpCAM, corresponded with the median fluorescence values (Figure 6). There were no significant differences in median fluorescence between CSC and parental cells. As opposed to detecting two distinct populations (positive and negative), cells were found to have a gradient from low to high expression of CSC markers (Figure 5).
Figure 5: Flow cytometry staining of CD166 and EpCAM in CSC and parental cell lines. Parental monolayer cells and CSC colonospheres were analyzed for the expression of CD166 and EpCAM. EpCAM expression was high in all cell lines, with any cells expressing EpCAM also staining positive for CD166. Virtually all HCT116 cells express both EpCAM and CD166 (upper right quadrant), whereas the percentage of dual labeled cells was much lower in SW480. CD166 expression was enriched for in SW480-CSCs.
Table II: Percentage of cells expressing EpCAM, CD166 and CD133. The expression of three colorectal CSC surface markers, EpCAM, CD166 and CD133 was examined using flow cytometry. The mean percentage of positive cells ± SEM (n=3) is shown for both parental cell lines and CSC colonospheres. Nearly all HCT116-P cells express CD166, CD133 and EpCAM. EpCAM expression was high in all cell lines (> 97%). The number of cells expressing CD166 was increased in SW480-CSCs compared to parental cells. The proportion of cells expressing CD133 was lower in CSCs compared to parental cell lines.

<table>
<thead>
<tr>
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<th>HCT116-P</th>
<th>HCT116-CSC</th>
<th>SW480-P</th>
<th>SW480-CSC</th>
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<tr>
<td>EpCAM</td>
<td>98.40 ± 0.86</td>
<td>97.23 ± 1.23</td>
<td>96.87 ± 0.90</td>
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<td>CD166</td>
<td>99.67 ± 0.09</td>
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<td>7.45 ± 1.25</td>
<td>23.83 ± 0.08</td>
</tr>
<tr>
<td>CD133</td>
<td>98.60 ± 0.31</td>
<td>64.13 ± 2.87</td>
<td>43.07 ± 8.99</td>
<td>28.90 ± 9.06</td>
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Figure 6: Median fluorescence and overlay histograms for CSC marker expression. The mean median fluorescence ± SEM for CD166, CD133 and EpCAM was determined by flow cytometric analysis (n=3). No statistically significant differences in median fluorescence were detected between the CSC and parental cell lines (p > 0.05).
Cell viability in response to chemotherapy treatment

For each individual cell line, the IC\textsubscript{50} values for 5-FU, cisplatin and epirubicin were determined by generating monolayer dose-response curves. Cells were treated with doses of chemotherapy ranging from 0 - 500 µM. PrestoBlue cell viability reagent was used to determine the number of metabolically active cells. Both CSC and parental cell lines responded in a similar manner to chemotherapy treatment (Figure 7). However, the CSCs did not grow as quickly as parental cell lines. This was evident by the lower absorbance values, even though the initial cell number was the same for all cell lines. SW480-P cells had the highest IC\textsubscript{50} value for both 5-FU (19.12 µM) and cisplatin (14.94 µM) (Table III). Although this IC\textsubscript{50} value was not drastically higher than for the other cell lines, a small proportion of SW480-P and SW480-CSCs were able to survive treatment with high doses of 5-FU (Figure 7). This finding provides evidence that the SW480 cell lines are more resistant than HCT116 cells to treatment with 5-FU. Overall, when cells were grown in monolayer no major differences in chemosensitivity were detected between the CSC and parental cell lines.

3D chemotherapy treatment was also performed to evaluate cell viability when cells were grown as colonospheres. After 7 days of growth, parental and CSC derived colonospheres were treated with 25 µM 5-FU, 5 µM cisplatin or 0.05 µM epirubicin, representing doses within the range of IC\textsubscript{50} values obtained by monolayer growth. The percentage of viable cells relative to control (untreated) colonospheres was determined using the PrestoBlue cell viability reagent (Figure 8). The monolayer IC\textsubscript{50} values did not lead to 50% inhibition of cellular viability when grown as colonospheres. HCT116 parental derived colonospheres were sensitive to treatment with 5-FU (**p ≤ 0.01) and
Figure 7: Monolayer dose response curves. PrestoBlue cell viability reagent was used to determine cell viability following 72 hours of chemotherapy treatment. Cells were treated with a range of doses (0 – 500 µM) of 5-fluorouracil (5-FU), cisplatin or epirubicin. CSC and parental cell lines responded similarly to treatment in a dose-dependent manner. A small fraction of SW480-P and SW480-CSCs survived high doses of 5-FU where HCT116-P and HCT116-CSCs were sensitive to 5-FU and high doses completely abolished all cells.
Table III: Summary of the IC$_{50}$ values derived from monolayer dose response curves. Cells were plated in monolayer and treated with a range of chemotherapy doses (0 – 500 µM) for 72 hours. PrestoBlue cell viability assay was used to derive dose-response curves and GraphPad Prism software was used to determine the IC$_{50}$ values for each cell line. The IC$_{50}$ value with the respective 95% confidence interval is shown. IC$_{50}$ values were similar for all cell lines with the exception of SW480-P cells having the highest IC$_{50}$ value for 5-fluorouracil and cisplatin than the other cell lines.

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<tr>
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<th>HCT116-P</th>
<th>HCT116-CSC</th>
<th>SW480-P</th>
<th>SW480-CSC</th>
</tr>
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<tbody>
<tr>
<td>5-FU</td>
<td>4.378 µM</td>
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<td>19.12 µM</td>
<td>7.679 µM</td>
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<td></td>
<td>(4.101 ± 4.674)</td>
<td>(2.593 ± 3.834)</td>
<td>(10.75 ± 34.01)</td>
<td>(5.360 ± 10.47)</td>
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<tr>
<td>Cisplatin</td>
<td>7.950 µM</td>
<td>4.140 µM</td>
<td>14.94 µM</td>
<td>6.489 µM</td>
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<tr>
<td>Epirubicin</td>
<td>0.02446 µM</td>
<td>0.01495 µM</td>
<td>0.04476 µM</td>
<td>0.07996 µM</td>
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<tr>
<td></td>
<td>(0.01858 ± 0.03221)</td>
<td>(0.01117 ± 0.02001)</td>
<td>(0.01632 ± 0.1228)</td>
<td>(0.01218 ± 0.5251)</td>
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cisplatin (*p ≤ 0.05) (Figure 8). A reduction in metabolically active cells was accompanied by visible colonosphere dissociation (Figure 9). SW480-CSC derived colonospheres were apparently resistant to all treatments (Figure 8) even with prolonged exposure. Colonospheres were also treated with doses lower and higher than the IC\textsubscript{50} values and dissociation was seen in response to high doses of chemotherapy and with prolonged exposure (data not shown).

Parental cells grown in monolayer, and CSC colonospheres were treated with chemotherapy for 48 hours prior to Annexin V/PI staining. Etoposide (50 µM), a topoisomerase II inhibitor, was included as a positive control as it is known to induce apoptosis. Annexin/PI staining confirmed the sensitivity of HCT116-P (Figure 10) to 5-FU. SW480-P and SW480-CSCs (Figure 11) were resistant to chemotherapy treatment, confirming the results from the PrestoBlue viability assays. HCT116 cell viability was significantly reduced (*p ≤ 0.001) by 5-FU and etoposide. HCT116-CSCs were unaffected by treatment (Figure 12). Cell viability was only significantly reduced in the SW480 cell lines (P and CSC) by etoposide treatment (*p ≤ 0.05) (Figure 12).

