CELLULAR MECHANISMS OF CANCER STEM CELLS

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DEDICATION

Dedicated to my late grandfather Sri Jaganath Bhagwandin and my dearest mother Sri Devmati Jaganath, for their motivation and relentless pursuit of success.

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by

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ABSTRACT

The concept of cancer stem cells is based on the presence of adult stem cells within tissues that can transform and give rise to tumors that also retain stem-like properties. Although advanced solid human tumors are treated with chemotherapy and/or radiotherapy, in most cases there are only partial responses and tumors generally recur. These observations suggest that there is a resilient 'rare' cancer cell type that survives therapy, which gives precedence to the following cancer stem cell hypothesis.

Hypothesis: Cancer cells retain stem-like characteristics that may reflect their degree of malignancy and resistance to conventional therapy.

The experiments presented in this thesis report focus on identification of cancer stem cells by exploiting conserved cellular mechanisms of normal stem cells such as; label retention (LRC) and transit amplification (TAC). Panc-1 human pancreatic cancer cells pulsed with BrdU gave rise to tumors that contain a 'rare' quiescent population of cells retaining their BrdU label up to one month *in vivo*. Transit amplification of these cells was explored *in vitro* by using a stem cell marker, the 'side population' (SP), that gave rise to two distinct cell populations after one week in culture. Isolated Panc-1 clones with varying degrees of the SP phenotype and metastatic potential demonstrated that loss of SP coincides with increased metastasis. Treatment of these clones with the chemotherapeutic drug gemcitabine showed that the presence of SP and decreased metastatic potential conferred resistance. Combined treatment of a telomerase inhibitor (GRN163L) and gemcitabine sensitized cancer stem cells *in vitro*. These findings suggest that cancer stem cells may retain stem-like characteristics such as label retention, symmetric and asymmetric divisions, and drug sensitivity to govern tumor progression and resistance. These finding may in the future allow the dissection of underlying molecular mechanisms regulating cancer stem cells and provide opportunities to discover therapies that specifically target these cancer stem cells.

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LIST OF ABBREVIATIONS

DNA – Deoxyribonucleic Acid

NOD/SCID – Non-Obese Diabetic / Severe Combined Immunodeficient

ALL – Acute Lymphoblastic Leukemia

AML – Acute Myeloid Leukemia

BTSC – Brain Tumor Stem Cells

PDAC – Pancreatic Ductal Adenocarcinoma

PanIN – Pancreatic Intraepithelial Neoplasia

LRC – Label Retaining Cells

BrdU – 5-Bromo-2-deoxyUridine

CFSE – 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester

DAPI – 4',6-Diamidino-2-Phenylindole

FACS – Fluorescence Activated Cell Sorting

In Vitro – In a culture dish

In Vivo – In an animal

TAC – Transit Amplifying Cells

NK – Natural Killer

STASIS – Stress or Aberrant Signaling Induced Senescence

ECM – Extracellular Matrix

H2B-GFP – Histone-2B linked Green Fluorescence Protein

FITC – Fluorescein Isothiocyanate

PFA – paraformaldehyde

HSC – Hematopoietic Stem Cells

MDR – Multi Drug Resistant

ALT – Alternative Lengthening of Telomeres

hTERT – Human Telomerase Reverse Transcriptase (Protein Component)

hTR – Human Telomerase RNA (Component)

PCR – Polymerase Chain Reaction

TRAP – Telomeric Repeat Amplification Protocol

5-FU – 5-Fluorouracil

CCD – Cooled Charge-Coupled Device

RLU – Relative Light Units

LET – Light Emission Tomography

MRI – Magnetic Resonance Imaging

FGF – Fibroblast Growth Factor

BMP – Bone Morphogenetic Protein

EGF – Epidermal Growth Factor

CHAPTER ONE
Introduction and Literature Review

ORIGIN OF CANCER STEM CELLS

The theory of neoplastic disease, 'Cancer', originating from developmentally undifferentiated stem cells was proposed by the cytology work of pathologist Julius Cohnheim in 1867 (Cohnheim 1867). His interpretation of karyotypic chromosomal differences between epithelial and mesenchymal tumors contributed to the characterization and understanding of tumor metastasis, which led to the belief that neoplasia, was a 'stem cell disorder'. This was further observed in 1890 by pathologist D. von Hansemann who determined that multipolar mitosis was a common phenotype of neoplasia and by today's nomenclature would be referred to as self-renewal or symmetric division, a conserved mechanism of normal stem cells (von Hansemann 1890).

During this era, cytologists were limited to microscopic observations of chromosomal aberrations by karyotyping, which they used to derive theories of origin and function. One of the more prominent proposals came from Boveri's Hypothesis: oncogeny by chromosomal mutation (Boveri 1914). The idea favored chromosomal number normalization and tumor evolution through accumulation of precise mutations and selective growth. Boveri's hypothesis and microscopic

techniques led to the reemergence of the 'stem-line concept' as proposed by O. Winge in 1930 (Winge 1930).

Almost a decade after Winge's proposal, but not realized as a stem cell mechanism at the time, Koch (Koch 1939) postulated that primary tumors were heterogeneous in nature and therefore could be enriched for highly metastatic sublines through *in vivo* selection. Koch demonstrated that peritoneal injections of Flexner Joblin rat carcinoma cells gave rise to highly metastatic ascites tumors. This landmark finding not only empowered researchers to overcome technical *in vivo* barriers of metastasis but also spurred a decade of studies focused on the biology of metastasis ranging from metabolism, growth, and enzymatic studies. This selective growth advantage of the ascites tumors may have been due to tumor evolution, as reviewed by Hauschka (Hauschka 1961). Hauschka reported that hypodiploid cells could not sustain survival due to poor metabolic adaptation, genomic instability and defective nuclei. In addition, he suggested that diploid tumors have a slow growth rate and survive due to retention of their parental normal stem cell properties. However rare these cells are, they may serve as the foundation of tumors consistent with the stem cell hypothesis (Hauschka 1958; Hauschka and Levan 1958; Revesz and Norman 1960; Revesz and Norman 1960).

In the early 1950's, George and Eva Klein of Sweden realized from their *in vivo* studies of Ehrlich ascites tumors (Klein 1950) that there was a limitation in this model for accurate characterization of carcinogenesis. They proposed that

since metastasis occurs from a primary tumor and not from injected cell suspensions, as demonstrated by Koch using the Flexner carcinoma model (Koch 1939), there should be a model that reflects the entire process from tumorigenesis to metastasis. To address this problem they developed a method to establish metastatic ascites tumors originating from primary solid tumors using the Krebs-2 cell line that was originally developed from the inguinal region of a male mouse. Through successive rounds of passage of tumor cells isolated from the ascites fluid, they created a cell line that could not only give rise to a primary tumor but also was capable of metastasizing to the peritoneum as an ascites tumor (Klein and Klein 1951; Klein 1953). This led to the characterization of growth rates, survival mechanisms, and rate of metastasis and metabolism of this cell line (Klein and Revesz 1953; Klein 1954; Klein and Klein 1954). This marked the first observation of a heterogeneous tumor cell population retaining its stem-like plasticity and multipotency in relation to neoplasia.

The establishment of several ‘tumor stem-lines’ and the development of a full progression model of tumorigenesis and metastasis allowed researchers to finally address Boveri’s hypothesis and Winge’s ‘stem-line concept’. The new concept suggested that a tumor originated from a normal stem cell where karyotypic changes in its chromosomes determined the stages of malignancy. This concept was revisited through experimentation in the late 1950’s with clonal and cytological findings of Shinji Makino, A. Levan, and Theodore S. Hauschka.

Their observations [reviewed by Hauschka (Hauschka 1961) and Foulds (Foulds 1958)] of ascites tumors, developed by Klein & Klein, suggested that nonmalignant stem cells (NSC), cells that contained karyotypic constancy and normality, undergo chromosomal alterations caused by environmental changes (i.e., carcinogens, radiation, and toxic compounds)(Walpole 1959) into a main stem line termed S (Figure 1.1) which was driven by 'endomitotic reduplication' of chromosomes (Levan and Hauschka 1953; Levan 1956; Levan 1956; Makino 1956). This stage was then followed by progression to a secondary stem line S^1 that was competent for transition to several cell fates synonymous to stem cell differentiation (Hauschka 1958; Hauschka and Levan 1958). They further elaborated that environmental selective pressures could cause chromosomal re-selection and progress the S^1 stem line through 'ascendant' mechanisms to an invasive state termed M resulting in malignancy (Schultz 1958). The M state retained plasticity for metastasis or transition to a drug resistant line (MDR) after accumulation of specific mutations. The S^1 stem line could also give rise to a radiation resistant line (RR) by chromosomal changes due to severe DNA damage and a selective growth advantage. Finally a variant of the S stem line, a 2S polyploid line, retained plasticity for alternative tumor progression that allowed for further evolution of tumors (Makino 1959).

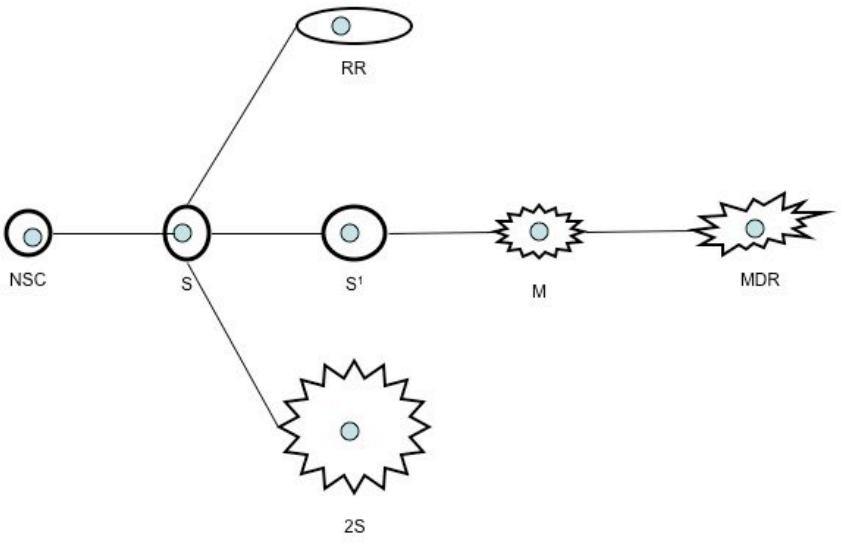

Figure 1.1 – Hauschka Model of Tumor Progression through Stem Lines. Adapted from Hauschka (Hauschka 1961). NSC = normal cell, S = main stem-line, S^1 =secondary stem line, M = malignant stem cell, MDR = multidrug resistant stem cell, RR = radiation resistant stem cell, 2S = polyploid stem cell variant.

‘MINIMAL DEVIATION’ OF CANCER STEM CELLS

Karyotypic characterization of the tumor stem-line concept in the 1950’s using transplantable tumor lines in metastatic ascites tumors was the first step towards identifying phenotypic changes in the early stages of malignancy. Chromosomal changes and growth rates were sufficient to understand uncontrolled growth and polyploidy, but not enough to determine cell of origin, metastatic potential, radiation resistance, and drug resistance. To describe these processes, further molecular characterization and *in vivo* cellular physiology of early stage tumors was necessary for understanding transformation. In the 1950’s researchers addressed this issue with the development of several hepatoma cell lines, the most common of these were the Dunning line established by ingestion of 2-acetylaminofluorene (Dunning, Curtis et al. 1950) and the Novikoff line established with ingestion of 4-dimethylaminoazobenzene (Armstrong, Gray et al. 1952; Novikoff 1957). Both lines resembled a normal phenotype based on microscopic and biochemical characterizations, but different from normal liver.

The Dunning and Novikoff lines were characterized extensively through the 1950’s for their growth rates, metabolic activity, and tumorigenicity, however they were unlike normal hepatocytes and therefore limited in the study of early stages of malignancy. In 1960 Harold P. Morris realized the limits of these cell

lines and established a rat hepatoma cell line by induction with the ingestion of n-(2-fluorenyl) pthalamic acid (2-FPA) (Morris, SIDRANSKY et al. 1960). An initial comparison of this hepatoma line, termed 'Morris hepatoma No. 5123' (Potter, PITOT et al. 1960), was microscopically determined to be similar to normal regenerative liver cells, implying stem origin. Morris characterized this line and compared it to normal liver, based on growth rates and enzyme related activities (Dyer, Gullino et al. 1964; Morris, DYER et al. 1964; Morris and Wagner 1964; Morris 1965; Lea, Morris et al. 1966; Sugimura, Ikeda et al. 1966; Wheeler, Alexander et al. 1966). Morris's findings showed that the No. 5123 transplantable hepatoma cell line had a slow growth rate and enzymatic activity nearly identical to normal liver cells. The heterogeneity of this population indicated both self-renewing (undifferentiated) and differentiated cells similar to normal stem cell phenotypes. From this he coined the term 'minimum deviation' to signify the existence of an early stage of transformation and malignancy (Potter 1961). Collaboration between the United States and Japan (Nishizuka, Hayaishi et al. 1966) resulted in comprehensive chromosomal karyotyping, carried out by Nowell and Yosida, to determine differences between other hepatoma cell lines. From their studies, it was determined that nearly six stem-lines had normal chromosomal number 42 with minimal abnormalities, however the six lines deviated in terms of growth rates and metabolic activity (Yosida, Imai et al. 1966; Nowell, Morris et al. 1967). This observation cemented Boveri's hypothesis of

oncogeny by chromosomal mutation in early transformation of a stem cell, rather than chromosomal loss or rearrangements found in leukemia (de Grouchy and de Nava 1968). These findings suggested that tumors arising from a common progenitor can give rise to primary tumors and can further progress to malignancy with ‘minimum deviation’ from the parental stem cell of origin, creating a model to study early stage malignancy and cancer stem cell differentiation.

CLONAL EVOLUTION OF A TUMOR

Koch's postulation in 1939 addressed heterogeneity within tumors and characterized a dominant but 'rare' aggressive cell population that could metastasize and that this rare subset of cells was established by *in vivo* selection. This was confirmed with findings of Klein & Klein in solid neoplasms transitioning to metastatic ascites tumors. However neither of these studies addressed whether the 'rare' but highly malignant cells existed as part of tumor formation or whether the rare malignant cells evolved by *in vivo* selection caused by microenvironment differences.

In 1976, Peter C. Nowell proposed a hypothesis that suggested clonality and existence of a variant tumor stem-line within a tumor either by progression or environment pressure for selection during malignancy. He proposed that most tumor stemlines have a common 'unicellular origin' based on karyotypic cytogenetic studies, they are also prone to mitotic errors due to DNA repair deficiencies, and that stemlines are influenced by their tumor microenvironment giving rise to heterogeneity and dominant cell populations (Nowell 1976).

In 1977, Isaiah Josh Fidler developed a new approach to address Nowell's hypothesis. The B16 melanoma cell line was used to establish a model of tumorigenesis and metastasis. In this model, cells injected into the tail vein of a rodent were grown for eighteen days to allow primary tumor growth and

metastasis to the lungs. In his earlier studies it was determined that most of the cells injected suffered acute death and only one in a thousand cells survived the injection that produced primary tumors and lung metastasis (Fidler 1970). This result suggested that tumor formation and metastasis was a rare and perhaps random (chance) event and it was by chance that progression occurred. To further address this question, cells injected into mice were monitored and the tumor cells that accumulated in the lung were isolated and subcutaneously injected into new mice. The cells maintained a constant increase of nodules in the lungs and the subcutaneously injected cells were more malignant than the original parental cell line. From this it was determined that highly metastatic cells do not occur by random stochastic events rather; they reflect a coordinated selective process for invasiveness, lymphocytic recruitment, rapid growth advantage, and increased angiogenic mediators (Fidler 1973; Fidler 1973; Fidler 1975). Fidler was interested in determining whether the heterogeneity of a primary tumor cell line was due to the preexisting nature of tumorigenesis or whether the cells re-established themselves according to the tumor microenvironment to confer metastatic potential. To address this, cells were selected *in vitro* based on colony formation in soft agar and injected as previously indicated and compared to the parental line (Figure 1.2). Fidler postulated that heterogeneity due to the origin of the tumor should give varied tumor incidence, however if heterogeneity occurs due to exogenous influences then the tumor incidence should be equal to that of

the line of origin. It was determined that a non-isogenic cell line gave rise to clones with varying degrees of tumorigenicity and metastasis. This validated the idea of metastasis being a coordinated and selective event rather than a random stochastic event (Fidler and Kripke 1977; Fidler 1978). In the following years Fidler explored more detailed experiments to gain further evidence of this discovery looking at, arrest, survival, growth, cell surface markers, and tissue selectivity of the established clones (Nicolson, Brunson et al. 1978; Poste, Doll et al. 1980; Poste and Fidler 1980; Raz, McLellan et al. 1980). His laboratory's findings were consistent with Nowell's hypothesis of heterogeneous tumor stem-lines giving rise to a varied population of sublines by preexisting mechanisms possibly due to mitotic errors or accumulation of precise mutations to the stem cell of origin. This is also consistent with the plasticity of normal stem cells where differentiation determines stem cell fate through a coordinated non-stochastic manner.

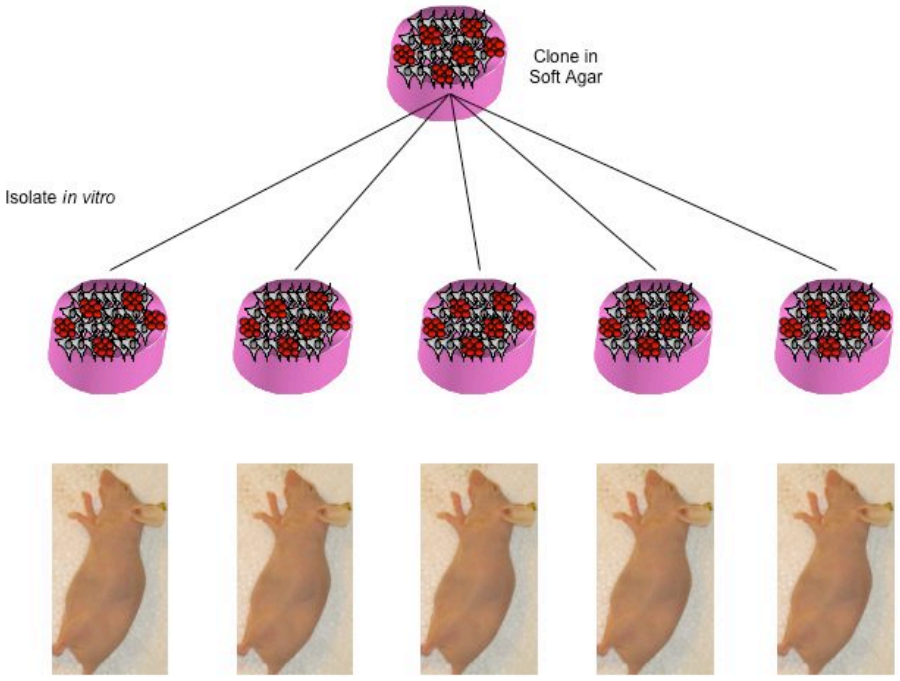

Figure 1.2 – The Fidler Method of Metastatic Clone Isolation. Adapted from Fidler (Fidler and Kripke 1977). Tumor cells from a non-isogenic B16 melanoma cells are grown in soft agar and clones are picked for mouse injections. After 18 days, pulmonary metastases are isolated and the procedure is repeated to enrich for a highly metastatic cell population.

LABEL RETAINING CELLS

The process of stem cell survival and longevity must be coordinated at the genetic level. To determine stem cell stability, the balance of stem cell pools and protection from somatic mutations need to be studied. From these concerns came two hypotheses: First stem cells maintain and dictate pools within tissues as required during turnover or tissue damage, and second stem cells retain their parental DNA strand during asymmetric division (differentiation) to increase lifespan and protect against DNA replication error prone mutations, historically referred to as the Cairns' hypothesis (Cairns 1975).

In 1981 Jackie R. Bickenbach reported 'label-retaining cells' as a hallmark of quiescent stem cells *in vivo*. She demonstrated that when mice (Bickenbach 1981) and hamsters (Bickenbach and Mackenzie 1984) were pulsed with tritiated thymidine on the fifth and sixth day post gestation, the dividing stem cells within tissue would incorporate the nucleotide and become 'marked'. Animals were then sacrificed after 69 days and processed for autoradiography. The results determined that cells containing ^{3}H-thymidine were located at the site of previously identified stem cell zones in the skin and oral mucosa at an 80% rate of occurrence. This spurred a trend over the next twenty years that became a criteria to identify stem cells in other tissues such as; skin keratinocytes and Langerhan

cells, embryonic fetal and adult epidermal stem cells, bulge area for follicular (hair) stem cells, mammary glands, and trachea (Mackenzie and Bickenbach 1985; Bickenbach, McCutecheon et al. 1986; Morris, Fischer et al. 1986; Bickenbach and Holbrook 1987; Cotsarelis, Sun et al. 1990; Morris and Potten 1994; Morris and Potten 1999; Borthwick, Shahbazian et al. 2001; Kenney, Smith et al. 2001; Ghali, Wong et al. 2004; Booth and Smith 2006; Cotsarelis 2006; Nijhof, van Pelt et al. 2007; Kimoto, Yura et al. 2008). The basis of labeling these stem cells was to identify a slow-cycling quiescent stem cell in G_0 that is ready for differentiation but may be involved in stem pool maintenance rather than transit amplification before differentiation. The commitment of these cells to exit G_0 was highly dependent on local stimuli.

In 1966 a novel concept (the Immortal Strand Hypothesis) was proposed and tested, of selective segregation of sister chromatids in primary mammalian cells synonymous to the defined segregation patterns in bacteria where a cell can be identified by template strands and newly synthesized strands (Lark, Consigli et al. 1966). It was also shown that carcinoma cells do not conserve this process probably due to deregulation of the mechanisms governing label retention and the fact that most tumor cells do not asymmetrically differentiate through divisions such as normal stem cells. Nearly ten years later in 1975, John Cairns hypothesized that this selective segregation process was conserved in stem cells as a barrier for tumor protection. He proposed that the error prone process of

DNA replication or oxidatively damaged DNA strands in normal stem cells have a high incidence of mutations that accumulate over time which will give rise to cancer. To test this hypothesis, embryonic cells were pulsed with ^{3}H-thymidine and allowed to divide. After one generation of division, there was a population of cells that were completely negative for ^{3}H-thymidine and the other was fully labeled. A model was proposed for the application to stem cells (Figure 1.3), and this has been the basis of several debates as to the validity of this process. These findings ignited explorations of this process in stem cells in many tissues such as the intestinal crypts, keratinocyte stem cells, mammary glands, neural stem cells, and muscle satellite cells (Potten, Hume et al. 1978; Potten, Wichmann et al. 1985; Merok, Lansita et al. 2002; Potten, Owen et al. 2002; Karpowicz, Morshead et al. 2005; Smith 2005; Shinin, Gayraud-Morel et al. 2006; Conboy, Karasov et al. 2007; Kiel, He et al. 2007; Rando 2007; Zhang, Ren et al. 2007). Their findings confirmed that stem cells use this mechanism for DNA damage protection, local stem cell pool maintenance and stem cell lifespan. Recent findings in the hematopoietic stem cells disprove the existence of this process for maintenance of the lineage (Kiel, He et al. 2007). These long-standing reports indicate that stem cells may or may not use this selective segregation process to protect their genomes from error prone replication or DNA damage that would normally induce malignant transformation depending on the tissue being studied.

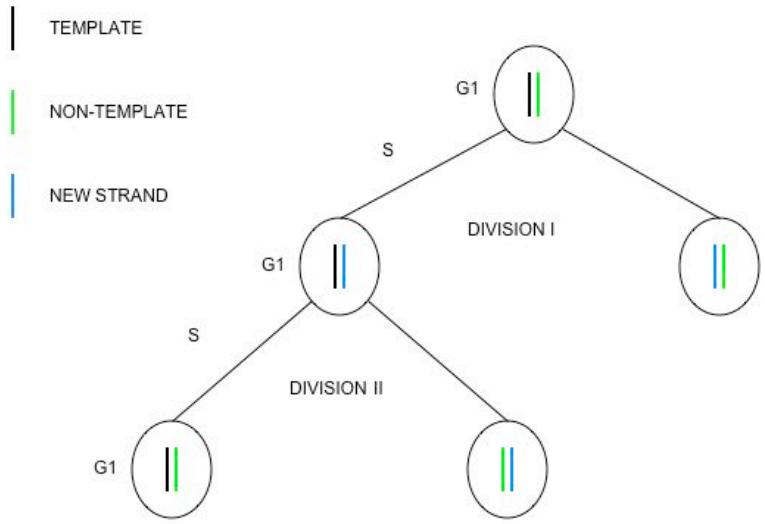

Figure 1.3 – Cairns Immortal Strand Hypothesis. Adapted from Cairns (Cairns 1975). The proposed concept states that stem cells retain their parental strand to protect their genomes from error-prone DNA replication and mutagenic transformation. Cells in G1 retain their parental DNA after S-phase and repeat the process to preserve the stem cell genome.

TUMOR INITIATING CELLS

In 1988 the development of an allogeneic model by John E. Dick used a nonobese diabetic severe combined immunodeficient (NOD/SCID) mouse that permitted the engraftment of purified human hematopoietic stem cells for the study of cell fate. These were conveniently called the SCID repopulating cells or SRC (Kamel-Reid and Dick 1988). Dick went on to establish a similar model of acute lymphoblastic leukemia (ALL) for the purpose of studying growth and progression of this disease as well as therapies directed towards human leukemia rather than rodent disease (Kamel-Reid, Letarte et al. 1989; Dick 1991). Since a limited number of the total population of cells injected gave rise to ALL, it was suggested that a rare leukaemic cell maybe the cause of the disease rather then the entire bone marrow. In 1994, this ALL model was adapted to an AML model to further characterize a more defined cell population based on markers of normal hematopoietic stem cells $CD34^{+}$ $CD38^{-}$, referred to as the 'tumor initiating cell' (TIC), because of its ability to graft with several orders of magnitude fewer cells compared to whole bone marrow (Lapidot, Sirard et al. 1994). Three years later he showed that these purified populations of tumor initiating cells were pluripotent enough to establish a heterogenic hierarchy after engraftment. This led

to the belief that transformation to leukemia occurred at a more primordial hematopoietic stem cell rather than a differentiated cell (Bonnet and Dick 1997).

CURRENT TRENDS IN CANCER STEM CELLS

The development of chimeric mice to study human leukaemic stem cells triggered a new era in tumor biology that was not truly realized until Tannistha Reya in 2001 published a review on hematopoietic stem cells, leukaemic stem cells, and reintroduced the idea of solid tumors containing 'rare' highly tumorigenic cell populations of stem origin (Reya, Morrison et al. 2001).

A couple of years after this proposal in 2003 Michael F. Clarke identified the first population of highly tumorigenic cancer cells originating from a solid tumor of the breast. Their findings validated the idea that a rare subset of $CD44^{+}CD24^{-}$ cells isolated from breast cancer patients were able to reconstitute tumors in immunodeficient mice versus cells $CD44^{-}CD24^{-}$ which did not give any tumors at all (Al-Hajj, Wicha et al. 2003). Clarke suggested these cells were the primordial stem cells of the tumor and characterization of these isolated populations could possibly explain growth advantage, survival, and relapse in patients treated with chemotherapy (Al-Hajj, Becker et al. 2004; Al-Hajj and Clarke 2004).

In contrast to the findings of Clarke, Peter B. Dirks was able to demonstrate that highly tumorigenic populations of human brain tumor stem cells (BTSC) from patients, isolated based on $CD133^{+}$, a marker of normal neural stem

cells, was not only able to reconstitute the tumor *in vivo* but was also able to give rise to differentiated neural cells indicative of the lineage of origin (Singh, Clarke et al. 2003). This was a paradigm shift from the Reya and Clarke concept of cancer stem cells by tumor initiation without differentiation that was initially demonstrated by Dick. However with Dirks' studies, he was not only able to demonstrate tumor initiation, but also differentiation. These findings raised the questions; what is a cancer stem cell and what is a tumor-initiating cell?

