IN VITRO AND *IN VIVO* CHARACTERIZATION OF CANCER STEM CELLS IN PRIMARY COLORECTAL CANCER MODELS

An Internship Report Presented to

the Faculty of the Biology Program

California State University Channel Islands

By

Rohit Gehani

September 25, 2010

APPROVED BY THE BIOLOGY PROGRAM

10/25/10

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10-25-10

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In Partial Fulfillment of

the Requirements for the Degree of

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ACKNOWLEDGEMENTS

To the casual observer, a master thesis may appear to be solitary work. However, to complete a research project of this magnitude requires a great network of support, and therefore, I am indebted to many people. I am grateful to my mentors, Justin Huard and Dr. Christophe Quéva, for their technical guidance. I am also grateful for the technical services of Esther Trueblood, Flow Cytometry Core Laboratory, and the other colleagues that supported me along the way at Amgen, Inc. Seattle, WA.

My deepest appreciation goes to my family. I would like to thank my parents, Mahesh and Heeru, for their guidance, support, and sacrifices throughout my education. I would like to thank my brothers, Aatish and Ashesh, for their motivation and encouragement.

A special thank you goes to Diana Traje, who has been my rock of support and inspiration throughout the years. I owe many thanks to my classmates and colleagues for their friendship, motivation, and encouragement.

I would also like to acknowledge CSUCI and California Institute of Regenerative Medicine for their support of my internship with the Bridges to Stem Cell Research Award.

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ABSTRACT

The existence of cancer stem cells (CSC) postulates that tumors are organized as a cellular hierarchy and that tumor initiation, growth and cellular heterogeneity are driven by a subset of cells with stem cell like properties. The CSCs are endowed with the ability to self-renew and thereby to proliferate indefinitely. At a functional level, CSCs are characterized by their ability to regenerate *in vivo* from a single cell into the full spectrum of histology of the tumor of origin and to form spheroid colonies *in vitro* in an anchorage independent environment.

The specific aims for this project include setting up assays that will enable the quantification and the characterization of CSCs and evaluating cell surface markers to enrich for CSC. Additionally, in our *in vivo* assays, we will compare different mice strains as hosts to our *in vivo* assays.

In order to assess the frequency of CSCs within a solid tumor, we established an *in vitro* and *in vivo* limited dilution assay (LDA). A known number of cells were seeded in a low attachment well with stem cell growth media, and the number of spheroids that grew was counted by Optronix GelCount. In an *in vivo* LDA, immune deficient mice were inoculated with a specific number of cells, and the number of tumor bearing mice was counted.

In order to optimize our methods, immortalized colorectal cancer (CRC) cell lines COLO205 and T84 were used as tools to develop LDAs. We were able to determine that the sphere and tumor-forming efficiency of COLO205 was superior to T84, resulting in the primary use of COLO205 cells for assay development. We also aimed to compare the tumor initiating capacity of the COLO205 cell line in order to investigate the effect of the mouse immune system on the read-out of the *in vivo* tumor formation assay. The result of this experiment was inconclusive; no tumors grew, which is a defect that we could pinpoint to a loss of viability of sorted tumor cells by FACS.

We worked with four colorectal cancer models derived from patient tumors and continuously passaged in immune deficient mice. Two models were established from primary tumors obtained from the Amgen Tissue Bank. These tumors were dissociated and cultured in serum-free stem cell growth media on low attachment plates. The other two models were obtained from a company that had continuously passaged the tumors in immune deficient mice. We used these two models to test mouse immune background and effects of irradiation on tumor initiation.

Antibodies to extracellular proteins epithelial cell adhesion molecule (EpCAM) and CD133 were used to enrich for a homogenous population of CSCs by sorting protocols. We could not confirm that either of these is a good marker but did find that FACS sorting may affect tumorigenicity and viability of CSCs in our CRC models. The outcome of the CSC enrichment procedure will be important to enable further characterization at the molecular and cellular level of CSC and the identification of potential therapeutic targets that could selectively eliminate CSCs.

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LIST OF ABBREVIATIONS

ALDH1: aldehyde dehydrogenase I

AML: acute myeloid leukemic

CFU: colony forming units

CRC: colorectal cancer

CSC: cancer stem cell

DMEM: Dulbecco's modified eagle's medium

EpCAM: epithelial cell adhesion molecule

FACS: fluorescence activated cell sorting

FCM: flow cytometry

HLA: human leukocyte antigen

IHC: immunohistochemistry

LDA: limiting dilution analysis

MHC: major histocompatibility complex

NK: natural killer

NOD/SCID: non-obese diabetic/ severe compromised immune deficient

NSG: NOD/SCID IL-2Rγ -/-

SC: stem cell

INTRODUCTION

Stem cells are cells that have the capability to self renew through asymmetric and symmetric cell division and differentiate into different cell types within the organism [4,7]. These stem cells reside in specific niches in the body and respond to signals to divide for normal tissue homeostasis or as required to repair organ injury[3].

In 1997, John Dick and colleagues similarly described a subset of acute myeloid leukemic (AML) cells (0.1-1% of total cells) that had a $CD34^+CD38^-$ phenotype, common to normal hematopoietic stem cells[7]. This subpopulation was able to regenerate AML in non-obese diabetic severe combined immunodeficient mice, thus sharing self renewal capability with hematopoietic stem cells. These leukemic cells were named "leukemic stem cells"[7]. In recent decades, these findings have been extended to other cancer cell types such as breast, head and neck, prostate and colorectal cancers, leading to the more general term "cancer stem cells (CSC)" or "tumor-initiating cells"[15]. Further serial transplantation studies showed that these tumorigenic cancer cells are critical to the development of the tumor's heterogenous makeup[3,12]. Heterogeneity refers to diversity in cancer cells within a single tumor that, in addition to CSC, is comprised of various progenitors engaged into an aberrant differentiation process. Transplantation of even a large number of these aberrant progenitors comprising the bulk of the tumor does not lead to new tumors[14,16]. However transplantation of as few as 20 cancer stem cells leads to the development of tumors identical to the tumor of origin[14].

The study of CSC has been facilitated by the generation of immune deficient mice that are used as host for tumors of human origin. Functional assays such as serial

transplantation assays use primary tumor models, primary indicating that the tumor was taken directly from the patient, serially passaged *in vivo* in mice and has not been in artificial growth conditions. Two assays are essential to the study of cancer stem cells. Serial transplantation in mice shows that only a specific subset of cells within a tumor is able to propagate tumor growth in immunodeficient mice, whereas other cells are unable to do so[8]. The CSCs are also able to generate *in vitro*, in colony formation assays, a colony, or sphere (also called a colosphere in colorectal cancer (CRC)) in low adherence plates with serum free media. These observations resulted in the cancer stem cell hypothesis stating that cancer tissue is organized as a hierarchy of cells mimicking normal tissue with CSCs endowed with self-renewal capabilities and present at the apex of an aberrant cell lineage differentiation process. Due to heterogeneity of the cells within a tumor, it is critical to optimize ways to enrich for this subset of CSC. Characterizing cell surface markers will help sort the CSC from the complex hierarchy of cells in a tumor and from endothelial cells, fibroblasts, macrophages and other cells forming the tumor stroma.