**Sphere formation is reduced following chemotherapy treatment**

The effect of chemotherapy treatment on self-renewal ability was assessed using limiting dilution analysis. The 1 cell/well density was included for initial experiments. However, chemotherapy treatment greatly reduced colonosphere formation and a density of 1,000 cells/well was needed to achieve colonosphere formation rates of > 10% for
Figure 8: 3D colonosphere chemotherapy treatment and cell viability. After 7 days of growth, colonospheres were treated with chemotherapy and PrestoBlue was used to determine cell viability at 48 (A,B) and 72 (C,D) hours post-treatment. The mean absorbance values ± SEM are expressed relative to control (untreated cells) for both HCT116 (A,C) and SW480 (B,D) colonospheres. Treatment with 5-FU (25 µM) significantly reduced viability in HCT116-P derived colonospheres (**p ≤ 0.01). Treatment with cisplatin (5 µM) also significantly reduced viability in HCT116-P derived colonospheres after 72 hours of treatment (*p ≤ 0.05). SW480-CSC colonospheres were resistant to chemotherapy treatment with no change in cell viability relative to control. SW480-P derived colonospheres were also resistant to chemotherapy treatment as viability was not significantly reduced compared to untreated cells.
Figure 9: Dissociation of colonospheres following chemotherapy treatment. Representative images of colonospheres are shown 48 hours following chemotherapy treatment. Dissociation of HCT116 colonospheres was seen in response to 5-FU (25 µM) treatment corresponding with a decrease in cell viability. CSC derived colonospheres were consistently bigger than colonospheres derived from parental cells, even though the initial plating density was only 50 cells/well for CSCs and 100 cells/well for parental cells.
Figure 10: HCT116 Annexin V/Propidium iodide staining. HCT116 parental monolayer cells and HCT116-CSC colonospheres were treated with 5-FU (25 μM), cisplatin (5 μM) or epirubicin (0.05 μM) for 48 hours. Etoposide (50 μM) was used as a positive control to detect apoptosis. Early apoptotic cells are located in the lower right quadrant and dead cells in the upper quadrants. HCT116-P cells were resistant to treatment with cisplatin or epirubicin. Treatment with 5-FU or etoposide induced cell death in HCT116-P cells, with only a small proportion of cells in early apoptosis after 48 hours. There was no obvious effect of treatment on HCT116-CSC cell viability although there was a significant proportion of dead cells in both control and treated samples. This is likely a result of aggressive dissociation of the spheres in order to achieve single cell suspensions.
Figure 11: SW480 Annexin V/Propidium iodide staining. SW480 parental cells were treated with 5-FU (25 μM), cisplatin (5 μM) or epirubicin (0.05 μM) for 48 hours. Etoposide (50 μM) was used as a positive control to detect apoptosis. Early apoptotic cells are located in the lower right quadrant and dead cells in the upper quadrants. SW480-P cells were resistant to all treatments with the exception of etoposide. Treatment with etoposide induced cell death in both SW480-P and SW480-CSCs, with only a small proportion of cells in early apoptosis after 48 hours. SW480-CSCs were resistant to all treatments with the exception of etoposide.
Figure 12: Percentage of viable cells following chemotherapy treatment determined by Annexin v/Propidium iodide staining. The percentage of viable cells was calculated relative to control for each individual cell line. HCT116-P cell viability was unaffected by treatment with cisplatin or epirubicin (A). However, 5-FU (25 μM) and etoposide (50 μM) treatment significantly reduced the percentage of viable cells in HCT116-P relative to control (*p ≤ 0.001). HCT116-CSCs were unaffected by treatment. Both SW480-CSC and parental cells were resistant to treatment with 5-FU (25 μM), cisplatin (5 μM) or epirubicin (0.05 μM) (B). Etoposide (50 μM) significantly reduced the percentage of viable cells by ~50% in both SW480-CSC (*p ≤ 0.05) and parental cells (**p ≤ 0.01).
HCT116 cells treated with 5-FU or cisplatin (Figure 13). Cisplatin significantly reduced the ability of HCT116 and SW480 cells to form colonospheres at both the 10 and 100 cells/well densities (*p ≤ 0.05) (Figure 13). Colonosphere formation was also reduced by treatment with epirubicin, although it had the least impact in both cell lines with a mean colonosphere formation frequency of 72% for HCT116 and 38% for SW480. Colonosphere formation after treatment with 5-FU was higher in SW480 than HCT116 cells supporting the sensitivity of HCT116 to 5-FU demonstrated by the PrestoBlue and Annexin V/PI cell viability assays.

**Chemotherapy treatment reduces colony formation ability**

The effect of chemotherapy treatment on adherent cell colony formation was determined using the clonogenic survival assay. The ability of parental cells to form colonies was greatly reduced by 48-hour treatment with 5-FU (25 µM), cisplatin (5 µM) or epirubicin (0.05 µM). Chemotherapy treatment impaired both the plating efficiency (number of colonies formed per number of cells plated) (Table IV), and the survival fraction (number of colonies formed relative to control) (Figure 14). All treatments significantly reduced the survival fraction for SW480-P cells (*p ≤ 0.05). Chemotherapy treatment also reduced colony formation in HCT116-P cells however this finding was not statistically significant (p > 0.05). Epirubicin had the least impact on colony formation in both cell lines. Overall, chemotherapy reduced both adherent colony and sphere formation, providing evidence that treatment affects the cell's ability to self-renew.
Figure 13: Limiting dilution analysis post-chemotherapy treatment. Parental cell lines were treated with chemotherapy for 48 hours prior to plating in 96 well ultra-low adhesion plates at densities of 10, 100 or 1000 cells/well. After 10 days of growth, wells were scored for the presence or absence of a colonosphere. The colonosphere formation frequency is expressed as the mean (%) ± SEM (n=3). Colonosphere formation was 100% for control (untreated) HCT116 (A) and SW480 (B) cells when plated at 100 cells/well so the 1000 cell/well density was not included for control cells. Cisplatin (5 µM) significantly reduced sphere formation ability in both HCT116 and SW480 cells at the 10 and 100 cell/well density (*p ≤ 0.05, **p ≤ 0.01). Treatment with 5-FU (25 µM) significantly reduced sphere formation in HCT116 cells at the 10 cell/well density and also decreased sphere formation at the higher densities in both HCT116 and SW480 cells. Epirubicin (0.05 µM) had the weakest impact on sphere formation in both cell lines.
Table IV: Plating efficiency for clonogenic survival assay. The optimal cell density was determined and the plating efficiency was calculated by calculating the number of adherent colonies formed relative to the number of cells plated. The plating efficiency is shown as the mean (%) ± SEM. The fraction of cells able to form colonies was reduced following 48-hour chemotherapy treatment, in both HCT116 and SW480 parental cells. Treatment with epirubicin (0.05 µM) had the weakest impact on colony formation in both cell lines.

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<thead>
<tr>
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<th>HCT116</th>
<th>SW480</th>
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<tr>
<td>Control</td>
<td>8.5 ± 3.8%</td>
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<tr>
<td>5-FU (25 µM)</td>
<td>0.012 ± 0.0023%</td>
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<td>Cisplatin (5 µM)</td>
<td>0.32 ± 0.0075%</td>
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<td>Epirubicin (0.05 µM)</td>
<td>0.89 ± 0.40%</td>
<td>1.1 ± 0.48%</td>
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</table>
**Figure 14: Chemotherapy treatment reduces adherent colony formation in parental cell lines.** The clonogenic survival fraction was calculated following 48 hours chemotherapy treatment. The plating efficiencies were normalized to control (untreated cells) and the mean ± SEM is shown (n=3) for each treatment for both HCT116 (A) and SW480 (B) parental cells. 5-FU (25 µM), cisplatin (5 µM) and epirubicin (0.05 µM) reduced colony formation by > 90% in HCT116 cells (p > 0.05). 5-FU, cisplatin and epirubicin significantly reduced colony formation in SW480 cells (*p ≤ 0.05, ***p ≤ 0.001) by 96.1%, 81.2% and 47.6% respectively.
Colonospheres have higher expression of drug resistance related genes

Expression of \( \text{ABCG2} \) was significantly higher (\( \sim 35 \) fold) in HCT116-CSCs compared to HCT116-P cells (*\( p \leq 0.05 \)) (Figure 15). HCT116-CSCs also had higher expression of \( \text{ABCB1} \) than HCT116-P cells, although this was not statistically significant (*\( p > 0.05 \)). Expression of \( \text{ABCB1} \) was significantly higher (\( \sim 50 \) fold) in SW480-CSCs compared to SW480-P cells (*\( p \leq 0.05 \)) with no change in \( \text{ABCC1} \) or \( \text{ABCG2} \) expression (Figure 15). SW480-CSCs had the highest expression of \( \text{ABCB1} \) among the cell lines tested (Table V). \( \text{ABCC1} \) was expressed at low to moderate levels in all cell lines tested. Except for moderate expression in HCT116-CSCs, \( \text{ABCG2} \) was expressed at low levels in all cell lines (Table V). These results support the heightened expression of \( \text{ABCB1} \) and \( \text{ABCG2} \) in CSCs.

