IDENTIFICATION AND CHARACTERIZATION OF NON SMALL CELL LUNG CANCER STEM CELLS

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Dedicated to

Brenda, Tim, Alex and Laura

# IDENTIFICATION AND CHARACTERIZATION OF NON SMALL CELL LUNG CANCER STEM CELLS

by

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The discovery of rare tumor cells with stem cell features first in myeloproliferative disease and later in solid tumors has emerged as an important area in cancer research. Through these studies the cancer stem cell model has emerged, which postulates that many tumors are initiated and progressed by a population of self-renewing malignant stem cells, referred to as cancer stem cells. This new tumor growth paradigm suggests that tumor metastasis and recurrence may be driven by a residual population of highly aggressive cancer stem cells. Furthermore this model argues that complete cancer remission may only be achieved by eradicating the malignant stem cell population charged as the source of tumor cell renewal.

Lung cancer is the most commonly lethal form of cancer in the world with about 90% of the nearly one million new cases succumbing to the disease. While progress is being made in understanding lung cancer pathogenesis and improving therapy, prognosis remains poor. One approach to improving outcome in lung cancer has been to therapeutically target a unique, phenotypically defined lung cancer stem cell population. However despite the relatively rapid pace of cancer stem cell research in solid tumors such as breast, brain and colon cancers, similar progress in lung cancer remains hampered in part due to an incomplete understanding of lung stem cell hierarchy and the complex heterogeneity of the disease.

To address this challenge, putative lung cancer stem cells were prospectively isolated from patient lung tumors and lung tumor cell lines using methods that have been reported to enrich for other stem cell populations in other cancers. As a result, a subpopulation of cells with elevated aldehyde dehydrogenase (ALDH) activity within many NSCLCs was identified with properties indicative of a cancer stem cell population including enhanced tumorigenicity in xenograft models, clonogenicity in culture and the capacity for self-renewal. In support of this, analysis of 282 clinically annotated non small cell lung cancer samples found elevated ALDH1A1 expression, the protein that drives ALDH in lung cancer, was associated with poor patient prognosis. Finally, molecular characterization of isolated $ALDH^+$ lung cancer cells revealed elevated expression of stem cell transcripts including Notch signaling transcripts, suggesting enhanced pathway activity. Suppression of Notch signaling through chemical inhibition or knockdown of the proto-oncogene *NOTCH3* resulted in a significant reduction in

clonogenic $ALDH^+$ cells indicating the importance of Notch signaling in lung cancer stem cell homeostasis and as a potential target for lung cancer stem cell directed therapy.

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LIST OF ABREVIATIONS

**95%CI** – 95% confidence interval

**AC** – adenocarcinoma

**ALDH** – aldehyde dehydrogenase

**AML –** acute myeloid leukemia

**BAAA** – BODIPY®-aminoacetaldehyde

**BAA** – BODIPY®-aminoacetate

**BADJ** – bronchioalveolar duct junction

**BASC** – bronchioalveolar stem cells

**BLI** – bioluminescent imaging

**CCSP** – Clara cell secretory protein

**CD** – cluster of differentiation

**CML** – chronic myeloid leukemia

**CSC** – cancer stem cell

**DAPT** – N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester

**DEAB** – diethylaminobenzaldehyde

**DMEM** – Dulbecco's modified Eagle's medium

**DMSO** – dimethylsulfoxide

**EGFR** – epidermal growth factor receptor

**EMT** – epithelial to mesenchymal transition

**FACS** – fluorescent activated cell sorting

**FBS** – fetal bovine serum

**GFP** – green fluorescent protein

**HBSS** – Hank's balanced salt solution

**HR** – hazard ratio

**IHC** - immunohistochemistry

**KRAS** – V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

**KSFM** – keratinocyte serum free medium

**NOD/SCID** – non-obese diabetic/severe combined immunodeficiency

**NSCLC** – non small cell lung cancer

**PBS** – phosphate buffered saline

**PCR** – polymerase chain reaction

**PI** – Propidium Iodide

**PNEC** – pulmonary neuroendocrine cells

**qPRC** – quantitative PCR

**RPMI** – Roswell Park Memorial Institute medium

**SCC** – squamous cell carcinoma

**SCLC** – small cell lung cancer

**shRNA** – short hairpin RNA

**SP** – side population

**SPC** – surfactant protein C

**TMA** – tissue micro array

# CHAPTER ONE

# THE ROLE OF STEM CELLS IN LUNG CANCER

## 1.1 Introduction to the Cancer Stem Cell Model

Cancer has been described as a "developmental disease," in which normal developmental pathways have been co-opted by oncogenic processes in cancer pathogenesis. In this context the development of a tumor is analogous to the development of an aberrant organ. During normal organogenesis, the self-renewal and differentiation of stem cells is carefully orchestrated to produce a functional organ with heterogeneous cellular phenotypes. In tumorigenesis the processes of self-renewal and differentiation become deregulated resulting in the production of hyper-proliferative, aberrantly differentiated cancerous tissue. In normal development and in cancer initiation, the stem cell processes of self-renewal and differentiation play a conspicuous role in the formation and homeostatic maintenance of a normal organ and a tumor. In adult tissues these processes are reserved only for a small population of resident organ stem and progenitor cells. However, where and how tumors have co-opted the capacity for self-renewal and differentiation has been a subject of ongoing intense debate.

The cancer stem cell hypothesis provides explanations for the origins of tumor self-renewal and heterogeneity (Clarke and Fuller, 2006; Reya et al., 2001; Wicha et al., 2006). One component of the cancer stem cell hypothesis is that cancers arise from stem cells that have acquired sufficient oncogenic mutations for transformation. Therefore, the

tumor cell of origin, also referred to as a tumor initiating cell, is believed to be a stem or progenitor cell that already is capable of self-renewal and differentiation.

### *1.1.1 The Cancer Stem Cell Model for Tumor Initiation*

The concept of stem cells as cancer initiating cells dates back almost as far as the discovery of somatic stem cells in the hematopoietic system. The hematopoietic system has served as the proving grounds for modern stem cell research, as much of what is known about general stem cell biology (and cancer stem cell biology) has come from 50 years of diligent investigation of human and murine hematopoietic systems. In the 1950s and 1960s, seminal studies in mice established fundamental concepts regarding the nature of the hematopoietic system and provided a foundation for later studies in human hematopoiesis. Principally these studies, which involved the transplantation of bone marrow cells into irradiated recipient mice, found that only a fraction of the total bone marrow cells were capable of reconstituting hematopoietic tissues, including the formation of multilineage cell colonies (containing myeloid and lymphoid cells) in the spleen (Ford et al., 1956; Till and Mc, 1961). Using radiation induced chromosomal markers to trace donor cell participation in hematopoiesis, it was found that each spleen colony was formed from a single donor cell and that these cells were capable of generating new colonies in secondary recipients, indicating their capacity for self-renewal (Becker et al., 1963; Siminovitch et al., 1963). This key property, together with the capacity for multilineage differentiation and extensive proliferation, suggested that these cells were enriched in hematopoietic stem cells.

While these early studies established the clonal nature of the hematopoietic system, later improvements in cell isolation techniques and functional assays enabled

researchers to resolve the hematopoietic stem cell hierarchy in greater detail. It is now well established that the human and mouse hematopoietic system is organized in a hierarchy that is ultimately sustained by a rare population of long-lived, pluripotent hematopoietic stem cells, capable of self-renewal (symmetric cell division, producing a duplicate stem cell) and differentiation (asymmetric cell division, producing a phenotypically different cell with limited replicative potential). These cells differentiate into a class of lineage restricted, multipotent hematopoietic stem cells (also referred to as progenitor cells) with limited self-renewal capacity. These cells in turn amplify and differentiate into vast numbers of fully mature, non-proliferating and short-lived blood cells. The hierarchical nature of the hematopoietic system provides for a lifetime of blood cell production without exhausting the regenerative pool of long-lived hematopoietic stem cells, as the bulk of the proliferation is performed by lineage restricted progenitor cells. Indeed similar stem cell hierarchies have also been confirmed in other adult mammalian organ systems, including the breast, brain, colon and lung (Gage, 2000; Leedham et al., 2005; Rawlins, 2008; Rawlins and Hogan, 2006; Villadsen et al., 2007).

A stem cell hierarchy has a number of advantages when it comes to maintaining proper organ development and tissue homeostasis. For example, by limiting replication to only a small fraction of bone marrow cells, hematopoiesis is centralized in stem cell niches and thus easier to regulate, whereas regulation of decentralized replication would be more challenging. Stem cells often reside in distinct regions of an organ and are surrounded by supportive niche cells which provide pro-survival, replication and

differentiation signals to nearby stem cells either through direct cell contact or via secreted factors.

A tightly controlled stem cell hierarchy may have evolved to protect against cancer development. The evolution of single celled organisms into longer-lived, multicellular organisms, comprised of many specialized cell types required the development of strict controls over cell differentiation and proliferation. If a cell were to escape or override these controls, the resulting hyperproliferation could kill the organisms or limit its Darwinian fitness. Therefore, long-lived, multicellular organisms have evolved many mechanisms to control cell proliferation and to protect against cancer. For a cancer to form, numerous oncogenic mutations must be accumulated within the same cell lineage in order to circumvent or override these protective mechanisms. For example, progression to cancer may arise through the loss of control over proliferation, leading to hyperplasia, or through the inactivation of failsafe mechanisms that compel normal cells to die or terminally differentiate (Fearon and Vogelstein, 1990; Hanahan and Weinberg, 2000). An organ stem cell hierarchy presents an additional check on developing cancer. In essence, the development of cancer boils down to statistics. For example, during the lifespan of a normal human body, trillions of cells are generated. If each of these cells possessed the capacity to replicate then there would be a reasonable chance that at least one cell would accumulate sufficient mutations that would lead to cancer formation at an early age. To reduce the probability of a proliferating cell turning cancerous, organisms have developed a strategy to limit the number of long-lived cells with self-renewal capacity. In addition, the limited self-renewal capacity of short-lived progenitor cells may also work to reduce the chance of a proliferating cell accumulating

sufficient mutations for oncogenic outgrowth (Clarke and Fuller, 2006). Thus, while stem cells provide an organism with a means of organized development and tissue rejuvenation, they also present a potent and corruptible cellular source for tumorigenesis.