Further analysis of these two concepts led to identification of tumor initiating cells in several tissue types such as, lung, prostate, melanoma, ovary, colon, head & neck, pancreatic, nasopharyngeal, and thyroid (Hemmati, Nakano et al. 2003; Singh, Clarke et al. 2003; Tzukerman, Rosenberg et al. 2003; Singh, Clarke et al. 2004; Singh, Hawkins et al. 2004; Collins, Berry et al. 2005; Fang, Nguyen et al. 2005; Kim, Jackson et al. 2005; Patrawala, Calhoun et al. 2005; Stanger, Stiles et al. 2005; Liu, Dontu et al. 2006; Patrawala, Calhoun et al. 2006; Szotek, Pieretti-Vanmarcke et al. 2006; Zhang, Grindley et al. 2006; Dalerba, Dylla et al. 2007; Kelly, Dakic et al. 2007; Li, Heidt et al. 2007; Mitsutake, Iwao et al. 2007; O'Brien, Pollett et al. 2007; Patrawala, Calhoun-Davis et al. 2007; Prince, Sivanandan et al. 2007; Ricci-Vitiani, Lombardi et al. 2007; Wang, Guo et al. 2007; Zucchi, Sanzone et al. 2007). Although the findings in all of these tissues are consistent, they only answer the question of tumor initiation and not stem cell origin and chemotherapy resistance.

PANCREATIC STEM CELLS AND CANCER

In order to understand the origin of cancer stem cells it is important to identify the normal stem cells within the tissue of interest and the development process of that organ. The model system of pancreatic development and pancreatic ductal adenocarcinoma (PDAC) will be discussed in this section.

The pancreas maintains glucose homeostasis and produces enzymes for food digestion, these systems are known as the endocrine and exocrine systems respectively. The following summarizes the interpretation of Helena Edlund (Edlund) on pancreatic development. During embryonal development, premature pancreatic tissue arises from the endodermal compartment around the ninth day of development. The endoderm gives rise to two bulges, the ventral and dorsal portions, which contain the necessary progenitors to make a complete pancreas. By the tenth day of development the two portions start to join around the bile duct and duodenum. On day 12, the pancreas is defined where the ventral portion becomes what will be known as the head of the pancreas closest to the bile duct and stomach. The dorsal end becomes what will be known as the tail of the pancreas most distal from the digestive track. Both the dorsal and ventral portions of the pancreas contain the essential stem cells to form both the endocrine and exocrine compartments of the organ. Further development of the organ gives rise

to a series of branch and duct-like structures composed of acinar cells surrounded by centroacinar cells and ductal epithelium, which secrete digestive enzymes during food consumption. Within the parenchyma of the organ lies patches of cells called the 'Islet of Langerhans' that are involved in the secretion of insulin, somatostatin, and glucagon into the bloodstream for glucose homeostasis [reviewed in (Hezel, Kimmelman et al. 2006)].

There are three major mediators of pancreatic development that determine cell fate of the progenitors from the foregut endoderm. The initial commitment of the endoderm to the premature pancreatic bud involves the loss of sonic hedgehog pathway activation and an increase expression of the homeobox gene PDX-1. Final establishment of a complete organ involves stem cell fates to four major cell types by loss of notch signaling. Each unique cell type increases expression of a protein, which correlates with its differentiation from the stem cell of origin or lineage specificity. For example, the islet cells produce insulin predominantly, acinar cells express amylase, ductal cells express cytokeratin-19, to indicate differentiation, while centroacinar cells express HES1 an indication of stem cell phenotype (Apelqvist, Li et al. 1999; Jensen, Pedersen et al. 2000; Hald, Hjorth et al. 2003; Murtaugh and Melton 2003; Murtaugh, Stanger et al. 2003; Esni, Ghosh et al. 2004; Esni, Stoffers et al. 2004; Kadesch 2004; Lai 2004; Hezel, Kimmelman et al. 2006).

Islet precursor cells have been shown to express a protein known as neurogenin-3. In a model of partial duct ligation an expansion of islets cells were observed, confirmed by staining of insulin and co-stained with neurogenin-3, implying that a stem cell had undergone transit amplification to replenish the loss of islet cells from the ligation procedure (Xu, D'Hoker et al. 2008).

In 2005, Douglas Melton characterized a model of pancreatic ductal adenocarcinoma (PDAC) of stem cell origin. The model was developed using the Cre-recombinase gene driven by the PDX-1 promoter to specify expression to the pancreatic compartment, and an animal was also established with a PTEN knockout construct flanked by loxP sites for excision. After crossing the two strains, he observed an expansion of centroacinar cells, increased expression of HES1, and decreased expression of amylase from the surrounding tissue. The expansion of centroacinar cells eventually progressed to PDAC (Stanger, Stiles et al. 2005). These findings established centroacinar cells as the stem cell of the acinar and ductal cells and perhaps the precursor cells that are transformed prior to PDAC.

The characterization of the developing pancreas and identification of precursor cells was useful in understanding the origin of PDAC, but in order to understand carcinogenesis the molecular process of tumor progression had to be identified. In 2000 Ralph H. Hruban, proposed a molecular model of pancreatic tumor progression, synonymous to Bert Vogelstein’s famous colon cancer

progression model known as the Vogelgram. Based on pathology data, Hruban was able to model the molecular changes throughout the process of pancreatic intraepithelial neoplasia (PanIN lesions) to carcinoma in situ (Hruban, Wilentz et al. 2000). Normal pancreatic ductal epithelium is cuboidal in nature. PanIN-1A lesions are identified by a loss of cuboidal shape to a columnar cytoplasm and polarized nucleus with relatively few differences from normal ductal epithelium. The most prominent and earliest molecular event in this stage is the loss of telomeres (van Heek, Meeker et al. 2002). PanIN-1B lesions are identified easily due to their micropapillary characteristic and the most commonly seen mutation of K-ras in codon 12, either a G12D or a G12V constitutively activates the K-ras protein. PanIN-2 lesions are papillary with large abnormal nuclei, and partial stratification. These lesions increase cyclin-D1 expression and lose p16, which may explain the increase in mitosis. PanIN-3 lesions have a high papillary characteristic similar to 'budding', abnormal nuclear polarity, polarized mucinous cytoplasm resembling carcinoma by without basement membrane penetration. These lesions lose their p53, DPC4, and BRCA2 expression while increasing Ki-67, a marker of proliferation. The only difference between PanIN-3 and carcinoma in situ is invasion, which is absent in PanIN-3 lesions. As well, it has been reported that there is a proliferation difference that correlates with these lesions, starting with normal cells being either quiescent or slow dividing to

PanIN-3 and invasive cells that are rapid dividing cells (Hruban, Adsay et al. 2001; Hruban, Takaori et al. 2004; Takaori, Hruban et al. 2004).

CHAPTER TWO
Identification of Label Retaining Cells

INTRODUCTION

Identification of 'label-retaining' stem cells in normal tissues (Potten, Hume et al. 1978) signified the importance of maintaining a tightly regulated stem cell pool in which a 'quiescent' cell in G_0 reserved the option to reside in its non-proliferative 'slow cycling' state or enter the cell cycle in order to carry-on normal stem cell functions. During the proliferative state a stem cell may coordinate either symmetric or asymmetric divisions to balance stem cell pools 'self-renewal', or maintain tissue homeostasis 'differentiation' (Fuchs and Raghavan 2002; Reya and Clevers 2005). In contrast, the regulation of cell cycle checkpoints within cancer cells has been considerably 'loosened' by mutagenic transformation thereby allowing G_0 exit and unregulated cell cycle progression (Malumbres and Barbacid 2001). By definition, most chemotherapeutic drugs target cancer cells based on their proliferative nature compared to normal cells. However, if a cancer cell is held in a state of 'quiescence' (G_0) either due to lack of stimulus or due to inhibition of cell cycle entry it will significantly diminish drug response and may eventually permit tumor recurrence. This does not include resistance, as 'resistance' is defined as the genetic tolerance of chemotherapeutics in

proliferating cells. A ‘rare’ slow cycling cancer cell that stalls in G_0 or never enters the cell cycle was traditionally identified by pulse-chase experiments with tritiated thymidine (3H-thymidine or 3HTdR) followed by autoradiography to detect label-retention (Lark, Consigli et al. 1966). This is referred to as the ‘quiescent’ label-retaining cell (LRC) commonly seen in normal stem cells. The idea of comparing LRC experiments in normal stem cells to define ‘quiescence’ in cancer stem cells has been limited to one report (Zhang, Ren et al. 2007). This set the precedence for further investigations into defining the origin of cancer stem cells in several other tumor types.

Label-retaining cells can only be identified in non-isogenic cell lines or patient biopsies of heterogeneous origin. In order to identify, in real-time, LRC *in vitro* without genetically altering parental heterogeneity, DNA incorporation of a traceable compound is necessary so that divisions of the cell is linked directly to the duplication of the genome. Traditionally tritiated thymidine (3H-thymidine or 3HTdR) was used, but was limited to detection of total population proliferation (Lyons and Parish 1994). Alternatively, 5-Bromo-2-deoxyUridine (BrdU) has been used, but is restricted to the detection of cells with limited divisions, as the sensitivity and resolution of BrdU labeling cannot identify two-fold changes, but is sufficient for long-term *in vivo* studies with no signs of decay or degradation (Gratzner 1982; Houck and Loken 1985; Lyons 1999; Borthwick, Shahbazian et al. 2001; Merok, Lansita et al. 2002; Smith 2005; Zhang, Ren et al. 2007; Kimoto,

Yura et al. 2008). A robust dye was needed to label live cells and trace proliferative differences in populations relating to label-retaining quiescent cells and rapid dividing cells. Several reports have shown that 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA SE or CFSE) dye can be used to evaluate divisions within a cell population at a single cell resolution (Weston and Parish 1990; Lyons and Parish 1994; Lyons 1999; Schorl and Sedivy 2003), and is known for its dual modality of detection via fluorescence activated cell sorting (FACS) and epifluorescence microscopy. CFSE is a cell-permeant lipophilic compound that is readily taken up by cells in a non-fluorescent state. Following cleavage by intracellular esterases CFSE is converted to a fluorescent state which becomes considerably less lipophilic than the uncleaved form allowing the diffusion of the compound to be extraordinarily slow or not at all. This cleavage reaction also leaves a highly reactive amine group to react with intracellular proteins. When the cell divides the covalently linked proteins to CFSE are equally distributed between the two daughter cells. The resulting daughter cells have 50% of the fluorescence as the parental cell (Parish 1999). Its direct correlation to cell division, rapid delivery into cells (ten minutes), and long half-life (two months) makes it an important compound for following dividing and quiescent cells in long term cultures. The dye is applied to an unsynchronized population of cells and requires 24-hours to be processed and retained within the cell. Importantly, 100% of the cell population can be labeled yielding a

homogeneous baseline fluorescence. Having all the cells labeled equally at time zero allows a population differential to be determined over a set period of time. The idea that is being tested is that as cells divide some cells in the population of will enter the cell cycle and lose their fluorescence versus other populations that will remain quiescent (G_0) and retain their fluorescence label (Figure 2.1). This, unfortunately, does not rule out the possibility of a 'sick' cell or a cell that has undergone cellular stress. Also, it has been reported that the CFSE dye will decay approximately 10% over time, up to one week, but considering that cells are labeled to homogeneity at time zero, the rate of decay will effect the entire population being studied and therefore will act as an internal control.

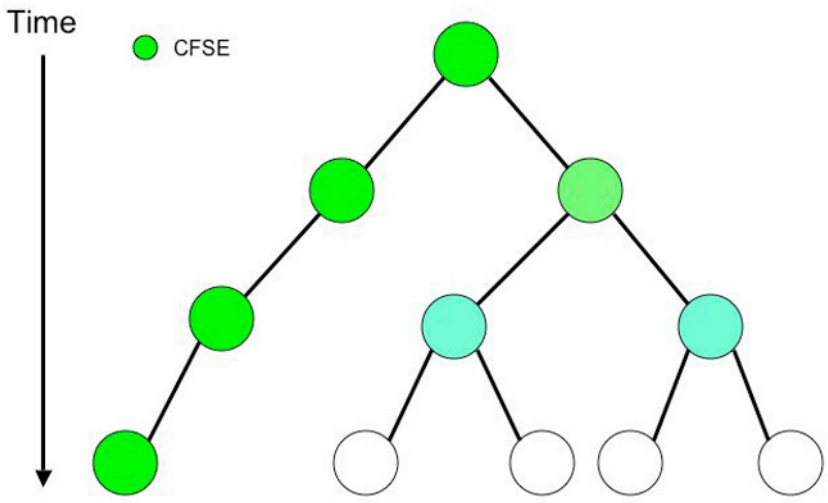

Figure 2.1 – The use of CFSE dye for Tracing Label Retaining Cells *in Vitro*. Cells are labeled with CFSE. Fluorescence microscopy (at the cellular level) and FACS (at the population level) are used to quantify rates of division by measuring the loss of fluorescence.

It is well documented that culturing stem cells on charged plastic results in induction of a differentiation process, premature senescence, and possible apoptosis (Pera 2005; Xu, Peck et al. 2005). Therefore, it was imperative to study LRC, not only in cells cultured on plastic, but also anchorage independent cultures *in vitro*. Panc-1 cells cultured on plastic at time zero compared to one week after initial labeling and then analyzed by fluorescence microscopy, revealed 1:20,000 label retaining frequency, this loss of CFSE fluorescence demonstrate the existence of a 'rare' population of LRC. CFSE labeled Panc-1 cells cultured in anchorage independent conditions (spheroids) were analyzed by three-dimensional fluorescence confocal microscopy. Under these conditions several spheroids of LRC were found one week of culture, thus confirming the existence of a 'rare' but detectable population of LRC *in vitro*. The existence of label retaining cells in colonies is not well understood and will need further characterization for interpretation.

RESULTS

Evaluation of sensitivity and labeling efficiency of CFSE on Panc-1 cells

Observations of tumors from patients reveal a heterogeneous pattern, therefore the heterogeneous pancreatic ductal adenocarcinoma (PDAC) cell line Panc-1 was used for this experiment. In order to exploit the CFSE dye-tracking system, the labeling efficiency and baseline fluorescence of the cells was first determined. Panc-1 cells were either labeled with CFSE or not (control) and allowed to equilibrate for 24 hours (for more details refer to the material and methods section at the end of this chapter). After 24 hours the cells were washed extensively to remove any residual CFSE dye. The cells were imaged by fluorescence microscopy and analyzed by FACS to determine labeling efficiency. Fluorescence microscopy imaging (Figure 2.2B) demonstrated that cells that are exposed to CFSE are labeled and there are no detectable negative cells. However, fluorescence microscopy may not be as sensitive as flow cytometry, and therefore a second modality of fluorescence detection must used to quantify differences between endogenous cellular auto-fluorescence and bona fide CFSE fluorescence. A FACS profile obtained of the labeled and unlabeled cells (Figure 2.2A) demonstrates the sensitivity of flow cytometry compared to fluorescence

microscopy and that the Panc-1 cell line does have a relative amount of ‘baseline’ auto-fluorescence, but the shift in fluorescence caused by the CFSE dye is considerable, and therefore valid experiments could be conducted *in vitro*. There were no signs of dye leakage from the cells, or significant decay within the first 24 hours of culture based on fluorescence microscopy.

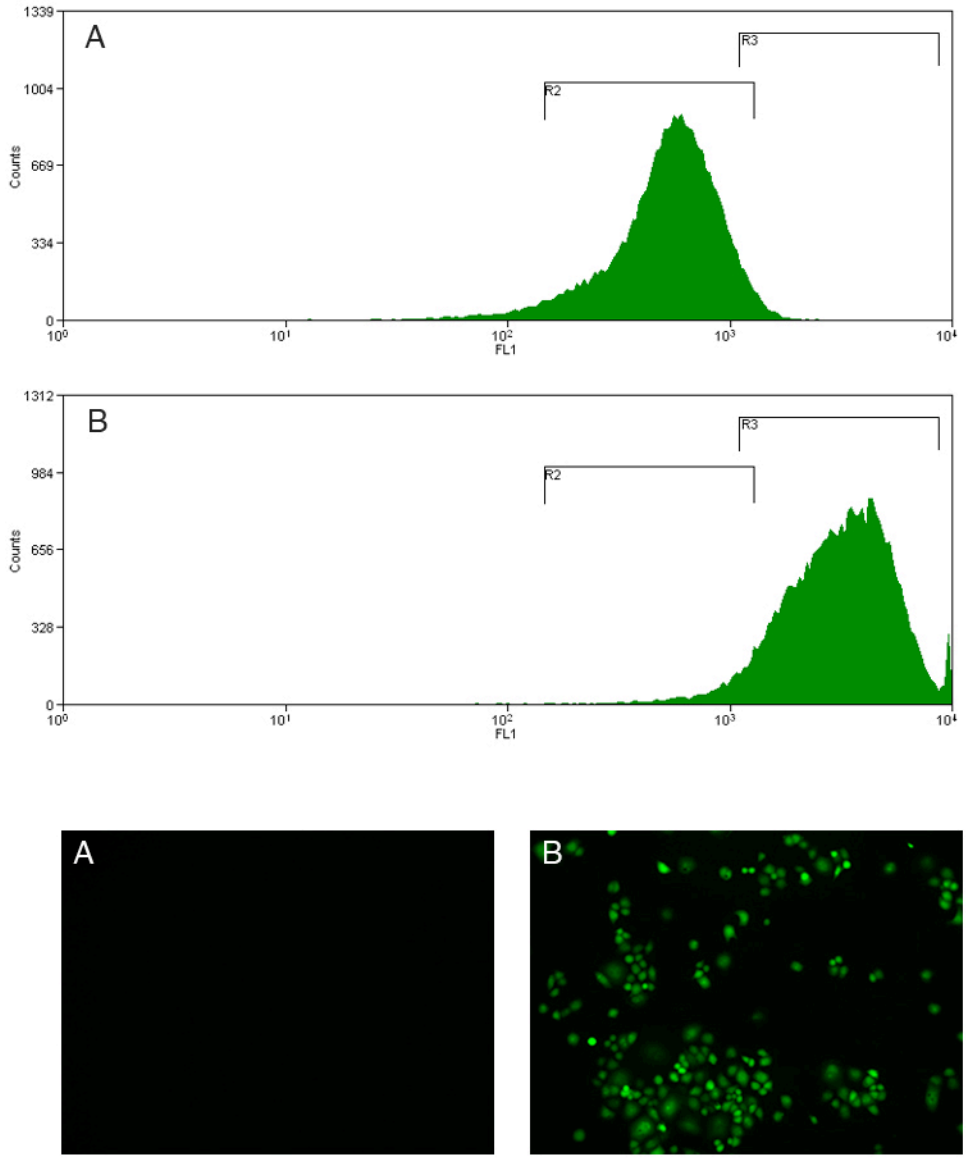

Figure 2.2 – FACS Analysis and Fluorescence Microscopy of Labeling Efficiency with CFSE dye. (A) Unlabeled Panc-1 cells treated with 'media only'. (B) Labeled Panc-1 cells 24 hours after exposure to CFSE dye.

Label-retaining cells detected in Panc-1 cell line labeled with CFSE grown on plastic

There are several reports of normal stem cells grown on plastic undergoing differentiation, premature senescence and apoptosis (Pera 2005; Xu, Peck et al. 2005). It was important to determine whether LRC could be studied in the context of plastic culture dishes and whether culture dishes can induce differentiation. Panc-1 cells were labeled with CFSE and grown on charged tissue culture dishes for one week and then analyzed by fluorescence microscopy and FACS. Analysis by microscopy at a total magnification of 200X revealed that the majority of the cell population had undergone division compared to time zero (Figure 2.3) leaving only one cell in twenty thousand that retained its label. This type of rapid cellular division is synonymous with transit amplifying (TA) cells seen in normal stem cells and established cell lines. FACS analysis confirmed that majority of the population had divided, and that there was a 'rare' population of cells in which cell division had 'stalled' possibly due to quiescence (G_0), comparable to the ratio observed by microscopy. Furthermore, fluorescence intensity of cells retaining their label was equivalent to cells at time zero, implying an LRC phenotype.

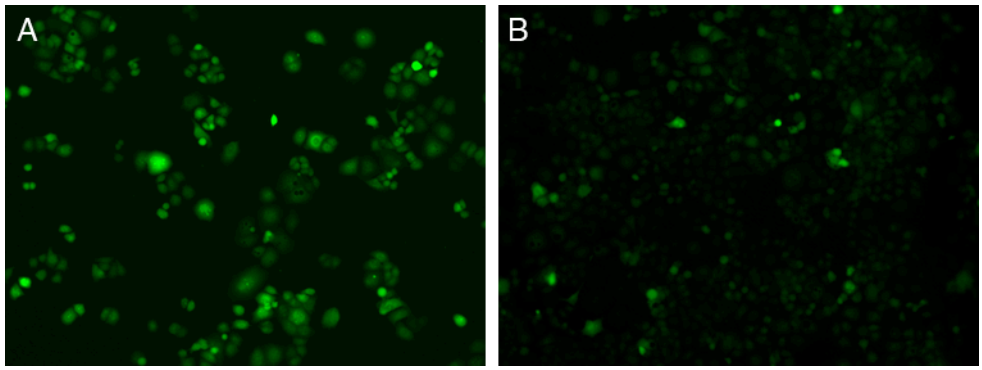

Figure 2.3 – Identification of Label Retaining Cells *in Vitro*. Panc-1 cells were labeled with CFSE dye, plated onto tissue culture dishes and analyzed by fluorescent microscopy, (A) 24-hours, and (B) one week after labeling.

Panc-1 cells cultured as spheroids contain LRC

In order to preserve the endogenous signals required to maintain a stem-like phenotype *in vitro*, another system was tested to reflect *in vivo* conditions. Previous reports demonstrate a model to study neurogenesis *in vitro*, where neuronal stem cells are placed in anchorage independent spheroid structures to mimic *in vivo* conditions (Singec, Knoth et al. 2006; Chen, Woodward et al. 2007). This technique has been extended to several cancer stem cell models (Mueller-Klieser 1987; McLeod, Beischer et al. 1997; Hamilton 1998; Sipos, Möser et al. 2003). To reconstitute an *in vivo* environment in culture an anchorage independent spheroid formation assay was established. Initially cells were grown on uncharged bacterial Petri dishes, but only a portion of the cells grew as anchorage independent structures, but most formed attached clusters (Figure 2.4A). Following this, a modification was made where, Panc-1 cells were labeled with CFSE for 24 hours and re-plated onto bacterial petri dishes in complete culture media containing methylcellulose, which induced anchorage independent spheroids (Sipos, Möser et al. 2003; Dalerba, Dylla et al. 2007). After growing pre-labeled spheroids for one week, the structures were analyzed by three-dimensional confocal microscopy. Due to limitations in the instrumentation, analysis was restricted to qualitative observations of label retaining anchorage independent colonies rather than individual cells. Grid analysis of the confocal

image (Figure 2.4B) indicated that Panc-1 spheroids contain label-retaining colonies with fluorescent intensities equivalent to that of cells labeled at time zero, and the decrease in fluorescence of the remaining spheroids was evidence of division, a reflection of what may be occurring *in vivo*.

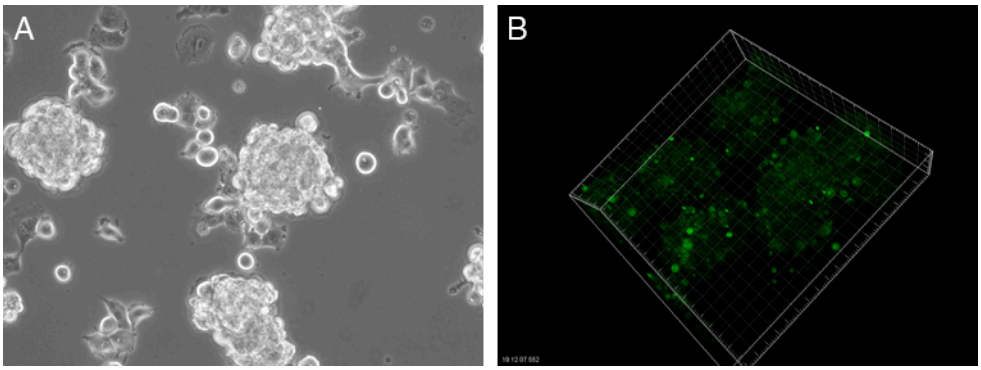

Figure 2.4 – Identification of Label Retaining Colonies in Spheroids. (A) Spheroids were established using Panc-1 cells plated in complete DMEM supplemented with methylcellulose on bacterial culture dishes. (B) Panc-1 cells labeled with CFSE dye were plated as spheroids for one week and analyzed by three-dimensional confocal fluorescence microscopy.

Determination of labeling efficiency during pulse BrdU incorporation

After detection of LRC *in vitro*, similar experiments were reconstituted *in vivo* to confirm these findings. Previous reports demonstrated LRC in mice and hamsters by direct injection into the animal as a single pulse. These established models are suitable for detection of LRC of endogenous normal stem cells, but they are susceptible to interpretation errors. For example, cells pulsed with BrdU that have not incorporated BrdU into their genome and then undergo apoptosis will release their BrdU to other cells which are not stem cells but have the capacity to incorporate the free BrdU. Although this occurrence may be rare, it is still however a concern in terms of background and interpretation of results. Also, BrdU administration to a whole animal may induce a certain level of toxicity and therefore may alter the endogenous stem cell physiology, which may not actually reflect normal stem cell biology creating an artifact of label retention. It is clear that in the case of cancer stem cells, *in vivo* labeling of cells to study LRC would be insufficient due to the fact that infiltrating inflammatory cells can incorporate BrdU during divisions and could contribute to the background within the tumor. In order to efficiently label and detect BrdU label retaining cells *in vivo*, a 'clean' system was developed in which Panc-1 cells were labeled *in vitro* until close to 100% of the cells were positive by fluorescence anti-BrdU staining. Cells were plated at 10% of complete confluency on regular tissue culture dishes containing

multiple coverslips. The cells were pulsed 24 hours later with increasing amounts of BrdU 10 μg/ml, 30 μg/ml, and 100 μg/ml to titer the tolerated dose of BrdU growth inhibition and coverslips were collected and fixed everyday for five days. The cells were then stained by fluorescence with anti-BrdU antibody (see Materials and Methods for details) to determine labeling efficiency. After 24 hours nearly 80% of all the cells were labeled positive for BrdU (Figure 2.5 A, D). At 48 hours nearly all of the cells were labeled but a few cells were still negative (Figure 2.5 B, E). Finally by 72 hours of incubation with 30 μg/ml of BrdU the entire population of Panc-1 cells was labeled (Figure 2.5 C, F). This labeling standard was used for the next series of *in vivo* experiments.

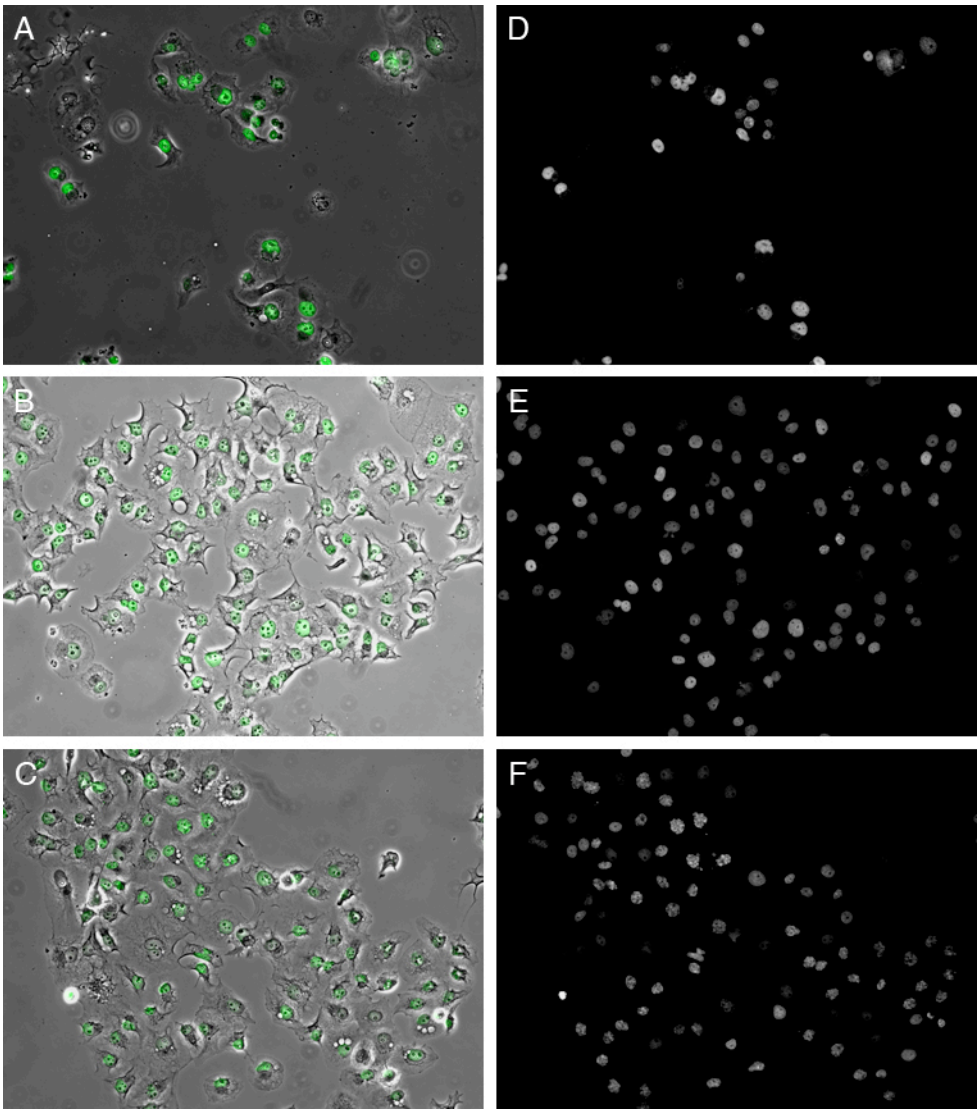

Figure 2.5 – Determination of BrdU Labeling Efficiency *in Vitro*. Panc-1 cells were plated at a density of 8 x 10^5 on a 15 cm dish containing 13 mm glass coverslips and pulsed with 30 μg/ml of BrdU for up to one week. Coverslips were collected and stained by anti-BrdU immunofluorescence, (A, D) 24 hours, (B, E) 48 hours, and (C, F) 72 hours after pulse. Panels (A, B, C) represent fluorescence emission of anti-BrdU staining from each day and combined with phase contrast microscopy at 200X magnification. Panels (D, E, F) are DAPI stain of the same fields.