Current cancer treatments are often not effective in ridding the body of most advanced tumors. The potential existence of cancer stem cells leads to question possible therapeutic implications and to suggest explanations for treatment failure and cancer relapse. The resistance to current chemotherapies and radiation might be explained by cancer stem cells' relative quiescence (the state of slow cell proliferation or "dormancy") [4,12]. Cytostatic drugs work on the cell division, and therefore, might be less effective on a quiescent CSC population. Also, stem cells express higher level of membrane

transporters that export drugs out of the cell compared to normal tissue and thus may be less sensitive to chemotherapy[3]. Indeed, fluorescence activated cell sorting (FACS) has allowed for the discovery of a "side population" in lung, breast, and ovarian cancer cells that have the ability to efflux Hoechst DNA stain out of the cell more efficiently than other cells in the tumor[5]. This side population encompasses CSC and may explain the relative resistance of CSC to standard chemotherapies and therefore disease recurrence[5]. The characterization of CSC is therefore very important from a therapeutic point of view if they can be targeted selectively without impacting the normal SC population. Their elimination may lead to long term remission and possible cures.

The purpose of this research project was to study cancer stem cells within solid colorectal cancer models with a goal of expanding on characterization and quantifying techniques in scientific publications. Immortal cell lines were used to develop the characterization and quantification assays. To expand the CSC population in our CRC models, tumor cells were characterized by their surface molecule expression of CD133 and epithelial cell adhesion molecule (EpCAM or CD326), two surface markers previously used in published literature[2,15]. In order to count these cells, limiting dilution analyses were developed and optimized to calculate the potential to form a tumor *in vivo* and form a clonal colony *in vitro*. The project also analyzed enrichment methods that were published for other cancer types, such as FACS and antibody-bead separation in addition to specialized stem cell culturing methods. Lastly, extrinsic factors such as FACS, host immune system, and radiation were studied for their effects on tumorigenicity of immortal cell lines and CSCs in primary models.

LITERATURE REVIEW

The references used in this review and thesis are from professional journals and reviews and primarily published within the last five years. They relate to the subjects of tumor growth, colorectal cancer, and characterization of CSC. The references were accessed from internet searches and Amgen online libraries.

The literature review will be divided into three sections. The first section will describe the background of the CSC theory versus the clonal evolution of tumorigenesis. The second section will highlight previous work performed by other scientists to support the CSC theory and to characterize and count these cells. Lastly, the third section will bring to light the challenges and limitations of quantifying the frequency of CSCs in tumors.

Background

There has been much debate on tumor initiation and the cellular heterogeneity seen in a tumor. The two debated theories are the clonal evolution and the cancer stem cell model. The clonal evolution model argues that all undifferentiated cells have similar tumorigenic potential[10]. Clonal evolution explains the increased proliferation rate and the resistance to cell death observed in cancer by genetic mutations leading to an over activity of genes that signal for cell proliferation and to the inactivation of tumor suppressor genes[10]. Conversely, the existence of cancer stem cells argues that only a small number of cells within a tumor are endowed with unlimited replicative capacities and have the ability of form a tumor. Clonal evolution and CSC have been supported by experimental evidences and are probably in effect to shape the tumor cells' behavior.

These forces are therefore not mutually exclusive and cancer stem cells may as well be the fruit of clonal evolution. However, the existence of CSCs best explain the cellular hierarchy observed in many tumors and provides novel insights into the mechanisms of tumor initiation, metastasis and recurrence, and the relative resistance to current therapies[3,7,14].

While the pioneering work in CSC theory was performed in leukemia, a subset of cells with similar properties has been described in solid tumors including breast, lung, and colorectal cancers[16]. Cancer biologists have tried to find answers to questions about the existence of CSC: "what are the proportions of CSCs in different tumors? Can progenitor or differentiated cells acquire the property of self renewal?" The existence has been proven in many different cancers, and differing numbers are found in different cancers. For example, Quintana and colleagues used xenotransplantation assays to enumerate melanoma CSC. Surprisingly they found that 27% of human melanoma cells grew into tumors (from a single cell injection) in an immune compromised mouse[2,9]. Other studies in melanoma found one CSC in 1e6 human metastatic melanoma cells which is a number closer to what has been observed in colon CSC[11].

Enrichment tools such as cell culture, FACS, and transplantation assays have allowed us to improve the characterization of CSCs by quantifying the frequency of cells capable of self renewal and tumor propagation with *in vitro* and *in vivo* models.

Progress in Enrichment and Characterization

Cell sorting has been used to sort out tumorigenic CRC cells by combinations of surface molecules that are thought to be expressed by CSC such as CD44, EpCAM,

CD133 and CD166[2]. Scientists found that CSC in breast cancer and CRC tissue expressed high levels EpCAM and CD44 compared to normal colon tissue[1]. Additionally, they showed that 200 to 500 $EpCAM^{+}/CD44^{+}$ cells consistently formed a tumor while $EpCAM^{low}/CD44^{-}$ from a tumor sample failed to grow a tumor when injected into a NOD/SCID mouse. Similar evidence of CD166 as a marker for CRC stem cells was shown.

As discussed earlier, a "side population" of cells noted by Hoechst efflux allows enrichment sorting of stem cells that have a greater number of membrane transporters[5]. This method has been used to enrich for CSC in many tumor types such as lung and ovarian cancers.

In vitro colony formation assays were considered a convenient surrogate for *in vivo* tumor implantation assays. They showed that by seeding a specific number of cells in low attachment media such as soft agar, one could see that colony formation was initiated by a certain population of cells, rather than all the cells seeded in this "bioassay"[6]. This *in vitro* colony formation assay was critical for the study of hematopoiesis, the generation of new blood cells. A normal hematopoietic stem cell was able to self renew and give rise to a progenitor cell which differentiated into a mature blood cell. Now, multiple *in vitro* assays exist including serial colony formation assays and label retention assays to enable high throughput visualization and quantification. While these *in vitro* assays remain powerful tools for analysis, John Dick and colleagues performed limiting dilution transplantations to show that only a subset of AML cells could engraft in immune compromised mice[7]. Testing enrichment by *in vivo* implantation

remains the gold standard to evaluate the number and capabilities of normal and cancer stem cells.

In conjunction with limiting dilution analyses and transplantation assays, the benefits of FACS have enabled the separation and enrichment of cells by specific membrane-associated markers and activities. Similar to normal stem cells, cancer stem cells in various types of cancer have been proven to have different marker expression phenotype. Colorectal cancer CSCs have been previously sorted by markers such as CD133 (Prominin-1), CD44, CD166, and by the enzyme activity of aldehyde dehydrogenase 1 (ALDH1). The sorted subsets can be used downstream in *in vitro* clonogenic and *in vivo* tumorigenesis assays to characterize cancer stem cells.

In the past few years, CD133 has been the most popular surface molecule to be both used and criticized in the sorting and enrichment of CRC. One paper published in 2007 showed that 1 in 262 CD133+ cells has tumor formation capacity, while 1 in $5.7x10^4$ unsorted tumor cells could form a tumor[8]. Additionally, the CD133+ fraction was able to regenerate a tumor of similar heterogeneity in serial transplantation[8]. Another group in the same year published that the CD133+ population makes up approximately 2.5% of CRC cells[15]. While not stating a frequency of tumor formation capacity, the authors showed that the CD133+ population formed a tumor that was morphologically the same as the original tumor. The two previous publications showed that CD133- cell fraction was not tumorigenic[8, 15]. While these and other researchers use CD133 as an isolation tool for CSCs in CRC, Quintana and colleagues published in 2008 showing that CD133- cells could form a tumor similar to CD133+ cells in NOD/SCID IL-2Rγ -/-[9,12].