While study of the hematopoietic system has provided important and fundamental insights into stem cell biology, the study of hematopoietic malignancies such as chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) have greatly contributed to the establishment of modern concepts of cancer development. In 1960, the study of CML patients revealed that nearly all replicating leukemia blasts possessed the same chromosomal abnormalities, indicating that all leukemia cells were derived from a common ancestor (the cancer initiating cell) with the same genetic manipulation (Baikie et al., 1960; Nowell and Hungerford, 1960). In addition to suggesting that CML is clonally derived, these studies also revealed that inherited genetic alteration was important to the pathogenesis of the disease and not simply correlative.

Hematopoietic disease models have also been the proving grounds for the establishment of the cancer stem cell model for tumor initiation and progression. The cancer stem cell model for tumor initiation suggests that cancer arises from the oncogenic outgrowth of transformed stem or progenitor cells. Again, this concept has been best demonstrated in myeloproliferative disorders where the selective oncogenic mutagenesis (for example, loss of the tumor suppressor gene *JunB* in mice) of primitive hematopoietic stem cells gives rise to CML blast crisis, whereas the same mutation in mature hematopoietic cell populations does not (Passegue et al., 2004). Similarly, the ectopic expression of some oncogenic fusion genes has been reported to enhance and/or reactivate hematopoietic stem cells and lineage committed progenitor cell self-renewal,

leading to leukemogenesis (Cozzio et al., 2003; Krivtsov et al., 2006). Importantly these studies revealed the potential for some protooncogenes to effectively dedifferentiate progenitor cells to a more primitive state that allowed for stem cell-like self renewal. In CML and in most types of AML, leukemogenesis is often caused by fusion proteins that act as aberrant transcriptional regulators, signaling proteins and/or disruptors of differentiation. It is now known that while these fusion proteins can stimulate hematopoietic stem cell and progenitor cell self-renewal, their effects on cell disease progression and self-renewal vary depending on the type of leukemia and stem cell.

In 2004, Huntly and colleagues compared the ability of two leukemia associated gene fusions, BCR-ABL and MOZ-TIF2, to transform hematopoietic stem cells, common myeloid progenitors, granulocyte-monocyte progenitors and bone marrow mononuclear cells (Huntly et al., 2004). Using retroviral delivery method to transfer the fusion genes into purified stem and progenitor cell populations, Huntly et al. used *in vitro* replating assays and transplantation of infected cells into lethally irradiated mice to functionally measure self-renewal and leukemogenesis. As a result, it was discovered MOZ-TIF2 and not BCR-ABL endows myeloid progenitors with the capacity for self-renewal and potential to generate AML. Interestingly BCR-ABL, was only capable of stimulating leukemogenesis in stem cells that already possessed the capacity for self-renewal, and the resulting leukemia was a CML. These data would suggest that while transformed stem and progenitor cells are the source of leukemogenesis, the oncogenic manipulations that stimulate or reactivate self-renewal have a profound effect of the progression of the disease (Huntly et al., 2004).

While the stem cell model for cancer initiation has taken root in hematopoietic malignancies, new evidence also suggest that many other cancer types arise from transformed adult stem and/or progenitor cells. Recently it was reported that tumor formation in astrocytoma mouse models lacking the tumor suppressors *p53*, *Nf1* and/or *Pten*, is initiated in the subventricular zone of the hippocampus and the lateral ventricals in the adult brain (Alcantara Llaguno et al., 2009). Importantly, these regions are known to facilitate neurogenesis and are enriched in adult neural stem cells and the loss of these tumor suppressors in neural stem/progenitor cells resulted in the stem/progenitor cell hyperproliferation, aberrant differentiation and tumorigenic outgrowth (Alcantara Llaguno et al., 2009; Gage, 2000).

In many cancers types, hyperplastic growth is first observed to occur in known or suspected regions of stem cell activity. The resultant tumors often express stem cell associated factors, many of which are functionally important for the survival of the tumor (Hu et al., 2008; Tai et al., 2005). These stem cell factors include the expression of human telomere reverse transcriptase gene (*hTERT*). As stem cells divide, small terminal portions of linear DNA are unable to be synthesized by the DNA replication machinery. This is known as the "End-Replication Problem" (Sfeir et al., 2005). As a result of the End-Replication Problem, the terminal ends of chromosomal DNA, known as telomeres, are made progressively shorter in newly replicated DNA. This erosion of the telomeres is compounded over serial cell divisions, eventually resulting in chromosomal instability, cell senescence and/or cell death. Stem cells have circumvented this limitation on long term proliferation through the expression and activation of telomerase (encoded in part by the *hTERT* gene), which adds telometric RNA to the 3' end of linear DNA (Harrington,

2004). In addition, many cancers also express *hTERT* and it has been proposed that up-regulation or re-expression of telomerase may be a critical for continuous tumor cell growth (Holt and Shay, 1999). While it is likely that in some tumors, telomerase activity was activated by oncogenic mutations, it is also likely that the acquisition of telomerase activity in many tumors is inherited from a transformed stem or progenitor cell (Shay and Wright, 2010).

Recent studies demonstrating the similarities between stem cells and cancer, as well as the functional studies linking stem cell aberration and carcinogenesis, have helped to define some cancers as a stem cell disease. The cancer stem cell model for tumor initiation has important ramifications for basic and translational cancer research, as well as for stem cell research. Thus, it can be expected that further characterization of the mechanisms that govern stem cell biology will no doubt contribute to a greater understanding of the events that lead to cancer.

### *1.1.2 The Cancer Stem Cell Model for Tumor Progression*

The second component of the cancer stem cell hypothesis is that tumor progression is driven by a subpopulation of self-renewing tumor cells. This view comes from the well documented observation that most tumors are comprised of functionally heterogeneous cell sub populations, including tumor cell populations that differ in their ability for limitless proliferative potential and repopulation ability *in vivo* and *in vitro*. The cancer stem cell model for tumor progression differs from other cancer models in how it explains the source and mechanism that drive tumor cell propagation and heterogeneity.

The clonal evolution model for tumor progression argues that mutant tumor cells with a selective growth advantage are the dominant cell populations that comprise the bulk tumor (Nowell, 1976). In this model, stochastic accumulation of genetic events, as well as factors such as tumor epigenetics and microenvironment, conspire to influence tumor cell heterogeneity. While these tumor paradigms are likely to exist in many cancers, the cancer stem cell model postulates that tumor cell heterogeneity is driven by an intrinsic tumor stem cell hierarchy (Figure 1.1) (Reya et al., 2001). However it is important to note that these models are not mutually exclusive, as tumor stem cells are also susceptible to the same selective pressures of a dynamic tumor environment.

Among the first experimental reports of a stem cell hierarchy in cancer came in 1997 when Bonnet and coworkers observed that only primitive $CD34^{+}CD38^{-}$ leukemia blasts isolated from mice with AML possessed the capacity to transfer the disease, whereas the majority of leukemic blasts could not propagate the disease in recipient mice (Bonnet and Dick, 1997). This capacity for sustained neoplastic growth in $CD34^{+}CD38^{-}$ leukemia blasts is due to the leukemia stem cell's ability to self-renew. This stem cell feature is considered the key discriminating difference between cancer stem cells and non cancer stem cells.

Since the discovery of cancer stem cells in human leukemia, a great amount of effort has been made to identify and characterize cancer stem cells in other cancer types. The first report of a cancer stem cell population in a solid tumor came in 2003 when Al-Hajj and colleges screened isolated breast tumor cells for their tumorigenicity in immunocompromised mice, based on their expression of a panel of tumor associated surface markers. This study found that many tumor cell populations existed in human

tumors based on their expression of surface markers, however only a small population of $CD44^{+}CD24^{-}$ tumor cells were capable of consistently and efficiently transplanting the disease in recipient immunocompromised mice (Al-Hajj et al., 2003). Later that year, brain cancer stem cells were reportedly identified in human medulloblastomas and gliomas. Similar to normal neural stem cells, brain cancer stem cells were enriched in spheroid colonies cultured in serum free, stem cell mitogen containing media, and also expressed the stem cell surface marker CD133. Furthermore, $CD133^{+}$ tumor cells (and not $CD133^{-}$ tumor cells) were observed to self-renew and differentiate, providing the cancer with a renewable source of heterogeneous tumor cells (Singh et al., 2003).

To date, human leukemia, breast, brain and colon cancer stem cells are among the best characterized cancer stem cell populations. This is due in large part to the relatively well documented normal stem cell populations in these tissue types. Because stem cells and cancer stem cells share many traits, it has been possible to identify and isolate cancer stem cells by their expression of stem cell factors or by growth selection methods that enrich for their normal stem cell counterparts. These identifying traits are often conserved in stem cells and cancer stem cells of various tissue types. For this reason, researchers have been able to utilize markers that select for normal or malignant stem cells in one tissue, for the identification of cancer stem cells in another tumor type (Table 1.1).

Analogous to their normal stem cell counterparts, most cancer stem cells are highly clonogenic, and compared to non-cancer stem cells, have a greater capacity for long term proliferation. Coupled with the capacity for self-renewal and differentiation, cancer stem cells are considered to be principle agents in tumor cell heterogeneity and

tumor progression (Figure 1.2). It is also hypothesized that migratory cancer stem cells may also be the source of distal tumor metastases in some cancers. For a tumor cell to produce a metastatic lesion, it must first detach from and survive outside of its niche, invade and navigate through neighboring tissues, and re-associate with and proliferate in a supportive microenvironment. To achieve this, tumor cells utilize a process important in organogenesis and tissue architecture known as epithelial to mesenchymal transition (EMT) and the reverse of this process, mesenchymal to epithelial transition (MET). During EMT, the epithelial features of a cell, such as the expression of the attachment molecule E-cadherin, are suppressed in favor of a more motile phenotype (Thiery et al., 2009). Recently it has been shown that signaling pathways important for stem cell maintenance and tumorigenesis are also active in the EMT process. In addition, tumor cells undergoing EMT have been found to be enriched in cancer stem cell-associated features, suggesting that the tumor stem cell plasticity facilitates the EMT and MET processes during metastasis (Brabletz et al., 2005). The connection between tumor "stemness" and EMT has become an increasingly researched topic and has provided evidence for and against the current articles of the cancer stem cell model (which will be reviewed in greater detail in Chapter Six).