The gene expression of \( \text{ALDH1A1, ALDH1A2, ALDH1A3} \) and \( \text{ALDH3A1} \) was also examined using RT-qPCR. Both parental and CSC enriched cell lines had weak expression of \( \text{ALDH1A1} \) and \( \text{ALDH1A2} \) (Table VI). \( \text{ALDH1A3} \) and \( \text{ALDH3A1} \) were expressed at moderate to high levels in both HCT116 and SW480 (Table VI). There were no significant differences in \( \text{ALDH1A3} \) or \( \text{ALDH3A1} \) expression between CSC and parental cell lines, but SW480-CSCs had significantly higher expression of \( \text{ALDH1A2} \) (*\( p \leq 0.05 \)) (Figure 16).

Drug resistance related gene expression changes following chemotherapy treatment

Parental monolayer cells, and CSC colonospheres, were treated with 5-FU (25 \( \mu \text{M} \)), cisplatin (5 \( \mu \text{M} \)) or epirubicin (0.05 \( \mu \text{M} \)) for 24 hours. RT-qPCR analysis was used to determine if the population surviving treatment had higher gene expression of \( \text{ABCB1} \),
Figure 15: RT-qPCR analysis of ABC transporter gene expression. Gene expression of three ABC transporter family members, \( ABCB1 \) (A), \( ABCC1 \) (B), and \( ABCG2 \) (C) was quantified in untreated cells. Target gene expression was normalized to the expression of two reference genes, \( \beta\)-actin and 18S ribosomal RNA. The mean fold change in gene expression ± SEM is shown for the CSC cell lines relative to their respective parental cell lines (n=3). CSC and parental cell lines had similar \( ABCC1 \) levels. HCT116-CSCs had higher \( ABCB1 \) (p > 0.05) and \( ABCG2 \) (*p ≤ 0.05) expression compared to parental cells. SW480-CSCs had higher \( ABCB1 \) (*p ≤ 0.05) expression compared to parental cells, with no change in \( ABCC1 \) or \( ABCG2 \) expression (p > 0.05).
Table V: Mean Ct values for ABC transporter gene expression in untreated cells.
The gene expression of three ABC transporter family members, \textit{ABCB1}, \textit{ABCC1}, and \textit{ABCG2} was determined using RT-qPCR in untreated parental and CSC enriched cell lines. The average Ct ± SEM and categorical expression (low to very high) is shown (n=3). \textit{ABCC1} was expressed at similar levels in all cell lines. SW480-CSCs had the highest expression of \textit{ABCB1} and HCT116-CSCs had the highest expression of \textit{ABCG2}.

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<tr>
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<th>HCT116-P</th>
<th>HCT116-CSC</th>
<th>SW480-P</th>
<th>SW480-CSC</th>
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<tr>
<td>\textit{ABCB1}</td>
<td>30.02 ± 0.63</td>
<td>26.76 ± 0.24</td>
<td>29.26 ± 0.37</td>
<td>23.48 ± 0.37</td>
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<td>Low</td>
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<tr>
<td>\textit{ABCC1}</td>
<td>29.31 ± 0.88</td>
<td>28.42 ± 0.65</td>
<td>30.23 ± 0.27</td>
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<td>\textit{ABCG2}</td>
<td>33.30 ± 0.53</td>
<td>27.56 ± 0.47</td>
<td>34.69 ± 0.50</td>
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Table VI: Mean Ct values for aldehyde dehydrogenase gene expression untreated cells. The gene expression of \textit{ALDH1A1}, \textit{ALDH1A2}, \textit{ALDH1A3} and \textit{ALDH3A1} was determined using RT-qPCR in untreated parental and CSC enriched cell lines. The average Ct ± SEM and categorical expression (low to very high) is shown (n=3). \textit{ALDH1A1} and \textit{ALDH1A2} expression was low in all cell lines. HCT116-CSCs had high expression of \textit{ALDH1A3} compared to all other cell lines showing moderate expression. \textit{ALDH3A1} was moderately expressed in SW480 cells, with higher expression in SW480 parental cells, and lowly expressed in HCT116 cells.

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<th>HCT116-P</th>
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<th>SW480-P</th>
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<tr>
<td>\textit{ALDH1A1}</td>
<td>34.81 ± 1.19</td>
<td>30.90 ± 0.20</td>
<td>34.08 ± 0.37</td>
<td>35.33 ± 0.87</td>
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<td>\textit{ALDH1A2}</td>
<td>39.67 ± 1.70</td>
<td>36.99 ± 1.41</td>
<td>39.90 ± 0.50</td>
<td>35.40 ± 0.99</td>
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<td>\textit{ALDH1A3}</td>
<td>29.32 ± 1.35</td>
<td>23.88 ± 1.06</td>
<td>27.25 ± 0.36</td>
<td>27.94 ± 0.40</td>
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<td>\textit{ALDH3A1}</td>
<td>31.22 ± 1.26</td>
<td>29.95 ± 0.59</td>
<td>26.14 ± 0.91</td>
<td>29.48 ± 0.67</td>
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Figure 16: RT-qPCR analysis of aldehyde dehydrogenase gene expression. Gene expression of ALDH1A1 (A), ALDH1A2 (B), ALDH1A3 (C), ALDH3A1 (D) was quantified in untreated cells. Target gene expression was normalized to the expression of two reference genes, β-actin and 18S ribosomal RNA. The mean fold change in gene expression ± SEM is shown for the CSC cell lines relative to their respective parental cell lines (n=3). SW480-CSCs had significantly higher ALDH1A2 expression compared to parental cells (*p \leq 0.05). HCT116-CSCs had higher expression of ALDH1A1, ALDH1A2 and ALDH1A3 compared to parental cells. SW480-CSCs had lower expression of ALDH3A1 than SW480 parental cells.
*ABCC1, ABCG2, ALDH1A3* and *ALDH3A1*. The expression levels in treated cells were compared to control (untreated) samples. A fold-change greater than 1.5 was considered up-regulated, and a fold-change less than 0.5 was considered down-regulated.

Treatment did not affect *ALDH1A3* expression in any of the cell lines tested (Table VII). *ABCC1* expression was also not affected by treatment, except for a down-regulation in HCT116-P cells in response to 5-FU (**p ≤ 0.01**) (Table VII). *ABCB1* was up-regulated in both parental cell lines in response to all treatments. Although increased in response to treatment, *ABCB1* expression was still lower than levels detected in untreated CSCs. Cisplatin treatment up-regulated *ALDH3A1* expression in all cell lines tested with significant up-regulation in HCT116-P and SW480-CSCs (*p ≤ 0.05*) (Table VII). *ALDH3A1* expression was lower in untreated HCT116-P and SW480-CSCs compared to their respective CSC and parental cell lines (Figure 16). *ABCG2* was also up-regulated in response to treatment with 5-FU, cisplatin or epirubicin (Table VII), although these changes were not statistically significant (p > 0.05). Collectively, these results provide evidence that chemotherapy treatment induces the expression of drug-resistance related genes in both CSC and parental cells.
Table VII: Changes in ABC transporter and ALDH gene expression in response to chemotherapy. Gene expression was quantified using RT-qPCR in cells treated with 5-FU (25 μM), cisplatin (5 μM) or epirubicin (0.05 μM) for 24 hours. Target gene expression was normalized to the expression of two reference genes, β-actin and 18S ribosomal RNA. Gene expression was normalized to control (untreated) cells. A mean fold change less than or greater than 1.5, corresponds to down-regulation (↓) and up-regulation (↑) respectively. NC refers to no change in gene expression (n=3). ABCC1 expression was unaffected by treatment, with the exception of a significant down-regulation in HCT116-P cells by 5-FU (**p ≤ 0.01). ALDH1A3 expression was not affected by any treatment in any of the cell lines, however ALDH3A1 expression was induced by treatment. Cisplatin significantly up-regulated ALDH3A1 expression in HCT116-P and SW480-CSCs compared to untreated cells (*p ≤ 0.05). Treatment also induced expression of ABCB1 in parental cells, and ABCG2 and ALDH3A1 in CSC and parental cells as well, although these findings were not statistically significant.