Other activities are being characterized to purify CSCs. Cells with low proliferating potential such as stem cells can be enriched by treatment with a membrane dye like PKH26 (Sigma Aldrich), cultured in serum-free growth conditions, and sorted by dye retention. PKH26 works by incorporating into the lipid bilayer of the cell membrane. As the cell divides, the amount, or intensity, of the PKH26 decreases accordingly. Cancer stem cells are more quiescent, and therefore will retain more PKH26 labeling. Progenitor and mature cells will have less PKH26 labeling. The varying intensities can be detected and separated by flow cytometry. Membrane labeling with dyes such as PKH26 currently shows the most promise in purifying a homogenous CSC population[1]. One group studying breast cancer stem cells used PKH26 to separate cells within mammospheres to compare frequency of self-renewing divisions in p53 knockout mice and ErbB2 overexpressing cancer cell line[1]. Sorting for the cells with the highest intensity of PKH26 allowed for highest purification of the CSC population.

Another feature of stem cells, normal and cancer alike, is the activity of ALDH1. ALDH1 is an enzyme that catalyzes the conversion of certain types of aldehydes into carboxylic acids[13]. These conversions can be responsible for proliferation and cell survival; therefore, measuring activity of ALDH1 may lead to enriching for a self renewing cell population like CSCs. ALDH1 levels are identified by a ALDEFLUOR (STEMCELL Technologies) system in which ALDEFLUOR reagent diffuses into cells and reacts with ubiquitous ALDH1 resulting in a green fluorescence. This fluorescence that can be detected by flow cytometry, and cells of higher enzyme activity (CSCs) can be enriched for by FACS.

Challenges in Determining CSC Frequency

While enrichment tools such as sorting for specific cell markers are in place to isolate subsets of cells, there is still work to be performed to isolate a homogenous population of CSCs. This will be difficult because expression of cell surface markers may be heterogenous across patients. Cell expression profile might also be affected by the clonal selection of more aggressive tumor phenotype. Until a stable phenotype is defined, the only way to define CSC is functionally by colony formation *in vitro* and tumor engraftment *in vivo.*

In vitro protocols for CSC quantification assays require serum-free growth conditions on low attachment plates. Most cells require serum because it supplies them with growth factors such as epidermal growth factor and insulin-like growth factor for normal function to preserve homeostasis. Normal and cancer stem cells do not require serum and growth factors, and therefore can be enriched in a serum-free culture where all other non-stem cells die off.

Once the CSCs are enriched in the serum free media on low attachment plates, counting the colonies that grow from the CSC can be an arduous process. Typically, one would seed cells, wait until colonies develop, and visualize under a microscope to manually count cells by eye. Counting and visualization has become easier with the use of plate readers that can scan and interpret colonies based on size parameters. To improve on the scanning accuracy, cells can be darkened by metabolic stains which are converted in the cell into a dark color can be read more easily by scanning apparatus.

The gold standard in determining CSC frequency in solid tumors is serial transplantation in animal models[11]. The obvious challenge with animal models is that tumor engraftment can take months and can be very costly with considering husbandry and labor. Another challenge with animal models is that different host strains can affect tumorigenicity and tumor take rates. A group studying melanoma showed that using mouse strain NOD/SCID IL-2Rγ -/- that are deprived of T, B and NK cells as host to tumor engraftment assays highly influences tumorigenicity of melanoma cancer cell[9]. This group found that 1 in every 4 melanoma cells can grow tumors in NSG mice in single cell transplants, fueling the ideas that the low frequency of CSC might be an artifact of the mouse model being used in transplantation studies.

The evidence of CSCs suggests that one cell is responsible for making up the entire histology of a tumor through asymmetric cell division and aberrant differentiation. In other words, there is a hierarchy of differentiated cells within a tumor stemming from just a small subset of cells. In order to fully realize the importance of CSCs and their therapeutic implications, it is important to isolate and enrich them by their characteristics such as phenotypic surface molecule profile, slow proliferation, and ability to efflux chemotherapeutics. From this point, we can build on assays to evaluate the success enrichment.

MATERIALS AND METHODS

Cell culture for CSC characterization and quantification assays. Colorectal cell lines COLO 205, T84, Caco-2 and DLD-1 were obtained from American Type Culture

Collection (ATCC) and grown according to culture conditions recommended by ATCC, unless otherwise stated.

Enzymatic Tumor Digestion. Patient tumor samples and xenograft tumors were digested with an enzyme cocktail (Hank's Balanced Salt Solution, 33mM $CaCl_2$, 2% FBS, 200 units/mL Type II Collagenase, 300 units/mL type IV Hyaluronidase, 50 units/mL DNase, 1,000 units/mL Dispase) for 45 minutes to dissociate tissues to make a single cell suspension. Versene (trypsin-EDTA) is used for 5-10 minutes to further reduce clumping of the single tumor cells before subjecting the cells to a 45um filter before downstream assays.

Limiting dilution analysis: Limiting dilution analyses (LDA) are designed for measuring an unknown frequency of cells in a population that give a positive response, in this case the capacity for colony formation *in vitro* and tumor formation *in vivo*. Limiting dilution analysis uses Poisson distribution to determine frequency of response. Frequencies are calculated by L-Calc, a program from STEMCELL Technologies (http://www.stemcell.com).

***In vitro* limiting dilution analysis:** Two colorectal cancer cell lines, COLO205 and T84 cells, were used to develop and optimize assays. *In vitro*, single cell suspensions were serially diluted 1:2 from 10,000 cells to 5 cells in a 96-well low attachment plate (Corning®) in stem cell growth media (Serum-free DMEM/F12 , 6 mg/ml Glucose , 1mg/ml $NaHCO_3$, 5 mM Hepes, 2mM L-Glutamine, 4ug/ml Heparin, 4mg/ml BSA, 100ug/ml apotransferrin, 25ug/ml insulin, 9.6ug/ml putrescin, 30nM sodium selenite anhydrous, 20nM progesterone and Penicillin/streptomycin) to investigate the frequency

of cells to form spheres. Cells grew in clonal spheroid colonies over one week, and the colonies were counted by the Oxford Optronix GelCount. In order to enhance the contrast of the suspended spheres, all wells were treated with the metabolic stain Iodonitrotetrazolium chloride (Sigma Aldrich) at 2 mg/ml for 18 hours prior to scanning.

***In vivo* limiting dilution analysis:** , Tumor cells (Colo205 (ATCC) or single cell suspension prepared from tumor fragment were subcutaneously injected 1:1 with Matrigel (Becton, Dickinson and Company) and serum-free RPMI media (with penicillin/ streptomysin) into the right flank of CrTac:NCr-*Foxn1*^{nu} or CB17 SCID (C.B-*Igh-1*^{b}/IcrTac-*Prkdc*$^{scid)}$ mice or NOD/SCID IL-2Rγ -/- (Jackson Laboratories) mice in four groups receiving- 100,000, 10,000, 1,000, and 100 cells. Another group of mice were injected with T84 cells with dose groups of 200,000, 20,000, 2,000 and 200 cells. Tumors arising from these cell inoculations were measured using calipers. A positive response was scored when tumor volume reached 200 mm^3. Animals were housed in sterilized cages on a 12-h light/dark cycle with food and water provided *ad libitum* in compliance with the recommendation of the Association for Assessment and Accreditation of Laboratory Animal Care. All procedures were conducted in accordance with federal animal care guidelines and were approved by the Amgen Institutional Animal Care and Use Committee.