The clinical implications of a tumorigenic hierarchy within a cancer become apparent when considering that therapies selected for their rapid reduction of tumor size are not selected for their discriminatory ability to treat tumor initiating cell subpopulations. Therefore if a therapy fails to eliminate all self-renewing cancer stem cells, residual surviving cancer stem cells are able to repopulate the disease, causing tumor relapse. This problem is compounded by the fact that some cancer stem cells, such

as $CD34^{+}CD38^{-}$ leukemia cancer stem cells for example, are relatively resistant to conventional chemotherapies and express drug effluxing pumps such as MDR-1 and ABCG2 (Costello et al., 2000; Misaghian et al., 2009; Wulf et al., 2001). In 2006, a study by Bao and colleagues found that $CD133^{+}$ glioma cancer stem cells persisted after radiation therapy, whereas the bulk of the tumor which was $CD133^{-}$ more readily succumbed to the treatment. In addition, Bao et al. found that glioma cancer stem cells achieved their radioresistance by preferentially activating DNA damage checkpoints, thereby allowing for the cell to stall cell death signals and repair damaged DNA (Bao et al., 2006).

Because of these therapy resistant traits, the development of novel targeted therapies is needed to more effectively eliminate cancer stem cells, thereby removing a prime source of tumor heterogeneity, progression, metastasis and tumor recurrence after therapy. However, targeting cancer stem cells remains a relatively new and underdeveloped field. The prevailing strategy for the development of new cancer stem cell therapies has been to identify targetable signaling factors that are not only preferentially activated in, but functionally important for, the survival and expansion of cancer stem cell populations. By these criteria, the signaling pathways that govern cancer stem cell self-renewal have come under increasing study for their potential as a cancer stem cell Achilles' heel (Misaghian et al., 2009; Sullivan et al., 2010).

For the most aggressive and lethal forms of cancer, the identification and characterization of cancer stem cells in these malignancies may provide the best leads for the development of new and more effective therapeutics. Although lung cancer is among the most commonly lethal forms of cancer in the world, comparatively less is known

about the biology of lung cancer stem cells compared to other solid tumor stem cells (Minna et al., 2002). Lung cancer remains the most lethal form of cancer in both men and women in the United States, and improvements in standard chemotherapy have been mostly palliative with a one year survival of only 35% (Jemal et al., 2009; Sun et al., 2007). Consequently, there is a pressing need for the development of new therapeutic agents that better manage the progression of highly aggressive lung cancer cells. However, the methods to identify and isolate self-renewing lung cancer stem cells are still being developed and the exact marker identity of human lung cancer stem cells remains controversial. This is due in part to the complexity of the disease in terms of its phenotypically diverse and regionally distinct types of neoplasia. Lung cancer is comprised four major histological types: small cell lung cancer (SCLC) and three types of non-small cell lung cancer (NSCLC) including squamous cell carcinoma (SCC), adenocarcinoma of the lung (AC), and large cell carcinoma (LC). In humans, SCLC and lung SCC occur in the proximal region of the respiratory tract whereas AC of the lung is distally located. The histological and regional diversity found in lung cancer may partly be due to the presence of diverse pools of self-renewing stem cells in the adult lung epithelium.

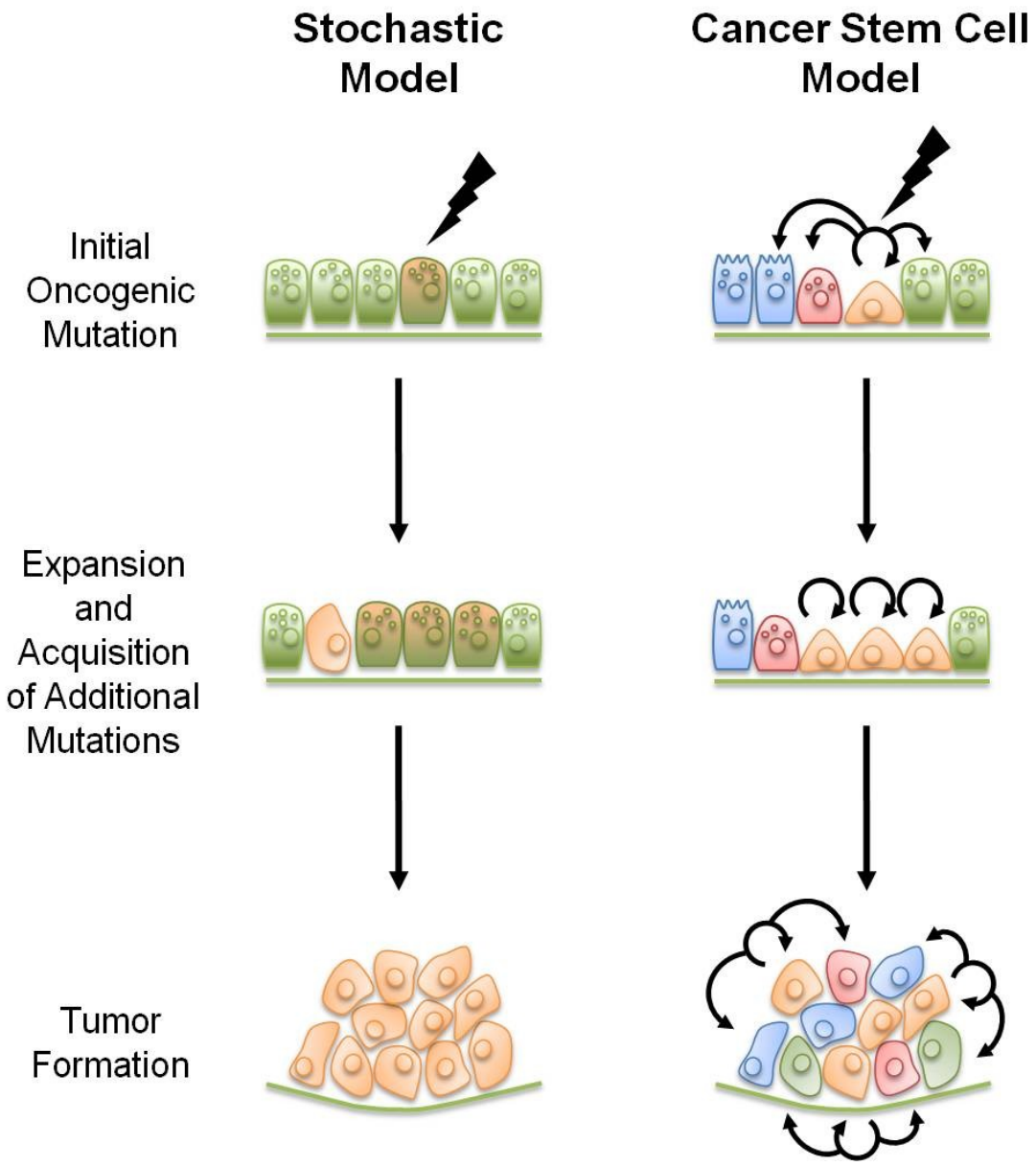



**Figure 1.1: The stochastic and stem cell models for tumorigenesis.** A simplified diagram of the stochastic and cancer stem cell models for tumor initiation and progression. In the stochastic model diagram, a differentiated cell (green) acquires an oncogenic mutation (jagged arrow) and begins to proliferate (represented by color change) into a hyperplasia. Over time, additional mutations are acquired and cells with growth advantages (orange cells) are selected to form what will eventually become a tumor. The cancer stem cell model suggests that the tumor initiating cell is a stem cell and that a stem cell hierarchy persists during cancer progression. In the cancer stem cell model diagram, a stem cell (orange), capable of self-renewal (circular arrow) and differentiation (directional arrow) acquires an oncogenic mutation that alters these stem cell functions. Expansion of the stem cell compartment is associated with an increase in self-renewal. The resulting tumor is driven by a population of cancer stem cells.

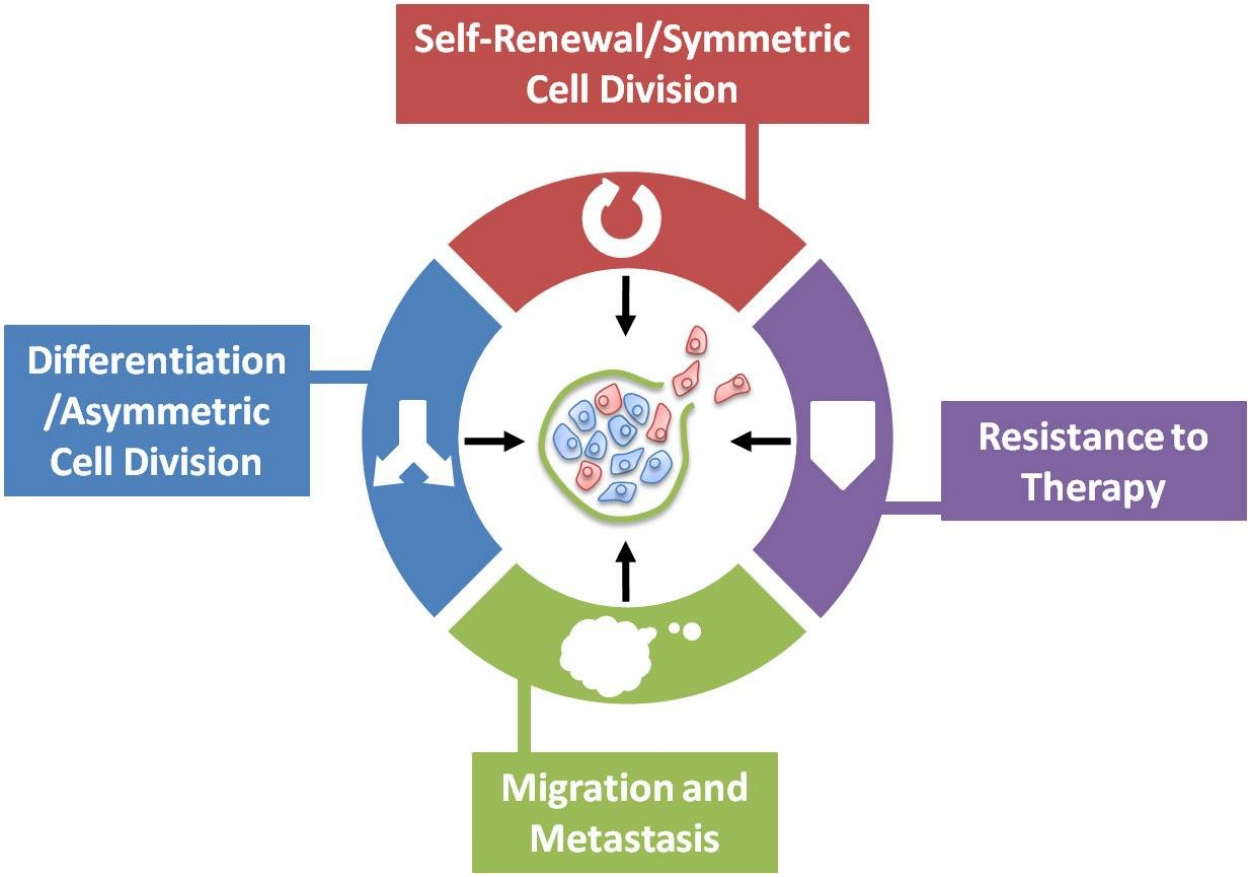



**Figure 1.2: Hallmarks of cancer stem cells.** A diagram illustrating the principle properties of a stem cell population in cancer.