Establishing a in vivo Pancreatic Orthotopic Xenograft Model

Xenograft transplantations of human tumor cell lines and biopsies have been the cornerstone of preclinical development for novel therapeutic cancer drugs. These models, despite their problems, have been useful in validating drugs to the clinic for more than twenty years. However, there is a growing demand for models that represent the molecular character of the tumor being studied in order to provide 'tailored' therapy. One approach is to deliver human cultured cell lines or human biopsies to the organ of origin in an immunodeficient rodent (orthotopic xenografts), such as an athymic nude mouse (nu/nu) or a severe combined immunodeficient mouse (scid/scid). Most xenograft tumor studies *in vivo* are conducted using subcutaneous model where a suspension of cells are injected into the subcutaneous layer under the skin, but as complexities and failures arise, these models have become less predictive and created a demand for organ specific transplantations (McLeod, Beischer et al. 1997; Morton and Houghton 2007). A recent report used a subcutaneous model to identify LRC in a nasopharyngeal cell line pre-labeled and delivered *in vivo* (Zhang, Ren et al. 2007). This report did not take into account the involvement of molecules influencing stem cell physiology in cancer progression and therefore cannot make any definitive conclusions about the correlation between LRC and cancer stem cells.

An organ specific orthotopic xenograft model was established for pancreatic cancer. Panc-1 cells were pulsed with BrdU using the *in vitro* method, discussed earlier in this chapter. Athymic nude mice were irradiated prior to surgery to eliminate rejection of human tumor cells by NK cells or macrophages. The following day animals were prepared for surgery and anesthetized (Figure 2.6A). The pancreas was exposed and a suspension of BrdU labeled Panc-1 cells was injected directly subcapsular into the head of the pancreas. Tumors were allowed to grow up to two months.

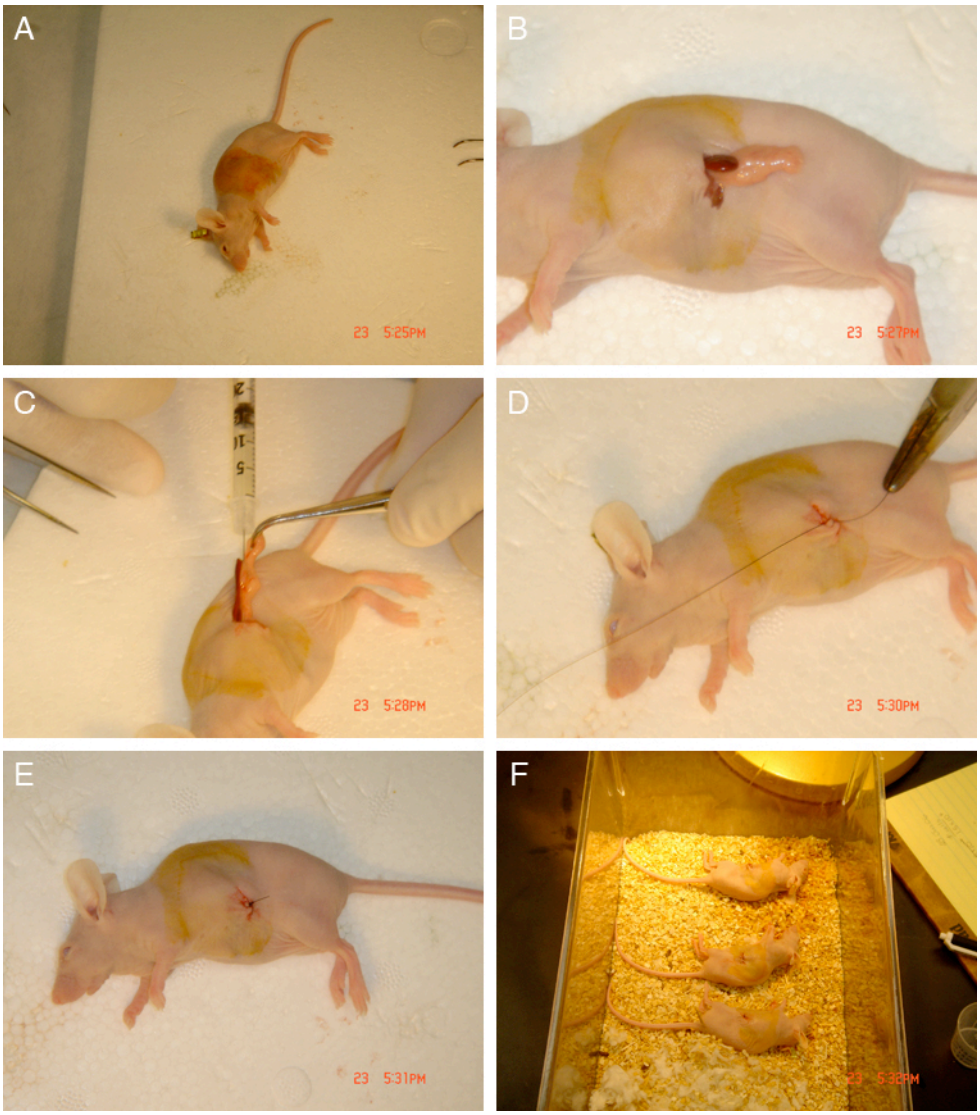

Figure 2.6 – Pancreatic Orthotopic Xenograft Surgery. (A) Athymic nude are anesthetized and sterilized at the site of incision, (B) incision is made below the costal margin and the pancreas is exposed, (C) 1 x 10^6 Panc-1 cells in 50 µl of saline are injected into the head of the pancreas, (D) the wound is sutured, (E) antibiotic ointment is applied to the wound, and (F) animals are placed in a warm recovery unit.

Presence of label retaining cells in an orthotopic xenograft model of pancreatic cancer

After the labeling efficiency of BrdU in Panc-1 cells was determined and an orthotopic pancreatic cancer model was established, the existence of label retaining cells *in vivo* was examined. BrdU labeled Panc-1 cells were injected into the pancreas as indicated and tumors were allowed to progress for two days, one month, and two months. The two day time point served as a label control and validation of the anti-BrdU antibodies, as well it was used to determine, the influence of acute apoptosis on the labeled cells, and the integrity of BrdU immediately after injection or shortly thereafter.

As short as two days was enough to establish a tumor bed and organization of a tumor-like structure (Figure 2.7 A, B). Serial sections of the pancreas from the mice sacrificed two days post tumor cell injection were stained with anti-BrdU and visualized by fluorescence microscopy (Figure 2.7 C, D). Imaging shows that the entire tumor contained BrdU signal, with a few exceptions, that could be related to the plane in which the signal arose, therefore validating the model for long-term label retaining studies.

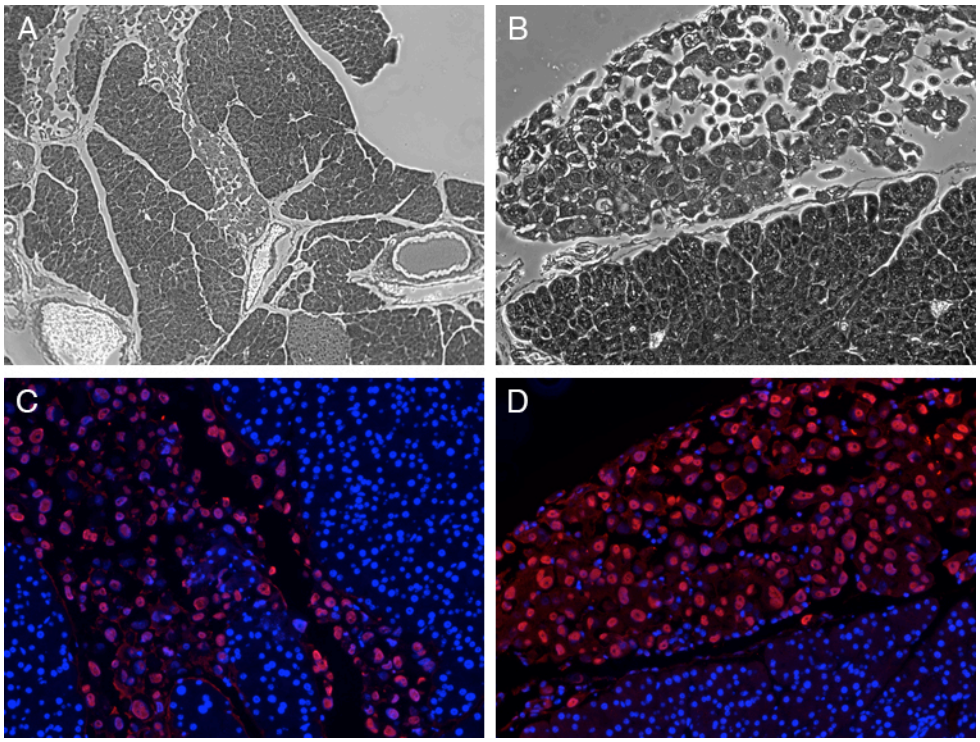

Figure 2.7 – Detection of BrdU *in Vivo*. Panc-1 cells labeled with BrdU in vitro were injected into the pancreas of mice and allowed to establish a tumor for two days. (A, B) 200X light microscopy images of H&E stain of the pancreatic tumor established in two days, (C, D) 200X fluorescence images of Panc-1 cells containing BrdU.

Further long-term analysis was performed on mice after one or two months post tumor cell injection. Serial sections of tumors harvested at one or two months, and stained with anti-BrdU antibody revealed label-retaining cells (Figure 2.8 A-D). Due to the significance of this finding it was important to quantify the presence of these cells. Stained tissue sections were placed on a deltavision fluorescence microscope and total tumor area was calculated by vector panel count. Approximately 700 panels were counted. A random five panels were quantified for Hoechst 33342 staining of nuclei to determine average total cell count per panel. The average total cell count per panel was 40 cells, yielding 28,000 thousand total cells per tumor per section. The label retaining cells were counted by 'eye' due to the infrequency of the cells. The count for the LRC was 19 cells per tumor per section. Therefore, the ratio of LRC and non-LRC cells is approximately 1 in 1500 cells. It has been shown that mouse cells have smaller nuclei than human cells, and therefore the presence of infiltrating inflammatory cells and other mouse cells could be accounted for by counting these cells. Each of the random five panels was quantified for the presence of small nuclei, which occupied 25% of the field, and normalized to the quantification of LRC. The intensity of the LRC was equivalent to the intensity of cells in the day 2 tumors. These findings do not rule out the influence of STASIS (STress or Aberrant Signaling Induced Senescence) or a dying cell transferring its label. However, STASIS does imply a distressed environment, but based on hematoxylin and

eosin staining there was no indication of necrosis or hypoxia. In the case of transference, a dying cell cannot transfer an incorporated label because the nucleoside has become a nucleotide by intracellular phosphorylation.

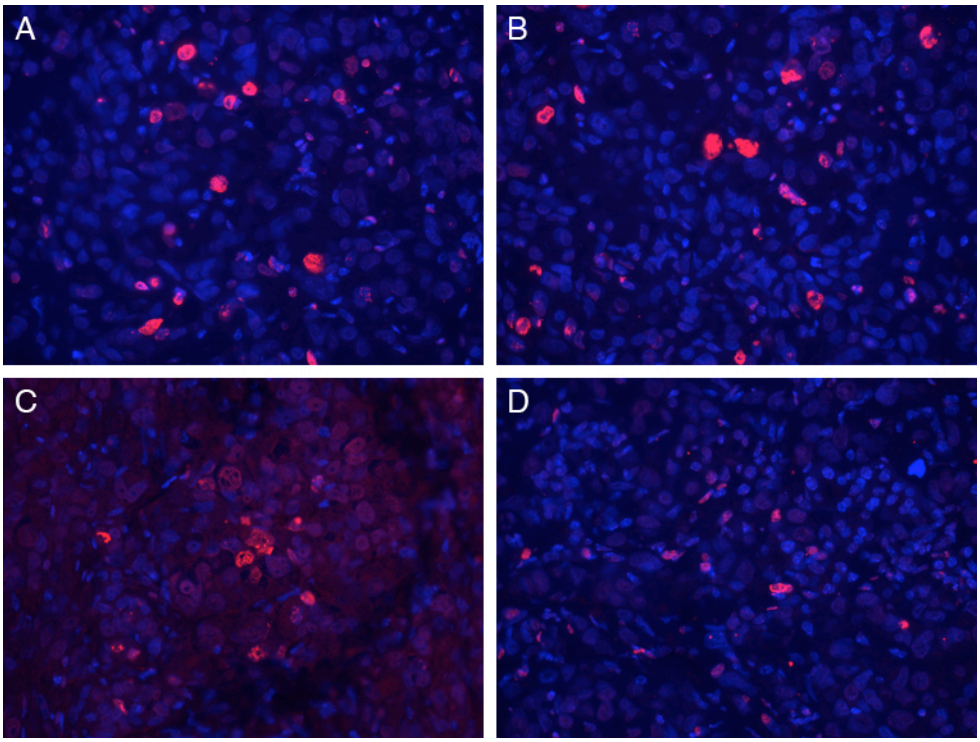

Figure 2.8 – Identification of Label Retaining Cells *in Vivo*. Panc-1 cells labeled with BrdU were injected into the pancreas and allowed to grow for up to two months. The pancreas was collected after one month (A, B) mouse #6819, and (C, D) mouse #6820, and two months.

DISCUSSION

Nearly forty years from the time that label retaining cells (LRC) was proposed as a hallmark trait of normal stem cells (Lark, Consigli et al. 1966), this is the first report of this trait in the context of human cancer stem cells. The activated fluorescent dye CFSE previously used to trace hematopoietic cell lifespan, migration, and proliferation was used to detect label-retaining cells in a non-isogenic cancer cell line. Fluorescence microscopy and FACS validated the tight connection of the dye to the proliferative capacity of these cells, nearly a log fold difference in fluorescence compared to unlabeled cells. Panc-1 cells grown on plastic or in anchorage independent spheroids underwent rapid asymmetric division within one week, similar to their normal stem cell counterpart. This rapid amplification is also accompanied by slow cycling of label retaining cells, likely the result of quiescence, by either cells that have entered G_0 or cells that have not entered the cell cycle possibly due to lack of stimulus or inhibition of signals (Malumbres and Barbacid 2001). Most importantly, Panc-1 cells have been shown to contain approximately 1 in 1500 ratio of label-retaining cells compared to non-label retaining cells in an *in vivo* orthotopic xenograft model of pancreatic cancer. The combination of *in vitro* and *in vivo* identification of label retaining cells in cancer strengthens the hypothesis that cancer cells may contain a subpopulation of

quiescent cells similar to normal adult stem cells validating the concept of 'minimum deviation' and this may be an additional trait of cancer stem cells.

Although the identification of LRC in cancer points to the origin of normal adult stem cells, the concern is whether the understanding of LRC can give insights into drug targeting and cellular mechanism of tumor progression. In the case of cells grown on plastic, we see a 10-fold decrease in frequency of LRC compared to *in vivo*, this is confirmed by several reports that tissue culture experiments do not directly correlate with what is observed *in vivo*. This idea was further tested with anchorage independent spheroids, where the ratio of LRC decreased, but it was substantially less then *in vivo* observations. Given that our *in vitro* models do not contain ECM proteins, vasculature, growth factors, hypoxia, acidity, and many other components of the tumor microenvironment, one interpretation is that *in vitro* models of LRC are limited in their application to study cellular mechanisms of tumor progression but may be sufficient for preliminary drug screens. The identification of LRC *in vivo* extends the application of studying tumor progression, but does not exclude the possibility that the cells have undergone early senescence, tumor dormancy, quiescence, or toxic stress which has kept the cells in infinite G_0 arrest.

These studies have identified label-retaining cancer stem cells and suggest new approaches for how anti-cancer drugs should be designed and targeted towards neoplastic disease. The *in vitro* model can be a first line screen to identify

therapeutics that forces the LRC to enter the cell cycle rapidly (i.e. mitogenic depletion of cancer stem cells), therefore allowing traditional chemotherapeutic drugs to have an effective response. There is a possibility of identifying molecular components that contribute to the quiescent phenotype, but due to a 10-fold increase in LRC ratio, the mechanisms may be different, *in vivo*. Furthermore, the results of the *in vivo* findings support the hypothesis that cancer stem cells may come from an adult stem cell origin thereby allowing analysis of signaling pathways that govern stem cell renewal, potency, proliferation, fate differentiation, and recurrence. Additional experiments would involve labeling cells with a genetic marker of H2B-GFP and tracing LRC in real-time *in vivo* models. Genetically labeled cells could be isolated easily and characterized for transplant experiments. The observations in this chapter characterize new ways of understanding quiescence in tumor progression and provide new assays for drug screening but do not explain why these LRC occur and what is the mechanism underlying this cellular state, paving the way for further investigation.

MATERIALS AND METHODS

Cell Culture

The human pancreatic ductal adenocarcinoma cell line Panc-1 was obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Hyclone #SH30243.02) supplemented with 10% fetal bovine serum (Hyclone).

CFSE Labeling

CFSE labeling experiments were carried out as previously described (Schorl and Sedivy 2003). Briefly, Panc-1 cells were harvested with trypsin and normalized to 3 x 10^7 cells/ml in DMEM with 10% fetal bovine serum and 10 μM CFSE (5-(6)-carboxyfluorescein di-aetate succinimidyl ester) for 10 min. at 37°C with mild agitation, then the suspension was quenched with media on ice for 5 min. and reseeded onto culture dishes or directly placed into spheroids (see Spheroid Assay for details).

Spheroid Assay

Panc-1 cells were harvested by trypsin and plated on non-tissue culture Petri dishes in complete DMEM media containing 20% of a 1.2% stock methylcellulose / DMEM mixture following the exact protocol (developed by

Spherogenex Inc.) at a density of 1 x 10^6 per plate. The cells were allowed to grow for up to 4 weeks before confocal and FACS analysis.

Flow Cytometry

The analysis of CFSE labeling was performed on a MoFlo flow cytometer. CFSE labeled cells were detached with trypsin, counted (Beckman Coulter, Z1 coulter counter), and normalized to 1 x 10^6 cells per ml in HBSS supplemented with 2% fetal bovine serum, washed, resuspend in HBSS containing 0.5 μg/ml DNAse I, 3% BSA, 2 μg/ml propidium iodide and then filtered through a 70 μm filter immediately before flow analysis. Unstained cells were used to determine baseline autofluorescence and a gate was set for analysis of signal above baseline. Labeled cells were analyzed and quantified based on the unlabeled gate to determine labeling efficiency.

BrdU Pulse Labeling

Panc-1 cells were seeded on coverslips in 10cm dishes at a density of 200K cells per dish. After 24 hours the cells were pulsed with 10 μg/ml, 30 μg/ml, or 100 μg/ml of BrdU (Sigma #B5002) and one coverslip was recovered everyday for up to five days and stained. After optimal label efficiency was determined, Panc-1 cells were scaled-up at a density of 7.5 x 10^5 cells per 15 cm dish for animal experiments. The cells were continuously pulsed with 10 μg/ml of BrdU for 72

hours and then collected in 0.9% sterile saline at a concentration of 1 x 10^6 cells per 50 μl for injection.

Orthotopic Pancreatic Surgeries

Athymic nude mice 4-5 weeks old were obtained from the NIH and irradiated with 350 rads 24 hours prior to surgery day. The next day, mice were anesthetized by I.P. injection using a 50 μl cocktail of 25 mg/ml Ketamine, 2.5 mg/ml Xylazine, 0.5 mg/ml Acepromazine 20 min. prior to surgery. The site of incision was sterilized with Betadine and cleared with an alcohol swab. An incision was made under the costal margin after which the head of the pancreas was exposed and 50 μl of the labeled the BrdU cell suspension was injected subcapsular using a 30-gauge needle. The pancreas was placed back in to the peritoneum and the incision was sutured and treated with antibiotic ointment. All animals were given Buprenorphine as a post-surgical analgesic with Tylenol supplemented in their feeding water. All mice were kept on a 37°C warm pad from the time of drug administration through recovery. The mice were monitored twice a week for up to 8 weeks by palpation for tumor growth. All animal experiments were in full compliance of the University of Texas IACUC rules and regulations.

BrdU Staining

All stains were conducted in hydrated chambers. BrdU pulsed Panc-1 cells grown on coverslips were washed three times in PBS, fixed for 20 min. in 4% PFA, permeablized in 80% Methanol at 4°C for 5 min., 100% Acetone at -20°C for 2 min. and washed three times in PBS. The cells were denatured in 2N HCl at 37°C for 1 hour, neutralized in 0.1M $Na_2B_4O_7$ (Borax) pH8.5, briefly washed in PBS. The coverslips were stained with a primary monoclonal anti-BrdU (Sigma # B2531) antibody diluted 1:1000 in 20% Aquablock/DMEM mixture (EastCoast Bio, #PP82-K3151) at 37°C for 30 min., washed three times in PBS, followed by a secondary goat anti-mouse-FITC (Molecular Probes) antibody diluted 1:1000 in 20% Aquablock at 37°C for 30 min., washed in PBS three times and counterstained in Hoechst 33342 diluted at 1 μg/ml for 5 min. at room temperature to visualize nuclei, then rinsed in water before mounting with Mowiol.

The tissues were fixed in 4% PFA, embedded in paraffin, and into 5 μm sections onto superfrost slides. The sections were deparaffinized in xylene, hydrated through graduated alcohols, and residual peroxidase activity and auto-fluorescence were quenched with 3% H_2O_2. The slides were then denatured in 2N HCl, for 30 min. at 37°C, neutralized in 0.1M $Na_2B_4O_7$ (Borax) at room temperature, washed three times in PBST, enzymatically digested in 0.4% Pepsin at 37°C for 30 min, washed again and finally placed in a blocking solution

containing 20% Aquablock at 37°C for 30 min. For the primary stain the tissue was stained with sheep anti-BrdU antibody (Capralogics, #P00013) diluted at 1:100 in PBST and incubated at 4°C overnight in a hydrated chamber, followed by three washes in PBST. For the secondary stain the tissue was stained with rabbit anti-sheep (Millipore, #AP147R) diluted at 1:500 in PBST, incubated for 1 hour at 37°C and then washed three times in PBST. The sections were counterstained in Hoechst 33342 diluted at 1 μg/ml for 5 min. at room temperature and rinsed with water before mounting with Mowiol.

Fluorescence Microscopy

All tissue stains were visualized on a Zeiss Epifluorescence microscope. The Hoechst dye was visualized with the DAPI filter and the Rhodamine on the Rhodamine filter. Fluorescence from stained tissue was captured using a Delta Vision fluorescence microscope as separate panels and stitched together to represent the whole tissue. Fluorescence emission was quantified by Image J software.

CHAPTER THREE
Self-Renewal and Transit Amplifying Cells

INTRODUCTION

The demand for therapeutic regenerative medicine has fueled the discovery of the 'side population', which was initially used to identify a pluripotent hematopoietic stem cell compartment within the bone marrow. These cells have the potential to repopulate their own 'self-renewing' pool and manage hematopoietic stores, or differentiate into lineage specific lymphoid and myeloid cells to replenish the turnover of cells within the immune system (Goodell, Brose et al. 1996). The identification of the side population offered new insights into identifying and characterizing stem cells in tissues such as the lung and breast (Otto 2002; Smalley and Clarke 2005). The purpose was to efficiently purify pluripotent stem cells from patients for regeneration of damaged tissue in congenital diseases or replacement of absent stem cell compartments due to chemotherapy.

It was proposed that these isolated 'side population' stem-like cells have evolved mechanisms to avoid natural xenobiotic toxicity by expressing ATP dependent cell surface pumps referred to as ABC (ATP binding cassette) tranporters (Gottesman and Pastan 1993; Krishnamurthy and Schuetz 2006). The fact that the expression and activity of ABC transporters are elevated in normal

stem cells allows the exploitation of these pumps to efflux Hoechst 33342, a fluorescent DNA intercalating dye, to identify 'side population' cells. The cells when pulsed with Hoechst 33342 dye for a short period of time removed the dye from the cytosol rendering a low emission in fluorescence. When the two emissions of Hoechst 33342 are plotted against each other on a log plot using a fluorescence activated cell sorter (FACS), they form a "hook-like" population of cells that display a low emission of fluorescence after UV excitation, hence referred to as Hoechst^{low} (Figure 3.1). These cells can be isolated for experimental or clinical purposes. Alternatively, this efflux capacity can also be analyzed by using Rhodamine 123, a mitochondrial staining dye that is a substrate for ABC pumps and has been shown to be effective in isolating HSC equivalent to Hoechst 33342 without the additional toxicity (Chen, Li et al. 2003; McKenzie, Takenaka et al. 2007). In cancer, the cells have either upregulated their transporters or retained the expression from the cell of origin providing additional evidence that a subset of cancer cells may be side population stem cells and may be important in the sustained growth of cancer as well as evasion from chemotherapeutics. In fact, analysis of AML cells by the side population assay revealed that cells that efflux Hoechst 33342 also co-labeled with cells carrying normal HSC markers and correlated with poor patient outcome (Feuring-Buske and Hogge 2001; Wulf, Wang et al. 2001). In the case of chemoresistance, it has been shown that several cancers have accumulated mutations or genetic changes to increase pump activity,

predominantly the MDR-1 transporter and to a lesser degree the MDR-3 pump (Gottesman and Pastan 1993). Chemotherapeutic drugs such as paclitaxel, cisplatin, etoposide, and vincristine, have been shown to be substrates for these transporters, shedding light on the underlying mechanism that may be important in relapse or poor initial drug responses. ABC transporters have become a target for therapeutic development in cancer. In one case, verapamil a calcium channel blocker normally used for hypertension was used to inhibit ABC pumps active on cancer cells, rendering them vulnerable to mitoxantrone (a toposiomerase II inhibitor) toxicity (Dean, Fojo et al. 2005). These findings are evidence that cancer cells have inherited or have the capacity to inherit a conserved molecular mechanism of survival against xenobiotics or chemotherapeutics by expressing dye effluxing ABC transporters.

Identification of the earliest state of progenitor differentiation prior to oncogenic transformation, and the characterization of plasticity within these states may help understand tumor progression and metastasis. It is important to understand exogenous cues that regulate these states. Oxidative stress has been one of the biggest concerns in stem cell research due to the induction of premature senescence rendering cells incapable of maintaining stem cell reserves within mature tissue (Kenyon and Gerson 2007). The influence of stem cell stromal interactions and extracellular matrix proteins are also important in understanding temporal and special cues during differentiation (Hackney, Charbord et al. 2002).

Classically, the tumorigenic potential of cancer cell has been tested by the ability of a single cell to form colonies *in vitro* (Fidler and Kripke 1977). The application of colony formation using cells from the side population may validate this assay for diagnostic systems. Normal stem cells are known to have a transit-amplifying phase in order to replenish cells in response to turnover or damage (Fuchs and Raghavan 2002; Reya and Clevers 2005). In cancer, this phenomenon may contribute to tumor bulk and may provide insight on how to transition cells out of a quiescent state making them vulnerable to antiproliferative nucleoside analogs. All of these cues may provide insights into the plasticity of a cancer cell for advanced development of chemotherapeutic drugs.