Developing Colorectal Cancer (CRC) models. Two primary colorectal cancer tumors were received from Amgen's tissue bank and passaged into CB17 SCID (C.B-*Igh-1*^{b}/IcrTac-*Prkdc*^{scid} Taconics) mice to develop primary CRC models (Table 2). Tumor model 082907 was initiated by subcutaneous implantation of a primary tumor fragment.

Model 032708 was initiated by subcutaneous implantation of CD133+ sorted cells from a primary tumor that was enzymatically digested. After these first tumors reached 600-1000 mm^3, they were divided into ~2mm3 fragments that were further implanted into new recipient CB17 SCID mice by subcutaneous implantation. Additionally, two sets of previously passaged primary CRC tumor fragments (ID# TCO 0613 and TCO 0753) were obtained from Onco*design* Biotechnology in order to provide and establish two new models to study cancer stem cells in CRC. The TCO 0613 tumor, previously engrafted in CB17 SCID, was subcutaneously passaged four times; the TCO 0753 tumor, previously transplanted in Balb/c nu/nu, was subcutaneously passaged four times in SCID mice at Oncodesign. Upon reception at Amgen, these tumor fragments were thawed and grown in NOD/SCID IL-2Rγ -/- (NSG) (Jackson Laboratories), NSG with irradiation, and CB17 SCID with irradiation to determine the best host for initial tumor implant. The mice were exposed to 180 radiation units (rads) at 53 rads per minute. A primary tumor cell bank was established with the tumor fragments and cell suspensions.

Cell staining and sorting. For the 032708 CRC model, the CD133 Isolation Kit from Miltenyi biotec was used to positively selected CD133+ cells separated by use of a magnet. For all other flow cytometry and sorting methods, antibody staining was performed at 2-8°C for 20-30 minutes. For the phenotypic characterization, fluorochrome-conjugated antibodies to human CD326 (anti-EpCAM, antibody EBA, BD and 9C4, BioLegend), to human CD133/2 (Antibody 293C3 and AC141), to human leukocyte antigen (HLA-A, -B, -C) (antibody G46-2.6) to human CD44 (antibody G44-26) and to human CD24 (antibody SN3, Life Technologies) were used to label

extracellular surface molecules. In order to eliminate mouse cells from single cell suspension prepared from tumor fragments passaged in mouse, we also used mouse monoclonal antibody specific to mouse MHC class I molecule H-2k^{d} (antibody SF1-1.1). All antibodies used for flow cytometry and sorting were from BD Biosciences except where noted. Flow cytometry and sorting were performed on the BD FACSCalibur and FACSAria. When sorting for human EpCAM, the top 10-20% of EpCAM expressing cells was collected for downstream LDA. Sorting and isolation of epithelial tumorigenic cells were also attempted by immunomagnetic separation using Dynal CELLection Enrichment kit (Life Technologies).

Histological Characterization of primary tumor #082907. Tumor fragments derived from the primary tumor (ID# 082907) were evaluated by immunohistochemistry (IHC) for the expression of CD133, a surface marker enriched in CSC fractions in CRC[5]. Mouse monoclonal antibody to CD133/1 (AC133, Miltenyi biotec) and rabbit monoclonal antibody to CD133 (C24B9, Cell Signaling) were used to stain and characterize the paraffin-embedded colorectal cancer tumor section. Also, sections were stained with the appropriate isotype control to assess background signal.

RESULTS

Immortal CRC cell lines as an assay development tool.

In order to establish *in vitro* LDA for our primary models, we first used fast proliferating CRC cell lines, Colo205 and T84 (ATCC). COLO205 was established from ascites taken from a patient with colon carcinoma, while T84 was established from the lung metastasis of a colon carcinoma. COLO205 cells and T84 cells were plated in serial dilution in low

adherence plates and in serum free growth media. Ten days after seeding cells in serial dilution, the cells and growing colonies were counted by the Optronix GelCount scanning equipment and analysis system. We set the Gelcount colony counting software to count a colony as the size of approximately 20 cells but could not get an accurate scan and count of the colonies because the cells were not dark enough to contrast them from the media and cell debris in the wells. We then stained all the cells and colonies with the metabolic stain Iodonitrotetrazolium chloride to enhance contrast and allowed us an improved evaluation of sphere count using the Optronix GelCount (Figure 1). The L-calc software (Stem Cell Technologies) calculated that 1 in every 476 Colo205 cells has the ability to form a colony- a comparable characteristic of CSCs. The estimate of the frequency of colony forming units in colorectal carcinoma T84 cell line was calculated to be 1 in 1,581 T84 cells.

Figure 1a. In vitro Limiting Dilution Analysis of COLO 205 model

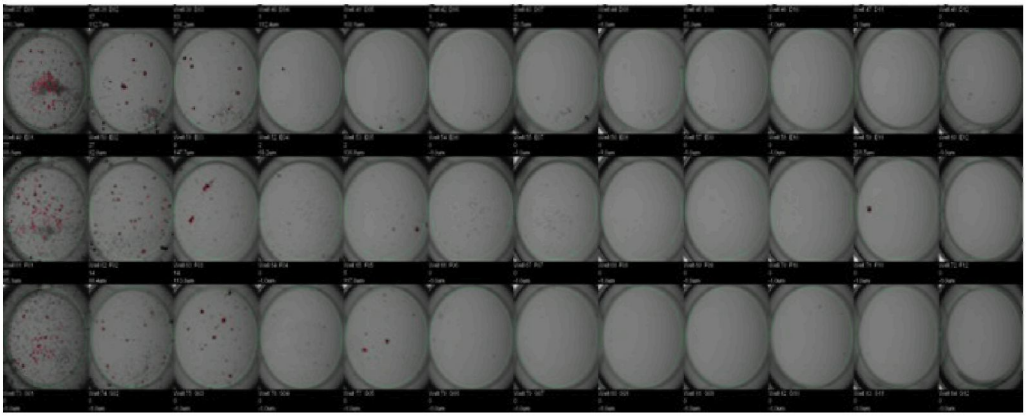
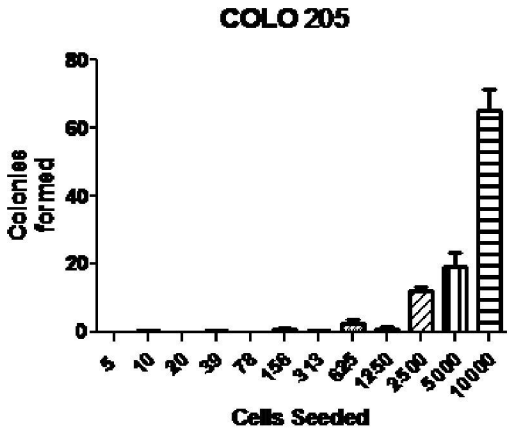


Figure 1b,c. Sphere formation assay – COLO 205



Cells seeded	average colonies formed
10,000	65
5,000	19.3
2,500	12
1,250	1
625	2.7
313	0.3
156	0.67
78	0
39	0.3
20	0
10	0.3
5	0

Figure 1a: Optronix GelCount Scan of the colonies formed from COLO 205 cells seeded in stem cell growth media in low attachment plates. From left to right, wells were seeded diluting 1:2 from 10,000 cells to 5 cells (in triplicate). The software allowed us to define parameters to establish what constituted a colony. Figure 1b, c: Bar graph representation of the average number of colonies formed after 10 days post seeding. Using L-calc, we calculated that 1 in 476 COLO 205 cells had the ability to form a colony.