| Cancer Stem Cell Type | Surface Marker Phenotype | Other Cell Phenotypes | References |
|---|---|---|---|
| AML | $CD34^+CD38^-$, $CD34^+CD71^-HLA\text{-}$, $CD90^-$, $CD117^-$, $IL\text{-}3Ra^+$ | Hoechst 33342 exclusion, $ALDH^+$ | Bonnet et al., 1997; Blair et al., 1997; Blair et al., 1998; Blair et al., 2000; Jordan et al., 2000; Feuring-Buske et al., 2001; Pearce et al., 2005 |
| Glioblastoma | $CD133^+$ | Hoechst 33342 exclusion, Sphere colony, $ALDH^+$ | Singh et al., 2003; Patrawala et al., 2005 |
| Medulloblastoma | $CD133^+$ | Sphere colony | Singh et al., 2003 |
| Pilocytic Astrocytoma | $CD133^+$ | Sphere colony | Singh et al., 2003 |
| Breast Cancer | $CD44^+CD24^-$ | Hoechst 33342 exclusion, Sphere colony, $ALDH^+$ | Al-Hajj et al., 2003; Patrawala et al., 2005; Ponti et al., 2005; Ginestier et al., 2007 |
| Prostate Cancer | $CD44^+$, $CD133^+$, $CD44^+\alpha_v\beta_3{}^{high}CD133^+$ | Hoechst 33342 exclusion, Sphere colony | Patrawala et al., 2005; Collins et al., 2005; Patrawala et al., 2006 |
| Ovarian Cancer | $CD44^+CD117^+$ | Hoechst 33342 exclusion, Sphere colony, $ALDH^+$ | Szotek et al., 2006; Zhang et al., 2008; Deng et al., 2010 |
| Endometrial Cancer | $CD133^+$ | | Rutella et al., 2009 |
| Colon Cancer | $CD133^+$, $CD44^+$, $CD166^+$ | Hoechst 33342 exclusion, Sphere colony, $ALDH^+$ | O'brien et al., 2007; Ricci-Vitiani et al., 2007; Huang et al., 2009 |
| Pancreatic Cancer | $CD44^+CD24^+ESA^+$, $CD133^+$ | Hoechst 33342 exclusion, Sphere colony, $ALDH^+$ | Li et al., 2007 |
| Hepatocellular Cancer | $CD133^+$ | | Ma et al., 2007 |
| Head and Neck SSC | $CD44^+$ | $ALDH^+$ | Prince et al., 2007; Clay et al., 2010 |
| Melanoma | $CD20^+$, $CD44^+$, $CD133^+$ , $ABDG5^+$ | | Fang et al., 2005; Schatton et al., 2008 |
| Bone Sarcomas | $CD44^+$, $Stro\text{-}1^+$, $CD105^+$ | $ALDH^+$ | Suvá et al., 2009; Wang et al., 2010 |

**Table 1.1: Cancer stem cell identities in human cancers.** Cancer stem cell marker identities from various tumor types, not including lung cancer, are represented in the table above. Surface markers used to identify and isolate cancer stem cells were detected by flow cytometry and cell populations were isolated by FACS. Alternative methods for identifying and isolating cancer stem cells listed in the above table include: the isolation of fluorescently dim, Hoechst 33342 effluxing cells, cells grown as sphere colonies in serum free, stem cell mitogen containing media, and cells with elevated aldehyde dehydrogenase activity (ALDH).

## 1.2 Sites of Self-Renewal in Lung Epithelium

The lung epithelium consists of a large variety of morphologically and functionally different cell types, whose roles include facilitating gas exchange, balancing fluids in the lung, detoxifying and clearing foreign agents, and the activation of inflammation due to injury (Knight and Holgate, 2003; Mercer et al., 2006). The varieties of lung epithelial cell lineages are organized along the pulmonary tree to facilitate the specialized role in each region of the lung. The proximal and airway of the respiratory tract includes the pharynx, larynx, trachea and bronchi. The tracheal airway epithelium consists primarily of ciliated columnar and mucus-secreting goblet cells that work to lubricate the surface of the proximal airway epithelium and to trap and clear foreign particulates. As the pulmonary tract of the lung branches into the smaller bronchioles and alveoli of the distal airway, the columnar epithelial cells transition into a more cuboidal morphology. Here the lung epithelium is lined with Clara cells which serve to protect and detoxify the bronchiolar epithelium. The most distal regions of the lung contain the terminal bronchioles and alveoli where gas exchange from the alveolar sacs into capillary beds occurs. The alveoli consist of type 1 and type 2 pneumocytes. The flat morphology of type 1 cells aids in diffusion of gas into the adjacent capillary beds. Type 2 pneumocytes are more cuboidal, and secrete surfactant proteins that serve to regulate lung fluid balance and provide elasticity to the lung epithelium (Knight and Holgate, 2003).

Unlike tissues with rapid cell turnover such as the blood, gastrointestinal tract and skin, turnover of the lung epithelium is less frequent, occurring every 30 to 50 days (Bowden, 1983; Kauffman, 1980; Rawlins and Hogan, 2008). However during injury,

cell turnover is accelerated in distinct regions in the lung where it is believed self-renewing stem cells reside (Giangreco et al., 2009). Although the existence of regional stem cell populations in adult lung epithelium has long been accepted, controversy remains as to the exact cellular identity and the capacity for self-renewal and lineage differentiation of some putative lung stem cells (Rawlins and Hogan, 2006).

In the epithelium of the trachea and bronchi, cell turnover is thought to be driven by a class of lung stem cells known as basal cells. Unlike the more differentiated columnar cells of the proximal airway, basal cells reside along the basement membrane and do not extend to the apical surface of the epithelium (Evans et al., 2001). The deposition of basal cells in rodents is primarily restricted to the trachea, whereas in human lungs, basal cells are ubiquitous in human conducting-airway epithelium (Boers et al., 1998; Schoch et al., 2004). Basal cells can be discriminated by their expression of cytokeratins 5 and 14 (*KRT5*/*KRT14*), as well as their expression of the transcription factor *Trp-63* (p63) (Nakajima et al., 1998).

The first clear evidence for the role of basal cells in the steady state maintenance of airway epithelium came from pulse-chase experiments using tritiated thymidine ($^{3}$H-TdR), where it was found that basal cells and Clara cells could divide and give rise to ciliated cell progeny (Bowden, 1983; Breuer et al., 1990; Donnelly et al., 1982; Kauffman, 1980). However these studies did not differentiate between the roles of these two progenitor populations in renewing ciliated airway epithelium. Tracing discrete airway cell types has been aided by the improvement and utilization of transgenic lineage reporter mice and cell sorting technology (Rawlins, 2008). Using KRT14-creER mice, it was found that after naphthalene ablation of both ciliated cells and Clara cells, basal cells

became activated, expanded, and subsequently differentiated into secretory and ciliated cells (Hong et al., 2004a, b). The regenerative properties of basal cells was also confirmed when isolated basal cells from rat and human trachea were found to repopulate a fully differentiated, pseudostratified airway epithelium *in vitro* as well as in denuded rat tracheas (Hajj et al., 2007; Randell et al., 1991). These findings suggest that basal cells are enriched multi-potent progenitor cells, capable of airway epithelial renewal in steady state maintenance and lung injury (Figure 1.3A). In support of this, mouse KRT5-GFP$^+$ and human p63$^+$ basal cells possess the ability to self-renew and well as differentiate *in vitro* (Rock et al., 2009). Therefore, the discovery that basal cells possess the capacity for multi-potent differentiation as well as self-renewal suggests these cells may be more "stem-like" than "progenitor-like." According to the cancer stem cell model for tumor initiation, basal cells may be a candidate population for the tumor initiating cells. The expansion of KRT14 expressing basal cells during tracheal hyperplasia in squamous cell carcinoma support this model; however, more stringent lineage tracing experiments will be needed to confirm the connection between basal stem cells and tumor initiation (Figure 1.1B) (Barth et al., 2000).

Clara cells, named after Max Clara the researcher who made their discovery, are domed-shaped secretary cells that are found throughout the airway epithelium, but are more concentrated in the bronchioles (Boers et al., 1999). Clara cells are discriminated in rodent lung epithelia by their expression of the secretoglobin, *scgb1a1*, also known as CCSP or CC10 (Nakajima et al., 1998). Clara cells also possess multi-potent capacity for differentiation; however it is believed that most Clara cells do not possess the ability to self-renew. An exception to the rule has been found in a subpopulation of naphthalene

resistant Clara cells, referred to as variant Clara cells. Variant Clara cells lack the expression of the cytochrome P450 2F2 isozyme (CYP2F2) that is responsible for the generation of toxic metabolites of naphthalene (Buckpitt et al., 1995). Both variant Clara cells and nearby pulmonary neuroendocrine cells (PNECs) in the bifurcation zone of the bronchioles have been shown to proliferate and participate in rodent airway epithelium renewal after naphthalene ablation of the airway epithelium (Reynolds et al., 2000a; Reynolds et al., 2000b; Stevens et al., 1997; Stripp et al., 1995). However when airway epithelial regeneration was measured after the selective ablation of variant Clara cells in mice lungs, PNEC expansion failed to reconstitute the full component of stratified lung epithelia (Hong et al., 2001). This indicates that while variant Clara cells and PNECs possess the ability to expand and self-renew, only variant Clara cells have the capacity for multi-potent differentiation (Figure 1.3A), however this observation has not been confirmed in human lung epithelia.

SCLC is a particularly aggressive and highly metastatic form of lung cancer, accounting for approximately 15% of total lung cancer cases (Simon et al., 2004). Furthermore, SCLC is generally intractable to therapy after relapse, which attributes to its dismal prognosis (Turrisi and Sherman, 2002). Similar to PNECs, SCLC exhibit primitive neuroendocrine features, such as the expression of calcitonin gene-related peptide, and commonly develop in the midlevel bronchioles. For these reasons, it is hypothesized that malignant self-renewing PNECs may be the origin of SCLC (Figure 1.3B) (Giangreco et al., 2007; Watkins et al., 2003).