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<th>ABCB1</th>
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DISCUSSION

Tumour heterogeneity is a significant issue affecting the reliable identification of CSC markers. Numerous studies have used cell-sorting methods to identify CSCs, which have revealed variable expression of markers in both primary patient samples[8], [9], [95] and established CRC cell lines [85], [109]. Therefore, sorting colorectal cancer cells based on their expression of proposed CSC markers, is not the most robust or universally applicable method. My results provide evidence that the functional sphere formation assay can be used to enrich for CSCs. This method successfully enriched for a subpopulation of cells with CSC-related gene expression and heightened self-renewal ability. This held true even in the HCT116 cell line, which already has a high intrinsic CSC population. When only 1 cell was plated in a low adhesion environment, the enriched HCT116-CSC population had a ~2.5 fold increase in sphere formation ability compared to the parental cell line. Other studies have also confirmed that the sphere formation assay is a successful and robust method to enrich for CSCs from HCT116 [17], [93] and established prostate cancer cell lines [12].

HCT116-CSCs also expressed CD44, CD166 and EpCAM at higher levels than the parental cell line. The expression of all genes in this study was also assessed for the CSCs grown in monolayer to determine if the stem cell media (SCM) used for colonosphere culture influenced gene expression. Expression levels were maintained regardless of the culture media used (Appendix IV: Supplementary Figure 1) suggesting no confounding effect of the growth factors used in the stem cell media.

Although sphere formation ability and CSC marker gene expression was increased, my results support the proposal that HCT116 is composed mainly of a CSC
population [85]. This is best demonstrated by the colonosphere formation frequency of 88% when HCT116 parental cells were plated at only 10 cells/well. Other CRC cell lines, such as HT-29 [104], have been shown to be comprised mainly of CSCs based on their tumourigenic ability and expression of CSC markers. Prolonged cell culture could select for a resilient phenotype and therefore it is expected that established cell lines would have a higher intrinsic CSC population than that found in primary patient tumours [89]. HCT116 cells can initiate tumour formation when only 200 - 1,000 cells are implanted into NOD/SCID mice [85]. For SW480, tumour formation can occur when as few as 2,000 cells are implanted [96]. However, in primary patient samples, the CSC subpopulation comprises a very small percentage of tumour cells and therefore more cells are needed to achieve tumour formation [8]. Established CRC cell lines still remain a useful tool to study CSCs despite their high intrinsic tumourigenic ability. These cell lines are representative of primary tumours and are well characterized [98], [178]. Since the mutational status of cell lines is known, their use could elucidate mutations correlated with CSC properties. In addition, CRC cell lines can form tumours in mice that consist of a heterogenic population of cells and are representative of primary colorectal tumours [10].

The results shown here suggest that SW480 is a more suitable cell line to study enrichment for CSCs than HCT116, as the enrichment for a CSC population from SW480 cells corresponded with ~13 fold increase in sphere formation ability. SW480-CSCs also had higher gene expression of CD166 and EpCAM compared to SW480 parental cells. The low level of CD166 gene expression in SW480-P cells corresponds with the flow cytometry results showing that only a small percentage of SW480-P cells express CD166.
CD166 has a role in maintaining hematopoietic stem cells [179] and is a proposed ISC marker [46]. In addition, increased levels of CD166 are associated with poor outcome in CRC [111]. My study found that the percentage of cells expressing CD166 was higher in SW480-CSCs, corresponding with an increase in CD166 gene expression compared to parental cells. This finding suggests that CD166 may be a useful CSC marker in the SW480 cell line.

CD133 was the first marker used to identify colorectal CSCs [8], [9]. However, subsequent studies have demonstrated that both CD133+ and CD133− populations can form tumours in immune deficient mice [107], [109]. The CD133− population has also been shown to generate CD133+ cells in vivo [109] a property that was initially only associated with the positive population. In my study, the percentage of cells expressing CD133 was lower in both HCT116-CSCs and SW480-CSCs compared to parental cells, although this finding was not statistically significant. Other studies have reported lower expression of CD133 in HCT116 cells than those detected in this study [104], [109], [180]. The use of different CD133 antibodies could explain the inconsistent results when used to identify CSCs. In addition, the commonly detected epitope of CD133 may undergo a conformational change in differentiated CRC cells, preventing efficient binding [181]. It is evident that the antibody used to determine CD133 expression influences results obtained from flow cytometry, which stresses the need for reliable detection methods.

Most of the contradictory results for colorectal CSC identification are a result of relying on a single marker and therefore dual staining for multiple markers is a more reliable method. For instance, the CD133+ subpopulation generally represents a larger
proportion of tumour cells than the CD44+ subpopulation [95], [104] and CD44+ cells are usually CD133+ [95]. Dual labeling with multiple CSC markers can reveal correlations such as these and is a more suitable method to correctly identify the CSC subpopulation.

CD44 is a reliable CSC marker in breast [6], [99] and prostate [11] cancers. CD44 also appears to be a useful marker for identifying colorectal CSCs [10], [94]. In both primary CRC tumours and established cell lines, sorting for CD44 or ALDH activity generated cell populations with increased tumourigenicity whereas sorting for CD133 did not [10]. CD44+ cells divide slowly compared to CD44− cells and can give rise to tumours that contain both CD44+ and CD44− cells [10]. In addition, knockdown of CD44 in breast CSCs resulted in their differentiation and lower tumourigenic potential [99]. Therefore CD44 appears to have a functional role for CSCs. I found that SW480-CSCs had significantly higher gene expression of CD44 compared to parental cells, but this differential expression of CD44 was not explored further by flow cytometry as the change did not appear to be as great as that seen for CD166 or EpCAM. In addition, CD44 has a number of splice variants and there have been documented discrepancies between mRNA levels and antibody binding [103]. It was recently discovered that minute differences in the collagenase method used to digest breast cancer tissues greatly influenced CD44 expression profiles [182]. Standardized methods across laboratories could aid in the reproducibility of cell sorting methods and CSC identification.

It is evident that EpCAM is not a suitable colorectal CSC marker as it is highly expressed on the majority of CRC cells, in both primary [95] and established cell lines [104]. In fact, over 99% of cells in many of the commonly used CRC cell lines including, HCT116, SW480, SW620, Colo205, DLD-1 and HT-29 express EpCAM
I found that EpCAM was expressed on over 96% of cells in both HCT116 and SW480 parental cell lines. Although an up-regulation in EpCAM gene expression was detected between the CSC enriched and parental cell lines, this finding was not statistically significant. In addition, there was no shift in the median fluorescence suggesting the increased gene expression is not reflected at the protein level. EpCAM median fluorescence was decreased in SW480-P cells compared to other cell lines, although this finding was not statistically significant. Other studies have reported higher EpCAM protein expression in HCT116 cells compared to SW480 [104]. The lack of concordance between protein and mRNA, could be explained by the fact that the flow cytometry data is only reflective of EpCAM surface expression as the cells were not permeabilized prior to staining. EpCAM could also have been expressed in the cytoplasm of these cells [93], [120], [183], [184].