As discovered in chapter two, hallmark characteristics appear of normal stem cells seem to be engaged in cancer cells such as, quiescence, activation of telomerase, emergence of a side population in the context of self-renewal, transit amplification and differentiation. Being that SP is an established criterion for purifying pluripotent stem cells, the side population was used as a marker to characterize putative cancer stem cells in this chapter.

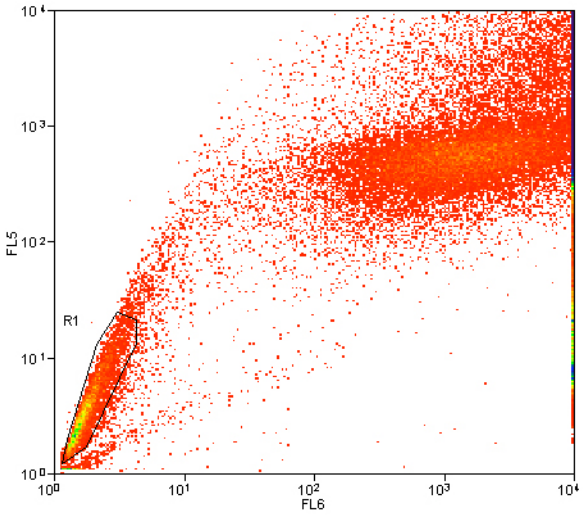

Figure 3.1 – The 'side population' Assay. Live cells are stained with Hoechst 33342 dye, and gated for live cells based on forward and side scatter, followed by the 'R1' gate designating the 'side population' (SP). The side population consists of a low emission of Hoechst 33342 dye with a hook-like pattern connecting to a large main population with low efflux capacity.

RESULTS

Titration of Hoechst 33342 toxicity and identification of 'side population' through Verapamil inhibition

The criterion for 'side population' is the ability to efflux Hoechst 33342 in an efficient manner devoid of cytotoxicity and inhibition by verapamil (a broad range ABC pump inhibitor). Panc-1 cells grown to 70% confluency were titrated with three concentrations of Hoechst (see materials and methods for details), 5 μg/ml, 7.5 μg/ml and 10 μg/ml to determine optimal conditions that produced the least amount of cell death with clear separation from non-SP cells. Labeled cells were analyzed by a flow cytometer equipped with a UV laser. The two emissions from Hoechst (blue and red) were plotted against each other on a log scale. In order to determine the precise location of SP, cell were treated with verapamil to inhibit the ABC pump and eliminate SP. Prior to labeling with Hoechst, cells were exposed to 100 μM verapamil, labeled with Hoechst and analyzed to determine the actual location of SP on the graph. Gates were set according to a side population reported previously, along with comparison to the verapamil treated control (Figure 3.2D) (Goodell, Brose et al. 1996). Hoechst was tolerated optimally at 5 μg/ml with 90% cell viability, at 7.5 μg/ml and 10 μg/ml cell viability was reduced to 75% and 60% respectively (Figure 3.2 A-C). The SP fraction at 5 μg/ml Hoechst was 10%.

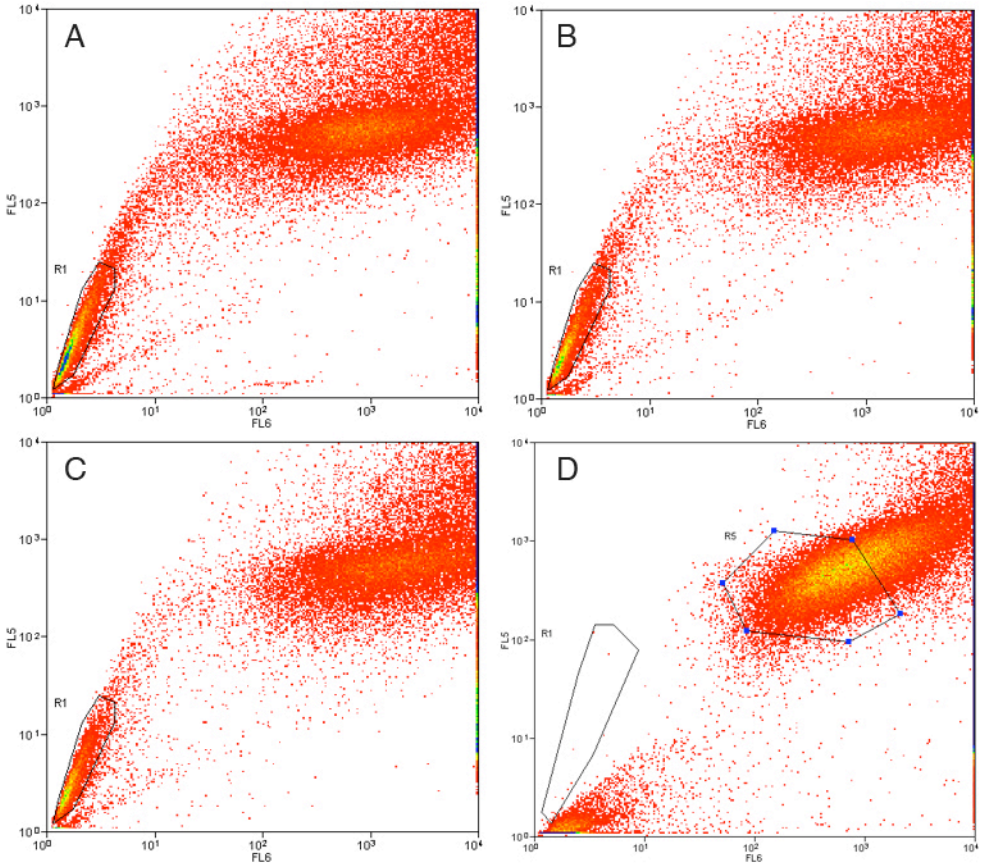

Figure 3.2 – Titration of Hoechst 33342 dye for SP Assay. (A-C) The DNA intercalating dye Hoechst 33342, also a substrate for ABC transporters was titrated using the following concentrations 5 μg/ml, 7.5 μg/ml, and 10 μg/ml respectively. Increasing amounts of Hoechst 33342 dye provides better separation of SP and MP. (D) A broad ABC transporter inhibitor, verapamil, was used at a physiological concentration of 100 μM to validate ABC activity involved in the SP assay.

Survey of three PDAC and one Sarcoma line for Side Population

In order to determine the prevalence of SP phenotype in the progression of cancer, three pancreatic ductal adenocarcinoma (PDAC) cell lines with differing levels of invasiveness were surveyed. In addition, a sarcoma line (Susm1) known to be negative for telomerase (ALT; alternative lengthening of telomeres) and considerably less invasive than the PDAC lines was assayed. All cell lines were grown to 70% confluency and assayed by standard SP analysis (see material and methods). Mia-Paca-2, a highly invasive and metastatic cell line known to cause liver metastasis contained no SP before or after verapamil treatment (Figure 3.2B). Current literature suggests that SP gives rise to highly tumorigenic and invasive cells tumors, so this finding was unexpected. When BxPC3 was tested, a moderately tumorigenic and invasive line, it contained an SP population of approximately 1.3%, comparable to published results (Figure 3.3C) (Kondo, Setoguchi et al. 2004). However, the SP in this cell line was not sensitive to verapamil inhibition making the SP profile a potential artifact, therefore disqualifying this line as a model for studying cancer stem cells. The Panc-1, a moderately tumorigenic and invasive PDAC cell line, became of interest due to its high SP profile of 10% (Figure 3.3A). Finally, it was important to determine whether sarcomas (soft tissue tumor cells) retain a stem-like origin, the sarcoma line Susm1 was assayed by SP giving a 3% SP profile, which was resistant to verapamil inhibition, therefore failing the test for SP (Figure 3.3D).

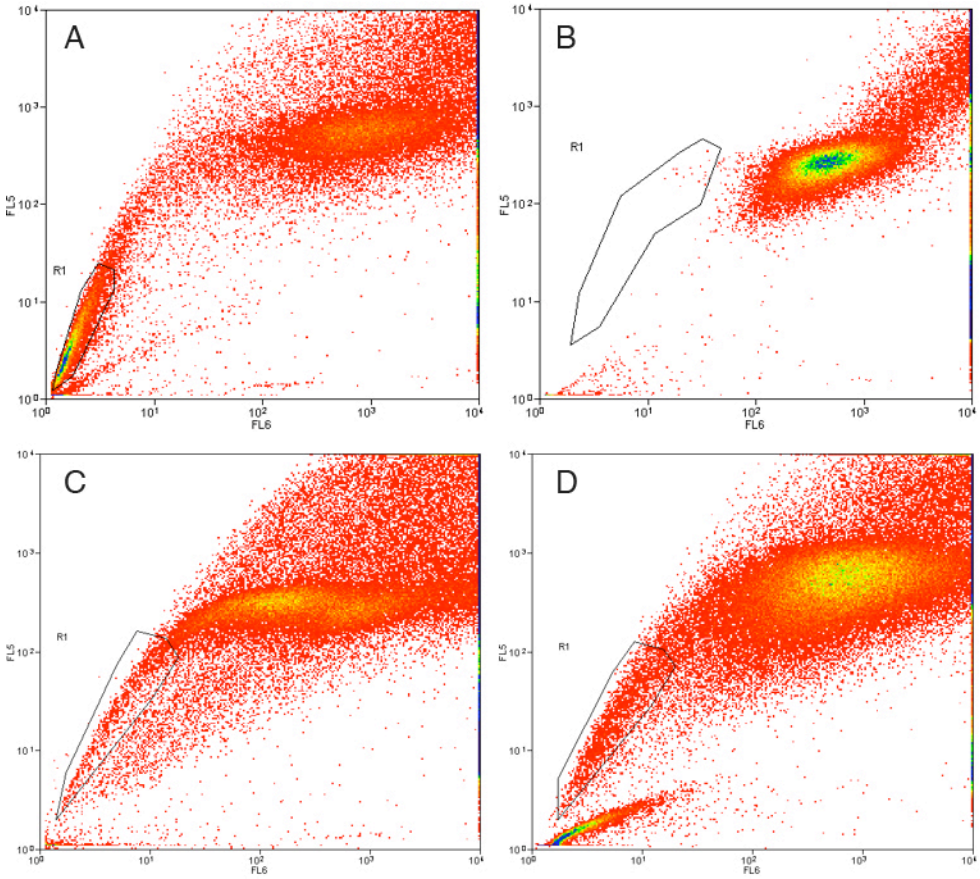

Figure 3.3 – Survey of Cell Lines for SP. The 'side population' assay was used to survey, (A) Panc-1 a moderately invasive, (B) MiaPaca-2 a highly metastatic, (C) BxPC3 moderately invasive, and (D) Susm1 moderately invasive cell line's.

Experiments conducted in hematopoietic stem cells have implicated the ABCG2 pump to be the molecular identity of the SP phenotype, and in chemoresistant cancer cell lines the ABCB1 pump (MDR-1/P-gp) contributes to SP (presented in the introduction). Considering Panc-1 cells contained high SP compared to other cell lines, the next experiment was to identify whether the expression of these two known pumps were contributing to the SP phenotype. Panc-1 cells were labeled with Hoechst 33342 as indicated in the materials and methods, followed by labeling with anti-ABCG2 antibody to identify and localize the presence of this pump in the context of SP. This was also done with MDR-1 protein. Surprisingly neither pump stained positive on Panc-1 cells, indicating that other ABC transporters are involved in the SP phenotype of non-chemoresistant cancer cell lines. Given that there are many ABC transporters, it is not inconceivable that Panc-1 cell line has chosen an alternative non-traditional mechanism for conferring the SP phenotype, suggesting that these pumps may have significant roles in tumorigenesis.

Cloning efficiency of SP cells

Cancer stem cells have been identified using several markers in a variety of tissues, and their tumorigenic potential has been tested either by clonogenicity *in vitro* or by xenograft transplants *in vivo*. Clonal efficiency is defined as the ability of a single cell to confer independent growth. Panc-1 SP, MP (main population that does not contain SP), and TP control (total population) cells were sorted onto 10 cm tissue culture dishes at densities of 100 and 300 cells in triplicate, and allowed to form colonies for two weeks. The plates were then fixed with methanol, stained with Giemsa, and counted manually. The colonies observed on the SP plate were nearly identical to the MP and TP control (total population) plates (Figure 3.4 A-C). Further detailed analysis (colony counting), could not differentiate significant differences between SP and MP, but did discriminate with TP by approximately 30% (Figure 3.4 D). An important finding to note, despite all fractions being processed through the flow cytometer, the colonies from the TP fraction were more diverse and larger than SP and MP colonies, an indication of heterogeneity, possibly explaining lower clonal efficiency.

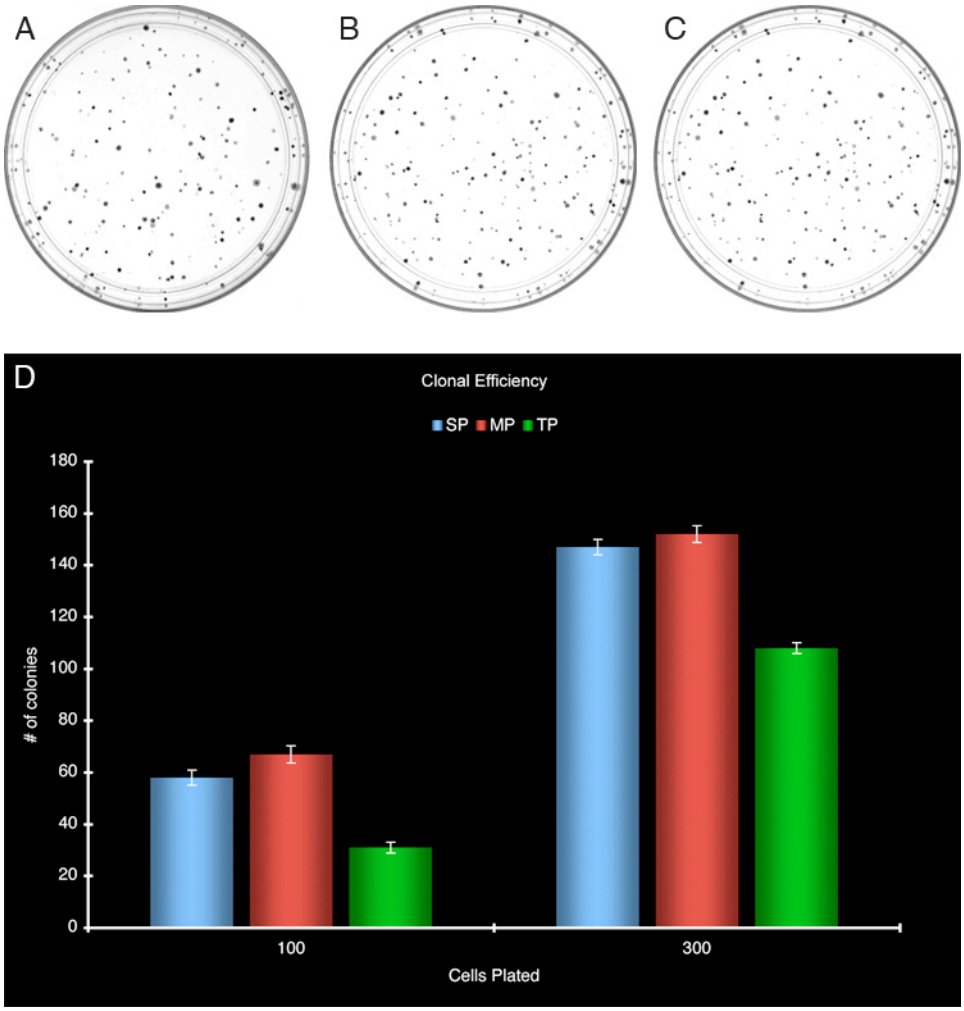

Figure 3.4 – Colony Formation Assay of Stem-like Fractions in Panc-1 Cells. The independent growth potential of a single cell from the SP, MP, and TP fractions were tested, by plating 100 and 300 in triplicate directly from an SP FACS sort and cultured for 14 days.

The effect of low oxygen on 'side population'

Stem cells are normally grown at atmospheric oxygen in standard tissue culture incubators and maintained in defined media and 5% CO_2 or on feeder layers. Although the self-renewal capacity of stem cells is maintained there are no data on their longevity. During transformation it is thought that cells under oxidative stress may accumulate mutations and lose their ability to repair damaged DNA and thereby reducing their 'stemness' and lifespan (Kenyon and Gerson 2007). If cancer stem cells inherit phenotypes from normal adult stem cells, then their ability to self-renew or differentiate may be influenced by changes in oxygen. If cancer stem cells have overcome the sensitivity to oxidative stress and lost their DNA repair mechanisms, then cells grown under different oxidative conditions will have no effect. Panc-1 SP and MP cells were sorted onto dishes and cultured in either 20% O_2 (hyperoxia) or 2% O_2 (normoxia) conditions for one week and then analyzed by FACS for changes in SP (Figure 3.5A). In the first round there were moderate changes in the SP sorted dishes, increasing the SP fraction to 20% in both normoxic and hyperoxic conditions. The MP sorted cells maintained a 6%-8% SP fraction in both O_2 conditions. In the second round, however, the SP sorted cells were enriched to 38% in 20% O_2 and 52% in 2% O_2, whereas there was no change in MP sorted cells indicating a persistent SP population in MP, either due to contamination or dedifferentiation of MP cells.

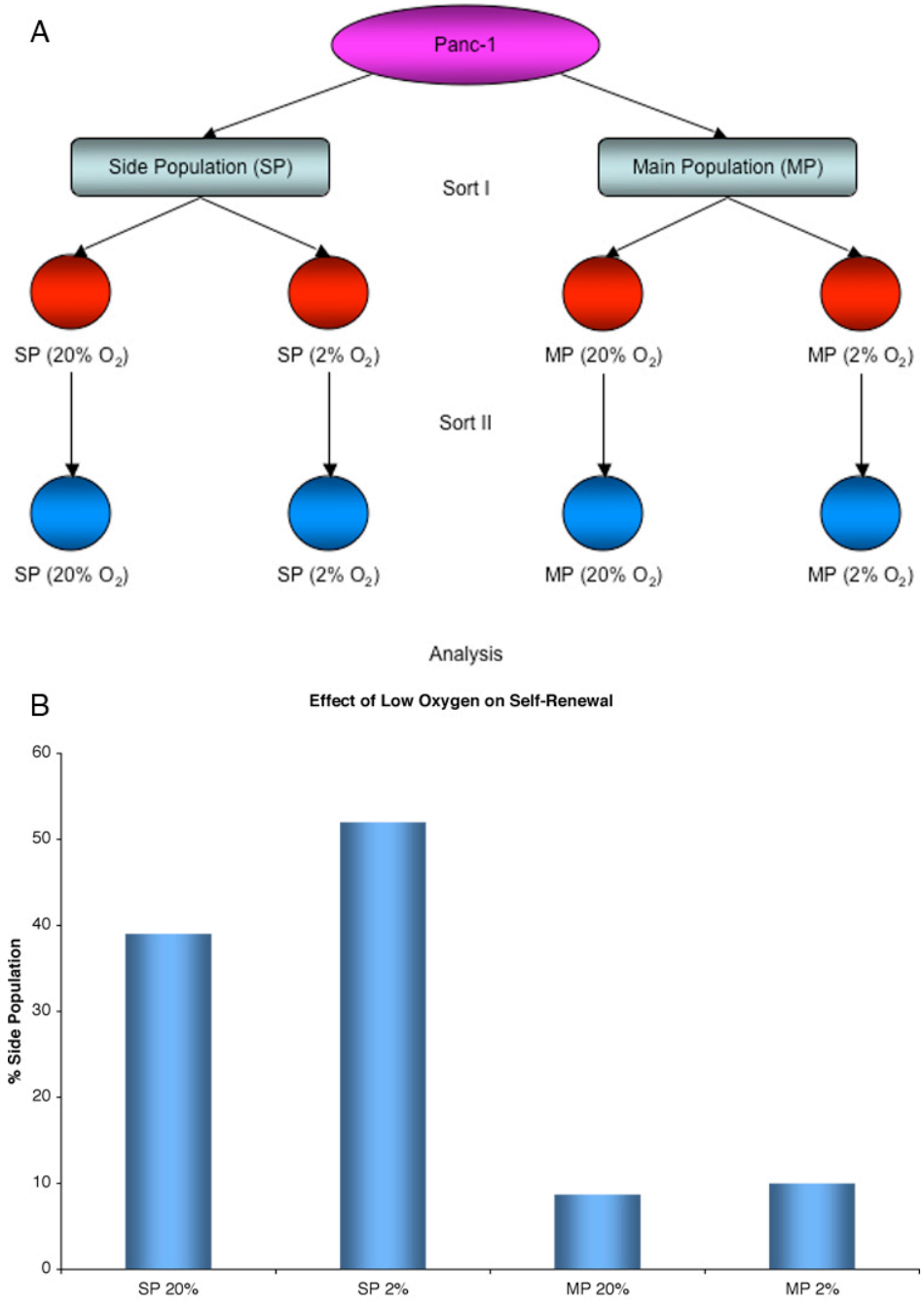

Figure 3.5 – Effect of Low Oxygen on Self-Renewal. Panc-1 cells were sorted by SP assay and cultured in low oxygen for up to four weeks by sub-culturing every week. (A) Strategy to analyze the effect of low oxygen on the SP compartment. (B) SP analysis of cells cultured in low oxygen for four weeks.

Effect of feeder layers co-culture on 'side population'

The maintenance of stem cells in culture, historically, has normally required growing them on fibroblast feeder layers. The secretion of proteins from these feeder layers helps retain their pluripotency and inhibit differentiation (Pera 2005; Xu, Peck et al. 2005). In order to enrich for SP it was necessary to further understand this stem cell characteristic for animal experiments. Sorted Panc-1 SP cells were co-cultured with NIH 3T3 feeder layers for one week and analyzed for changes in SP (Figure 3.6A). Surprisingly the feeder layer induced an increase in the MP phenotype, changing the fraction of SP from 10% to less than 2% (Figure 3.6B), suggesting that feeder layers in the context of cancer may cause differentiation, which has never been reported.

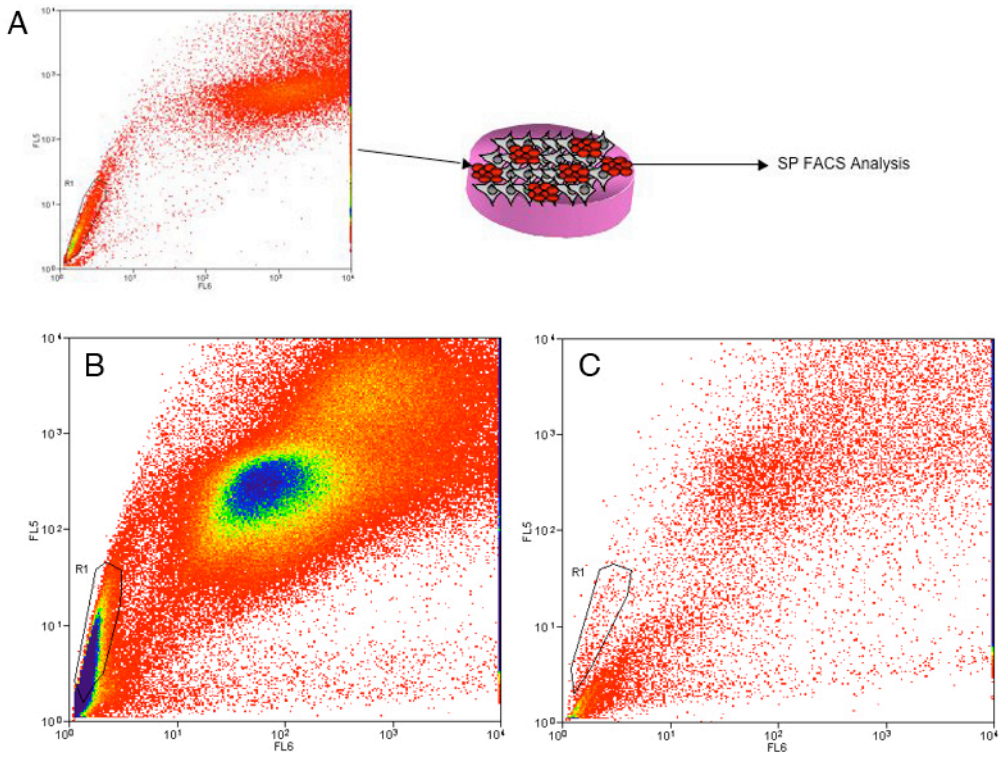

Figure 3.6 – Effects of Feeder Layers on the 'side population'. (A) FACS sorted SP cells plated on mitomycin C treated NIH 3T3 cells for one week. (B) FACS sort of SP cells before exposure to feeder layer, (C) FACS sort of SP fraction after one week of culture with feeder layer *in vitro*.

Persistent SP cells arising from purified MP cells

There have been reports of genetic reprogramming of fibroblast cells or skin keratinocytes to an embryonic stem cell state, implying de-differentiation (Wernig, Meissner et al. 2007). Although this is not commonly seen, the possibility of the existence of this phenomenon cannot be ignored. In light of this and due to a persistent SP fraction in purified MP cells found in the low oxygen experiments, Panc-1 MP cells were purified and serially passaged each week to observe any changes to the MP phenotype. After every passage and purification of the MP fraction, the analysis of the SP fraction was a consistent 6-8% fraction of SP, which may suggest contaminating SP cells (Figure 3.7 A, B) or dedifferentiating cancer cells.

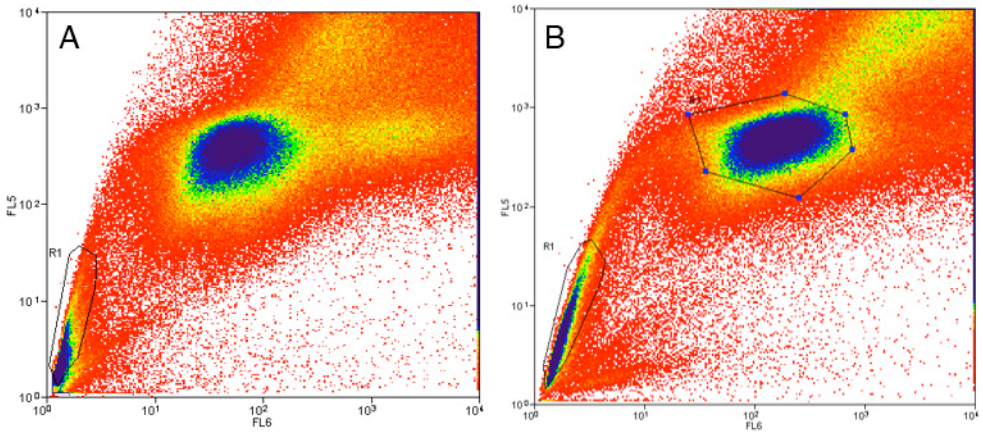

Figure 3.7 – Identification of SP in MP. Panc-1 cells were sorted using the SP assay where MP cells were sorted to purity sub-cultured for four weeks, (A) SP fraction of initial sort, and (B) SP fraction after serial passages for four weeks.

Effect of synchronization on 'side population'

The dye, Hoechst 33342, is a DNA binding dye, which intercalates in between the bases of DNA. This function provides a direct link to the cell cycle; therefore Hoechst 33342 can be used for cell cycle analysis, as previously shown (Parish 1999). During the use of Hoechst 33342 dye for SP analysis there is a possibility that the cell cycle may influence the fraction of SP and that the discovery of SP cells in the MP fraction and vice versa may be in part an artifact of cell cycle status, not true cellular differences. To test this, aphidicolin a chemical that stops the growth of cells in the G1/S phase of the cell cycle was used to synchronize cells. After synchronization for 24 hours there was no difference in SP profile compared to logarithmically growing cells. This suggests that contaminating SP in the MP fraction is due to overlapping wavelengths of the Hoechst red versus Hoechst blue. During SP analysis there are two wavelengths emitted by Hoechst 33342 upon UV excitation, the blue emission, which is recorded with a filter set at 450nm, and a red emission, which is recorded with a filter set at 675nm. However these emissions represent a range not a finite emission, therefore based on spectral analysis there is overlap of the emissions detected within these filters that may explain the contaminating SP within MP.