In the most distal region of the lung, the terminal bronchioles transition into sac-like alveoli. The alveolar epithelium is comprised of a thin layer of flattened type I

pneumocytes and cuboidal type II pneumocytes. Type I pneumocytes are terminally differentiated cells that facilitate gas exchange between the alveoli and the adjacent capillary bed. Interspersed among the type I pneumocytes are the cuboidal, type II pneumocytes whole roles include the secretion of surfactants (such as surfactant protein C or SPC) that modulate surface tensions. Early pulse-chase experiments using $^{3}$H-TdR in injured rodent lungs revealed type II pneumocytes may also have to capacity to differentiate into type I pneumocytes (Adamson and Bowden, 1975; Evans et al., 1975). Further investigation of isolated type II pneumocytes from injured rat lungs revealed the existence of up to four phenotypically different type II pneumocytes populations, each expressing differing markers and telomerase activity (Buckley et al., 1998; Reddy et al., 2004). This suggests that only some type II pneumocytes are progenitor cells with uni-potency, although the exact marker identity of these cells remains to be elucidated.

Another class of lung stem cells was recently discovered in the putative stem cell niche of the bronchioalveolar duct junction (BADJ) of mice. After naphthalene-induced alveolar cell ablation, pollutant resistant, BADJ associated cells expressing the Clara cell marker CCSP were observed to expand and regenerate the alveoli. Unlike the variant Clara stem cells, these BADJ associated Clara-like cells were not in proximity to the PNEC stem cell niche (Giangreco et al., 2002). In addition, these cells also expressed the type II pneumocyte marker SPC and the pan-murine stem cell marker Sca-1. The capacity for bronchioalveolar epithelium regeneration (indicating self-renewal and differentiation capacity), as well as the unique spatial location and expression of stem cell markers, suggested that these CCSP$^{+}$CC10$^{+}$Sca-1$^{+}$ BADJ cells are a new pedigree of lung stem cell, termed bronchioalveolar stem cells (BASCs) (Figure 1.3A) (Kim et al., 2005).

In the same study that identified BASC in mice, it was also discovered that BASC outgrowth driven by the *K-ras* oncogene contributed to the formation of atypical adenomous hyperplasia, a precursor lesion for adenocarcinoma of the lung (Jackson et al., 2001; Kim et al., 2005). Bronchiolar adenocarcinomas and progressed bronchioalveolar cell carcinomas of the peripheral airways are among the most common types of lung cancer in the world presently (Minna et al., 2002). In murine models of lung adenocarcinomas, tumors arise from the BADJ and typically exhibit alveolar differentiation (Fisher et al., 2001; Politi et al., 2006). Taken together, these studies would suggest that the tumor initiating cell of murine adeno- and bronchioalveolar carcinomas are self-renewing BASCs, however this remains somewhat controversial and has yet to be determined in human lungs (Giangreco et al., 2007).

### 1.3 Stem Cells in Lung Cancer

According to the cancer stem cell hypothesis, a stem cell hierarchy exists within a tumor and only a sub-population of stem-like cancer cells possess the ability to self-renew, differentiate and proliferate indefinitely. Often this function is measured in xenotransplantation studies, where putative cancer stem cell populations are assayed for the capacity to transplant secondary and tertiary tumors that reproduce the heterogeneity of the primary disease. A more stringent derivation of this assay has been the serial xenotransplantation of limiting cell dilutions. Considered the "gold standard" for tumorigenicity and tumor "stemness", as few as one to 10,000 prospective cancer stem cells are injected (either orthotopically or paratopically) into recipient

immunocompromised mice and the incidence of secondary tumor formation is evaluated (Clarke, 2005; Clarke et al., 2006).

In a recent study performed by the group that discovered murine BASCs, the serial transplant assay was employed to determine if the same cell type that initiated adenomas (the precursor to adenocarcinomas) in mice, possessed the cancer stem cell ability to transplant the disease in recipient mice. The authors chose to investigate the tumor propagating capacity of cells that expressed BASC markers in three oncogenetic models of murine lung adenocarcinoma: mutant *K-ras*, mutant *K-ras* with a *p53* deficiency (*K-ras*;*p53*-flox), and mutant EGFR-driven lung adenocarcinomas. Remarkably each of these tumor oncogenotypes produced adenocarcinomas containing similar frequencies of $CD45^{-}CD34^{-}Sca\text{-}1^{+}$ BASC-like cells; however the tumorigenicity of these isolated cells differed greatly between the three lung tumor oncogenotypes. In adenocarcinomas driven by mutant *K-ras* alone, Sca-1 expression was not associated with tumor transplant activity, whereas in *K-ras*;*p53*-flox adenocarcinomas, Sca-1 expression was associated with a greater than 5-fold enrichment in tumorigenicity. Importantly, *K-ras*;*p53*-flox $Sca\text{-}1^{+}$ tumor cells fit the profile of a population enriched in lung cancer stem cell as they were found to form robust tumors in secondary and tertiary mice from as few as 100 cells. Conversely, the few secondary tumors that formed from *K-ras*;*p53*-flox $Sca\text{-}1^{-}$ cells were relatively small, had no detectable $Sca\text{-}1^{+}$ cells, and could not generate tertiary tumors. In striking contrast, the selection of $Sca\text{-}1^{-}$ tumor cells from EGFR driven lung adenocarcinomas was found to greatly enrich for cancer stem cell activity. Taken together these findings suggest that while lung cancer stem cells can be identified using markers that are expressed on the likely tumor initiating cell, the

oncogenotype that drives tumorigenesis can have a deciding effect on the marker identity and biology of cancer stem cells (Curtis et al., 2010; Sullivan and Minna, 2010).

The presence of a stem-like clonogenic subpopulation in human lung cancer was demonstrated almost 30 years ago, when Carney and colleagues observed that only a very small proportion (<1.5%) of SCLC and lung adenocarcinoma cells from patient samples could generate colonies in soft agar (Carney et al., 1982; Carney et al., 1980). When inoculated into athymic nude mice, these soft agar colonies generated SCLCs and lung adenocarcinomas similar to their primary lesions, suggesting that these rare, clonogenic tumor cells were enriched with lung cancer stem cells (Carney et al., 1982; Gazdar et al., 1981). Since then, researchers have attempted to prospectively isolate human lung cancer stem cells using markers that discriminate between intra-tumoral cell heterogeneity. This strategy has proven successful in several solid tumor types, such as breast, brain and colon cancers where cancer stem cells can be identified and isolated via FACS by their expression of stem cell specific cell surface markers (Visvader and Lindeman, 2008). In lieu of adequate and selectable markers for human lung stem cells, researchers have also turned to other FACS based stem cell isolation techniques to identify and isolate lung cancer stem cells.

The Side Population assay, first described by Goodell and colleagues to select for human hematopoietic stem cells (Goodell et al., 1996), relies on the ability of ABC transporters expressed in stem cell populations (Zhou et al., 2001), to efflux the fluorescent Hoechst 33342 dye. Hoechst 33342 dye excluding cells, termed Side Population cells (SP cells), have been described in a variety of tumor types as being enriched in stem-like properties (Hirschmann-Jax et al., 2004). In acute myeloid

leukemia, neuroblastoma, glioma, and ovarian cancer, isolated SP cells transplanted into recipient immunodeprived mice display a significantly greater capacity for tumorigenic growth than bulk non-SP cells, implying that SP cells are enriched in cancer stem cells (Feuring-Buske and Hogge, 2001; Kondo et al., 2004; Patrawala et al., 2005; Szotek et al., 2006; Wulf et al., 2001). In human lung cancer as few as 1,000 isolated SP cells from lung cancer cell lines produce robust xenografts in mice, whereas non-SP cells failed to generate tumors with similar numbers of SP cells (Ho et al., 2007). SP cells were also found to self-renew and express elevated levels of hTERT, compared to bulk non-SP cells (Ho et al., 2007).

While these reports support the notion that the Side Population assay selects for cancer stem cells in lung tumors, several criticisms have been raised regarding the use of Hoechst 33342 dye to isolate stem-like population cells. For example, because Hoechst 33342 is a DNA binding dye, cells that are unable to efflux the dye may suffer from its cytotoxic effects. In the breast cancer cell line MCF7 and ovarian cancer cell line SK-OV3, treatment with Hoechst 33342 dye resulted in a decrease in the ability of these cell lines to form clones, suggesting the cytotoxicity of Hoechst 33342 dye could be a biasing variable in the Side Population assay (Zhong et al., 2007). Furthermore, because of the assay's sensitivity to experimental variables such as incubation time, dye concentration, cell concentrations, and gating variability, SP phenotypes often vary between experiments (Montanaro et al., 2004; Platet et al., 2007). These observations throw into question the nature of lung cancer SP cells and suggest that further stringent experimentation is needed to determine the ability of this assay to isolate cancer stem cell populations (Wu and Alman, 2008).

Another common strategy for isolating human cancer stem cell populations in solid tumors has been the use of flow cytometry to sort tumor cells for the extracellular portions of surface stem cell markers. CD133 (*PROM1*) is a cell surface glycoprotein that consists of 5 transmembrane domains and 2 large glycosylated extracellular loops (Mizrak et al., 2008). CD133 and its glycosylated epitope, AC133, have been useful markers in the selection of both human hematopoietic stem cells and neural stem cells (Uchida et al., 2000; Yin et al., 1997). Similarly, expression of CD133 and AC133 has been reported to select for brain (Singh et al., 2004), colon (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), pancreatic (Li et al., 2007) and most recently, lung cancer stem cells (Eramo et al., 2008).

In 2008, Eramo and colleagues identified rare, highly tumorigenic, self-renewing $CD133^+$ cells in both NSCLC and SCLC specimens (Eramo et al., 2008). Similar to other cancer stem cell studies, some of these patient lung tumor derived $CD133^+$ cells could be expanded *in vitro* as floating tumor spheres cultured in defined serum free media (Eramo et al., 2008; Ghods et al., 2007; Lee et al., 2006; Ponti et al., 2005). These $CD133^+$ enriched spheres were refractory to short term chemotherapy, suggesting that putative $CD133^+$ lung cancer stem cells are resistant to conventional chemotherapy. This drug resistant feature of $CD133^+$ lung cancer cells was confirmed in experiments that revealed long term chemotherapy *in vitro* as well as *in vivo* could enrich for $CD133^+$ lung cancer cells (Bertolini et al., 2009; Levina et al., 2008).