We next investigated the ability of Colo205 and T84 to engraft in immune compromised mice. Previous experience with these cells lines was used to determine the maximum number of cells to inject as well as the mouse background to be used. NCr nude CrTac:NCr-*Foxn1*^{nu} (nude) mice were chosen as these cell lines are known to robustly grow in nude that they are only T-cell deficient. Nude mice were therefore implanted with a mixture of matrigel and cells. Colo205 cells were injected at cell groups of 1e6, 1e5, 1e4, and 1e3 while T84 cells were injected at cells groups of 2e6, 2e5, 2e4, and 2e3. We assessed tumor development by measuring tumor size using calipers. A tumor volume reaching 200mm^3 was given a positive score for the LDA assay. We had to wait 13 days for Colo205 cells to become measurable tumors and 19 days for T84 cells to become measurable tumors. In the *in vivo* limiting dilution assay, our frequency

calculation software calculated that 1 in 83 Colo205 cells grows into a tumor; and 1 in 93,108 T84 cells grows into a tumor.

FACS effects on tumorigenicity.

After successful *in vitro* and *in vivo* LDA, we next tested whether our sorting technique may be affecting the assays. In order to test this hypothesis, we tested the effect of sorting Colo205 cells by their surface EpCAM expression. All Colo205 do express EpCAM (Figure 2).

Figure 2. Characterization of cell surface molecule EpCAM on COLO205 cells. EpCAM is a good marker to test if sorting affects tumorigenicity.

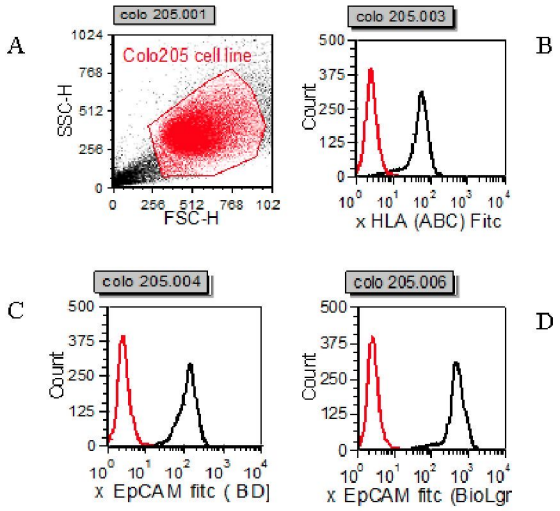


Figure 2: A) FACS FSC vs. SSC dot plot representing the COLO205 cells scatter for gating. B) COLO205 stained with anti-HLA (pan) FITC shows all cells positive for HLA. C,D) All cells stained with anti- Human EpCAM –FITC (from BD and BioLegend). Red overlay is representative of the isotype control staining to control for false positive staining.

The sorted cell fraction was injected into SCID mice (at the following cell numbers: 1e6, 1e5, 5e4, 1e4, and 1e3) to test tumorigenicity. SCID mice were used rather than nude mice because SCID mice have a lower immunity hurdle being void of T and B cells. We used a lower number of mice per group compared to the previous study as this

experiment was considered a pilot and we knew the robustness of Colo205 growth *in vivo*. We found that the antibody sorted cells were less tumorigenic in mice in a parallel LDA with unsorted Colo205 cells. Tumorigenic cell frequencies were calculated by the limiting dilution analysis software L-calc using the data from Tables 1a-1b. One in 2,100 unsorted Colo205 cells could engraft *in vivo*, and 1 in 85,284 EpCAM+ sorted Colo205 cells could engraft *in vivo*. Also it is important to note that tumors engrafted from the unsorted Colo205 cells became palpable after 13 days post injection, while the tumors engrafted from the EpCAM+ sorted cells were palpable at day 20 post injection.

Table 1a (top), b (bottom): 1a. Unsorted Colo205 cells in SCID mice. 1b. EpCAM sorted Colo205 cells in SCID mice

Dose (# cells)	Number injected	Positive response (Tumor growth = 200 mm^3)
1,000,000	3	3
100,000	3	3
50,000	3	3
10,000	3	3
1,000	3	1

Dose (# cells)	Number injected	Positive response (Tumor growth = 200 mm^3)
1,000,000	3	3
100,000	3	1
50,000	3	3
10,000	3	0
1,000	3	0

Data from *in vivo* LDA at day 29 post cell injection entered into L-calc program (STEMCELL Technologies).

Establishment of Primary CRC tumor models from Amgen tissue bank samples.

A number of tumor fragments from different patients were received from the Amgen tissue bank in 2007-2008, but only two out of 15 engrafted into CB17 SCID mice. Tumors formed approximately eight months post transplantation of fragments (model

082907) or inoculation of 1e6 CD133+ sorted cells (model 032708). Tumor growth curves shown in Figure 3 display the growth rates of the successive *in vivo* passaging of these two models from fragments of the initial tumor grown in mice.

Figure 3a, b. Successful establishment of 2 tumor models derived either from colorectal cancer tumor fragments (a) or CD133+ sorted cells (b)

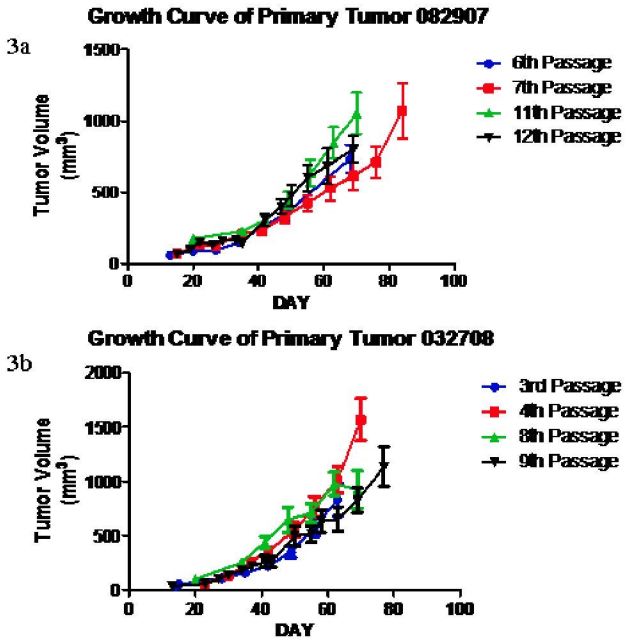


Figure 3a, b: Growth curve patterns of the two primary CRC tumor models indicate that post xenograft fragment implantation, tumor growth is comparable from passage to passage for the same tumor. However it is important to note that initial implantations (primary CRC model 082907 fragments and primary CRC 032708 sorted CD133+ cells) required a very long time (~8 months) to grow (Data not shown).