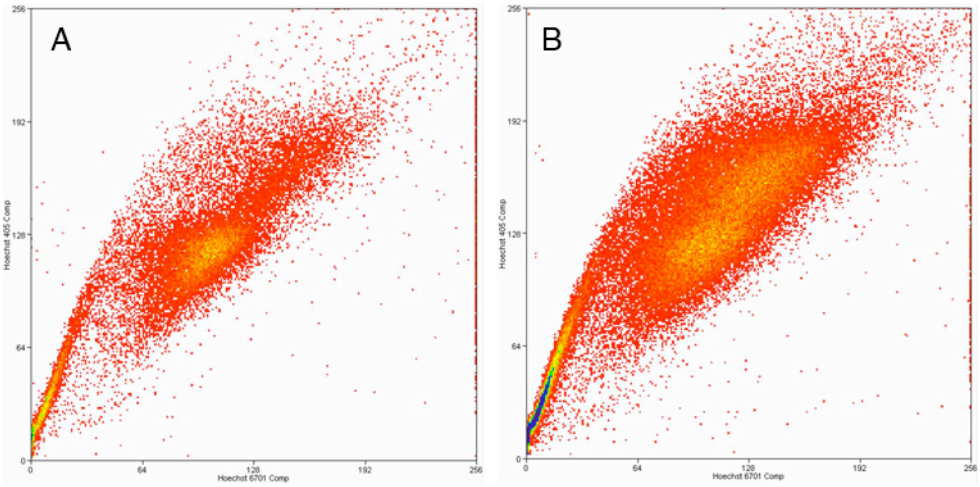

Figure 3.8 – Synchronization of the 'side population'. Panc-1 cells were exposed to Aphidicolin for 24 hours and then analyzed by FACS. (A) No Aphidicolin control, (B) Aphidicolin for 24 hours.

Identification of transit amplifying cells

It has been shown that bulge cells in hair follicles and certain stem-like cells at the base of colonic crypts undergo transit amplification (Fuchs and Raghavan 2002; Reya and Clevers 2005). This is defined by the ability of cells to transition from a stem cell to the target cell of the tissue. In the process these cells will rapidly expand the cellular pool to repopulate the tissue. Cancer stem cells however do not have such defined states since a differentiated cancer cell normally transitions to senescence rather than expansion (Eckhardt, Dai et al. 1994). Therefore, a physiological assay SP was used to identify transit amplifying cells within a tumor. Several groups have reported that SP can give rise to MP (Kondo, Setoguchi et al. 2004; Ho, Ng et al. 2007). To identify transit amplifying cells (TAC) in Panc-1 cells, purified SP cells were sorted directly into a dish and cultured for one week. If cancer cells do not transition to MP (non-SP) then only the SP fraction will expand and implying restricted self-renewal with limited plasticity. If a portion of the pure SP population gives rise to MP cells then it may be interpreted as TAC along with self-renewing capacity conferring CSC plasticity. The latter proved correct over the former. Purified Panc-1 SP cells gave rise to MP cells but also retained nearly 50% of its own population (Figure 3.9 A, B). These findings, demonstrate the plasticity of cancer stem cells independent of regulatory stimuli as shown in normal stem cells.

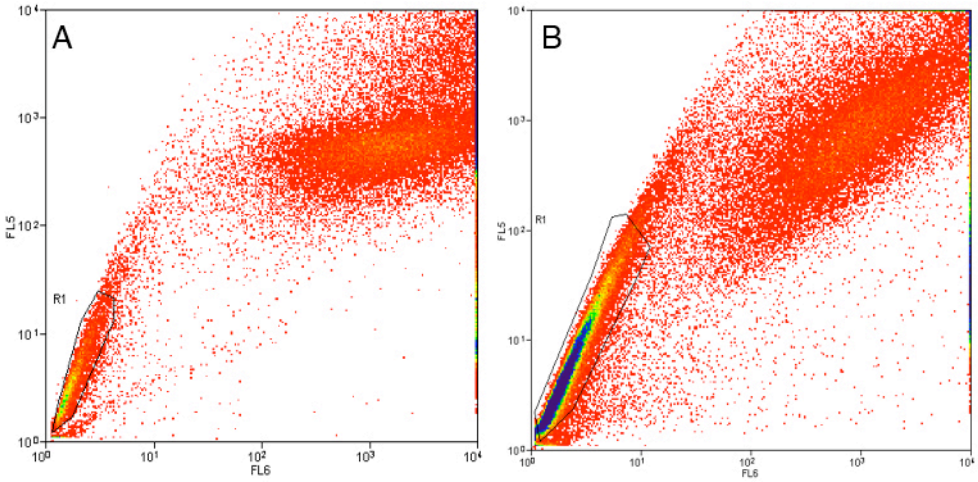

Figure 3.9 – Enrichment of SP by serial sub-culture *in Vitro*. (A) SP from Panc-1 cells were sorted onto culture dishes, (B) cultured for one week and reanalyzed.

Validation of a transit amplifying phenotype

Although serial passages of SP cells enriched for the SP phenotype (and that gave rise to MP), secondary assays are needed to validate this phenomenon. If pure SP gives rise to nearly 50% MP, then either MP cells are dividing faster than SP or SP cells are turning over to MP faster than SP self-renewal. An assay to eliminate rapid dividing cells was used to decipher this point. It has been shown that cells containing BrdU are 10,000 times more sensitive to Hoechst 33258 induced DNA damage than without [BrdU/Hoechst/Irradiation (BHI) method] (Stetten, Latt et al. 1976; Shay, West et al. 1992). Total Panc-1 cells were seeded and pulsed with BrdU for three days. On the third day the cells were pulsed with Hoechst 33258 for 4 hours and exposed to light irradiation for 30 minutes (Figure 3.10 A). After recovery, the cells were assayed for SP content. There was no change in SP compared to control on the first day post irradiation (Figure 3.10 B, C), but further evaluation of the population shows enrichment on day two and three post irradiation up to 30% (Figure 3.10 D, E). This suggests that either SP cells are turning over to MP rapidly or that MP cells have a faster doubling time than SP cells and therefore will be more sensitive to BHI selection than SP cells. A third approach to validate TAC in Panc-1 cells was employed. The chemotherapeutic drug gemcitabine, a nucleoside analog, is widely used for the treatment of PDAC and lung cancer (Schultz, Merriman et al. 1993). Gemcitabine incorporates into the genome of rapidly dividing cells and induces apoptosis due to genomic

instability caused by improper DNA replication. If there are two populations of cells dividing at different rates or if the turnover of one cell gives rise to the other then gemcitabine will be able to kill off the faster of the two populations without the complications of multiple DNA damaging agents and irradiation as done earlier. If there is a difference then the drug response of SP should be less sensitive than MP. Panc-1 cells were plated in log phase density and gemcitabine was added at the published IC50 concentration 16 ng/ml (Schultz, Merriman et al. 1993). The SP fraction was also enriched to 30% as shown in the BHI experiment suggesting that either MP cells are dividing faster than SP cells or the rate at which SP cells are self-renewing are equal to that for SP cells converting to MP cells.

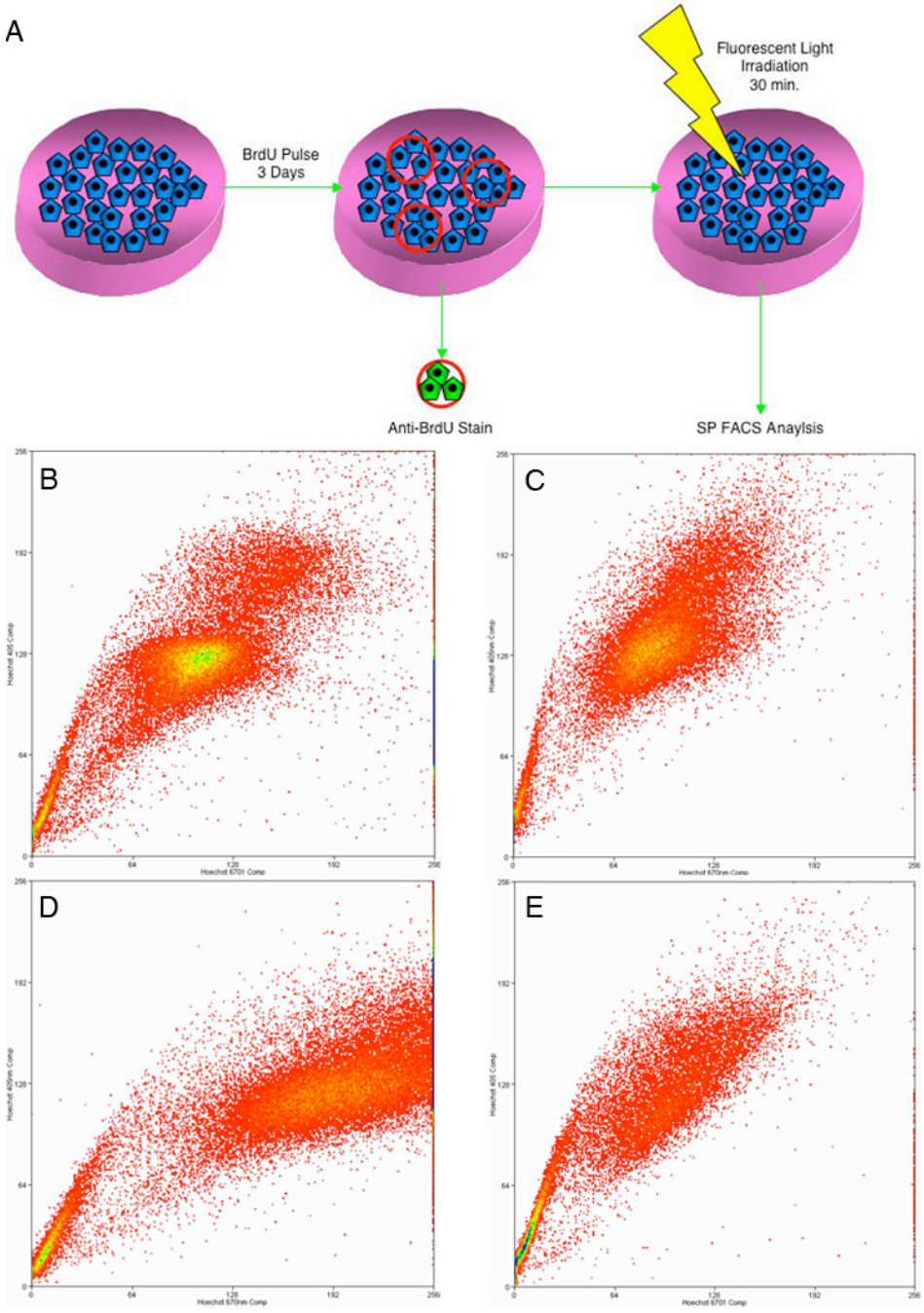

Figure 3.10 – BHI selection to Validate Transit Amplifying Cells. (A) Panc-1 cells were pulsed with BrdU for three days, followed by hoechst treatment for four hours, and 30 min. exposure to white fluorescent light. (B) Negative control without hoechst treatment, (C-E) SP FACS analysis for three days, respectively.

Heterogeneity of Panc-1 cells

It is only in non-isogenic backgrounds that cancer stem cells can be studied. Isogenic cell lines may have undergone dominate selection and therefore generally do not reflect the heterogeneity of the original tumor. In the case of Panc-1 cell line, it has been confirmed in this report that Panc-1 is a non-isogenic heterogeneous population of cells, which may reflect the pathology of the original tumor. Although this may be preferred for studying cancer stem cells, the restriction is that the SP is unstable and the MP has contamination with SP. Thus, it becomes difficult to resolve, which population contains stem cells and which population confers metastatic phenotypes. It is because of this hurdle that the Panc-1 cell line needed to be clonally isolated. The Panc-1 cells were plated at limiting dilutions onto 96-well plates and clones were selected based on their transformed phenotype (Figure 3.11).

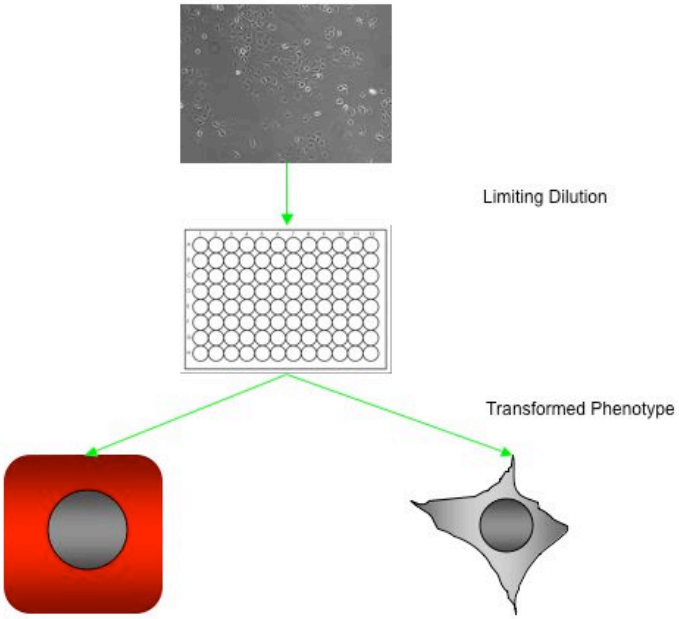

Figure 3.11 – Selection Process to Establish Panc-1 Subclones. Panc-1 cells are plated by limiting dilution into a 96-well plate and cultured for two weeks at which time clones were selected based on a transformed phenotype.

Four major clones were selected for analysis. The cell lines were designated as B10, C4, C5, F9, and were orthotopically transplanted into the pancreas of several mice to determine their tumorigenicity and metastatic potential (courtesy of the Brekken and White lab). As expected based on the selection criteria of a transformed phenotype, the B10 cells were tumorigenic but not metastatic, C4 cells were tumorigenic and moderately metastatic, C5 and F9 were highly tumorigenic and metastatic. The SP content was evaluated to correlate stem-like characteristics to these clones. The B10 clone contained 48% SP, C4 clone 45%, C5 clone 0.27%, and F9 clone 0% (Figure 3.12 A-D). This data, based on the current cancer stem cell hypothesis, was unexpected, suggesting that the plasticity of SP cells is linked to a primordial stem cell of origin driving tumor progression rather than the reported tumorigenic phenotype.

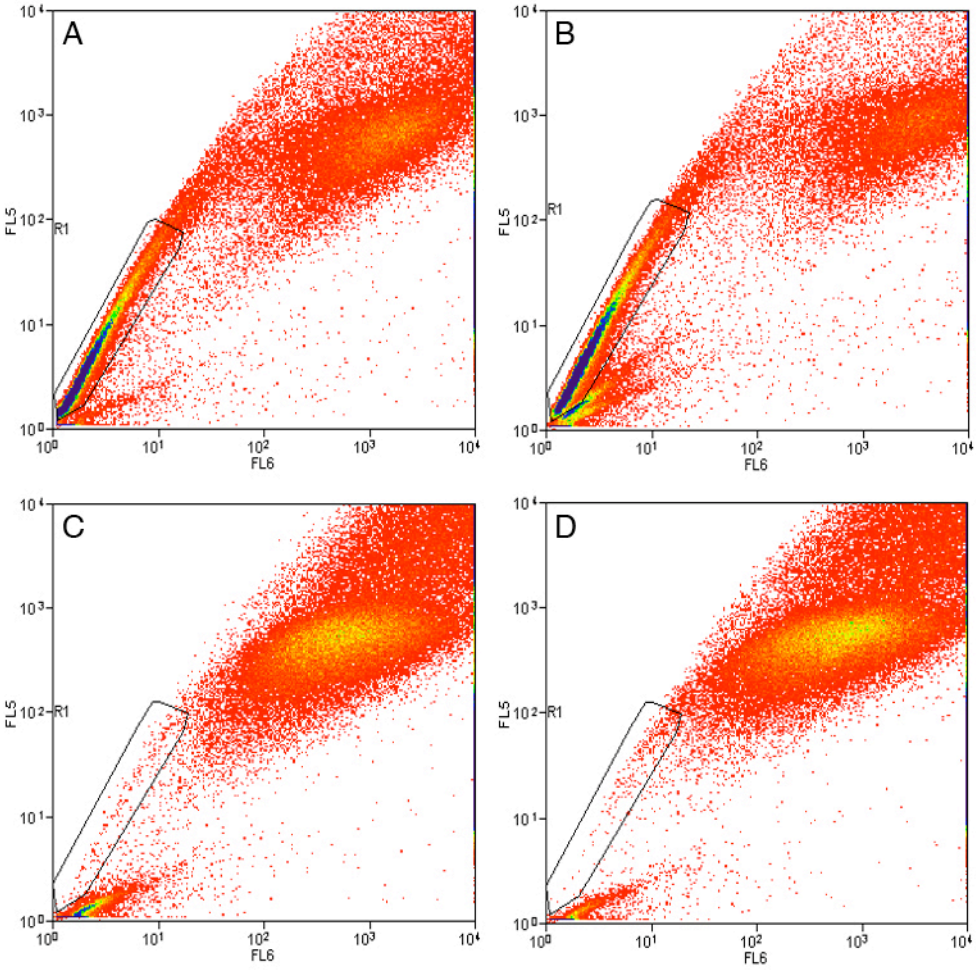

Figure 3.12 – Characterization of Panc-1 Subclones. Panc-1 clones (A) B10 tumorigenic, (B) C4 tumorigenic / mildly metastatic, (C) C5 tumorigenic / highly metastatic, (D) F9 tumorigenic / highly metastatic were analyzed by FACS for their SP content.

DISCUSSION

When stem cells differentiate they lose their side population suggesting it can be used as a marker for differentiation (Goodell, Brose et al. 1996; Majka, Beutz et al. 2005; Smalley and Clarke 2005). The definition of differentiation is when a stem cell loses its plasticity and give rise to a phenotypically and functionally different cell that may not continue to divide or self-renew. In order to test plasticity, SP cells were purified and cultured for one week and then analyzed for SP content. The SP profile had changed from 100% SP to 50% SP and MP, this finding was intriguing, but not unexpected as similar results in other tumor types had been reported, but in those cases slight changes were reported not 50% (Kondo, Setoguchi et al. 2004; Majka, Beutz et al. 2005). This suggests that SP cells retain their plasticity and have potential for 'transit amplification' a primordial phenotype related to non-tumor stem cells. Experiments to confirm this phenotype were performed by using BrdU, Hoechst 33258 labeling of cells followed by irradiation, or a single chemotherapeutic drug gemcitabine, to kill off rapidly dividing cells. The data confirmed a rapidly dividing population of cells that were enriched for SP three-fold after selection.

When Panc-1 cells were grown on NIH 3T3 feeder layers the cells unexpectedly lost 80% of their SP, suggesting that loss of SP is an indicator of

differentiation as seen in normal stem cells. However, our screen of other cell types revealed that highly tumorigenic and metastatic cell lines do not have a side population. These cell lines when assessed by pathologists are referred to as 'undifferentiated' tumor cells because of their similarity to each other, but obviously different when compared to non-malignant tissues. These findings offer a novel but counterintuitive hypothesis, that loss of SP correlates with loss of tumor plasticity, but gain of tumorigenicity, henceforth defined and termed here as 'tumorigenic conversion' by mechanism in the context of cancer stem cells, which contains an undifferentiated phenotype *in vivo*.

Based on the current study, if isolated SP cells are tumorigenic, then there should be a difference in their clonal efficiency compared to non-SP. However, the SP, MP, and TP fractions when compared in a colony formation assay formed equivalent colonies to each other. This finding suggests that in order to exploit SP as an assay for tumor plasticity and tumorigenic potential either an anchorage independent assay such as spheroids or orthotopic transplantations need to be used.

It is thought that oxidative stress may play a role in stem cell maintenance and lifespan, however there was no significant change in SP compared to MP controls when cultured in low oxygen versus atmospheric oxygen. During this study it was discovered that the non-SP (MP) fraction gave rise to SP, which suggested that tumor cells could de-differentiate. However, another interpretation

is that was pre-existing low levels of contaminating SP in the MP fraction. In conjunction with this finding, it is known that Hoechst is a DNA intercalating dye that can be used to analyze the cell cycle, therefore it was important to determine whether the states of SP and MP were stable under synchronization experiments and whether MP contained contaminating SP due to its cell cycle state. When cells were synchronized with aphidicolin a G1/S-phase blocking agent, the SP fraction was increased equal to the percentage that had contaminated the MP fraction and the MP fraction was devoid of any SP cells, suggesting that either the cell cycle state of SP can mimic the MP phenotype or that overlapping wavelengths will always contaminate the MP compartment, therefore identifying a minor flaw in the side population assay.

Previous studies in this chapter indicate that SP cells are unstable and differentiate too quickly to perform long-term experiments *in vitro*. Due to limitations in cell survival from FACS it is difficult to isolate enough cells for *in vivo* experiments. Therefore stable cells containing highly sustained fractions of SP were needed to perform further experiments. Panc-1 clones were established by clonal plating, similar to Fidler's work in1977, and assayed for varying degrees of tumorigenicity *in vivo*. The clones when analyzed by side population assay confirmed previous data in this chapter, that decreasing SP fraction correlates with a tumorigenic phenotype contradicting the findings in current literature, (Kondo, Setoguchi et al. 2004; Patrawala, Calhoun et al. 2005; Szotek,

Pieretti-Vanmarcke et al. 2006; Ho, Ng et al. 2007) of SP being a marker of tumorigenic cancer stem cell. To the contrary, based on the current findings SP is a marker of cancer stem cells that undergo 'tumorigenic conversion' (MP cells) to a more aggressive metastatic phenotype. The experimental findings in this chapter define the use of 'side population' as an assay to isolate and characterize cancer cells, that have plasticity transit amplification, differentiation potential, self-renewal, and tumorigenicity, based on *in vitro* exogenous cues and *in vivo* transplantations.

MATERIALS AND METHODS

The 'side population' Assay

Panc-1 cells were harvested with trypsin and normalized to 1 x 10^6 cells/ml in DMEM supplemented with 2% serum. Hoechst 33342 dye was added to a final concentration of 5 μg/ml or otherwise indicated. Controls were treated with a final concentration of 100 μM of verapamil for 10 minutes prior to addition of Hoechst dye. The samples were incubated at 37°C for 1 hour with slight agitation every 15 minutes. The cells were then collected by centrifugation, and resuspended in HBSS containing 0.5mg/ml DNAse I, 3% BSA, 2 μg/ml propidium iodide, and then filtered through a 70 μm filter into FACS tubes. In experiments that required staining the antibodies were added to the cells at vendor recommended concentrations for 20 minutes at 37°C followed by a PBS wash and filtered as previously indicated. Cells were then analyzed on a MoFlo flow cytometer equipped with a UV laser. The side population was determined by plotting the emission of hoechst red and blue against each other on a log plot gated for live cells by propidium iodide exclusion and forward and side scatter plots. The cells co-stained with antibodies were also gated based on their particular fluorophore.

Colony Formation Assay

Panc-1 cells were sorted directly onto 10 cm tissue cultures dishes at 100 or 300 cells per dish and cultured for two weeks. The plates were washed three times with PBS and fixed with 80% methanol. Following fixation the plates were stained with Giemsa stain diluted in PBS at a 1:20 ratio for 1 hour. The plates were rinsed in de-ionized water until all the unbound dye was removed, air-dried, imaged on an Alpha Imager gel doc system and counted.

Low Oxygen

Sorted Panc-1 SP and MP cells were plated onto 10 cm dishes and placed in modified low oxygen chambers. The modified chambers were gassed with a tri-gas mixture of 2% O_2, 7% CO_2, and 91% O_2 for two minutes (refer to the Shay/Wright lab) and sealed with silicone rubber plugs. The chambers were placed in a normal tissue culture incubator for one week prior to SP analysis.

Feeder Layers

NIH 3T3 cells were grown on 10 cm dishes and treated with 10 μg/ml of mitomycin C for two hours at 37°C in complete culture medium. The cells were then washed with warm PBS, collected by trypsin and quenched with serum containing medium. The feeder layer cells were mixed with Panc-1 cells at a ratio

of 2.5 x 10^5 feeder cells per 1 x 10^6 Panc-1 cells per 10 cm dish and cultured for one week prior to SP analysis.

Cell Synchronization

Panc-1 cells in log phase were synchronized by the addition of aphidicolin for 24 hours, washed three times with PBS, collected with trypsin, and analyzed for SP.

BrdU, Hoechst, Irradiation Selection (BHI method)

In order to select for slow cycling cells, the rapidly dividing cells had to be removed or killed off. Panc-1 cells were plated at a density of 7.5 x 10^5 cells per 150 mm dish. As soon as cells entered log phase they were pulsed with 30 μg/ml of BrdU until coverslip staining confirmed 100% labeling efficiency. In this case for three days followed by pulse labeling with 1μg/ml of Hoechst 33258 for three hours and finally irradiated with fluorescent light for 30 minutes. Cells were then recovered in complete medium; time points were assayed for SP up to three days after irradiation.

Gemcitabine Selection

An alternative to the BHI method was to exploit the cytotoxicity of gemcitabine to kill off rapidly dividing cells. Log phase Panc-1 cells were exposed to 16 ng/ml of gemcitabine and time points were assayed for SP for three days.

Establishment of Panc-1 Subclones

In order to determine the heterogeneity of Panc-1, the different populations had to be clonally isolated. Therefore a population of Panc-1 cells was diluted to single cells per well into a 96-well dish. After two weeks of culture, clones were selected based on visual criteria of a transformed phenotype and expanded. Clones were assayed for the ability to form colonies in a soft agar assay for tumorigenicity and also in orthotopic transplantations as described in chapter 2.

CHAPTER FOUR
Molecular Markers of Cancer Stem Cells

INTRODUCTION

The observation that a rare cell within tumors can initiate a malignant phenotype may be attributed to a conserved primordial cellular mechanism equivalent to its normal stem cell counterpart, where self-renewal is the maintenance of a cancer cell and differentiation is the bulk of a tumor. The molecular and cellular characterization of these cells may be critical in understanding tumorigenicity and metastatic disease and the elusive nature of cancer stem cells. A cancer stem cell in the context of leukaemia, is defined by the ability of a single cell, purified based on malignant markers such as $CD34^{+}$ / $CD38^{-}$, as well as functional markers such as the dye exclusion 'side population' assay, to repopulate a leukaemic phenotype *in vivo* (Lapidot, Sirard et al. 1994). Recently, there has been a new series of experiments to identify putative cancer stem cells in solid tumors of every tissue type in order to understand why current therapies fail. There are two approaches that are widely being used. The first is to isolate cells based on cell surface markers from patient samples which have tissue regenerative capacity and can give rise to differentiated cells, in this case a tumor. Second, to trace back the

earliest state of the cell in order to identify all the cellular mechanisms inherited for tumor survival, and therapy evasion.

When the first interpretation is used, several reports in breast and pancreatic tumors have shown that $CD44^{+}$ / $CD24^{+or-}$ cells isolated from a patient does reconstitute a highly tumorigenic phenotype *in vivo* (SCID mice) compared to $CD44^{-}$ / $CD24^{+or-}$ cells (Ponti, Costa et al. 2005; Li, Heidt et al. 2007). The CD44 marker also known as; Hermes, Pgp-1, H-CAM, hyaluronic acid receptor, lymphocytic homing receptor, is a receptor that predominantly binds to hyaluronate and is involved in cell-cell and cell-ECM interactions (Ponta, Sherman et al. 2003). Based on these findings many investigators have shown that CD44 acts as a cell homing receptor that promotes chemotaxis and in the case of cancer, invasion. Therefore, cells isolated based on this marker should be highly tumorigenic and invasive. The CD24 marker is a (GPI)-linked mucin-like adhesion protein that is heavily glycosylated, also known as the heat-stable antigen (HAS) and is thought to be the ligand for P-selectin. It was initially identified on hematopoietic cells, specifically B-cells and is involved in cellular rolling along P-selectin on activated endothelial cells and platelets. Many investigators have shown the function of CD24 to enhance the metastatic potential of invasive cells and can be used as a diagnostic marker of malignancy.

The second interpretation, the use of the side population (as discussed in chapter 3) as a conserved stem cell marker also shows a tumorigenic phenotype *in*

vivo. In many tumors, the expression of nestin, a filament protein and neuronal stem cell marker, has been characterized and may serve as a co-marker for SP (Fuchs and Weber 1994). Mucin-1, a known marker for invasiveness should localize into the population within the SP that is more invasive and which is more stem-like (Stanger, Stiles et al. 2005). Nanog, a marker that is involved in embryonic stem cell maintenance, is also a marker that is upregulated in tumors, implying that cells have either acquired expression or reactivated the gene for tumor maintenance.