The discovery of putative $CD133^+$ lung cancer stem cells in both SCLC and NSCLC indicate that CD133 may serve as a pan-lung cancer stem cell marker. However, several lines of evidence suggest that the ability of CD133 expression to discriminate

lung cancer stem cells may have been overstated. For example, in a limited study using lung cancer cell lines, Meng et al. demonstrated that isolated $CD133^-$ lung cancer cells also possess the ability to self-renew and generate robust xenografts outgrowth (Meng et al., 2009b). Furthermore, unlike gliomas where CD133 is a more established cancer stem cell marker, recent studies (including our own as described in Chaper Five) have determined that CD133 expression in lung cancer is not associated with patient prognosis (Howard and Boockvar, 2008; Salnikov et al., 2009; Tirino et al., 2009). While not necessarily a requisite characteristic of a cancer stem cell marker, the fact that CD133 is not associated with overall survival or patient prognosis suggest that CD133 is not indicative of an aggressive cell phenotype in all or most cases of lung cancer. In fact, in many lung cancer samples, CD133 is not detected, suggesting that if CD133 is a lung cancer stem cell marker it is only relevant to a subset of lung cancers (Bertolini et al., 2009; Salnikov et al., 2009; Tirino et al., 2009).

Recently some scientists have questioned the use of CD133 as a selective cancer stem cell marker in other solid tumor types, citing cases where CD133 negative cells also possess the capacity for self-renewal and cancer initiation (Shmelkov et al., 2008; Wang et al., 2008). Indeed our own investigation of CD133, described in Chapters Five and Six, has yielded little evidence to support this molecule as a lung cancer stem cell marker in most NSCLC cases. Furthermore, it has been suggested that the existence of variable CD133 isoforms, extrinsic environmental pressures on CD133 expression and the states of CD133 glycosylation, complicate the use of CD133 and AC133 as a pan-cancer stem cell marker (Bidlingmaier et al., 2008; Mizrak et al., 2008). Therefore, the utility of CD133 as a cancer stem cell marker in lung cancer remains controversial.

Another method for identifying and selecting stem cell populations is based on aldehyde dehydrogenase activity. Aldehyde dehydrogenase (ALDH) enzymes are a family of intracellular enzymes that participate in cellular detoxification, differentiation and drug resistance through the oxidation of cellular aldehydes (Moreb et al., 1996). In hematopoietic stem cells, ALDH activity is thought to preserve an undifferentiated cellular state by interfering with endogenous retinoic acid biosynthesis (Chute et al., 2005; Chute et al., 2006). Using flow cytometry to detect and isolate cells with elevated ALDH activity, hematopoietic stem cells and their leukemic counterparts have been identified in both human and murine samples (Cheung et al., 2007; Pearce and Bonnet, 2007; Pearce et al., 2005). ALDH activity has also been useful in isolating putative human brain (Bar et al., 2007), breast (Ginestier et al., 2007), colon (Huang et al., 2009a), and head and neck squamous cancer stem cell populations (Chen et al., 2009).

Evidence for ALDH as a relevant lung cancer stem cell marker was published first in 2008 with the discovery of elevated ALDH protein expression in putative lung stem cell niches during malignant transformation (Patel et al., 2008). Specifically in the Patel study, basal cell expression of ALDH1A1 and ALDH3A1 was observed to increase during disease progression in hyperplasic and malignant tissues, suggesting that basal stem cell populations that express ALDH1A1, ALDH3A1 or both, are expanded during oncogenensis.

In this study, the utility of ALDH activity and protein expression as prospective markers for lung cancer stem cells was examined. By isolating and characterizing the *in vivo* and *in vitro* growth properties of $ALDH^+$ and $ALDH^-$ lung cancer cells from tumors cell lines, it was found that $ALDH^+$ lung cancer cells are enriched for stem-like lung

cancer cells. Specifically, $ALDH^+$ cells were observed to be highly tumorigenic in mice, clonogenic in culture and capable of self-renewal and differentiation. In addition, analysis of 282 NSCLC samples (detailed in Chapter Four) revealed a significant association between elevated ALDH1A1 expression and reduced overall patient survival. Recently a similar study investigating ALDH in two lung cancer cell lines found ALDH activity to enrich for highly tumorigenic, $CD133^+$ cancer cells (Jiang et al., 2009b). Additionally, through a limited study of stage I lung adenocarcinoma, high levels of ALDH1 protein expression was correlated with poor patient prognosis (Jiang et al., 2009b). As described in later chapters, these studied provide compelling evidence for ALDH as a marker for a stem cell population in lung cancer.

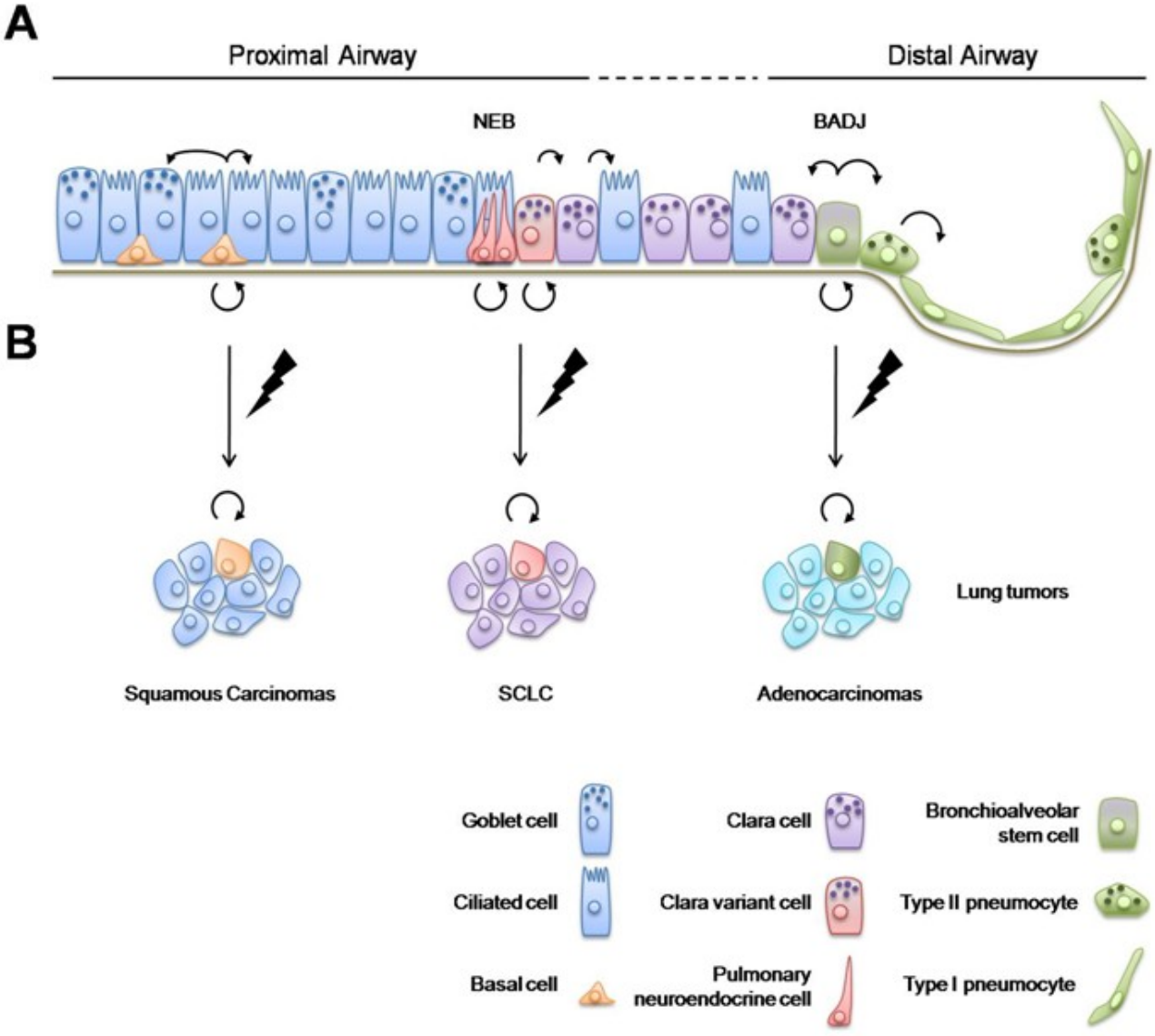



**Figure 1.3: Sites of self-renewal and tumor initiation in lung epithelia.** Basal cells of the proximal airway, pulmonary neuroendocrine cells (PNECs) which colonize as neuroendocrine bodies (NEBs), naphthalene resistant variant Clara cells, and bronchioalveolar stem cells of the bronchioalveolar duct junction (BADJ), are all proposed to be self-renewing stem cell populations in the lung epithelium (A). The accumulation of oncogenic mutations (represented by jagged arrows) in different stem cell compartments of the lung are thought to give rise to histologically different tumor types in the lung. Within each of these different lung tumor types, a distinct sub-population of self-renewing tumor cell (shaded tumor cells with circular arrows above) is hypothesized to populate and progress the disease (B).

## 1.4 Molecular Pathways of Self-Renewal in Lung Cancer

### *1.4.1 Wnt Pathway*

Stem cell self-renewal is a tightly controlled process that is governed by both signals from the stem cell niche as well as deliberate and regulated control of key developmental pathways such as the Wnt, Hedgehog and Notch signaling pathways (Figure 1.4). Tumor stem cells also undergo self-renewal, however unlike self-renewal in organogenesis, self-renewal in tumorigenesis is also thought to be achieved in part by the deregulation of these key pathways. Cancer stem cells comprise the self-renewing component of tumors; therefore it is hypothesized that the same pathways that govern normal stem cell self-renewal could also govern cancer stem cell self-renewal (Reya et al., 2001). For this reason, the prospect of targeting these developmental pathways in tumors has become an appealing strategy for treating tumors that are often intractable to conventional therapy alone (Al-Hajj and Clarke, 2004; Wicha et al., 2006). The Wnt/β-catenin pathway plays an important role in the regulation of hematopoietic stem cell self-renewal (Kirstetter et al., 2006; Reya et al., 2003), however the role of Wnt signaling in lung epithelial stem cells is less well understood (Stripp and Reynolds, 2008). Recently, it was discovered that activated Wnt/β-catenin signaling in the developing lung coincided with an expansion of BASCs and attenuated bronchiolar differentiation (Reynolds et al., 2008). Conversely, conditional Cre-mediated deletion of *Catnb* had no appreciable effect on the repair and maintenance of the bronchiolar epithelium, suggesting the role of Wnt/β-catenin signaling in lung stem cell self-renewal may be niche specific (Zemke et al., 2009). In lung cancers, evidence of activated Wnt signaling in lung tumors suggests

aberrant Wnt signaling may be important for tumorigenesis (Lemjabbar-Alaoui et al., 2006; Uematsu et al., 2003a; Uematsu et al., 2003b). Recently, inhibition of Wnt signaling by a Wnt-2 monoclonal antibody resulted in the induction of apoptosis in NSCLC cells (You et al., 2004b), as well as in other tumor types (Shi et al., 2007; You et al., 2004a). The prospect of Wnt signaling as a driver of lung tumorigenesis and stem cell self renewal make the Wnt signaling pathway an appealing target for therapy, however further studies will be necessary to define the context of activated Wnt signaling in lung cancer stem cells, as well as in different types of lung cancer (Daniel et al., 2006; Reya and Clevers, 2005).