The growth rate of tumors within the same tumor model is similar from passage to passage, but there is a slight distinction in growth rates between the two models: fragments from the tumors originally initiated by CD133+ sorted cells of 032708 grew faster than the transplanted fragments of tumor model 082907. The tumor take time for the two models was the same (~15 days) (Table 2).

Tumor Model	Initiation of model	Year of model establishment	Passage started at Amgen	Progress
082907	Subcutaneous implant of primary tumor frag (ATB) into CB17SCID mice	2007	Primary	IHC on primary and after xenograft for CD133; LDA in vitro and in vivo with EpCAM+ sorted
032708	Subcutaneous inject. of CD133+ sorted cells from primary tumor into CB17SCID mice	2008	Primary	LDA in vivo and in vitro with EpCAM+ sorted
TCO 0613	Subcutaneous implant of primary tumor frag into CB17 SCID	2007-2008	5	Growth rate assay in NSG, NSG+irradiation, NOD/SCID +irradiation
TCO 0753	Subcutaneous implant of primary tumor frag into Balb/c nu/nu	2007-2008	4	Growth rate assay in NSG, NSG+irradiation, NOD/SCID +irradiation

Table 2. Primary colorectal cancer model progress (Key: CB17SCID: severe compromised immune deficient; NSG: NOD/SCID gamma: NOD/SCID IL-2Rγ -/-)

Finding a suitable host two acquired primary models.

In addition to the models developed in house, we acquired 2 primary models from Oncodesign. Tumor fragments from TCO 0613 and TCO 0753 were established in SCID and Balb/c nude mice, respectively. We tested two mouse backgrounds as well as the effect of irradiation on take rate. Figure 4 is a line graph representing a growth curve of the initial transplantation of TCO 0613 colorectal Oncodesign solid tumors. The fragments grew in the naïve NSG mouse more vigorously than in the radiation treated NSG mouse and radiation treated SCID mouse (Figure 4) The TCO 0753 tumor fragments grew similarly in the naïve NSG mouse and radiation treated NSG mouse, but did not grow as well in radiation treated SCID mouse. The untreated NSG mouse became the xenograft host for Onco*design* tumors models.

Figure 4. Comparison of tumor TCOL-001 (TCO 0613) and TCOL-007 (TCO 0753) growth rates in different hosts

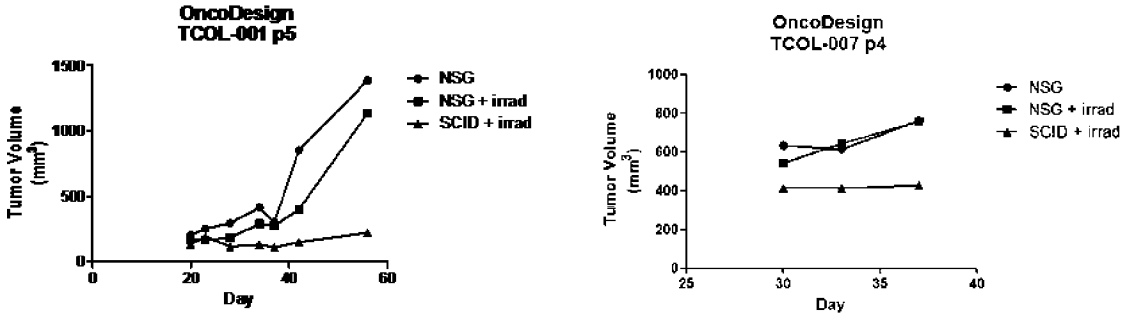


Figure 4: Growth curve patterns of the CRC tumor models TCOL-001 (TCO 0613) and TCOL-007 (TCO 0753) indicate that naïve NSG (not treated with radiation) are the best host in which to passage the tumor fragments to establish the model. Tumor fragments in radiation treated NSG mice grew similarly, but treated SCID mice did not provide a beneficial microenvironment for the tumors.

Surface marker expression on primary tumor cells.

In order to begin the enrichment process for CSCs we chose antibodies to surface markers that would allow separating the human cells from the mouse stroma. Anti-human leukocyte antigen (HLA), to detect cells expressing MHC class I protein; anti-human EpCAM to detect human epithelial tumor cells (not cross reactive to mouse EpCAM), and anti-mouse H-2k^{d} (mouse MHC protein) were used to differentiate human and infiltrating mouse cells from the stroma. In comparing the histogram staining intensities (Figure 5) of Human specific MHC class I and Mouse MHC class I, the signal shift is very minute indicating that antibodies to Human and mouse MHC class I proteins will not allow for good detection of their respective antigens. Human specific EpCAM appeared to be a better marker from initial FACS analysis, staining 14% of the cells positive. (Figure 5)

Figure 5: Characterization of cell surface molecules on 032708 tumor cells. Epcam reactivity may allow the isolation of human cells from the mouse stroma.

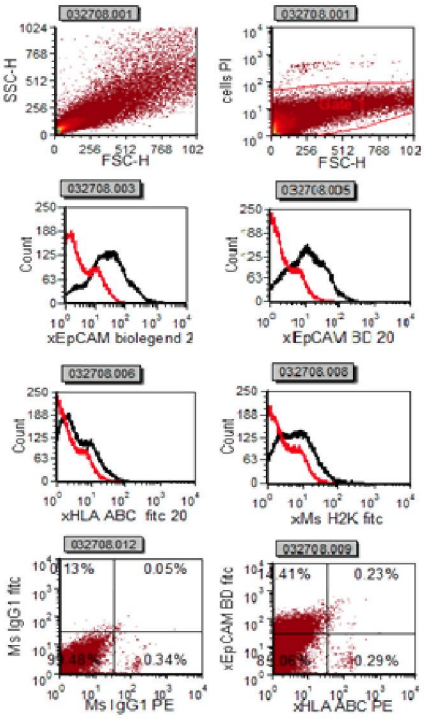


Figure 5: First row: Forward scatter (FSC) vs side scatter used to set initial gating of single cell suspension from 032708 tumor. FSC vs PI (propidium iodine stained cells) plot is used to gate live cells. The red overlays are representative of the isotype control. Second row: Staining of anti-Human EpCAM (from BD and BioLegend) comparison confirms expression of epithelial cell adhesion molecule (EpCAM) on the cells. Third row: Staining for expression of human and mouse MHC Class I proteins shows that there is not a strong reactivity to Mouse and Human cells MHC class I proteins. These antibodies will not allow a good separation of human cells from mouse cells. Bottom row: Dual antibody staining of EpCAM and HLA-A,-B,-C and corresponding isotype controls. Data shows that EpCAM as an extracellular marker on colorectal tumor 032708 cells and may allow the separation of human tumor cells from mouse stroma.

From these results, we moved to sorting for the top and bottom 20% of cells expressing human EpCAM, the positive and negative fractions respectively and performed *in vitro* and *in vivo* limiting dilution assays. These cells, unexpectedly, did not grow into definable colonies *in vitro* unlike what was seen when we seeded unsorted Colo205 cells in a colony formation LDA and primary models 082907 and 032708 in culture. The sorted human epithelial cells, from the primary tumors 082907 and 032708 were injected into SCID and NSG mice at the following cell numbers: 2e5, 1e5, 5e4, 2.5e4, 1e4, 5e3, 1e3, 5e2, and 1e2. Surprisingly, none of the mice developed any tumors

after seventy days. However, tumor fragments from these same models were growing consistently in continuous passaging in the same mouse strain.