In this chapter, the comparison of whether cells labeled for $CD44^{+}$ or $CD24^{+or-}$ localize with side population will be presented. If co-localization of $CD44^{+}$ with SP occurs it would validate the first interpretation. If not then extensive additional studies using stem cell specific markers need to be performed in order to link the cancer cell to a stem cell of origin as proposed in the second interpretation.

RESULTS

Localization of CD44 and CD24 to side population

The recent and extensive use of CD44 and CD24 as markers to isolate putative cancer stem cells from clinical samples has prompted further investigation into the experimental application of these markers for drug development. While the idea of chemotactic and proteoglycan adhesion markers to identify stem-like cells appears to be counterintuitive to understanding how cancer cells evade conventional therapy, it is plausible from a perspective of developing second and third generation conventional therapeutics for rapidly dividing malignant cells. The use of side population has proven to be useful to identify normal stem cells, and recently in identification of cancer stem cells. If both criteria lead to similar endpoints then each marker should localize to similar populations of cells. Panc-1 cells were labeled with Hoechst dye as indicated in chapter 3 to identify SP cells and then co-labeled with anti-CD44 or anti-CD24 antibodies. Unstained Panc-1 cells were used to control for autofluorescence. Post FACS analysis was performed using a negative gate on unstained Panc-1 cells to identify $CD44^+$ or $CD24^+$ populations on stained cells. This gate was also applied to the side population profile, nearly the entire population stained $CD44^+$. When the gate for CD24 was set, it localized to the non-SP (MP) cells, making CD24 a strong candidate for non-stem like cells (Figure 4.1A, B).

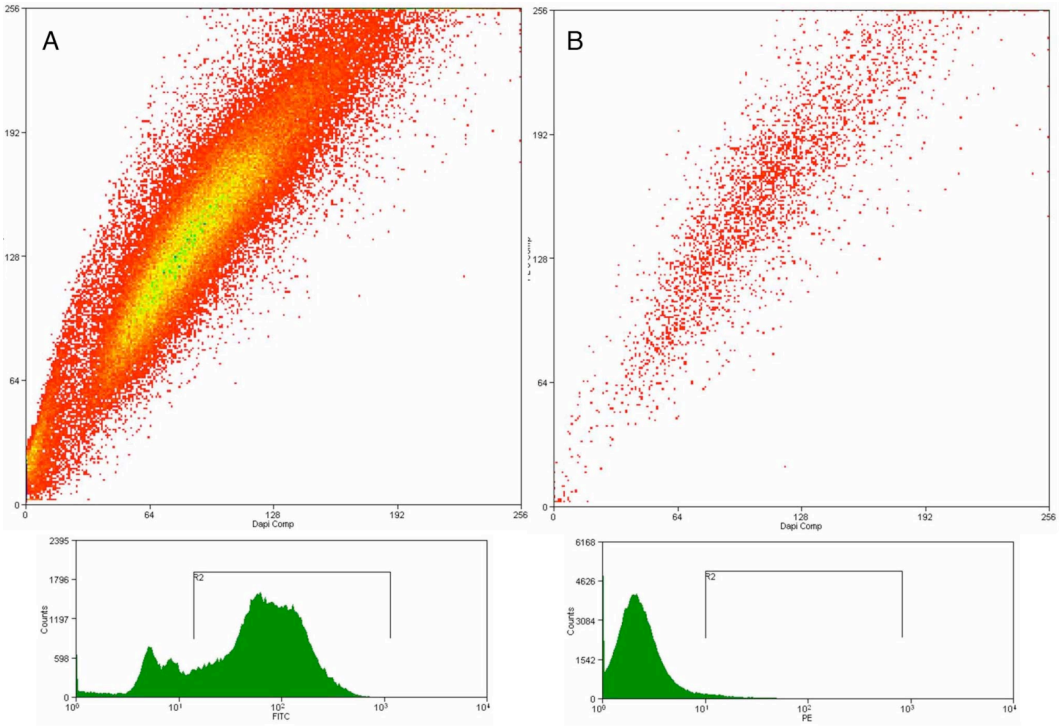

Figure 4.1 – The Localization of $CD44^+$ and $CD24^+$ cells to 'side population'. (A) Panc-1 cells were labeled with Hoechst for side population and co-stained with anti-CD44-FITC antibody. The $CD44^+$ cells were gated and localized to the SP and MP fractions. (B) Panc-1 cells co-stained with SP. The CD24+ cells were gated and localized to the SP and MP fractions. A gate was set on a negative control of unlabeled cells for autofluorescence.

Characterization of Stem Cell Markers on Panc-1 Cells

The molecular characterization of cancer stem cells may give insight into the state of a cell prior to transformation, implying that cells inherit cellular mechanisms for tumor survival and therapy evasion. The understanding of conserved protein mediators within a subset of cells may identify putative cancer stem cells. As discussed in the introduction, nestin, nanog, and muc-1 are all conserved proteins that are identified in normal stem cells, but are not usually expressed in pancreatic cancer.

Panc-1 cells were seeded on 13 mm coverslips for 48 hours. They were fixed and stained for CD44, nestin, nanog, and muc-1 (see materials and methods). Since CD44 is a cell surface marker, the cells have a distinct ring around the perimeter (Figure 4.2 A) signifying their surface expression. Nestin is associated with filament structures localized in the cytoplasm as shown (Figure 4.2C). Nanog a protein commonly seen and required in embryonic stem cell maintenance is highly localized to the cytoplasm (Figure 4.2B). Muc-1, an extracellular matrix protein is localized to the secretory vesicles and the surface (Figure 4.2D). All the markers stained positive in the entire population, giving evidence that cancer cells retain protein expression similar to normal stem cell.

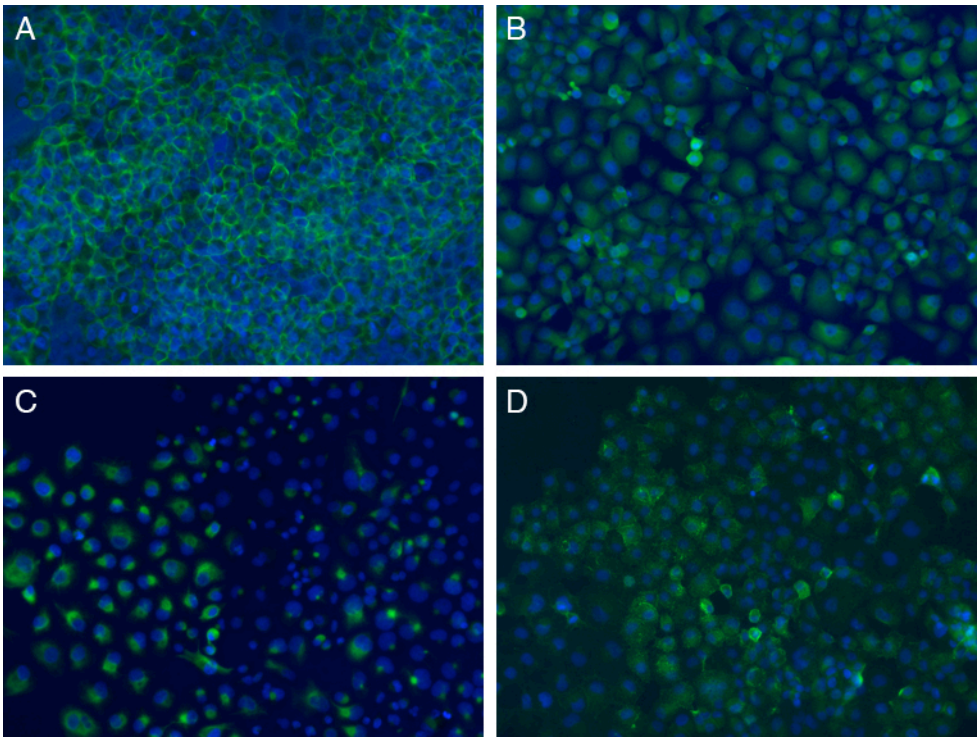

Figure 4.2 – Molecular Markers of Cancer Stem Cells. Panc-1 cells were plated on 13mm glass coverslips, fixed 48 hours after culture and stained for markers; (A) CD44, (B) Nanog, (C) Nestin, (D) Mucin-1.

DISCUSSION

The identification of putative cancer stem cells depends on the criteria used to isolate and characterize them. In previous reports, cells expressing $CD44^{+}$ and $CD24^{+/-}$ are identified as highly tumorigenic population of cells able to initiate tumors in rodents. Pathology studies show that CD44 correlates with poor prognosis and patient outcome (Rall and Rustgi 1995). This sorting strategy tests the hypothesis that cells isolated based on their regenerative capacity are the putative cancer stem cells similar to pluripotent stem cells, however it does not address the cause of poor drug response or relapse. As shown in chapter three, the side population was used to test whether cancer cells inherit mechanisms from their putative stem cell of origin for tumor survival by characterization of their plasticity and tumorigenicity. Considering that both (SP and CD44/CD24) strategies address similar hypotheses, it was important to test whether combining both them would converge to similar results. Unfortunately, SP cells that were characterized in chapter three for having plasticity, and previously published for being cancer stem cells did not co-label with CD44, a proposed cancer stem cell marker. Instead CD44 stained the entire population, leaving the interpretation that either cancer cells upregulate CD44 during cell culture adaptation, or that cells from clinical samples isolated using proteolytic methods are degrading CD44 considerably to create a ‘rare’ artifact, when in fact the entire tumor may be CD44

positive. Surprisingly, $CD24^+$ co-stained with non-SP (MP) cells, which confirmed a report that highly tumorigenic cells are $CD24^+$ in pancreatic cancer, as also shown in chapter three using Panc-1 clones negative for SP. These findings suggest that CD44 is not a good marker for Pancreatic cancer stem cell isolation and characterization, but can be used to isolate tumor initiators. The co-staining of $CD24^+$ with MP cells suggests that it can be used as a negative marker for 'cancer stem cells', or as a positive marker for Pancreatic 'tumor initiator cells' while SP and $CD24^-$ cells are essentially the putative cancer stem cell.

To address whether cancer cells originate from a normal stem cell that has become tumorigenic, it is important to identify markers that have remained for the maintenance of the cancer cell. It is known that nanog is required for ES cell maintenance, and nestin is primarily identified in neural stem cells, therefore staining cancer cells with these markers may give insight into their parental ancestry. The selectivity was limited in both of these stains, since essentially the entire population stained positive, there can be two interpretations. First, there is no differential staining therefore these markers cannot be used for cancer stem cell characterization. Second, the fact that all of the cells are stained for these markers indicates a common ancestral origin and that alternative markers may be needed for the identification of tumorigenicity and therapy evasion.

The results in this chapter confirm published results (Li, Heidt et al. 2007) that $CD44^+$ and $CD24^+$ are good markers for Pancreatic tumor initiators NOT

Pancreatic cancer stem cells, and that the cell line in this study relates back to a common ancestor based on ubiquitous staining of normal stem cell markers; nanog and nestin. However neither of these experiments explains the cause of therapy evasion or relapse.

MATERIAL AND METHODS

Co-labeling with 'side population'

Panc-1 cells were labeled with Hoechst 33342 dye as indicated in chapter 3 (the 'side population'). After labeling, the cells were washed with PBS and resuspended with DMEM supplemented with 2% FBS, either anti-CD44-FITC or anti-CD24-PE antibodies at 37°C for 20 minutes. The cells were then washed and resuspended in HBSS containing 0.5 mg/ml DNAse I, 3% BSA, and 2 μg/ml propidium iodide, and then filtered through a 70 μm filter into FACS tubes. The cells were then analyzed on a MoFlo flow cytometer equipped with a UV laser for excitation of the Hoechst dye, and a 488 nm laser for the FITC and PE fluorophore. The emission for side population was plotted as previously noted. Gates were set to identify autofluorescence on unstained and compared to cells that were stained with CD44 or CD24 and applied to the SP profile.

Immunocytochemistry

Panc-1 cells were plated in a 24-well tissue culture dish containing 13mm glass coverslips at a density of 1×10^4 cells per well. After 48 hours the cells were washed three times with PBS and fixed with 4% paraformaldehyde for twenty minutes and washed again. For direct cell surface detection, CD44 and Muc1, the coverslips were placed in Aquablock for 30 minutes at room temperature and then

antibodies were added at 37°C for 1 hour. In the case of Muc1, a secondary antibody conjugated to FITC was incubated at 37°C for one hour. The cells were then washed and nuclei were stained with Hoechst 33342 at 5μg/ml for five minutes at room temperature. The coverslips were washed with PBS and mounted onto frosted slides with Mowiol, an antifade-mounting medium. For intracellular staining the cells were then permeablized with 100% methanol at -20°C for 5 minutes and washed with PBS. The coverslips were then treated the exact same way as indicated here. All slides were visualized on a Zeiss inverted microscope.

CHAPTER FIVE
Therapy and Resistance in Cancer Stem Cells

INTRODUCTION

It has been frequent to see poor drug response or relapse in cancer patients after repeated treatments with traditional therapies, but the underlying cause behind this is not well known. One rationale for studying cancer stem cells is to potentially identify cells responsible for this problem and unravel the molecular mechanisms that govern their existence (Pardal, Clarke et al. 2003; Dean, Fojo et al. 2005). The most recurring causes of drug resistance occur in patients treated with gamma irradiation and chemotherapy due to poor drug delivery, or low drug response. In chapter three it was shown that the side population assay, an evolutionary conserved trait of stem cells, could be exploited to identify progenitors in cancer cells. The stem origin of cancer justifies the use of SP to assay the effects of gamma irradiation and chemotherapy, and whether the conserved stem cell response can explain the cause of relapse.

The purpose of using radiation for cancer therapy is to induce overwhelming double-strand breaks in the genomic DNA of cancer cells, which creates a critical mass activation of the apoptosis pathway and elimination of the tumor. The art is to accomplish this with minimal damage to the surrounding

normal tissue. The use of gamma irradiation adjuvant therapy is the second line of defense against invasive pancreatic adenocarcinoma after gemcitabine treatment, but prior to surgical resection. Unfortunately, the results are almost universally poor and in many cases there is recurrence due to radioresistance rendering the tumor incurable. Radiation studies with several pancreatic cell lines have shown the amplification of key DNA damage repair factors such as RAD51, Bcl-X, and Survivin in cells that confer radioresistance to conventional gamma irradiation. When these factors are down regulated or knocked down, the cells become radiosensitive (Maacke, Jost et al. 2000; Hezel, Kimmelman et al. 2006). During the analysis of these factors there have been few reports attempting to identify a 'rare' population of cells that resemble stem cells, rather total population studies are performed. The only account of cancer stem cells being involved in resistance has been characterized in glioblastoma where Chk1 and Chk2 enzymes have been implicated in radioresistance caused by the existence of a $CD133^{+}$ cancer stem cell (Bao, Wu et al. 2006). If cancer cells arise from normal stem cells and retain their molecular mediators of DNA damage repair, then studies need to be performed in the context of isolated stem cells for a thorough evaluation of radioresistance.

The stealth nature of pancreatic adenocarcinoma has made early detection difficult and treatment almost impossible. Due to this hurdle, the cancer is normally found in its late stages and is unresponsive to many conventional

therapies (Maitra and Hruban 2007). In many cases, after attempts to treat the disease, the tumor becomes chemoresistant resulting in the poor prognosis of most pancreatic cancer patients. There have been many studies characterizing the cause of this resistance. The common reason is the upregulation of ABC transporter pumps that does not allow most vinca alkaloid or taxanes to poison the cell. The findings that pancreatic cancer becomes resistant to gemcitabine is quite rare, but in the case of nucleoside analogs, which are not substrates for ABC transporters, the cause is an increase in Bcl-X_L expression that can allow normal nucleosides but not toxic analogs for incorporation (Shi, Liu et al. 2002). The central question is if a small 'rare' subset of stem-like cancer cells retains their transporter activity or anti-apoptotic genes to dominate the content of the tumor.

Telomerase (hTERT), the only reverse transcriptase in the human genome, has been shown to be active in 90% of all tumors and its activity is controlled by the cell cycle (Zhu, Kumar et al. 1996). Most stem cells have moderate to low activity of hTERT, but need the activity to maintain an extended lifespan (Flores, Benetti et al. 2006). It has been shown that when hTERT is repressed the telomeres become shorter over time in normal cells, and in cancer cells the tumors regress (Gellert, Dikmen et al. 2006). The idea that hTERT may contribute to the extended lifespan and tumorigenicity of cancer cells is obvious, but what is not known is whether the cancer stem cells also regulate hTERT for their quiescent state.

In this chapter resistance during radiation and chemotherapy will be examined in the context of cancer stem cells identified by the side population assay. Also, new therapies that inhibit hTERT will be used to circumvent resistance altogether by exploiting the conserved stem like mechanisms of cancer cell survival.

RESULTS

Gamma Irradiation in Cancer Stem Cells

The use of radiation to eradicate tumors has been used for decades, but has only been moderately effective in "curing" solid tumors. The increase in DNA damage repair mediators has been the largest reason for poor response and radioresistance. In these studies, what has not been explored is whether this response is due to an increase in protein expression or a clonal expansion of a cancer stem cell population to support tumor survival. The effect of gamma irradiation on cancer cells is most commonly measured by colony formation, the ability of a single cell to form an independent clone, post-irradiation. To evaluate this Panc-1 cells were sorted for SP and plated directly into 10 cm culture dishes at densities of 100 and 300 cells per dish in triplicate. After 24 hours, the dishes were gamma irradiated with 0 Gy, 1 Gy, 3 Gy, and 5 Gy. The dishes were cultured for two weeks and colonies were counted. The non-irradiated SP fraction had a significant increase in clonogenicity compared to MP or TP. However as the strength of radiation increased, the differences in clonal efficiency decreased. The MP and TP fractions eventually became indiscernible between each other, but the SP fraction maintained a noticeable difference to the MP and TP (Figure 5.1). The degree of difference between each fraction indicates that SP may not be a good indicator of radioresistance.

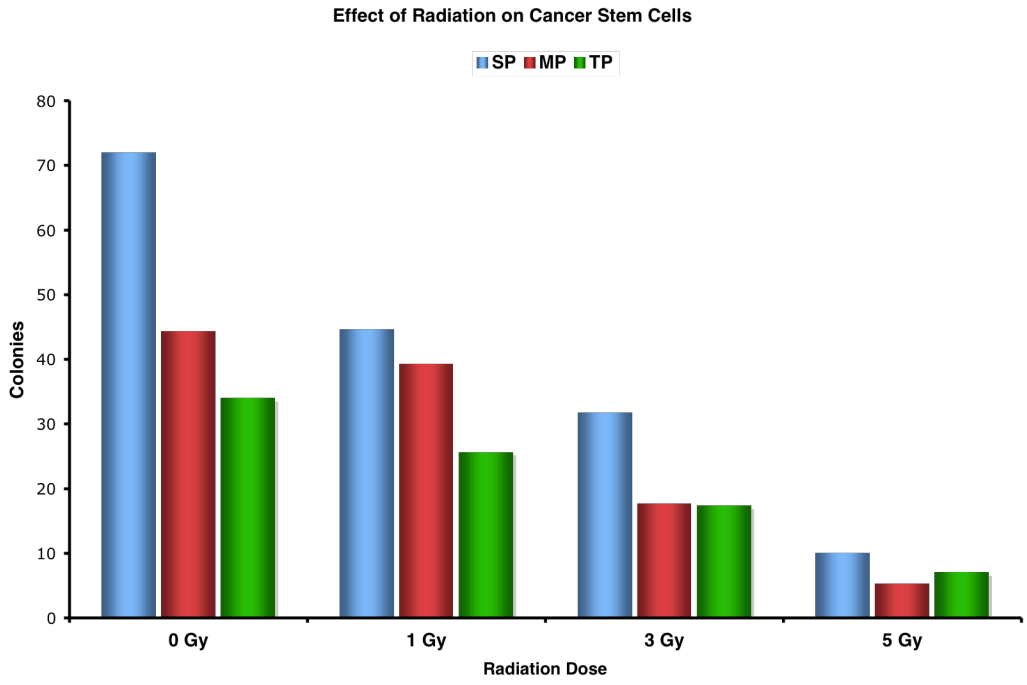

Figure 5.1 – Effect of Radiation on Cancer Stem Cells. Panc-1 cells were SP-FACS sorted directly onto 10 cm culture dishes at 100 cells per dish and irradiated 24 hours later with, 0 Gy, 1 Gy, 3 Gy, and 5 Gy respectively and cultured for two weeks. Total colony counts were plotted against each fraction per intensity of radiation.

Gemcitabine on Varying Tumorigenic Clones

Tumor resistance tends to be the most common reason that cancers relapse (Maitra and Hruban 2007). One rationale for studying cancer stem cells is to identify the source of minimum residual disease and the mechanisms that govern their existence. There are several reasons why tumors return, such as poor delivery of chemotherapeutics or rejection of vinca alkaloids and taxanes via ABC transporters. The use of gemcitabine has become the primary treatment for pancreatic cancer over surgical resection, however the response has been modest (Mercalli, Sordi et al. 2007). Gemcitabine is a nucleoside analog of cytidine and causes apoptosis due to genomic instability as the analog is incorporated into DNA (Kornmann, Butzer et al. 2000). In this study Panc-1 subclones were compared in their response to gemcitabine. The subclones B10, C4, C5, F9 along with Panc-1 cells as control were plated at a density of 1000 cells per well and treated with varying amounts of gemcitabine from 1 nM to 1 mM for one week. The drug response was determined by MTS assay and plotted as a function of cell growth versus drug concentration. The response from F9 was nearly the same as Panc-1 control, C5 was the most sensitive with a two-fold decrease cell growth and survival, and B10 & C4 (cell lines with ~40% SP) were the most refractory with a 30% increase in cell survival compared to Panc-1 control. While these are not dramatic differences, this confirms heterogeneity within a tumor cell line, but also validates that SP has an increased propensity for cell survival (Figure 5.2).

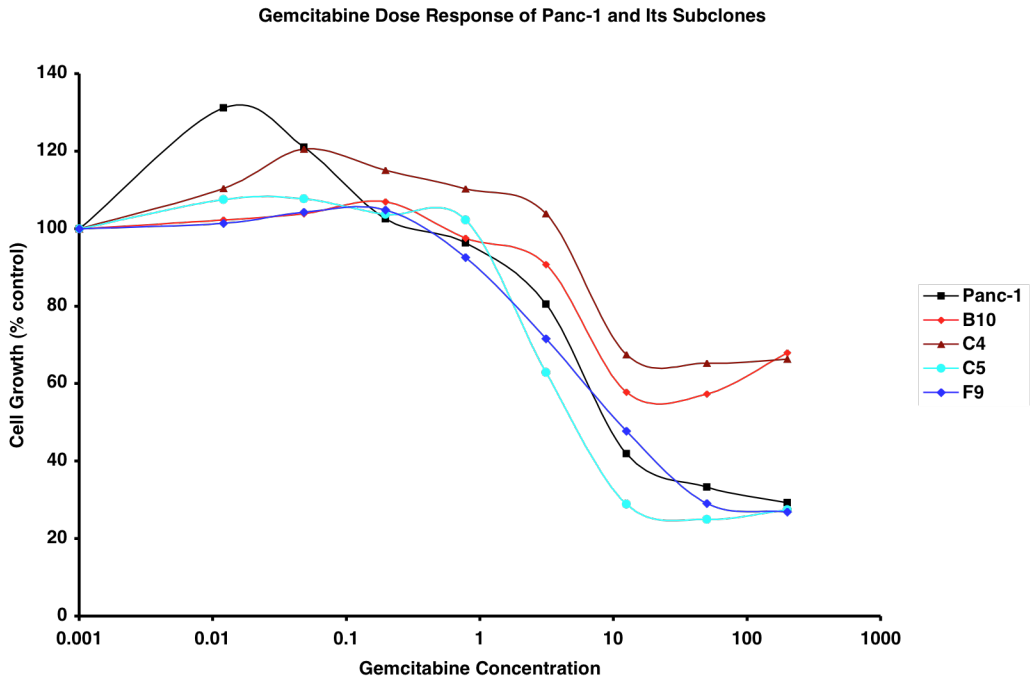

Figure 5.2 – Gemcitabine Dose Response Curve of Panc-1 and its Sub-clones. Panc-1 cells and its sub-clones were treated with several concentrations of gemcitabine and assayed for inhibition of proliferation by MTS assay.

Effect of GRN163L on Cancer Stem Cells

Telomerase has been implicated in most solid tumors of epithelial origin. Several attempts to use reverse transcriptase inhibitors have failed or are too toxic to the host for clinical applications (Strahl and Blackburn 1994; Strahl and Blackburn 1996; Hayakawa, Nozawa et al. 1999). Recent advancements in oligonucleotide chemistry has allowed development more applicable drugs with longer half-lives and decreased toxicity to the host (Gellert, Dikmen et al. 2006). The use of phosphoamidate backbones in oligonucleotides as well as lipidation has proven well tolerated in pre-clinical applications (Herbert, Pongracz et al. 2002; Pruzan, Pongracz et al. 2002; Akiyama, Hideshima et al. 2003; Herbert, Gellert et al. 2005). The recent development of a telomerase inhibitor using this technology has prompted its application to many cancers (Dikmen, Gellert et al. 2005). The development of TRAP (telomeric repeat amplification protocol) a PCR based assay to detect telomerase activity from tissues or cells has allowed automation and increased sensitivity down to the single cell level (Kim, Piatyszek et al. 1994). Briefly, cells are lysed with a detergent and added to an extension reaction where a 'bait' telomere is used as an activity trap for telomerase. The reaction is then placed in a PCR reaction where the extension products are amplified. There is an internal control for quantification and sensitivity (ITAS). SP sorted Panc-1 cells were treated with 1 μM of GRN163L for three days and assayed by TRAP. The SP cells had slightly less hTERT activity as shown by the complete retention

of ITAS. The MP cells had less ITAS indicating that there is more telomerase activity compared to the SP cells. ITAS is competitive with telomerase in this assay, thus the more telomerase that is present the weaker ITAS band becomes. The reduced activity in the SP population could be explained if the SP are initially cycling slower than the MP compartment. Irrespective, both fractions (SP and MP) were sensitive to the GRN163L treatment as shown by almost complete inhibition of the TRAP ladder. A titration of H1299 cells at 35, 350, 3500 cell, was used to quantify the results (Figure 5.3).

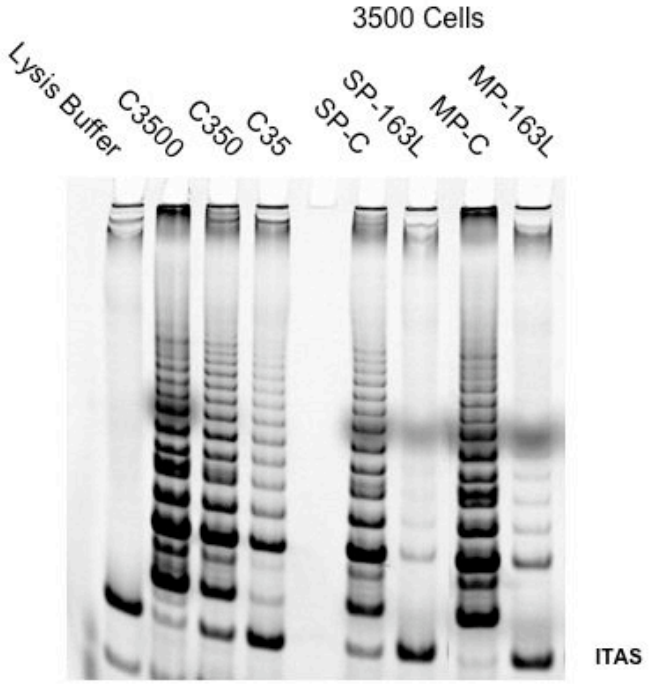

Figure 5.3 – Telomerase Activity in Cancer Stem Cells and Inhibition using GRN163L. Panc-1 cells were sorted by SP-FACS onto a 6-well culture dish and cultured for three days in the presence or absence of GRN163L drug and assayed for telomerase activity.

Effects of Combination Therapy on Pancreatic Cancer Cells

It has become more common to combine chemotherapies to increase overall tumor regression in order to have overall better patient responses. Gemcitabine is the standard chemotherapeutic in pancreatic cancer and therefore it would serve well to combine new experimental drugs to increase the effect of the drug. Panc-1 cells were treated with the telomerase inhibitor GRN163L, (1 μM) alone, gemcitabine (0.16 μg/ml), or the combination of the two and compared to untreated cells. To determine drug effects, the plates were analyzed by the MTT assay and measured by standard spectrophotometry. The GRN163L compound had very little effects on the population after five days of treatment, and gemcitabine treatment alone confirmed published results, but the combination of the two resulted in 40% cell death and reduction in growth rate. This pilot experiment indicates that targeting telomerase in combination with current antiproliferative nucleoside analogs may have additive effects in pancreatic cancer (Figure 5.4).