### *1.4.2 Hedgehog Pathway*

The Hedgehog (Hh) signaling pathway is activated when one of three extracellular Hh ligands (in mammals there are three: sonic hedgehog (SHH), desert hedgehog (DHH), and Indian hedgehog (IHH) ligands) binds to and inactivates its receptor patched (PTCH) (Figure 1.4). This binding event relieves the repressive function of PTCH on the downstream protein smoothened (SMO), allowing SMO to activate downstream targets through the GLI transcriptional effectors. The Hh signaling pathway is a key developmental pathway required for proper embryogenesis (Litingtung et al., 1998). In the developing lungs, activated Hh signaling is involved in pulmonary cell fate determination and branching morphogenesis (Bellusci et al., 1997; Pepicelli et al., 1998). Aberrations in expression and activation of this pathway lead to deformations in development as well as to contribute to tumorigenesis (Goodrich and Scott, 1998; Nilsson et al., 2000; Taipale and Beachy, 2001). During lung epithelial regeneration after

injury, activated Hh signaling is observed in regions of repair and in pulmonary neuroendocrine stem cell niches (Watkins et al., 2003). In the same study, cyclopamine-mediated suppression of aberrantly active Hh signaling in some SCLCs resulted in a dramatic drop in cell viability and tumorigenicity (Watkins et al., 2003). These findings provide evidence that SCLC is not only a malignancy that arises from a population of self-renewing PNECs that retain active Hh signaling as well as primitive neuroendocrine features, but that therapeutic targeting of the Hh signaling pathway may suppress stem-like tumor cell self renewal (Chi et al., 2006; Vestergaard et al., 2006; Watkins et al., 2003). Recently, activated Hh signaling has been implicated the self-renewal of myeloid leukemia (Peacock et al., 2007; Zhao et al., 2009), glioblastoma (Bar et al., 2007), and breast cancer stem cells (Liu et al., 2006). The mounting evidence for the role of Hh signaling in tumor cell maintenance and cancer stem cell self-renewal has prompted the development of better and more specific inhibitors of the Hh pathway, some of which are in currently in clinical trials for SCLC (Dlugosz and Talpaz, 2009; Hyman et al., 2009; Tremblay et al., 2009).

### *1.4.3 Notch Pathway*

The Notch signaling pathway is involved in cell fate determination, and during organogenesis and tissue homeostasis Notch-mediated cell-cell interactions dictates the preservation or differentiation of stem cells (Artavanis-Tsakonas et al., 1999). Activation of Notch signaling begins when membrane bound Notch ligands bind to receptors on adjacent cells. Upon binding, the intracellular domain of the receptor is cleaved by a gamma-secretase, allowing for the activation of downstream targets, such as the

inhibitory basic helix-loop-helix transcription factor Hes1 (Figure 1.4) (Artavanis-Tsakonas et al., 1999). In the developing lung, Notch signaling appears to be required for determining proximal and distal lung epithelial cell fates (Collins et al., 2004). In transgenic *Hes1* knockout mice, suppression of Notch signaling at the transcriptional level results in premature and promiscuous neuroendocrine differentiation during lung development (Ito et al., 2000). This may be due to the alleviation of Hes1 mediated suppression of Achaete-scute homolog like-1 (ASCL1) expression, a basic helix-loop-helix transcription factor required for proneural differentiation (Borges et al., 1997). Studies forcing the activation of Notch signaling in the developing lung tissue, either through the ectopic expression of intracellular Notch domains or through gamma-secretase activation, result in the accumulation of distal airway stem cells and a reduction in neuroendocrine and alveolar cell differentiation (Dang et al., 2003; Guseh et al., 2009; Tsao et al., 2008). This suggests that in some lung stem cells, activated Notch signaling functions to preserve a primitive and undifferentiated state. In lung cancer, elevated Notch signaling transcripts have been described in NSCLC, however the role of Notch in tumor maintenance remains poorly understood. Suppression of Notch signaling in some NSCLC cells by treatment with a gamma-secretase inhibitor induces cell death and decreased tumor growth in mice (Haruki et al., 2005; Konishi et al., 2007). Paradoxically, activation of Notch signaling in A549 cells through overexpression of Notch1 leads to a decrease in proliferation and tumorigenic growth in mice (Zheng et al., 2007). However, the apparent discrepancy between these results may be due to the perturbations of Notch signaling through different Notch receptors, as well as the oncogenic context of the tumor. As mentioned previously, tumor oncogenotype plays a

major role in defining cancer stem cell behavior, yet how a tumor's oncogenotype might affect or determine cancer stem cell self-renewal is still an open question. Recently a study of 176 resected NSCLCs revealed a correlation between activated NOTCH1 signaling and poor prognosis in a subset of NSCLC patients without a *TP53* (p53) mutation (Westhoff et al., 2009). This could mean that Notch signaling promotes self-renewal in cancer stem cells with a mutant *TP53* oncogenotype and that cancer stem cells with wild-type *TP53* achieve self-renewal through a different signaling pathway. Thus, the Notch pathway may be a potent therapeutic target for some cancer stem cell populations. For example, in a study of early passage human glioma specimens, the suppression of Notch signaling by treatment with a gamma-secretase inhibitor reduced the capacity for xenograft growth in mice, and dramatically reduced the proportion of $CD133^+$ glioma stem cells (Fan et al., 2006). Using a human specific DLL4 blocking antibody (anti-hDLL4) to suppress Notch signaling in human breast cancer xenografts, Hoey and coworkers observed a dramatic reduction in tumor growth as well as a significant decrease in $CD44^+$ breast cancer stem cells (Hoey et al., 2009). In addition, when host derived DLL4 was blocked with a separate monoclonal antibody (anti-mDLL4), tumor growth and tumor vasculature was dramatically reduced, suggesting a dual role for Notch signaling in tumor cell self-renewal and tumor angiogenesis (Hoey et al., 2009). Although it is not yet clear if lung cancer stem cells require Notch signaling for self-renewal, several reports suggest Notch signaling components are expressed in putative lung cancer stem cell populations and are required for tumor initiation capacity (Bertolini et al., 2009; Jiang et al., 2009c; Levina et al., 2008).

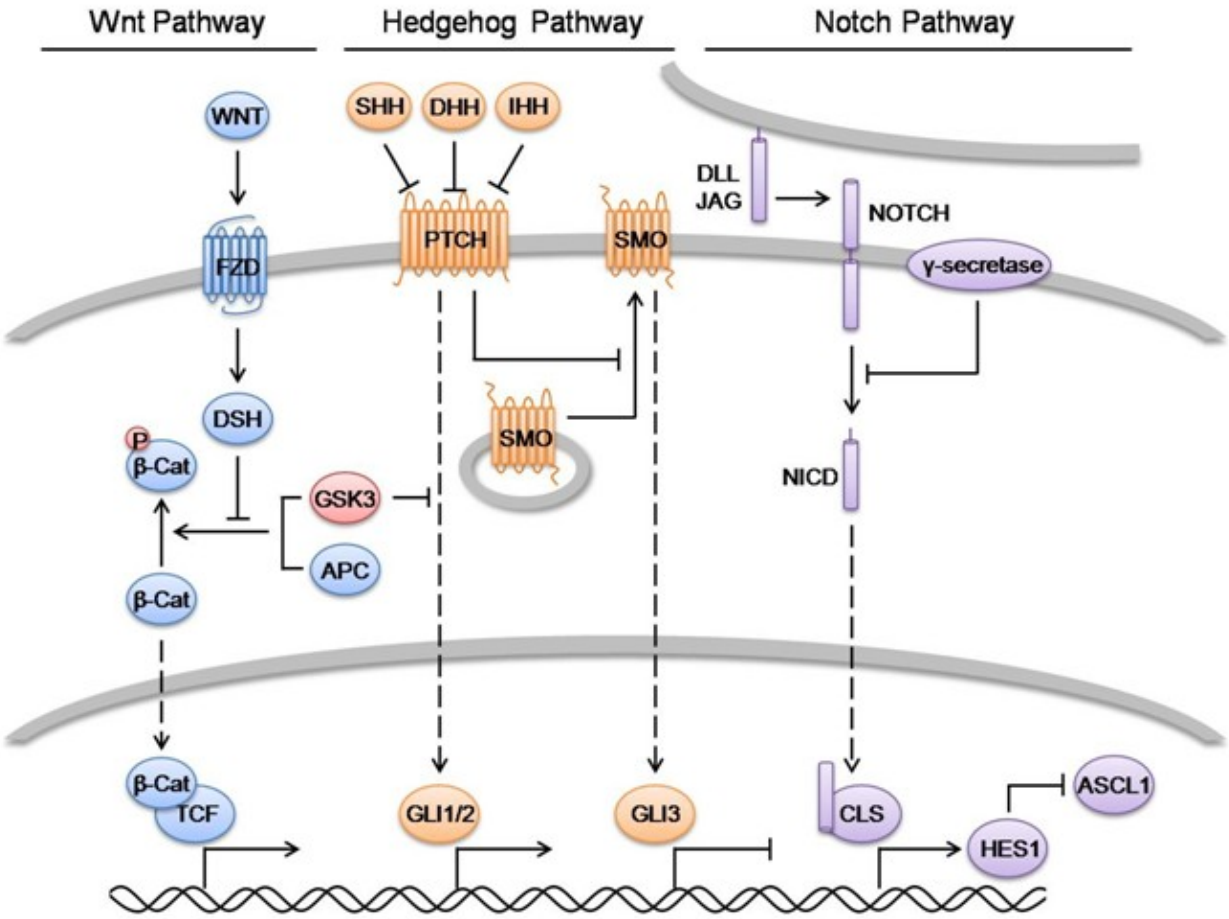



**Figure 1.4: The Wnt, Hedgehog and Notch signaling pathways.** Activation of the Wnt signaling cascade begins when secreted Wnt ligands bind to Frizzled (FZD) receptors resulting in downstream stabilization and nuclear translocation of β-catenin. In the absence of Wnt ligand, GSK3 mediated phosphorylation of β-catenin leads to the ubiquitinilation and β-catenin degradation. The binding of the Hedgehog receptor Patched (PTCH) to secreted ligands (SHH, DHH and IHH) alleviates PTCH repression of membrane translocation and activation of Smoothened (SMO). Activated SMO leads to the nuclear translocation of GLI transcriptions factors (GLI1 and GLI2). In the absence Hh ligands, GSK3 mediated phosphorylation of GLI1 and GLI2 lead to ubiquitinilation and degradation, whereas nuclear translocated GLI3 functions to repress target gene transcription. The binding of Notch receptors to membrane bound Notch ligands (DLL and JAG) activates a series of receptor-ligand cleavage events, such as γ-secretase mediated cleavage of the Notch intracellular domain (NICD). The free cytoplasmic NICD is translocated to the nucleus and binds to the CLS family transcription factor complex to activate target gene transcription. Notch signaling target genes include the Hes1 transcriptional repressor which functions to suppress Achaete-scute homolog like-1 (ASCL1) expression.