Immunohistochemistry (IHC) and flow cytometry (FCM) to detect CD133, a surface marker for CSC characterization in primary colorectal cancer tumor.

We next investigated the expression of CD133, a promising CSC marker, in our *in vivo* models using 2 methods, FCM and IHC. First, analysis by flow cytometry found that 1-2% cells from the primary tumor were positive for CD133 expression. Implanted fragments (082907) and injected cells (032708) regenerated tumors that were 2% of cells were positive for CD133. However, our IHC analysis (Figure 6) for CD133 expression on primary tumor 082907 indicated that few cells in limited areas in the tissue section express CD133 on their surface. After xenograft there is a notable increase in CD133 expression in various regions of the tissue sample (top right panel). This increased staining after xenograft was unexpected and is not in agreement with the previous flow cytometry analysis that shows a small population of CD133+ cells. According to the IHC, the antibody is staining non-specifically all luminal surfaces. The IHC on primary tumor 082907 was controlled by DLD-1, a cell line known to not express CD133[6]. As a positive control, we stained CRC cell line Caco2, known to express high level of CD133[6]. The IHC was specific with a strong signal detected in Caco2 and no staining observed with the negative control DLD-1 and isotype control.

Figure 6. Detection of a subset of CD133+ cells population by immunochemistry in the primary tumor sample and in the xenograft established from the same tumor.

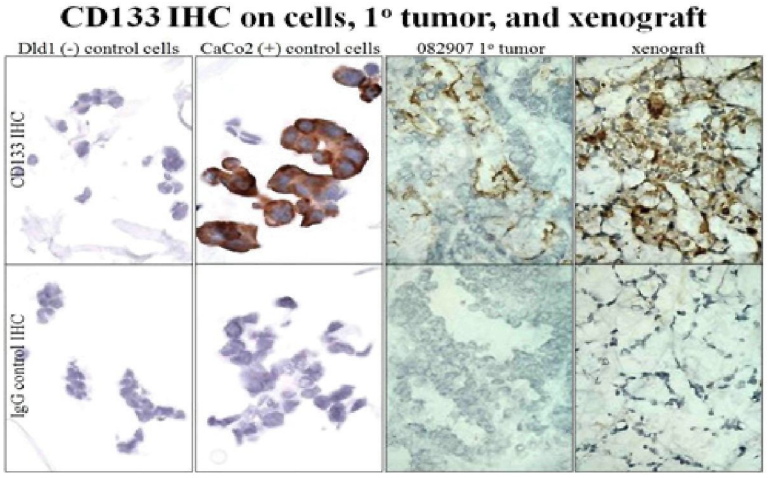


Figure 6: Top: IHC staining of mouse antibody to Human CD133/1 antigen (AC133 Miltenyi) shows that CD133 staining was preserved during *in vivo* passage DLD-1, a colorectal carcinoma known to be negative for expression of the CD133 antigen and CaCo2 cells known to express the CD133 antigen were embedded in collagen and used as controls for immunochemistry. CD133 staining of the primary tumor fragment #082907 was compared to 082907 xenograft established in CB 17 mice (C.B-*Igh-1*^{b}/IcrTac-*Prkdc*^{scid}). (Similar results were seen with a rabbit antibody to Human CD133 antigen from Cell Signaling.) Bottom row: Isotype control staining.

DISCUSSION

We developed and optimized in vitro and in vivo CSC functional assays with the immortal CRC cell lines Colo205 and T84 before moving to the primary tumors. While these cell lines have been propagated for a long time *in vitro*, their robust growth and tumorigenicity *in vivo* makes them ideal positive control for assay development. *In vitro,* the cells were seeded in a limiting dilution analysis with a goal of estimating the number of colony forming units (CFU) within a specific number of cells. We were able to show that the cell lines grew in spheroid colonies in defined stem cell growth media. This

spheroid growth in serum-free, anchor independent environment forced the immortal cell lines to grow similarly to how we know about stem cells grow *in vitro.* This allows us to continue assay development and translation to our primary CRC tumor models.

We used the L-calc program along with Optronix GelCount to provide a higher throughput way to calculate the frequency of CFU in Colo205 and T84 *in vitro*. We found the growth of Colo205 cells into colonies to be more aggressive than T84 cells which we expected based on experience with this cell line. It is also possible that Colo205, being derived from ascites, has adapted to grow with minimal attachment. However, the *in vivo* and *in vitro* CFU frequencies of Colo205 were expected to yield similar results but did not. A reason for the inconsistency between *in vivo* and *in vitro* CFU frequency in Colo205 could be due to human error as it was the technician's first time performing subcutaneous injections as well as the first trial of the *in vitro* LDA. After more trials, it is more likely that we would see a closer correlation between the *in vivo* and *in vitro* data comparing the two cell lines independently. Also, colony interpretation by GelCount can vary by the operator's definition of a colony by size parameters and use of a colorimetric metabolic stain. In repeating the *in vivo* LDA with Colo205 to test sorting effects on tumor proliferation, the CSC frequencies varied as well. This can be because a different strain was used (nude mice was used to develop assay; SCID was used in repeat); and a smaller number of mice was used in the repeat because it was a pilot study. Both factors may have affected the L-calc program calculation.

After establishing our LDA assays with the immortal cell lines, we turned our attention to EpCAM as a first candidate surface molecule for enrichment of human

epithelial cancer cells from mouse stroma. Antibody specific to human EpCAM, that is not cross reactive to the mouse homologue, would enable the separation of host (mouse) cells from human tumor epithelial cells. Indeed cells prepared from primary models and Colo205 express high levels of EpCAM. Cells from primary models were strongly 14-20% EpCAM+, and virtually all Colo205 were EpCAM+ deeming it to be a suitable marker. After more investigation with different tumor models and sorting methods, we found that the act of sorting was affecting the tumorigenicity of the tumor cell fractions. The limiting dilution analyses comparing the sorted versus unsorted Colo205 estimated that unsorted Colo205 cells were 40x more likely to form a tumor. Additionally, tumors from unsorted Colo205 cells became palpable 7 days sooner than tumors from Colo205 sorted for EpCAM (13 days compared to 20 days post injection).

In our two primary models that were established at Amgen from 2007-2008, we found tumor growth to be comparable from passage to passage, independent of specific model. While the initial implantations and injections required about 8 months to first engraft, once the model was established, tumor take rate for both models, 082907 and 032708, was the same (~15 days post injection or implantation). It was also interesting to note that in most of the passages, the 032708 model, initiated by injection of sorted CD133 cells from patient tumor, grew faster than the 082907 model, which was initiated by whole fragment implantation. This can be interpreted as CD133 possibly being a good marker to investigate further for enrichment of cancer stem cells, but this observation would need to be repeated in more models to be confirmed.