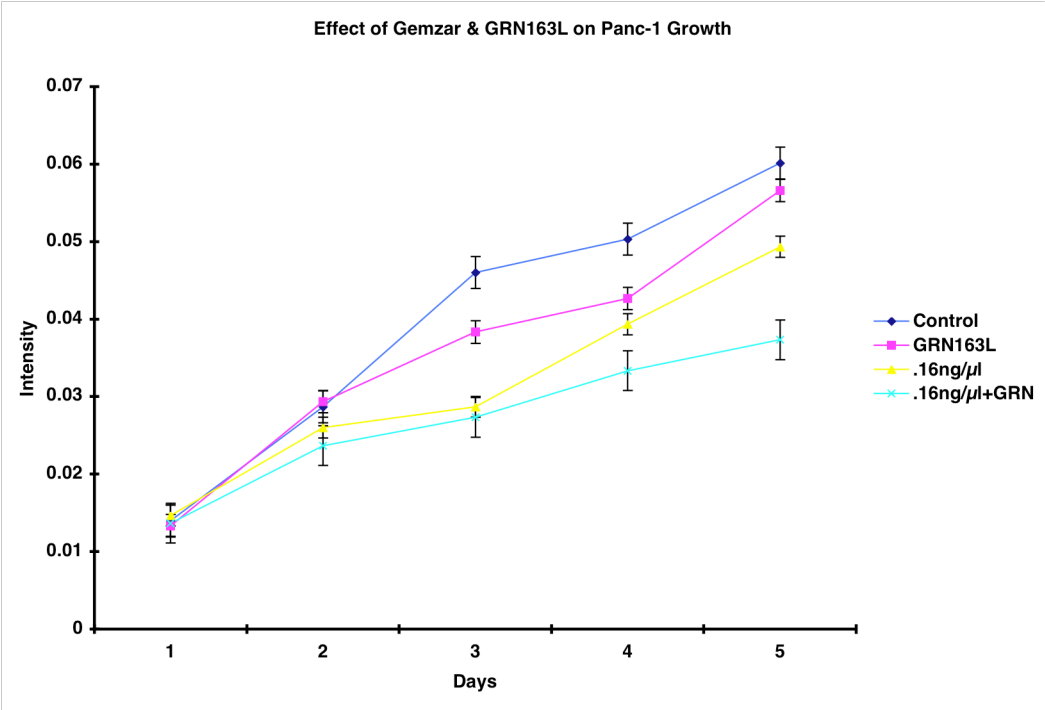

Figure 5.4 – Effect of GRN163L Combined with Gemcitabine. Panc-1 cells were treated with GRN163L, Gemzar or both for 5 days. Their proliferation was measured by MTT assay and plotted as a function of time. An approximate 20% decrease in by gemcitabine treatment and nearly 40% with the combination for GRN163L and gemcitabine.

DISCUSSION

This chapter has focused on the effects of known treatments for pancreatic cancer on isolated SP stem-like cells that may explain poor irradiation and drug response, and attempts to test new developments that circumvent the cancer stem cells altogether.

Radiation being a common but difficult approach to treatment has limitations of specificity and penetration (Shinchi, Maemura et al. 2007). In the colony formation assays, there were differences in the clonogenicity of Panc-1 SP, MP, and TP compartments, although the differences were minor at the higher radiation doses. This supports the concept that cancer cells retain stem cell mechanisms for survival. These minor differences may be attributed to the instability of SP in culture along with the contaminating SP cells in MP, which were characterized in chapter three. Therefore, this result should be interpreted with caution and gives basis for testing these affects in anchorage independent cultures and more so *in vivo* where the SP and MP compartments may be stable and thus more interpretable.

The use of nucleoside analogs to target tumors such as 5-FU or gemcitabine are common in lung and pancreatic cancer (Mercalli, Sordi et al. 2007). These drugs are more effective than classical drugs because they are not substrates for ABC transporters and resistance by mutation accumulation is quite

rare. That would imply that the cause of recurrence is either due to poor drug delivery or that a quiescent stem-like cell is able to evade treatment, giving rise to a new tumor. The difference of Panc-1 and its subclones in the response to gemcitabine was subtle but present. Subclones established from the same cell line with varying tumorigenicity would be expected to have varying grades of resistance if several of central tenets of the cancer stem cell hypothesis can be generalized. Although the differences were subtle, the results indicate that SP identifies a population of cells, which has decreased sensitivity to nucleoside analog gemcitabine due to slow cycling and stem-like quiescence.

Telomerase inhibition is a novel and almost universal oncology target and has been the subject of numerous preclinical studies. Unfortunately, most of them have been unsuccessful and have not proceeded into clinical trials (Strahl and Blackburn 1994; Hayakawa, Nozawa et al. 1999; Raymond, Soria et al. 2000). The discovery of phosphoamidate chemistry to create a competitive telomerase template antagonists that has longer cellular half-lives at 37°C and increased potency against their targets has proven worthy. In this case the telomerase oligonucleotide (GRN163L) was not used in an antisense manner since the target was the functional RNA (hTR) and not the TERT protein mRNA. Thus the GRN163L telomerase inhibitors act as a competitive template inhibitor for a human reverse transcriptase. Clinical diseases associated with telomerase dysfunction such as dyskeratosis congenita, sporadic bone marrow failure and

idiopathic pulmonary fibrosis indicate haploinsufficiency for telomerase may reduce telomere length and thereby lead to clinical disease (most likely due to depletion of normal stem cells). Clearly, but not surprisingly, the SP pancreatic cancer cells responded to GRN163L equally to the MP cells. In chapter three it was shown that SP cells cycle slower than MP cells, therefore being the sole reason as to why SP cells tolerate gemcitabine treatment. The response to GRN163L shows that the mode of action is independent of cycling state and validates the use of competitive inhibitors to telomerase for targeting cancer stem cells. This finding prompted the question; what would happen in combination with conventional therapy? Since gemcitabine is used for pancreatic cancer it seemed obvious to use it in combination with GRN163L to look for additive or synergistic effects. From the data presented here, there were no synergistic effects of the combination. However the two drugs alone did not affect cellular growth as effective as the two together, suggesting that there was an additive effect. Considering GRN163L has no major side effects even in early stage clinical trials, it is plausible to assume that the combination of this drug along with the clinically approved drug gemcitabine may improve tumor responses and patient outcomes.

Poor therapeutic response is the basis of why cancer stem cells were proposed for study. The data presented in this chapter highlights a heterogeneous cell line that contains inherited traits that confer tolerance to radiation, and retained plasticity that varies in drug response similar to its parental stem cell.

Previous studies also indicate this type of response, but they fail to resolve whether it's a total population event or a 'rare' subset of cells that is causing these problems, therefore misleading the authors into concluding that it's a ubiquitous effect. Further studies here have shown that telomerase inhibition may circumvent the cancer stem cell problem and combination with conventional drugs may help deplete the non-responsive tumor. These studies allow for molecular characterization of mediators that govern poor drug response and suggest new strategies for therapeutic development.

MATERIAL AND METHODS

Colony Formation and Irradiation

Fractions of Panc-1, SP, MP and TP, cells were plated on 10 cm dishes at densities of 100 and 300 cells per dish. The next day the plates were irradiated with no treatment, 1 Gy, 3 Gy, or 5 Gy. After two weeks the cells are stained, counted and plotted, as indicated in chapter three.

MTT or MTS Growth Assay

Panc-1 cells or subclones were plated at 1 x 10^3 cells per well and treated with varying amounts of Gemzar (Figure 5.2). After six days the cells are exposed to MTS compound at the vendor suggested concentration and quantified by fluorescence spectroscopy on a plate reader and plotted against each other.

TRAP Assay

The TRAP assay outlined in 'Current Protocols in Molecular Biology' was used to evaluate the telomerase activity. Briefly, cells are lysed with a mild non-ionic detergent and combined with a 'bait' template, which allows telomerase to extend the end of it. This extension reaction is placed in a PCR reaction to amplify the extension products, analyzed on a polyacrylamide gel and scanned on a 'Typhoon' (General Electric Biosciences) for detection.

CHAPTER SIX
Light Emission Tomography in Tumor Progression

INTRODUCTION

The advancement of non-invasive imaging technologies has brought on a new era to biomedical research where tumors can be visualized in real-time without the termination of animals. In the early inception of this technology, a protein isolated from jellyfish in the early 1970's (green fluorescent protein - GFP) that when excited by a 488nm light or laser emits green visible light was discovered (Spurlock and Cormier 1975). This allowed researchers to mark cells and follow their progression *in vivo*. This tool circumvented the need for histopathology and allowed for real-time live isolation and *in vivo* imaging of a disease. However, this technology had many difficulties during *in vivo* analysis (Contag and Bachmann 2002). First, tremendous autofluorescence emitted by the skin due to light scattering caused by the tissue and poor excitation of the fluorophore made it impossible to quantify data. In addition, GFP could not easily be visualized in organs deep within the peritoneal cavity such as the liver and pancreas. Following this came identification and cloning of the firefly luciferase gene, the basis of why fireflies emit light, served as a reporter for gene transcription and promoter activity analysis (Lorenz, McCann et al. 1991). When the substrate D-luciferin is

provided to the enzyme there is an ATP dependent catabolic reaction (chemiluminescent) that converts the compound to emit light only visible to highly sensitive CCD cameras (Sweeney, Mailänder et al. 1999). The advantages are no autofluorescence, considerably less scattering, no excitation required and longer half-life with no photobleaching (Herschman 2003).

In order to study cancer stem cells *in vivo* that utilize this technology, a lentivirus carrying the luciferase gene reporter placed under a strong promoter was used to label a population of Panc-1 cells. The result was a population of cells expressing high amounts of luciferase enzyme. These cells can be orthotopically implanted into the pancreas and periodically visualized to follow tumor progression using CCD cameras (Contag, Olomu et al. 1998). Initially the sensitivity of this system was tested *in vitro* and *in vivo* for practical purposes.

After the model was established and sensitivity was determined (Contag, Jenkins et al. 2000; Contag and Ross 2002; Edinger, Cao et al. 2002; El Hilali, Rubio et al. 2002; Burgos, Rosol et al. 2003; Marx 2003; Marx 2003), the study of cancer stem cells also must include full tumor progression. The model that show metastasis in living animals would be extremely valuable, thus experiments were extended further to reflect liver metastasis verified by two-dimensional imaging of the tumors. Although this technology has become the standard for most *in vivo* tumor models, there is still the limitation of getting full quantitative analysis of the tumor in 2D. If cancer stem cells are to be studied *in vivo* whole

tumor analysis should be the route to proper representation of self-renewal and differentiation. There have been several attempts to view tumors in three dimensions with little success (Weissleder, Tung et al. 1999). In this chapter, the sensitivity, and three-dimensional analysis of an orthotopic xenograft model of pancreatic cancer was established, using a combination of five CCD cameras in a tomogram to reconstitute a whole tumor (Figure 6.1). The goal of this was to develop technology that would allow for more detailed analysis of cancer stem cells progression *in vivo*.

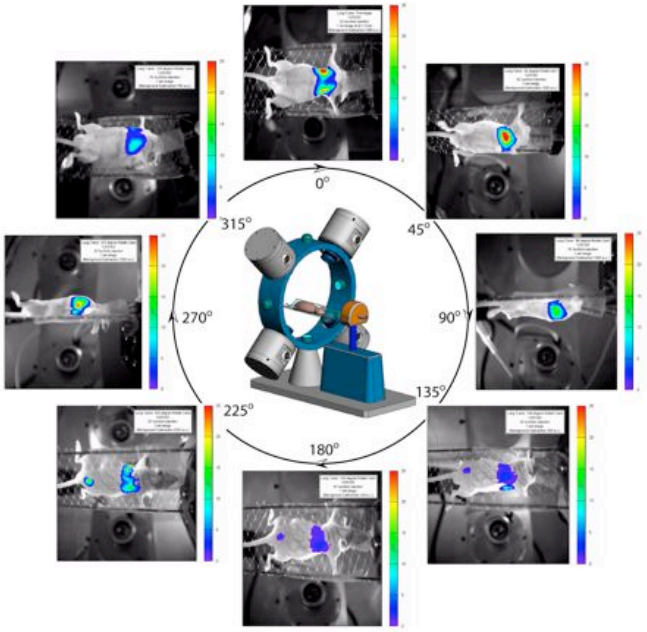

Figure 6.1 – Schematic of Light Emission Tomogram. A schematic diagram demonstrates the function of the LET apparatus. Images are taken from five CCD cameras representing all the angles within 360° and reconstructed into a 3D image.

RESULTS

Lentiviral Delivery of the Luciferase Gene

Many methods have been used to express luciferase in cell lines, ranging from standard transfection of plasmids to retroviral transduction. Unfortunately these methods fail to retain high expression levels for long periods of time, either due to weak promoter activity, integration into silenced regions of the genome or promoter methylation. A new method established by Geron Corporation (Menlo Park, CA), incorporates a MND promoter, which gives strong expression. This vector is less likely to be methylated, and the integration is dependent on a lentivirus, which has been shown to integrate into highly transcriptionally active sites within the genome. A four-plasmid strategy was used to transfect the packaging cell line HEK293 and viable viruses were harvested every eight hours for 72 hours. The viruses were used to infect Panc-1 cells sequentially for the duration of the viral harvest, essentially allowing for multiple integrations per cell. The infected cells were selected based on the neomycin marker for ten days and assayed for luciferase activity. Cell infected with luciferase were evaluated in light units, and found to express over 300 light units per cell compared to uninfected cells (Figure 6.2).

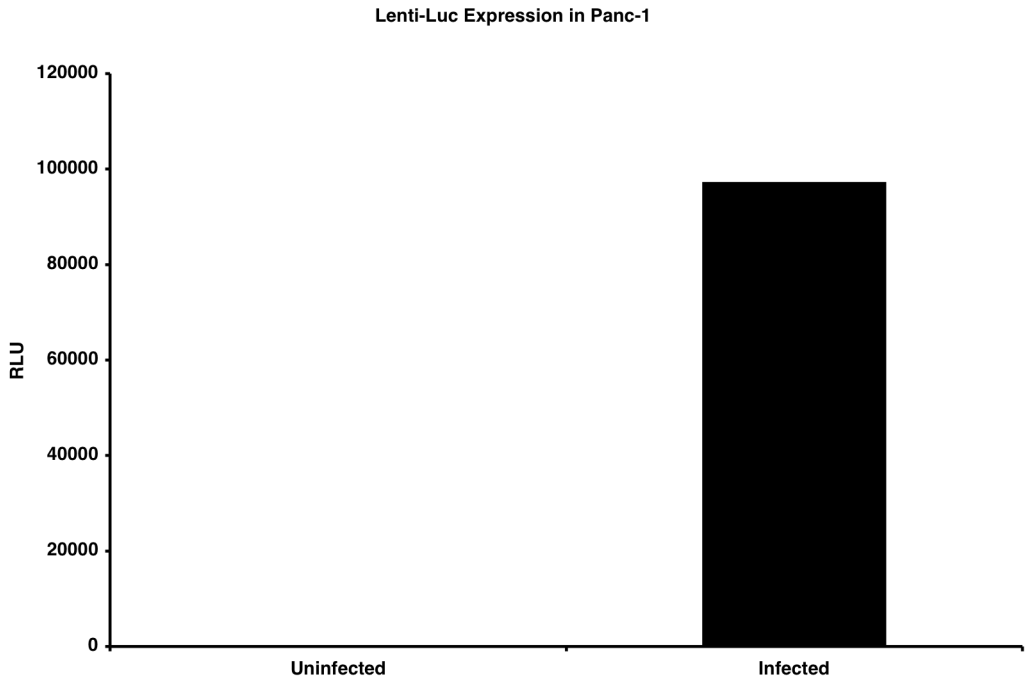

Figure 6.2 – Determination of Luciferase Expression. Panc-1 cells were infected with a lentivirus carrying a firefly luciferase gene under the control of a MND promoter. The activity was assayed with D-luciferin and plotted against cells uninfected with luciferase.

Titration of Cells Expressing Luciferase in Vivo

The cancer stem cell hypothesis states that a 'rare' cell must be able to reconstitute a tumor *in vivo*. To study cancer stem cell dynamics in live animals real-time, the RLU sensitivity of the cells must be determined. Labeled Panc-1 cells were titered ranging from 5×10^5, 5×10^4, and 5×10^3 cells per injection into the pancreas. The animals were imaged every week for three weeks by standard 2D chemiluminescent imaging (Figure 6.3). Images taken immediately and two days after transplantation showed light emission (2 min. exposure times) values of 3×10^6 RLU per animal in the 500K, and 50K animals, however no light could be detected in the 5K animal above background. Two weeks after injection, imaging resulted in 1.7×10^7 RLU (500K), 5×10^6 RLU (50K) and 2.8×10^5 RLU (5K). Three weeks after injection, imaging resulted in 6.7×10^7 RLU (500K), 2.6×10^7 RLU (50K) and nothing was detected for the (5K) animal.

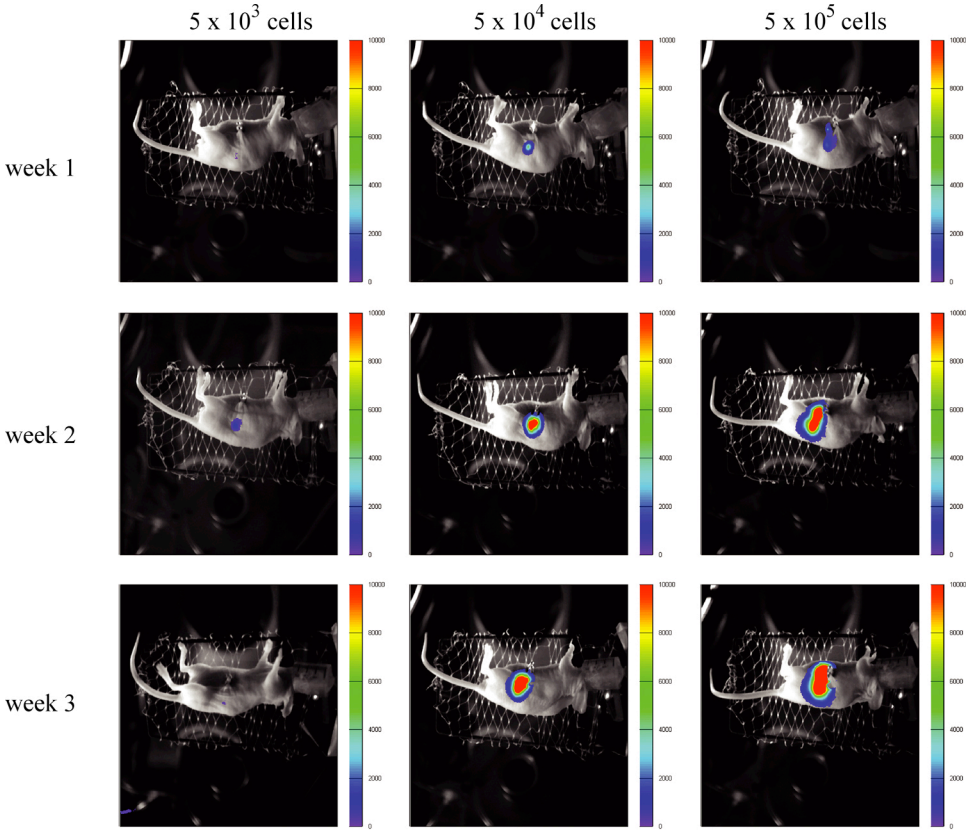

Figure 6.3 – Titration of LET Sensitivity. Stably expressing luciferase labeled Panc-1 cells were orthotopically injected into the head of the pancreas. 5k, 50k, 500k cells were injected and imaged weekly for three weeks.

Detection of Liver Metastasis

The imaging of a primary tumor is not enough to study cancer stem cells. There needs to be a representation of the entire progression from primary to metastasis. Many reports have shown that Panc-1 cells can, after two months, give rise to liver metastasis a reflection of human disease (Fleming and Brekken 2003; Katz, Takimoto et al. 2003; Katz, Takimoto et al. 2004). As a pilot experiment, 2×10^6 Panc-1 cells labeled with luciferase were injected into the pancreas and allowed to develop for two months. When the animal was surgically opened and imaged there were primary tumors as well as liver metastasis (Figure 6.4).

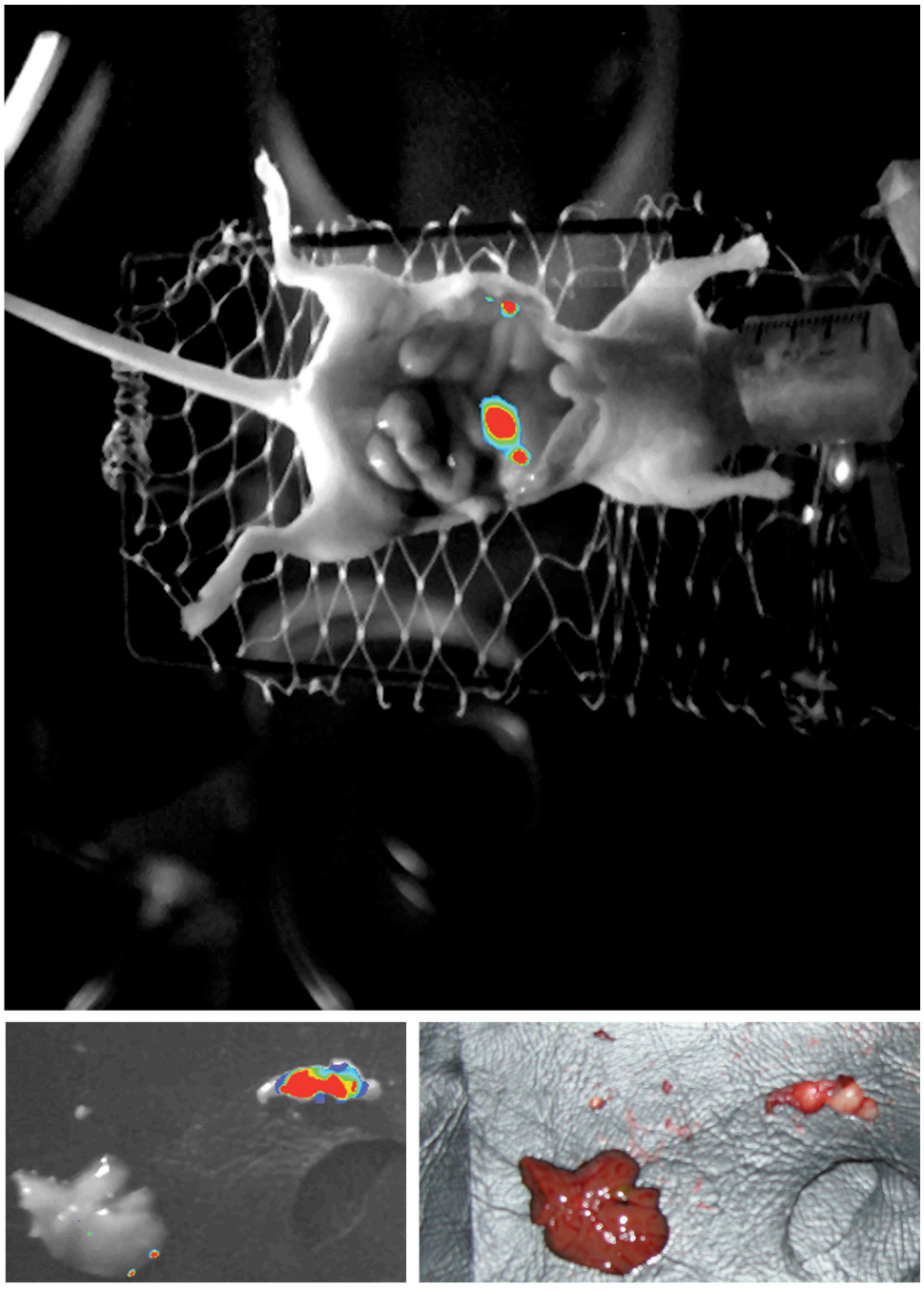

Figure 6.4 – Detection of Liver Metastasis. 1 x 10^6 Panc-1 cells were injected into the head of the pancreas and imaged every week for four weeks and then dissected for confirmation of primary pancreatic tumor and liver metastasis.

3-D Reconstruction of a Pancreatic Tumor in vivo

The titration of labeled cells provided information about the sensitivity of detection, but being in a 2D system did not address the status of the entire tumor. Therefore it was necessary to establish technologies to image an *in vivo* tumor in 3D for full representation of cancer. Collaboration with the Radiology Department prompted the development of a five CCD camera tomogram where an animal is placed in a MRI type apparatus and sequentially imaged at all 360° positions. The images were then integrated and reconstituted into a quicktime video using established algorithms by E. Richer N. Slavine and P. Antich in the Radiology Department. The video clearly identifies cells that would not traditionally be seen by 2D analysis.

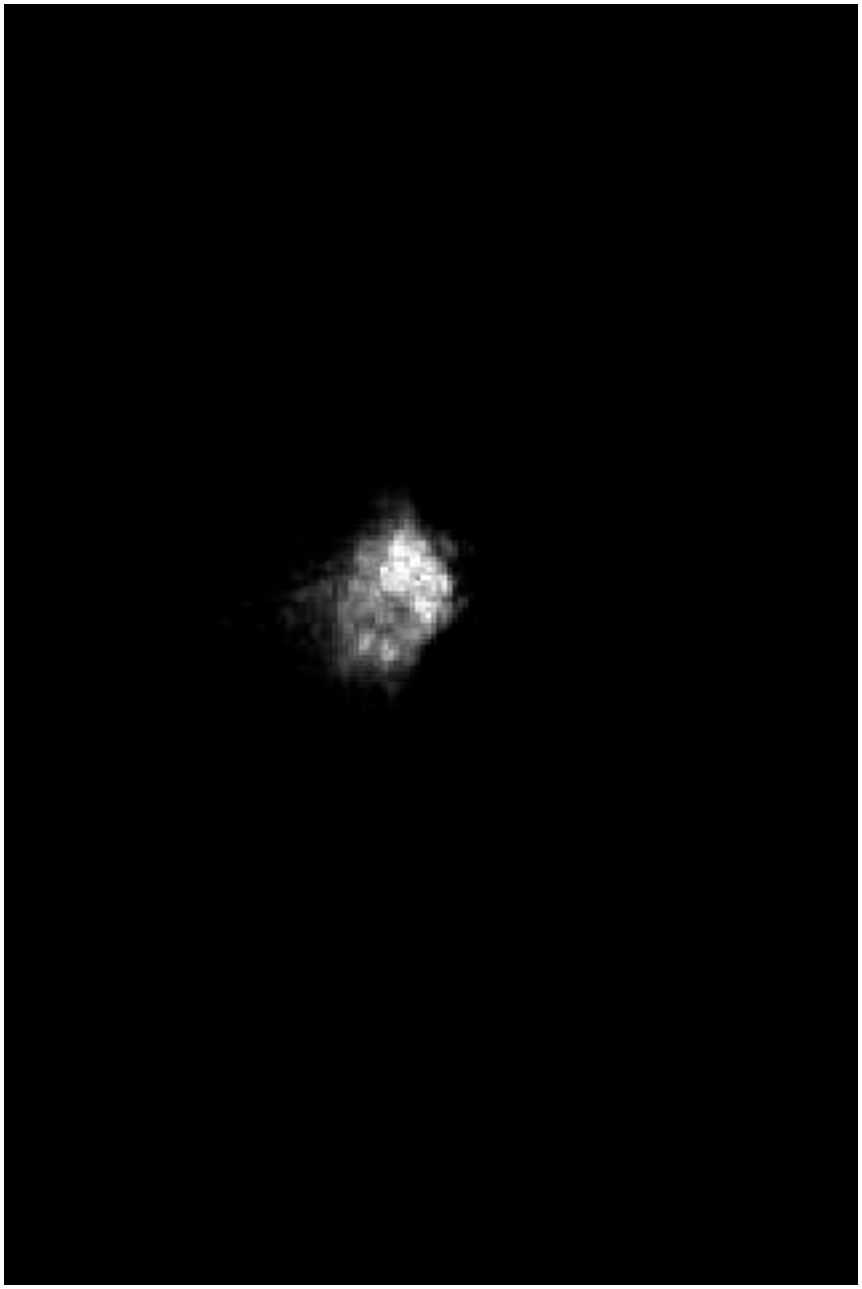

Figure 6.5 – 3D Imaging of a Pancreatic Tumor. 1 x 10^6 luciferase labeled Panc-1 cells were injected into the head of the pancreas and imaged by light emission tomography after four weeks.