EpCAM proves to be most useful when combined with other CSC markers to identify CSCs from primary tumour samples. In primary tumour samples immune infiltrating cells could also be present, but these cells would not express EpCAM as it is an epithelial marker [185]. Sorting primary cancer cells based on a CSC marker such as CD44, would not exclude immune cells as they also express this marker [186]. EpCAM could also be useful to identify human epithelial cells when analyzing CSC marker expression from PDX tumours, to eliminate the contamination of mouse cells as human specific antibodies could be used [109].

An important colorectal CSC marker, LGR5, was not examined in this study as it had been previously investigated in our laboratory (Farias, 2013). LGR5 is a normal ISC marker [31], [40] and therefore may serve useful in identifying colorectal CSCs. It was
determined via western blotting that there was no difference in LGR5 protein expression in HCT116 monolayer cells compared to colonospheres and that SW480 colonospheres showed a reduction in LGR5 expression [29]. These results were accompanied by activation of the Wnt pathway shown through increased phosphorylated β-catenin in HCT116 and SW480 colonospheres compared to parental monolayer cells [29]. It is apparent that the SW480 cell line consists of both adherent and non-adherent cell types. The non-adherent SW480 cells have been shown to express LGR5 at higher levels than adherent cells and silencing LGR5 led to morphological changes with the non-adherent cell population being lost [96].

Due to the variability in CSC surface marker expression there is a pressing need to identify more putative markers for colorectal CSCs. Stem cell transcription factors such as SOX2, Nanog or OCT4, have potential for CSC identification [12], [15], [45], [122]. Retrovirally introducing OCT3/4 and SOX2 into colon cancer cells induced CSCs properties including sphere formation, chemoresistance and tumourgenicity [188]. In the present study, the gene expression of SOX2 was determined to be very low in both HCT116 and SW480 parental monolayer and CSC colonospheres using RT-PCR (Appendix IV: Supplementary Table I), thus it appears that SOX2 is not of critical importance for the HCT116 and SW480 cell lines. Other studies have also found low mRNA and protein levels of SOX2 in HCT116 and SW480 cell lines in comparison to other human CRC cell lines including SW620 and Lovo [189].

It is evident from the results shown in this study, along with the literature, that multiple markers should be used to identify CSCs. If cell sorting methods are to be employed from initial primary patient samples, the best approach would be to first use
EpCAM to identify the CRC cells, then further sort using multiple CSC surface markers. However, due to the broad heterogeneity among CRC samples, the optimal markers to identify colorectal CSCs have not yet been determined. Recently a study generated a highly tumourigenic CRC cell line (CR4) derived from liver metastases, that has very high expression of CD133, CD44, EpCAM, CD166, LGR5 and SOX2 [122] highlighting the importance of multiple markers for CSC identification.

Most studies examining the sphere formation of CSC populations have first sorted cells based on CSC surface marker expression [94], [109]. It remains to be determined if sphere formation can enrich for a CRC cell population with the increased ability to establish cancers in mice. Prostate CSCs generated through an in vitro sphere formation strategy similar to the one I used show more aggressive growth in vivo than parental monolayer cells [190]. Xenograft experiments remain the gold-standard to truly identify a CSC subpopulation from both established and primary cell lines. Studies have determined that the ability to form spheres is not correlated with advanced stage, but is correlated with lymphatic invasion, in the original tumour [191]. High sphere formation also significantly correlated with shorter disease free survival [191]. The CSC cell lines enriched through the sphere formation assay in our laboratory have not been tested for their tumourigenic ability using xenograft experiments. The increased sphere formation ability at low cell densities suggests that these CSC enriched cell lines have a heightened self-renewal capacity and would be more tumourigenic in vivo.

Chemoresistance is an important property of the CSC population. Cancer cells that survive chemotherapy treatment are enriched with other CSC properties [192], [193]. I found that SW480 cells were more resistant to 5-FU both in monolayer and when grown
as colonospheres compared to HCT116. I generated 5-FU drug resistant SW480 cells through subjecting parental cells to two rounds of clonogenic survival, but there was no significant increase in the proportion of cells expressing CD133, CD166 or EpCAM in the 5-FU drug-resistant population (Appendix IV: Supplementary Figure 2). In fact, the 5-FU resistant SW480 cells actually contained a significantly smaller proportion of cells expressing CD166 and EpCAM compared to SW480-CSCs. Cisplatin and epirubicin drug-resistant cells were also generated for HCT116, and these drug-resistant HCT116 cell lines had similar CD133, CD166 and EpCAM expression as the HCT116 parental cell line (Appendix IV: Supplementary Figure 3). Other studies examining 5-FU or oxaliplatin resistant HT-29 CRC cells showed an enrichment for CD133+ cells [192]. However, these results are contradicted by the high proportion of CD133+ HT-29 cells reported in other studies [104].

Many studies have reported that the doses used in monolayer do not correlate with a similar reduction in cell viability when treating 3D spheres [194]–[196]. It is important to note that many 3D culture experiments use spheroid models where greater than 1,000 cells are plated [195]. 3D treatment is more representative of clinical studies than traditional monolayer experiments. The monolayer IC50 values determined in this study did not lead to 50% inhibition of cellular viability when grown as colonospheres, which supports the discrepancy between doses used in 2D and 3D culture.

Both CSC cell lines were more resistant to chemotherapy treatment when grown as colonospheres compared to the un-enriched cell lines. It is important to note that the sphere formation is restricted to the immature cell population while the differentiated cells that are adhesion dependent will not survive [90]. Therefore, the 3D treatment
experiments were actually comparing cell viability in passage 1 spheres versus high passage spheres. The conclusion drawn here is that prolonged colonosphere culture did not significantly enrich for a drug resistant population.

A more sensitive assay to detect viable cells is the flow cytometric analysis of Annexin V/Propidium iodide staining. In early apoptosis, phosphotidylserine translocates from to the outside of the plasma membrane from the inner layer. Fluorescently conjugated Annexin V has a high affinity for phosphatidylserine and can be used to detect cells in early apoptosis [197]. Propidium iodide (PI) is a red fluorescent dye that is impermeable to intact membranes. Viable cells, or cells in early apoptosis still have an intact cell membrane and will exclude PI. On the other hand, cells undergoing necrotic cell death, or cells that have died of apoptosis, will lose their membrane integrity and be stained positive by PI [197]. Based on these principles, dual staining with Annexin V and PI can separate viable cells (Annexin V/PI−), early apoptotic cells (Annexin V+/PI−) and dead cells (Annexin V+/PI+ or Annexin V−/PI+). Although Annexin V/PI staining is a more sensitive method than viability assays that rely on metabolic activity, it requires single cell suspensions. Trypsin was not used to dissociate the spheres as it led to cell lysis and therefore the spheres required mechanical dissociation. As demonstrated by the low cell viability in the HCT116-CSC control cells, aggressive mechanical dissociation greatly reduced the proportion of viable cells. Therefore flow cytometric analysis of colonospheres requires a different approach than utilized in this study. Accutase is a more gentle enzymatic dissociation than trypsin and has been effectively employed to dissociate neurospheres with improved cell viability over trypsin [198]. With this in mind it is difficult to compare the Annexin V/PI results using only the dot plots. The
decreased viability in the colonospheres can be accounted for by normalizing the percentage of viable cells in treated samples to control. This analysis confirmed the sensitivity of HCT116 cells to 5-FU. There were no major differences in the percentage of viable cells relative to control for SW480-CSC and parental cells. Both HCT116 and SW480 cell lines were sensitive to treatment with etoposide. Our laboratory has previously detected the presence of apoptotic bodies using immunofluorescence in HCT116 cells in response to 24 hour treatment with 50 µM etoposide [199].