In the process of establishing the two CRC tumor models, we discovered a number of other primary samples did not engraft in SCID mice. The failure to engraft could be attributed to mouse host immune background. For these concerns in establishing our two purchased CRC models, we compared tumor take and growth rates in mice of different backgrounds and radiation treatment. We found that NSG mice were superior host to SCID mice, which was not surprising because NSG are void of T, B, and NK cells and have been shown in melanoma studies to be exceptional hosts for single cell transplants giving rise to a tumor[9]. It was surprising to see that radiation of host animals was not beneficial for tumor growth. Radiation is used to reduce bone marrow activity thereby leaving the mouse host with virtually no immune cell activity. The tumor growth in the naïve (untreated) NSG was faster and more consistent, but these results are based on comparisons of 1 mouse per treatment. Repeating this host test and treatment with more mice can confirm our findings.

In spite of providing evidence that human EpCAM is superior to mouse MHC and Human MHC as a marker for enriching tumor cells from mouse stroma, we did not see engraftment by EpCAM sorted cells that were run through FACS. Sorting our primary models 082907 and 032708 for human EpCAM did not result in engraftment which again suggests that our sorting by FACS may be affecting the tumorigenicity of the CSCs. We had injected these cells in both NSG and SCID mice but could not confirm which is the best host considering immune background for these two primary tumor models that were established at Amgen.

This gives rise to two explanations; either EpCAM is not expressed on CSC or FACS dramatically decreases the ability for highly tumorigenic immortal cells and xenografts from primary tumors to form tumors in immunodeficient mice. There is evidence that magnetic affinity beads may be less harsh on cells compared to the high pressured, small diameter flow stream of FACS equipment. Tumor cells are large heterogeneous cell populations that are considerably less uniform than a typical cell line or lysed peripheral blood that are typically run through cytometers and FACS equipment.

Another possible reason for failed engraftment of antibody sorted EpCAM+ cells could be the chemistry that is seen in the mouse host. The immunoglobin isotype of the produced antibody to EpCAM is Mouse IgG1, which binds poorly to the Fc receptors on macrophages and natural killer cells. These are major components of immunity that work with T-cell mediated response to an antigen (in this case, tumor cells bound with antibodies to human EpCAM. Although we tried using NSG, devoid of B, T, and natural killer cells, as a low hurdle for tumor engraftment, there is a possibility that whatever is remaining of the mouse innate immunity is deactivating the EpCAM+ tumor cell and preventing adherence of the tumor cells in the mouse. Some recently published papers on melanoma used negative selection to enrich for the human epithelial tumor cells. They use an antibody cocktail that binds to mouse and human hematopoietic and endothelial cells, leaving a cell population of human epithelial tumor cells without antibody bound to it[8]. If the hydrodynamics of the sorter can be optimized for large tumor cells, a similar negative sorting protocol could be used to better enrich for CSCs within our colorectal cancer models.

Contrary to what our tumor model 032708 shows in comparison to the 082907 model, a number of papers have been published refuting CD133 as a good marker enrichment of CSCs in CRC. Prominin-1 is expressed on cells in glioblastomas, medulloblastomas, and also normal neural stem cells[13]. While there are populations of cells expressing CD133 in CRC, there has been evidence that CD133-/EpCAM$^+$ cells are capable of tumor growth[8]. Our IHC analysis of tumor 082907 upon reception of primary tumor and after one xenograft passage surprisingly shows CD133 expressed all over the luminal surfaces of the tissue from the xenograft. This can be attributed to non specific binding of the antibody used to different regions of the tumor sample. Further investigation by FACS and flow cytometry analysis did not agree with the IHC, giving evidence that CD133 expressing cells are ~2% of the total tumor cell population, which would be expected for a CSC marker. It is possible that the mAb to CD133 displays different properties by FACS or IHC. To resolve this issue, CD133+ sorted and CD133- sorted cells need to be assayed in *in vivo* and *in vitro* LDAs to establish their respective colony and tumor formation frequencies.

According to the publications and our own studies, the number of CSC varies amongst different types of cancer and also different patients of the same cancer. In developing two new colorectal cancer models from fragments from Oncodesign, we have given ourselves more opportunity to develop more accurate limiting dilution analyses in order to allow us to calculate frequency of cancer stem cells after enrichment steps to ensure CSC purity. Purifying this CSC population may give insight on how to

specifically target the subset and may lead to long term remission and to possible cures for cancer.

SUMMARY

1. Colorectal cancer lines Colo205 and T84, albeit not relevant to the study of cancer stem cells (CSC), are robust models to use for limiting dilution analysis assay development. Two limiting dilution assays have been set up: *in vitro* we studied the ability of tumor cells to grow as spheres in low attachment plates and *in vivo* we quantify the ability of cells implanted in immune deficient mice to form a tumor. Using these assays, we confirmed that Colo205 cells are more aggressive than T84 cells in regards to tumor initiation and proliferation.
2. Sorting of tumor cells based on plasma membrane protein markers affected the tumor take of Colo205 and CSC population in primary models. Possible reasons may include host reaction to bound antibody to cells or FACS process is too harsh on tumor cells affecting viability and engraftment.
3. Four models from primary CRC tumors are available for further use. Two models were established at Amgen from 2007-2008 and still display consistent growth in continuous passaging in SCID mice. Two models were bought from an external source. Thusfar, these two purchased models have been reestablished in NSG mice; radiation showed no benefit to tumor take rate and growth.
4. A diverse set of cell markers has been investigated to improve the purification of CSCs. Our flow cytometry analysis suggested that Human EpCAM was a better marker to sort for than Human MHC Class I and Mouse MHC Class I. This may be explained by the quality of reagents, but more studies are necessary to confirm.

5. CD133 is a published marker encompassing CSC in CRC. However, we observed discrepancies between our flow cytometry and IHC data comparing detection of CD133 on primary CRC tissue and after passage of the tumor as xenograft in mice.

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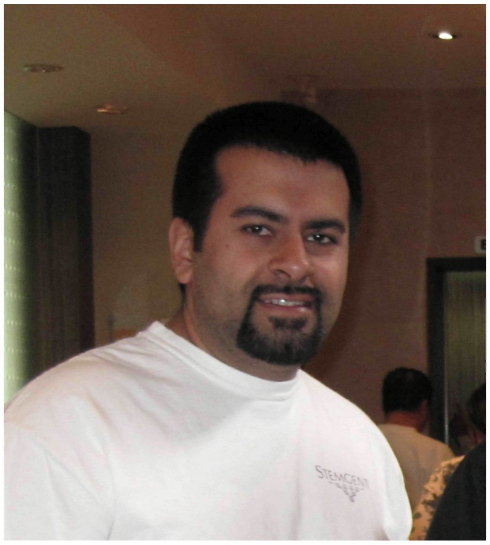

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Rohit graduated from the University of California, Davis, in 2005, receiving his BS in Biotechnology. Post graduation, he joined CALTAG Laboratories as an antibody purification technician. In October 2006, he returned to southern California and took a quality control position with Invitrogen Corporation, simultaneously playing a critical role in the relocation of CALTAG Laboratories' cGMP facility into Invitrogen's new site. In September 2008, Rohit entered the M.Sc. in Biotechnology program at California State University, Channel Islands, where a year later, he was awarded a CIRM Bridges to Stem Cell Research Grant and internship. In September 2009, after completing his M.Sc coursework, he left his post at Invitrogen to become a stem cell technology intern at Amgen Corporation in Seattle, WA. After completing his internship project in September 2010, he plans to utilize his experiences and pursue a career in stem cell biology and specialize in oncology and inflammation.