DISCUSSION

The use of bioluminescent imaging for the detection of tumor growth has proven to be valuable compared to fluorescence as it eliminates the requirement of excitation and reduces scattering and absorption within tissues. The detection of tumor growth and progression, in terms of localization and quantification, has been limited to two-dimensional planar imaging, and there is no spatial discrepancy between cells emitting light from the anterior or posterior portions of the animal. Since the tissues of an animal can interfere with the light emitted from tumor, the detection of a 'rare' cancer stem cell's growth and dissemination to other organs may be hindered. Therefore multiple highly sensitive cooled charge-coupled devices (CCD) were developed to acquire high-resolution three-dimensional reconstruction of tumors in small animals.

Due to the limitations of green fluorescence protein (GFP) for *in vivo* imaging of tumors (presented in the introduction), the firefly luciferase gene was virally expressed in the Panc-1 cancer stem cell line stably for orthotopic experiments. After selection cells were compared against uninfected Panc-1 cells. The infection of luciferase into the cells resulted in a total of 1.5×10^7 relative light units per 5×10^4 cells or 300 relative light units (RLU) per cell. It has been reported, on average, expression of transiently and stably expressed luciferase from plasmid, and retrovirally expressed luciferase range from 1 to 30 relative

light units per cell. This strategy established a stable cell line expressing firefly luciferase 10-fold more than any report to date.

To utilize this novel bioluminescent imaging instrument, the sensitivity of this *in vivo* detection system was established. Titration experiments of cells ranging from 5 x 10^5 (500K), 5 x 10^4 (50K), 5 x 10^3 (5K), were orthotopically transplanted per animal. In the first week of injection the cameras could not detect 5K cells but could detect 50K and 500K cells injected. One explanation of why the RLU for 50K and 500K were approximately the same (e.g. 3 x 10^6 RLU for each tumor). This may be due to the limitation of a planar two-dimensional imaging system or that the tumor has not been established and therefore preventing penetration of the D-luciferin and oxygen. Two weeks post injection tumors were imaged and a three-fold difference between 500K ($1.7e^6$) and 50K ($5e^6$) was observed as well as tumor formation in the 5K ($2.8e^5$) injection detected at 10 to 30-fold less RLU than the other two animals. Three weeks later, imaging of the 5K injection showed a complete loss of light emission, and the 500K ($6.7e^7$) and 50K ($2.6e^7$) maintaining a three-fold difference in light emission. This data suggests that the current CCD cameras are sensitive enough to detect 5K cells *in vivo* (possibly less considering Fidler determined that only ~0.1% of injected cells survive within one week of injection), although this amount of cells is not sufficient to support a tumor long-term. However, most reports use matrigel to support small amounts of cells for tumor propagation *in vivo*, therefore further

experiments using matrigel or even carrier fibroblasts to help establish the microenvironment may need to be performed in order to establish a detection method for cancer stem cells.

The study of cancer stem cells requires the identification of primary tumors as well as disseminated tumor cells that establish a metastatic lesion. If Fidler's survival calculation holds, then 0.1% of 5000 cells mean that the sensitivity of the CCD cameras has detection limits as low as 500 cells. A mouse injected with $2e^6$ Panc1-Luc cells was allowed to grow and metastasize for two months. Open animal imaging of the tumor showed primary tumor formation and metastatic lesions on the surface of the liver. At minimum, based on the titration of these cells *in vivo*, the disseminated tumor contains 500 cells possibly more. This data suggests that the CCD cameras are sensitive enough to detect cells distal to the primary tumor at less than 500 cells per lesion.

Finally, the purpose of titrating the sensitivity of the CCD cameras was to provide the maximum image resolution for three-dimensional (3D) reconstruction of the tumor. The metastatic model above was used prior to dissemination for the purpose of 3D capture and reconstruction. Images were captured by three cameras compiling all the angles within 360° of rotation at 2 min. intervals per angle. An algorithm, adapted from techniques used for single-photon emission tomography, by the Radiology Department (E. Richer, N. Slavine, P. Antich) was used to reconstruct the planar images into a 3D movie. The reconstruction demonstrates

the sensitivity of the method by preventing scattered light from entering into other planes from which it did not originate. Further experiments will need to be conducted to determine the reconstruction capabilities of this apparatus as applied to cancer stem cells. However this pilot experiment demonstrates that detection of small numbers of cells (e.g. cancer stem cells) invading into distal tissues is feasible and with future development of more sensitive detectors may prove to be a useful too for visualization of even smaller numbers of disseminated tumor cells and hopefully help in development of new cancer therapies.

MATERIALS AND METHODS

Lentiviral Labeling of Cancer Cells

A four-plasmid transfection system was used to make lentivirus. Plasmids obtained from Geron Corp. encoding tet-rev, gag-pol, vsv-g, and pMND-luc were transfected into HEK293 cell line using Fugene 6 following vendor transfection recommendations. The cells were recovered after 24 hours and viral supernatants were collected every 12 hours for 72 hours total. Supernatants were filtered using a 0.45μM filter and mixed DEAD-dextran at 4 μg/ml and added to Panc-1 cells for transduction. After 72 hours of sequential infections, the cells were recovered in normal culture media for three days. The cells were then split at a 1:10 ratio and placed under G418 selection at 1mg/ml for 10 days. Live cells were collected and lysed using Promega's luciferase lysis buffer system for 30 minutes on ice and then measured on a luminometer.

CHAPTER SEVEN
Discussion

The 'cancer stem cell' hypothesis suggests that a 'rare' cancer cell, originating from a normal stem cell, within a tumor has the potential to reestablish a new tumor and thus may be the basis of tumor growth and recurrence. The study of cancer stem cells and the identification of conserved cellular regulatory mechanisms may improve the development of early cancer detection and the development of novel therapeutic drugs. This chapter discusses putative cancer stem cells and new approaches for future experimentation.

QUIESCENCE AND TUMOR DORMANCY

Quiescence, a trait of normal stem cells, is speculated as being important for cancer stem cells and may explain in part cancer stem cell resistance to therapy. The non-isogenic Panc-1 cell line labeled with CFSE *in vitro* was grown within organ-like structures termed 'spheroids' (Mueller-Klieser 1987; McLeod, Beischer et al. 1997; Hamilton 1998; Sipos, Möser et al. 2003) synonymous to embryoid bodies used with normal stem cells (Singec, Knoth et al. 2006). Cells that divided rapidly aggregated as well as the label retaining cells (LRC) with a

frequency of one label retaining spheroid unit (LRPU) per every three hundred spheroid units (SU) or (~0.3%). One interpretation of this could be an *in vivo* artificial non-native environment devoid of stimuli that would normally maintain homeostatic quiescence such as adaptations of the local niche (Shen, Goderie et al. 2004) as discussed in chapter 2. At the time of this experiment another report demonstrated the presence of LRC in subcutaneous tumors of nasopharyngeal carcinoma (NSC) with a frequency of (~0.3%) (Zhang, Ren et al. 2007). Experiments using neurospheres, mammospheres, and prostaspheres (Patrawala, Calhoun et al. 2005; Calabrese, Poppleton et al. 2007; Zucchi, Sanzone et al. 2007) validate similar label retaining patterns and imply a heterogeneous growth population as suggested by Morris, Nowell, and Fidler (discussed in the introduction). A 'clean' system was developed by performing orthotopic surgeries using *in vitro* BrdU labeled cancer cells that circumvented host labeled stem cell artifacts as would occur if animals were pulsed with BrdU after injection of tumor cells (details in chapter 2). The frequency of LRC within orthotopic tissue occurred at one cell per 1500 cells per tumor, or (0.00067%). In contrast, normal label retaining stem cells occur from 0.4% to 1% depending on the tissue (reviewed in chapter 2). The current *in vivo* observation was 5-fold less as compared to the above and 10-fold less than reported with normal stem cells *in vivo*. The decrease in the frequency of label retention may be related to the

genetic alterations of the cancer cell line as well as an enhancement of its stem-like label retaining phenotype prior to transformation.

Lark demonstrated the segregation of sister chromatids in 1966, followed by John Cairn's 'immortal strand hypothesis' in 1975, where a normal stem cell protects its genome by segregation of its parental strand of DNA to the daughter stem cell and not to the differentiated daughter cell. This could provide one explanation for the observations of label retention in this present research. It could be that during transit amplification the putative cancer stem cell retains its BrdU labeled parental strand while giving rise to cells that lose their BrdU and compose the bulk of the tumor. Fidler determined the survival of cells during a graft to be approximately 0.1% of the total cells injected and current observations indicate that cancer stem cells makeup 1-10% fraction of a tumor or cell line (previously reported). If this holds true then 0.1% x 1-10% = 0.1-1% of cancer stem cells injected should retain their label. In this model one million BrdU labeled cells are injected and therefore 1000 – 10,000 cells should retain their label. The tumors in this experiment were approximately 8 mm^3 in size and the average count of LRC was 20 cells per 5 μm section. Hypothetically, if there were 40 section x 20 cells per section = then there should be 800 LRC per tumor. Based on the results of this hypothetical calculation it is possible that cancer stem cells use immortal strand segregation to confer quiescence. In contrast if cancer cells accumulate mutations more frequently than normal stem cells due to DNA error (reviews by Nowell),

then the reason for retaining strand parental segregation is nullified, however if the cells are ‘minimally deviated’ from the parental stem cell then the ‘immortal strand’ hypothesis may hold true and serves as basis for further investigation.

The presence of label retaining cells within a tumor may suggest cell cycle arrest due to lack of stimuli or inhibition to enter the cell cycle. It has been shown that cellular dormant cancer cells are G_0-G_1 arrested and therefore may explain the occurrence of LRC at the earliest point of tumorigenesis. Tumor dormancy occurs when a primary tumor or disseminated tumor cell has entered G_0 and has delayed its ability to exit or reenter the cell cycle. After drug treatment the bulk of tumors are reduced, but a dormant population may exist as minimal residual disease that can reemerge years later. Most metastatic events occur due to activation of dormant disseminated tumors cells at distal organs, it is this event that likely increases the mortality in most tumor patients. There are three identified cellular mechanisms of tumor dormancy [reviewed by Aguirre-Ghiso (Aguirre-Ghiso 2007)]. First is immunosurveillance where innate and humoral immune responses prevent the disseminated dormant tumor population from expanding, escape from this immunosuppression causes growth of metastatic disease. Second is angiogenic dormancy where disseminated tumor cells are held dormant due to constant balance between pro and anti-angiogenic factors, termed the ‘angiogenic switch’. An increase of pro-angiogenic factors allows expansion of the dormant tumors. Third is cellular dormancy where primary or disseminated

tumor cells are dependent on their microenvironment and held at G_0-G_1 arrest. An unknown biochemical change in the local environment releases these populations from arrest. The observation of label retaining cells within a tumor may be explained by cellular dormancy and its release to transit amplification may depend on the biochemical change in the tumor stem cell niche, yet to be defined, similar to normal stem cells (Talpaz, Estrov et al. 1994; Tumbar, Guasch et al. 2004). This G_0 dormant state of label retaining cells may allow characterization of why tumor cells evade therapy and give insights on how to induce dormant cancer cells out of the G_0 state and make them vulnerable to conventional anti-proliferative therapy.

The persistence of quiescent label retaining cells in these tumors may be due to cellular stress and not dormancy or strand segregation. This state is termed 'STASIS' (STress or Aberrant Signaling Induced Stress), defined as metabolically active cells held in growth arrest either by oxidative stress, DNA damage, and inappropriate culture conditions by plastic or media (Shay and Wright 2005). Since tumors in this model are established with one million cells there is equal opportunity for the entire population to undergo STASIS, however this is not the case as the infrequency of LRC in a tumor is too low (0.06%) to explain this mechanism.

TRANSIT AMPLIFICATION

It is thought that the bulk of a tumor may be attributed to the transit amplifying nature of cancer stem cells where the cell undergoes a transient rapid proliferative state during asymmetric division possibly due to exit from cellular dormancy (G_0-G_1 arrest). However in cancer stem cells the mechanism for this is unknown and served as the reason for further investigation. In this report, SP cells (putative cancer stem cells as defined here) cells were purified and sequentially passaged for four weeks *in vitro* and analyzed for changes in SP and MP ratios. The results showed that the SP fraction could be enriched from 10% to 50%, but further purification and passages could not exceed this amount. By gross observation of cellular divisions one can speculate on a mechanism. First, it may be that a small population of cells had underdone asymmetric division, giving rise to a rapidly dividing MP cell, and after one week the MP fraction was equivalent to the SP fraction. Another explanation could be that the rate at which the SP cells are dividing symmetrically is equal to the rate at which SP cells are asymmetrically transitioning to MP cells. These possibilities were investigated by using a BrdU, Hoechst, irradiation selection method to remove rapidly dividing cells, where BrdU containing cells were 10,000 times more sensitive to fluorescent light after administration of Hoechst dye. The acute increase in SP fraction to 30% in three days demonstrates the presence of transiently proliferative population of cells,

however it does not resolve whether it's due to rapid proliferation of MP or rapid transition to MP. Recently, there have been reports of transit amplification of lung, glioma, prostate, and breast cancer stem cells (Kondo, Setoguchi et al. 2004; Clarke, Spence et al. 2005; Patrawala, Calhoun et al. 2006; Ho, Ng et al. 2007). In every report SP cells were between 1-10% of the total population and were purified to homogeneity, then subcultured *in vitro* for at least one week, which resulted in the maintenance of SP percentage not an increase of the SP fraction as shown in this report. The observation of a five-fold increase in SP (reported in chapter 3) may contribute to the invasive and malignant phenotype of pancreatic cancer. Both previous reports and observations here appears to be synonymous to transit amplifying cells reported in the skin and intestinal epithelia (Taipale and Beachy 2001; Fuchs and Raghavan 2002).

Several reports from Elaine Fuchs laboratory characterized the plasticity of stem cells of the skin epithelium to self-renew, transiently amplify and differentiate. The epidermis is made up of layers of cells representing stages in stem cell progression. It is in the basal layer that contains the putative stem cells of the skin. The spinous layer is an intermediate layer of differentiating cells followed by the granular layer, which contain further differentiated cells, and the stratum corneum that contain the dead cells of the skin. The basal layer makes up the portion of the epidermis next to the dermis separated by a basement membrane. The epidermis also contains the transit amplifying cells that mature to

give progeny that will eventually give rise to the other layers and eventually slough off and die (Fuchs and Raghavan 2002; Alonso and Fuchs 2003; Fuchs 2007). Briefly the molecular mechanisms that govern skin stem cell differentiation are as follows: During development, ectodermal progenitors commit to neuronal cells if exposed to FGF and are deprived of BMP signals, however if Wnt proteins act on the ectodermal progenitors the cell will allow BMP signaling to occur and will commit to the epidermal lineage. Further maturation of these cells to stratified epidermis occur if BMP and notch signaling are turned on, although further Wnt and EGF exposure results in development to hair follicles (Alonso and Fuchs 2003). Deregulation of this pathway leads to basal cell carcinoma.

A similar stem cell development pattern has been observed in the intestinal epithelium. Hans Clevers laboratory has characterized the stem cell maturation of colonic stem cells residing in the crypts. The intestinal tube gives rise to the large and small intestine. The large intestinal epithelium is comprised of structures referred to as the crypts of Lieberkuhn that include multipotent stem cells of the intestinal epithelium. The crypt is composed of Paneth cells at the base, followed by the stem cells, which undergo rapid proliferation referred to as the transit amplification cell population that moves further up the walls of the crypt where they differentiate into goblet cells and differentiated intestinal absorptive cells [review (Pinto and Clevers 2005)]. The maintenance of crypt

epithelial stem cells is due to the constant activation of the canonical Wnt pathway predominantly at the base of the crypt. There is a descending gradient that reduces the Wnt ligand from the bottom of the crypt to the top, hence deactivating the pathway for differentiation (Taipale and Beachy 2001; Reya and Clevers 2005). Inactivation of this pathway leads to colon cancer.

Taken the observations of stem cells in the skin and intestinal epithelium, it is plausible to model pancreatic development and cancer in a similar way. In pancreatic development there is a loss of SHH (sonic hedgehog) signaling to give rise to the pancreatic bud, and further loss of notch signaling to give rise to the endocrine and exocrine cells. A report of a Pten knockout mouse, a tumor suppressor and phosphatase of Akt phosphorylation, specifically in the pancreas demonstrates the expansion of centroacinar cells and increased expression of the target gene of the notch pathway Hes1 which further gives rise to PDAC (Stanger, Stiles et al. 2005). This result suggests that centroacinar cells may be where the putative stem cells of acinar and ductal epithelia reside. It could be that when centroacinar cells differentiate they undergo transit amplification to give rise to ductal or acinar cells. If PDAC cells retain mechanisms different from its normal stem cell of origin by restricting differentiation then it may be possible that they also retain the ability to undergo transit amplification that composes the bulk of the tumor.

MARKERS OF CANCER STEM CELLS

The characterization of cancer stem cells may allow the identification of novel genes and molecular mechanisms that govern the persistent and evasive properties of tumorigenesis. In order to identify putative cancer stem cells several groups have adopted methods of isolation based on cell surface markers from primary tumors removed from patients during resection.

The first accomplishment of this was the work of John Dick in 1994 where $CD34^{+}/CD38^{-}$ cells isolated from acute myeloid leukemia (AML) patients were able to initiate AML in immunodeficient mice *in vivo*. The established AML demonstrated self-renewal by expansion of $CD34^{+}/CD38^{-}$ and differentiation by asymmetric division to blast cells, eosinophils, and basophils. The next successful demonstration of cancer stem cells was in brain tumors based on the work of Peter Dirks in 2003 where $CD133^{+}$ cells, referred to as brain tumor stem cells (BTSC), were isolated from patients and assayed *in vitro* for self-renewal and differentiation. The established method of culture and spheroid formation allowed the identification of self-renewal by expansion of the $CD133^{+}$ cells and differentiation by asymmetric division to astrocytes, neurons, and oligodendrocytes. Both of these results determined that cells isolated based on specific cell surface markers were able to retain their plasticity from the stem cell of origin. These findings validate ‘Boveri’s hypothesis: oncogeny by

chromosomal mutation' and the concept of 'minimal deviation' where genetically altering mutations in brain tumors or genetic inversions (Bcr-Abl) in leukaemic cells occur earlier in the stem cells lineage thereby retaining their plasticity, as proposed by Morris, Potter and Nowell in the 1960's (reviewed in the introduction).

The previous studies were conducted with disease of the hematopoietic lineage, and neuronal lineage and therefore further studies since then have been done in five major organ tumor tissues to establish whether the above findings would hold true in other tumor cell types of epithelial origin. In the lung and prostate side population (SP) cells were isolated (Patrawala, Calhoun et al. 2005; Ho, Ng et al. 2007) and injected into SCID mice to initiate tumors, the population maintained a persistent SP of 0.1% (prostate), 8% (lung) and gave rise to non-SP cells as well *in vivo*. The injected non-SP cells failed to do the same. However there was no report of stem cell differentiation to other cell types, thus these more recent reports do not fulfill the definition of a cancer stem cells which includes the ability to differentiate. In the breast, prostate, colon, and pancreas it was determined that $CD44^+/CD24^-$ (breast) (Al-Hajj, Wicha et al. 2003), $CD44^+/\alpha_2\beta_1^{high}/CD133^+$ (prostate) (Collins, Berry et al. 2005; Patrawala, Calhoun et al. 2005; Patrawala, Calhoun et al. 2006; Patrawala, Calhoun-Davis et al. 2007), $CD133^+$ (colon) (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007) and $CD44^+/CD24^+$ (pancreas) cells could initiate a tumor, as compared to

negative cells that could not *in vivo*. These isolated cells were able initiate a tumor and maintain their population by self-renewal and give rise to marker negative cells as referred to as differentiation, but there was no report of stem-like differentiation to other cell types as shown in the leukemia and brain tumor models.

The differences in tumorigenesis and connection to stem-like phenotypes presented between these reports are inherently and dramatically different and therefore may have alternative interpretations. If cancer cells originated from stem cells that have accumulated mutations to drive transformation (refer to introduction of Boveri's Hypothesis), they may be referred to as 'minimally deviated' cells. In the context of leukemia and brain tumors, the observation of multi-lineage differentiation suggests that transformation occurred further up (earlier) in the stem cell lineage therefore maintaining a minimal deviated phenotype and can be referred to as the putative 'cancer stem cell'.

The proposed hypothesis of Koch in 1939 of the clonal evolution of a tumor states that tumors are made up of heterogeneous populations of cells ranging in their tumorigenicity. These cells can be isolated from tumors or cancer stemlines and enriched for a highly tumorigenic population by *in vivo* selection. This hypothesis was re-proposed by Nowell in 1976 and tested by Fidler in 1977. Fidler determined that clones isolated from a heterogeneous cell line could be enriched for a tumorigenic population and hold stable for several generations. It is

known that solid tumors are heterogeneous and contain cells at different stages of transformation, mutation accumulation, and independent cell survival where cells have acquired the ability to survive without other cells of similar origin. The threshold to gain independent cell survival and mutations to cause dissemination resulting in metastatic lesions requires tremendous changes within the cell and is a 'rare' event in its own right. In the context of epithelial tumors, the current observation of isolated cells from patient samples and their ability to initiate a tumor may be these 'rare' cells and re-defines the work established by Fidler with associated metastatic markers, and therefore should be referred to as 'clonally evolved' tumor cells or 'tumor initiators'.

TARGETING CANCER STEM CELLS

The purpose of studying cancer stem cells as proposed initially was to identify and characterize mechanisms that govern tumor resistance resulting in patient relapse and to perhaps help explain why cells reemerge as highly malignant tumors years if not decades after therapy and surgical resection.

The work presented in chapter five attempted to address these problems from a standpoint of anti-telomerase therapy combined with conventional therapy as applied to pancreatic cancer. The increased survival of SP cells *in vitro* post-irradiation indicates a unique mechanistic ability (reviewed in introduction) that may confer radioresistance within a purified population of tumor cells rather than the total population and requires more detailed characterization in the future. The nominal differences in IC_{50} for gemcitabine between isogenic cell lines isolated from Panc-1 cells indicate that resistance to nucleoside analog therapy relies on the increase in anti-apoptotic genes (reviewed in introduction), which normal stem cells may also use for survival, and therefore the conserved stem cell marker SP cannot be used to assess this effect. However, it has been reported that most pancreatic cancers do not respond to vinca alkaloids and taxanes, which are substrates of ABC transporters (Panc-1 cells contain a 10% SP), therefore experiments using current approved pump inhibitor's such as verapamil, reserpine along with mitoxantrone, vinblastine, and paclitaxel to target pancreatic cancer

cells may require further investigation, and the SP biomarker may be used to help develop newer therapies. Since the loss of SP identifies cells that comprise the bulk of a tumor and are highly metastatic, at minimum it may be possible to target these cells with standard vinca and taxanes therapy and reduce tumor dissemination, although this does not address the SP stem cell problem nor does it resolve cellular dormancy.

The anti-proliferative effects of gemcitabine have been well characterized and approved for use against pancreatic cancer, but due to enhancement of anti-apoptotic genes the response is very low. The development of a competitive template inhibitor of telomerase (hTERT) GRN163L has brought on a new approach to anti-cancer therapy. The findings in chapter 5 demonstrate a two-fold increase in tumor *in vitro* reduction when gemcitabine combined with GRN163L is compared to gemcitabine alone. It has been shown that a subset of cancer cells such as in about 20-30% of sarcoma's lack telomerase expression and use a mechanism referred to as alternative lengthening of telomeres (ALT) to maintain their telomeres. This mechanism generates ongoing DNA damage due to recombination and critically short telomeres. However, it has also been shown that over expression of exogenous (hTERT) dominates the ALT mechanism by reestablishing traditional telomere maintenance and reduces DNA damage to undetectable levels (Ford, Zou et al. 2001). It may be that the increase of anti-apoptotic genes during gemcitabine treatment are resolved by telomerase

inhibition by reduction of the expression of these genes due to ‘uncapping’ of the telomeres (uncapped telomeres create a DNA damage signal), and therefore suggests telomerase as a ‘hinge-pin’ for targeting bulk tumor cells and perhaps cancer stem cells.

The characterization of the development of the pancreas identified two major stem cell regulatory pathways for differentiation. The first was the hedgehog pathway where loss of hedgehog signaling led to pancreatic bud development (Hebrok, Kim et al. 1998; Ingham and McMahon 2001; Hebrok 2003). Several reports show a reactivation or retention of hedgehog signaling after transformation giving rise to tumors ‘trapped’ in the process of self-renewal (Berman, Karhadkar et al. 2003; Pasca di Magliano and Hebrok 2003; Thayer, di Magliano et al. 2003). Second was the notch pathway where loss of notch signaling gave rise to endocrine and exocrine cells (Apelqvist, Li et al. 1999; Jensen, Pedersen et al. 2000; Hald, Hjorth et al. 2003; Murtaugh and Melton 2003; Murtaugh, Stanger et al. 2003; Esni, Ghosh et al. 2004; Esni, Stoffers et al. 2004; Kadesch 2004; Lai 2004). Several reports show a reactivation or retention of notch signaling after transformation to PDAC (Miyamoto, Maitra et al. 2003; Radtke and Raj 2003). The loss of the tumor suppressor Pten identified a multipotent stem cell lineage; the centroacinar cells (Stanger, Stiles et al. 2005) and most PDAC have a retained or reactivated notch pathway. If these pathways are intact and responsive, then targeting the mediators within these pathways may

be beneficial. One approach may be a depletion model where the self-renewing phenotype driven by this active pathway is abrogated using a small molecule inhibitor such as a γ-secretase inhibitor which generates the notch induced cellular domain (NICD) activator in the notch pathway (Curry, Reed et al. 2005; van Es and Clevers 2005; van Es, van Gijn et al. 2005) or cyclopamine, a competitive inhibitor of SHH for the PTC receptor in the hedgehog pathway (Taipale, Chen et al. 2000). Induction of cancer stem cells out of their self-renewing mode may make then susceptible to conventional therapies as well allowing for development of new ones.

CONCLUSIONS

In summary, this thesis project tested some aspects of the hypothesis that pancreatic tumors or cancer cells lines contain 'rare' populations of cells that use stem-like cellular mechanisms for the progression of cancer and resistance.

The data presented here demonstrates very rare populations of cells within the Panc-1 cell line confer stem-like quiescence observed through label retention by a mechanism that still remains to be elucidated. Further results in this body of research suggest that Panc-1 cells retained a primordial stem cell marker (SP), and by this phenotype it was determined that this population could self-renew through symmetric divisions and could undergo transit amplification by asymmetric divisions. Next, it was confirmed that an invasive asymmetrically divided non-SP cell contained a recently identified cancer stem cell marker $CD24^{+}$. Additional experiments determined that anti-telomerase therapy could be used to target stem and non-stem cells for cancer therapy and the combination with nucleoside analogs improved drug response. Finally, a sensitive method was developed to view the initiation and progression of tumorigenesis *in vivo*.

The findings in this report identify a small population of cells that use stem-like cellular mechanisms for the progression of pancreatic cancer and resistance and provide insights into further investigations and therapeutic development.

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Vitae

Devmati Jaganath immigrated from a small village in Suva, Fiji Islands to San Mateo, California in 1971 where she permanently settled and gave birth to Vikash Jaganath Bhagwandin on the 30th day of October, 1972. Growing up in San Mateo, Vikash attended Aragon High School and in 1990 entered College of San Mateo where he earned A.S. degrees in Biology, Chemistry and Physics. In 1994 he transferred to the University of California at Santa Cruz and received a Bachelor of Arts degree in Biology with an emphasis on Biochemistry and Pharmacology. During the summers of his undergraduate career he completed five internships at Cell Genesys Inc., Tularik Inc., Genentech Inc., Dendreon Corporation, and Geron Corporation, Inc. After completion of his undergraduate degree he worked as a technician at UCSF under the supervision of Dr. George H. Caughey M.D. in the Cardiovascular Research Institute, Pulmonary Division where he published his work on secreted tryptic serine proteases. In 2002, Dr. Steven L. McKnight recruited him to the Ph.D. program in the Division of Cell and Molecular Biology at the University of Texas Southwestern Medical at Dallas. In 2003, he entered the Integrative Biology program and joined the lab of Dr. Jerry W. Shay and Dr. Woodring E. Wright for his doctoral research.

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