Drug resistance in CSCs has also been pinpointed through sorting cells based on CSC markers and treating the isolated populations. CD133+/CD44+ HCT116 cells were more resistant to radiation than unsorted cells [200]. For primary colorectal tumours the response to 5-FU was correlated with CD133 status, but the association conflicted depending on tumour stage [108]. Overexpression of LGR5 reduced the sensitivity of CRC cell lines to 5-FU and oxaliplatin [17]. In my study cells were not sorted based on their CSC marker expression and therefore I was unable to determine if cells expressing these markers are more drug-resistant. However, I did note that SW480 cells were more resistant to 5-FU treatment than HCT116 cells. Nearly all HCT116 parental cells expressed the CSC markers examined yet this cell line was the most sensitive to 5-FU. These results suggest that lower CD166 or CD133 expression as detected in SW480 cells is associated with resistance to 5-FU. However, the SW480 cell lines also had higher expression of \( ABCB1 \) than HCT116 cells which may contribute to 5-FU resistance. Other studies have shown that over-expression of LGR5 was associated with higher \( ABCB1 \) expression and resistance to 5-FU and oxaliplatin [17].
Chemoresistance has also been inferred through identifying the side population by flow cytometry. The side population is believed to be due to the function of ABC transporters, particularly ABCB1 [142] and ABCG2 [143–145], to efflux Hoescht dye. The side population has been shown to be enriched in CSC markers [146], [148], [151], sphere formation [148] and tumourigenic ability [146], [148], [149], [201]. Side population cells are reported to be in a quiescent state as the majority of cells exist in G0/G1 [148]. In addition, side population cells can differentiate into both side population and non-side population cells [148], [151]. Hoescht dye binds to the A-T regions in the minor groove of DNA to stain cells positive, uptake occurs in all cells however efflux is restricted to cells with high levels of ABC transporters [22]. This renders Hoescht dye exclusion a good functional model to identify a drug resistant subpopulation and CSCs.

In the HCT116 and SW480 cell lines, it has been shown that the side population cells do contain CSCs [148]. The SP SW480 is more resistant to 5-FU and cisplatin and has higher sphere formation and colony formation ability [148]. In addition the SW480 SP had higher expression of ABCB1, ABCG2, OCT-4, Nanog, SOX2, CD133 and CD44 at both the gene and protein levels [148] suggesting SW480 has properties of CSCs. Other studies have also shown increased ABCB1 and ABCG2 expression in SW480 SP cells in addition to their ability to self-renew, give rise to non-SP cells and demonstrate resistance to 5-FU, doxorubicin and gemcitabine [151].

In my study, HCT116-CSC colonospheres had ~35-fold increase in ABCG2 gene expression compared to parental cells. ABCB1 was also up-regulated in both HCT116 (~6 fold) and SW480-CSCs (~50 fold) compared to parental cells. The function of ABC transporter genes was not examined therefore I am unable to determine if the side
population is enriched in CSC colonospheres. Based on the gene expression levels of these ABC pumps, my results suggest that the ability to efflux Hoescht dye would be greater in the CSC enriched cell lines. HCT116 colonospheres have been shown to have increased ABCG2 protein levels associated with an 80% increase in dye exclusion compared to parental cells [93]. 5-FU and oxaliplatin drug resistant HCT116 cell lines also have ~2.5 fold higher Hoechst dye exclusion associated with a ~3 fold increase in ABCG2 compared to parental cells [202]. However these studies did not examine ABCB1 expression, which has also been shown to contribute to Hoechst dye efflux [142]. Higher expression of LGR5 in human CRC tissues is also associated with increased ABCB1 expression [17]. In lung xenografts, CD133\(^+\) derived tumours showed increased survival after treatment with cisplatin which was associated with the expression of ABC transporters specifically ABCG2 [18].

Other CSC markers may have a functional role in protection against chemotherapy. Chemotherapy induces the formation of ROS, which at high levels are toxic to cells [152]. Aldehydes are highly reactive and can cause significant damage to many cellular constituents [152]. The CSC marker ALDH can detoxify reactive aldehydes through catalyzing their conversion to carboxylic acids [23]. Specifically, members of the ALDH1A family and ALDH3A1 are proposed CSC markers [158] and both have been implicated in cyclophosphamide resistance in cancer cells [175].

ALDH expression or activity is a putative CSC marker in many cancer types. The ALDH\(^{\text{high}}\) population generally comprises a small proportion of tumour cells. In fact in CRC, CD133 and CD44 populations were ~7 times as large as the ALDH population and sorting for CD133 or CD44 formed tumours at a similar rate to ALDH\(^+\) [28].
addition, removing the ALDH\textsuperscript{+} population from CD133 or CD44 subpopulations eradicated the ability to form tumours, suggesting that ALDH has a critical functional role in CSCs [28]. Most studies have used the Aldefluor\textregistered assay, which measures ALDH activity to identify CSCs. Although this assay is proven useful, it is not able to separate the specific ALDH family members responsible for the enzymatic reaction. An inhibitor is often used that is specific to ALDH1 and therefore this assay can be reflective of ALDH1 enzymatic activity. The gene expression of gastric cancer cells sorted with high ALDH activity revealed only up-regulation in \textit{ALDH1A1} [167] suggesting this family member is responsible for Aldefluor activity. Theoretically an ALDH inhibitor that is specific to a different family member could be used to ensure that other family members do not also contributing to the fluorescent conversion of the substrate. There are many ALDH inhibitors that differ in their specificity for ALDH families [165]. The anti-alcoholic drug disulfiram has been under investigation for the ability to reverse drug-resistance in cancer cells. Disulfiram has been shown to be cytotoxic in breast cancer [203] and glioblastoma [173]. Disulfiram also reverses paclitaxel and cisplatin resistance in triple-negative breast cancer cells [171] accompanied by a reduction in CSC characteristics.

In addition to the Aldefluor assay, protein levels can be detected by western blotting immunohistochemistry or immunofluorescence or PCR can be used for gene expression. These approaches have shown the levels of ALDH to be up-regulated in CSCs, not just the activity [28]. Many studies focus on the 1A family. I found that \textit{ALDH1A1} gene expression was low in HCT116 and SW480 cells, supporting the undetectable ALDH1A1 protein expression previously reported in these cell lines [104].
Few studies have looked at ALDH3A1 in CRC cell lines [204], with no studies looking at HCT116 or SW480. In other cancer types including breast [205] and prostate [125], ALDH3A1 has been shown to be up-regulated in CSCs. In breast cancer, mammospheres derived from MCF-7 cells had significantly higher gene expression of ALDH3A1 compared to monolayer cells [205]. ALDH3A1 was also up-regulated at both the protein and genetic level in prostate cancer spheres compared to corresponding parental monolayer cells [176]. ALDH3A1 was also up-regulated in xenograft tumours derived from prostate CSCs compared to monolayer parental cells and was associated with tumour progression [176].

In my study, HCT116-CSCs had higher expression of ALDH3A1 compared to parental cells. However, SW480-CSCs had lower expression of ALDH3A1 compared to parental cells. The consequences of this differential expression between HCT116 and SW480-CSCs remain to be elucidated. The differences in ALDH3A1 expression between SW480 parental and SW480-CSCs did not correlate with differences in chemoresistance.

In addition to providing intrinsic chemoresistance, drug-resistance genes can also be induced by chemotherapy treatment [134]. Therefore the expression of ABC transporters and ALDH were evaluated following chemotherapy treatment. Cisplatin significantly induced ALDH3A1 expression in HCT116-P and SW480-CSCs and 5-FU, cisplatin and epirubicin induced the expression of ABCB1 in parental cell lines and ABCG2 in both parental and CSC enriched cell lines, although these findings were not statistically significant. Other studies have shown that epirubicin up-regulates ABCB1 and ABCC1 gene expression after 48 hours of treatment [134]. A natural compound, sinomenine, has been shown to sensitize Caco-2 cells to doxorubicin through down-
regulating the expression of ABCB1 [133]. Epirubicin/doxorubicin are known ligands for both ABCB1 and ABCG2 [20].

It is proposed that if CSCs are more drug-resistant they will survive chemotherapy and go on to form recurrent tumours [127]. Based on this principle, the CSC population would be expected to increase following treatment, but I found that self-renewal was significantly affected by cisplatin. 5-FU and cisplatin also reduced the ability of parental HCT116 and SW480 cells to self-renew but this finding was not statistically significant. However, due to the ability of CSCs to establish tumours when small numbers are implanted [9], [94], it is possible that the CSC population does not have to increase following treatment to be involved in tumour recurrence. The small number of CSCs surviving treatment could still go on to self-renew and form a recurrent tumour.

Although sphere and colony formation was greatly reduced by chemotherapy treatment in my study, a very small fraction of cells were still able to self-renew. Attempts to characterize the CSC marker expression of these surviving populations were made, however initial experiments failed to show any major differences and this experiment was not completed due to extenuating circumstances.

It is evident that CSCs are an important drug tolerant population that requires alternate therapy methods. Most of what is known about CSCs is their shared properties with normal stem cells. It has been shown that inhibiting stem cell signaling pathways can revert CSCs to a chemosensitive population [206]. However, inhibiting these properties clinically could lead to detrimental effects on the normal stem cell population. Finding unique properties of CSCs that allow the normal stem cell population to be spared would be a preferred approach. An alternative method is to induce the
differentiation of CSCs to make them sensitive to current treatments. Retinoic acid in combination with anthracycline chemotherapy has improved survival and prevented relapse in acute promyelocytic leukemia and acts through causing the leukemia cells to differentiate [160]. In CRC, down-regulation of Bmi-1 inhibits the ability of CSCs to self-renew [124]. Approaches such as these could improve the outcome of CRC and prevent disease recurrence.
IMPLICATIONS AND FUTURE DIRECTIONS

As discussed, CSCs are an important subpopulation of tumour cells that are implicated in tumour formation, metastases and disease recurrence. The ability to self-renew, in addition to the evasion of current chemotherapeutics, renders the CSC population a critical target in cancer research. The CSC markers CD44, CD133, CD166, EpCAM and ALDH1A have all been used, with varying success, to identify colorectal CSCs. High expression or activity of ABC transporters, particularly ABCB1, ABCC1 and ABCG2, is also a characteristic of the CSC population. This thesis characterized the human CRC cell lines, HCT116 and SW480, in regards to their CSC properties and attempted to elucidate the role of CSCs in chemotherapy resistance, and focused on functional CSC markers as potential drug resistance mechanisms. As opposed to sorting cells based on their expression of proposed CSC markers, this thesis explored the suitability of a functional sphere formation assay to enrich for CSCs.

The finding that HCT116 was composed mainly of CSCs is supported in the literature [85] as is the observation that EpCAM is not a suitable CSC marker for HCT116 or SW480 [104]. This is the first time that ABCB1, ABCC1 and ABCG2 expression have been examined simultaneously in HCT116 and SW480 monolayer cells versus colonospheres. The significant up-regulation of ABCB1 in SW480-CSCs and ABCG2 in HCT116-CSCs supports the hypothesis that the CSC population has heightened chemoprotective mechanisms. These data suggest that the side population identified via flow cytometry would also be enriched for using this sphere formation assay. Performing Hoechst dye exclusion experiments with the parental and enriched
CSC populations would determine if the side population was also enriched in the CSC lines used here.

The majority of the focus on ALDH as a CSC marker has been on the 1A family and particularly ALDH1A1. This thesis determined that ALDH3A1 is expressed at higher levels in SW480 cells compared to HCT116. In addition, I show for the first time that ALDH1A3 and ALDH3A1 are expressed at higher levels than ALDH1A1 or ALDH1A2 in these cell lines. ALDH3A1 is associated with CSCs in other tumour types including breast [205], and prostate [176] cancers. Other colorectal CSC markers are shared in common with breast cancer (CD44) and prostate cancer (CD44 and CD133). The results in this thesis warrant further exploration into ALDH3A1 expression in colorectal cancer.

5-FU, cisplatin and epirubicin were all shown to reduce the ability of parental cell lines to self-renew. These data suggest that chemotherapy exposure did not enrich for the CSC population. This finding could be insufficient as sphere formation was still achieved but at a lower frequency than in untreated cells. Hypothetically as long as a fraction of cells with self-renewal capacity survive chemotherapy treatment, they could repopulate the tumour.

The question of whether CSCs drive drug resistance or if drug resistance induces CSC properties could not be answered in this study. Future experiments evaluating the tumourigenicity of the parental, CSC enriched, and drug resistant cell lines would provide more evidence to answer this question.
LIMITATIONS

For the 3D colonosphere treatment, parental cells were plated at 100 cells/well and CSCs were plated at 50 cells/well. This was done to compare treating colonospheres of similar size. This experiment was repeated with the CSCs plated at 100 cells/well and similar results were obtained. For the flow cytometry experiments and gene expression analysis, colonospheres were grown in non-adherent plates plated as single cell suspensions at low cell densities. This proved to be a very cost-effective method that generated high enough yields to perform experiments. However, not every cell formed a sphere and there were a number of single cells affecting the analysis. Using 96-well ULA round bottom plates where only 1 colonosphere is formed per well would have been a more accurate, although more time consuming and costly approach.

In vivo experiments to assess the tumourigenic ability of the CSC enriched cell lines was not performed. Implanting the CSC enriched and parental cell lines into immunodeficient mice and evaluating their ability to form tumours is the gold-standard method to confidently identify a population of cells with tumour-initiating activity. Tumourigenicity was inferred through limiting dilution analysis and clonogenic survival, which effectively assess the ability of cells to self-renew. However, this approach is not as powerful as xenograft experiments.

Attempts were made to understand whether drug resistance drives stemness or vice versa. Drug resistant cell lines were generated and evaluation of their sphere formation ability and expression of CSC markers was planned. Due to insufficient time, resources and microbial contamination issues, these experiments were not completed. The available data are shown in Appendix IV:Supplementary Data. Drug resistant cell
lines were generated using the clonogenic survival assay as outlined in the materials and methods section, by collecting the colonies and exposing to a second round of chemotherapy treatment and colony formation. Other approaches to generate drug resistant cell lines, such as prolonged culturing of cells in chemotherapy media and slowly increasing the dose can also be used. Both of these methods have been used successfully in the literature and would have added an interesting angle to this thesis.

As mentioned previously, using primary patient samples or PDX models is the optimal way to study CSCs. However, I did not have access to primary samples and therefore used established human CRC cell lines. Although this approach is not ideal, many previous studies have shown that it is possible to enrich for CSCs from established cell lines. It is important to note that since established cell lines have adapted to culture the proportion of CSCs is likely increased compared to primary patient tumours.
SUMMARY AND CONCLUSIONS

Colorectal cancer is a highly prevalent disease with an estimated 25,000 Canadians expected to be diagnosed in 2015 [3]. The high recurrence rate and tendency for aggressive metastases has a negative impact on patient survival. CSCs are a critical subpopulation of tumour cells with the ability to self-renew and evade current treatments. The implication of CSCs in disease recurrence is due to the observation that this population has heightened chemoprotective mechanisms. This thesis explored the protective role of the functional CSC markers, ABC transporters and ALDH, against commonly employed chemotherapy.

Typically CSCs are identified using fluorescent activated cell sorting based on surface marker expression. This thesis explored the suitability of a functional sphere formation assay to enrich for CSCs. Serial colonosphere culture enriched for a population of cells with heightened CSC-related gene expression. CD44, CD166 and EpCAM were up-regulated in CSC enriched cell lines compared to parental cells. In addition, ABCB1 levels were increased in SW480-CSCs and ABCG2 levels were increased in HCT116-CSCs compared to parental cells. These findings suggest that sphere formation successfully enriches for cells with high expression of CSC-related genes. In addition, CSC enriched cell lines showed evidence of resistance to chemotherapy when treated as colonospheres. SW480 cells were found to have intrinsic resistance to 5-FU in comparison to the 5-FU sensitive HCT116 cells. This was accompanied by SW480 cells’ self-renewal ability being affected to a lesser extent by 5-FU than HCT116. Chemotherapy treatment did impact the ability of parental cells to
self-renew and altered the gene expression of the drug-resistance related markers \textit{ABCB1}, \textit{ABCG2}, and \textit{ALDH3A1}.

This study is the first to characterize the expression of CSC related genes in both monolayer and colonospheres derived from the HCT116 and SW480 cell lines. It is also the first to report higher expression of \textit{ALDH3A1} in these cell lines compared to the more commonly studied ALDH1A family. The findings discussed here warrant further investigation into the functional CSC markers, ABCB1, ABCG2 and ALDH in the HCT116 and SW480 cell lines in regards to tumourigenic ability and resistance to chemotherapy.

CSCs are a critical population of tumour cells that contribute to tumour formation, metastases and disease recurrence. Evidence supports that CSCs are not efficiently targeted by current chemotherapeutic strategies. In order to achieve disease remission, we must target the CSC population in addition to the bulk of the tumour cells. An alternative strategy could induce the differentiation of CSCs so they are effectively targeted by current therapies. It is crucial that both the bulk of tumour cells and the CSC populations are eradicated to ultimately achieve disease remission.
REFERENCES


L. Patrawala, T. Calhoun, R. Schneider-Broussard, J. Zhou, K. Claypool, and D. G. Tang, “Side population is enriched in tumorigenic, stem-like cancer cells,


APPENDIX I: CHEMICAL LIST AND SUPPLIERS

5-fluorouracil
(IV preparation 50 µg/mL)
96 well PCR Plates
Annexin V-Alexafluor488/Propidium
Iodide Apoptosis Kit
Aurum™ Total RNA Mini Kit
B27 supplement
Bovine serum albumin
Cell culture plates
CD133-PE conjugated anti-human antibody
CD166-Alexafluor488 conjugated anti-human antibody
Chloroform
Cisplatin (IV preparation 1 µg/mL)
Crystal Violet
Distilled water DNase, RNase free
DMEM/F-12 media
Gene Ruler™ 1kb DNA ladder
Dulbecco’s modified eagle’s medium
EpCAM-PerCP conjugated anti-human antibody
Epidermal growth factor
Epirubicin hydrochloride
(IV preparation 2 µg/mL)
Fetal bovine serum
Fibroblast growth factor
HCT116

iScript cDNA synthesis kit
Isopropranol
Microseal covers for PCR plates
Paraformaldehyde (4 %)
96-well PCR plates
Phosphate buffered saline (PBS)
PrestoBlue™ cell viability reagent
RedSafe™
RiboZol RNA extraction reagent
Sodium azide
Sodium pyruvate
SsoFast SYBR Green Master Mix
Standard petri dishes
TAE buffer

Hospira, Montreal, QC
Corning, Tweksbury, MA, USA
Life Technologies, Burlington, ON
BioRad, Mississauga, ON
Life Technologies, Burlington, ON
Amresco LLC, Solon, OH, USA
Sarstedt, Newton, NC, USA
Miltenyi BioTech, San Diego, CA, USA
R&D Systems Inc., Minneapolis, MN, USA
Sigma-Aldrich, Oakville, ON
Hospira, Montreal, QC
Thermo Fisher Scientific, Nepean, ON
Gibco, Life Technologies, Burlington, ON
Gibco, Life Technologies, Burlington, ON
MBI Fermentas, Burlington, ON
Sigma-Aldrich, Oakville, ON
R&D Systems Inc., Minneapolis, MN, USA
Sigma-Aldrich, Oakville, ON
Pharmaceuticals Partners of Canada, Inc.
Richmond Hill, ON
Life Technologies, Burlington, ON
Sigma-Aldrich, Oakville, ON
American Type Culture Collection (ATCC)
Manassas, VA, USA
BioRad, Mississauga, ON
Thermo Fisher Scientific, Nepean, ON
BioRad, Mississauga, ON
MJS BioLynx Inc., Brockville, ON
Corning, Tweksbury, MA, USA
Sigma-Aldrich, Oakville, ON
Invitrogen, Life Technologies, Burlington, ON
Intron Biotechnology, Korea
Amresco, LLC, Solon, OH, USA
Sigma-Aldrich, Oakville, ON
Sigma-Aldrich, Oakville, ON
BioRad, Mississauga, ON
Thermo Fisher Scientific, Nepean, ON
Life Technologies, Burlington, ON
Trypan blue
Trypsin-EDTA solution 10X
Ultra-low adhesion round bottom 96-well plates
Ultra-Pure™ Agarose

Thermo Fisher Scientific, Nepean, ON
Sigma-Aldrich, Oakville, ON
Grenier, Sigma-Aldrich, Oakville, ON
Invitrogen, Life Technologies, Burlington, ON
APPENDIX II: PREPARATION OF SOLUTIONS

Flow cytometry incubation buffer
Bovine serum albumin (BSA) 1.50 g
Phosphate buffered saline (PBS) 100 mL
Mix components and store at 4°C

Agarose gel for PCR product size confirmation
Agarose 1.50 g
1X TAE buffer 100 mL
RedSafe™ 5 µL
Mix agarose into 1X TAE buffer while heating until agarose is completely dissolved. Add RedSafe™ and mix thoroughly. Pour solution into gel cassettes and allow to cool and solidify.
## APPENDIX III: PRIMER SEQUENCES

<table>
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<th>Primer Set</th>
<th>Forward Sequence (5’→ 3’)</th>
<th>Reverse Sequence (5’→ 3’)</th>
<th>Amplicon Size</th>
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Supplementary Figure 1: Gene expression levels of CSC enriched cell lines grown in monolayer. CSC enriched cell lines were grown in a monolayer environment in either standard culture media or stem cell media (SCM). Gene expression levels were equal showing no evidence of the media used for colonosphere culture affecting gene expression (n=1).
**Supplementary Table I: SOX2 gene expression levels.** The gene expression of SOX2 was determined using RT-qPCR in untreated parental and CSC enriched cell lines. The average Ct ± SEM and categorical expression (low to very high) is shown (n=1). Both CSC enriched cells and parental cell lines had low expression of SOX2.

<table>
<thead>
<tr>
<th></th>
<th>HCT116-P</th>
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<th>SW480-P</th>
<th>SW480-CSC</th>
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<td></td>
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Supplementary Figure 2: CSC marker expression in 5-FU resistant SW480 cells. SW480 parental cells were treated with 25 µM 5-FU for 48 hours. Surviving cells were collected and plated at limiting cell densities. After 2 weeks colonies were collected and the process was repeated a second time to enrich for drug-resistant cells. Flow cytometric analysis of the CSC markers CD133, CD166 and EpCAM was performed (n=3). No significant changes in the percentage of cells expressing CD133 or the median fluorescence was detected. The percentage of cells expressing CD166 was significantly lower in 5-FU drug resistant cells compared to SW480-CSCs (p ≤ 0.05) with no change in median fluorescence. The percentage of cells expressing EpCAM was also significantly lower in 5-FU drug resistant cells (p ≤ 0.05) with no change in median fluorescence.
**Supplementary Figure 3: CSC marker expression in cisplatin and epirubicin drug-resistant HCT116 cell lines.** HCT116 parental cells were treated with 5 µM cisplatin or 0.05 µM epirubicin for 48 hours. Surviving cells were collected and plated at limiting cell densities. After 2 weeks colonies were collected and the process was repeated a second time to enrich for drug-resistant cells. Flow cytometric analysis of the CSC markers CD133, CD166 and EpCAM was performed (n=3). No significant changes in the percentage of cells expressing CD133 or EpCAM were detected. There was also no significant difference in the median fluorescence between the cell lines. The percentage of cells expressing CD166 was significantly higher in cisplatin drug resistant cells compared to HCT116-CSCs (p ≤ 0.05) with no change in median fluorescence.