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 Materials

### *2.1.1 Lung Cell Lines*

With the exception of A549, Calu-1, and Calu-6, which were purchased from the American Type Culture Collection (ATCC), all human lung cancer cell lines used in this study were established by the Minna and Gazdar laboratories, and maintained in RPMI-1640 (Life Technologies Inc.) with 5% fetal bovine serum (FBS) and grown in a humidified atmosphere with 5% $CO_2$ at 37°C (Phelps et al., 1996). Lung cancer cell lines that were established at the National Cancer Institute are denoted with the prefix H and lung cancer cell lines that were established at the UTSW Hamon Center for Therapeutic Oncology Research are annotated as HCC (Gazdar et al., 2010). Immortalized human bronchioalveolar epithelial cells (HBECs) expressing ectopic *CDK4* and *hTERT* were previously established in the Minna lab and cultured in KSFM supplemented with bovine pituitary extract and recombinant human epithelial growth factor (Gibco) (Ramirez et al., 2004).

Lung cancer cell lines expressing CMV promoter driven luciferase were established in the Minna lab and have the suffix –luc. All of the cell lines have been DNA fingerprinted for provenance using the PowerPlex 1.2 kit (Promega) and confirmed

to be the same as the DNA fingerprint library maintained either by the ATCC or the Minna and Gazdar labs. The lines were also tested to be free of mycoplasma by e-Myco kit (Boca Scientific).

Early passage murine lung tumor cell cultures were derived from double transgenic CCSP-rtTA/tet-o-K-ras$^{G12D}$ mice with an Ink4a/ARF$^{-/-}$ background as reported previously and cultured in DMEM complete medium with 10% FBS and 1 ug/ml doxycycline (Fisher et al., 2001).

### *2.1.2 Resected Patient Lung Tumor Samples*

Lung tumor samples were collected from consenting patients after surgery and mechanically/chemically disassociated. To obtain a tumor cell suspension suitable for flow cytometry, tumors were minced into ~1 $mm^3$ pieces, incubated for 1-2 hours with 1 mg/ml Collagenase I in HBSS (Gibco) at 37°C and passed through a 70 μm cell strainer. Cells were then spun down and resuspended in fresh PBS. A single cell suspension was confirmed by light microscopy.

To obtain a tumor cell suspension suitable for flow cytometry from lung cancer pleural effusions, the effusion was first spin down and resuspended in a hypotonic red blood cell lysis buffer containing 155 mM $NH_4Cl$ , 12 mM $NaHCO_3$ and 0.1 mM EDTA in distilled water. After 5-10 min incubation cells were spun down, resuspended and incubated for up to 1 hour in 1 mg/ml Collagenase I in HBSS at 37°C. To ensure a single cell suspension, resuspended cells were passed through a 70 μm cell strainer, counted and prepared for flow cytometric analysis.

### *2.1.3 NSCLC Tissue Micro Array*

Archived, formalin-fixed, paraffin-embedded tissues from surgically resected lung cancer specimens (lobectomies and pneumonectomies) containing tumor and adjacent normal epithelium tissues were obtained from the Lung Cancer Specialized Program of Research Excellence (SPORE) Tissue Bank at The University of Texas M. D. Anderson Cancer Center (Houston, TX), which has been approved by an institutional review board. The tissue specimens were histologically examined and classified using the 2004 World Health Organization classification system and 282 NSCLC samples (177 adenocarcinomas and 105 squamous cell carcinomas) were selected for our tissue microarray (TMA). TMAs were constructed using triplicate 1-mm diameter cores per tumor; each core included central, intermediate, and peripheral tumor tissue. Detailed clinical and pathologic information, including patient demographics, smoking history, smoking status, clinical and pathologic TNM stage, overall survival duration, and mutation status of *KRAS* and *EGFR*, was available for most cases.

## 2.2 Methods

### *2.2.1 Fluorescence Activated Cell Sorting and Flow Cytometric Based Assays*

***Expression Analysis***

Lung cancer cell lines and patient samples were analyzed for their expression of a panel of putative lung cancer stem cell markers by flow cytometry. Briefly, cells were detached and disaggregated using 0.5% Tripsin-EDTA (Gibco) and resuspended in HBSS+ (HBSS containing 2% FBS and 10 μM HEPES) at a concentration of 1 million

cells per ml. Cells were then stained with fluorescently conjugated antibodies against CD24, CD30, CD34, CD44, CD45, CD47, CD66, CD87, CD117, CD147, CD151, CD184, CD318 (BD Biosciences) and CD133 (Miltenyi Biotec) and incubated for 30 min at 4°C. Cells were then washed and resuspended in fresh HBSS+ and stained with Propidium Iodide (PI) to account for non-viable cells. Flow cytometric analyses were performed on a FACScan (Becton-Dickinson) or FACSCalibur flow cytometer (BD Biosciences) and figures were produced using FlowJo software (Treestar). Cell sorting was performed on a three laser, nine color MoFlow (Dako Cytomation) or a five laser, eighteen color BD Aria (BD Biosciences).

***Aldefluor Assay***

The Aldefluor kit (Stem Cell Technologies) was used to profile and separate cells with high and low aldehyde dehydrogenase activity (ALDH) as described previously (Ginestier et al., 2007). Cells were incubated in Aldefluor assay buffer containing the ALDH protein substrate BODIPY-aminoacetaldehyde (BAAA) for 45 min at 37°C. Cells that could catalyze BAAA to its fluorescent product BODIPY-aminoacetate (BAA) were considered $ALDH^+$. Sorting gates for FACS were drawn relative to cell baseline fluorescence, which was determined by the addition of the ALDH specific inhibitor diethylaminobenzaldehyde (DEAB) during the incubation and DEAB treated samples served as negative controls (Figure 2.1). After incubation cells were suspended in fresh assay buffer containing 1 μg/ml PI to mark non viable cells. $ALDH^+$ and $ALDH^-$ cells were sorted by a MoFlow or BD Aria and the purity of sorted cells was assayed after the sort was completed. In co-staining experiments, cells were incubated with monoclonal

anti-CD133-APC (Miltenyi Biotec), anti-EpCam-PE (BD Biosciences) or anti-Sca-1-PE (BD Pharmigen) antibodies in Aldefluor assay buffer for 20 min at 4°C, as described in the Aldefluor kit insert. ALDH gating in patient tumor samples was performed on $EpCam^+$ cells to exclude potential hematopoietic or stromal $ALDH^+$ cells.

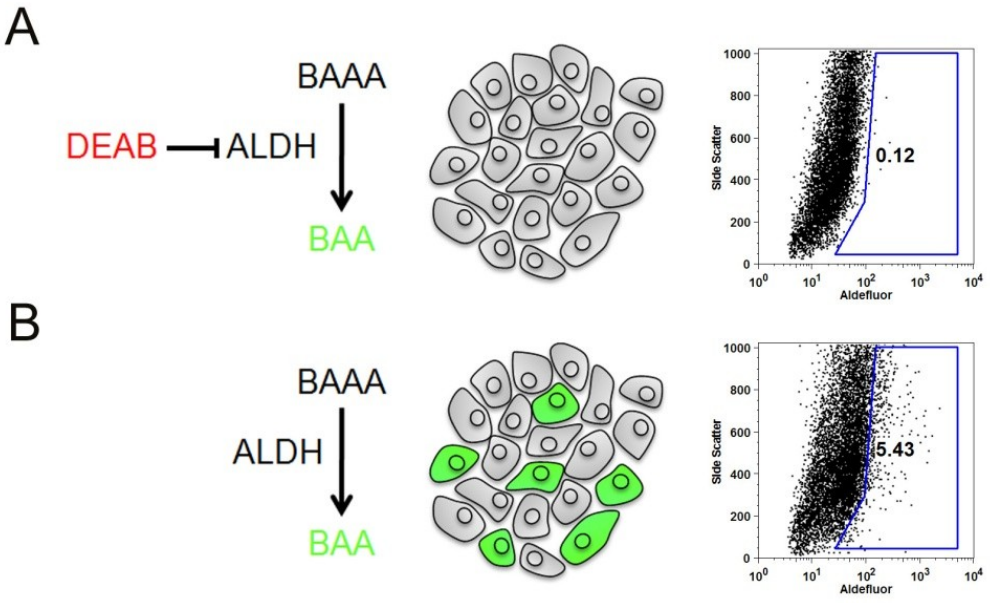



**Figure 2.1: The Aldefluor assay.** DEAB inhibits the ALDH mediated production of fluorescent BAA from the substrate BAAA. Cell fluorescence is plotted as a dot-plot where the x-axis is a measure of Aldefluor based fluorescence and the y-axis is a measure of cell complexity/granularity, referred to as "Side Scatter". To determine the proportion of fluorescent $ALDH^+$ cells within a population, an aliquot of cells are first incubated in DEAB as a negative control to determine their baseline fluorescence by flow cytometry (A). In the absence of DEAB, ALDH mediated fluorescence is generated in cells that express the requisite ALDH protein and fluorescent cells are detected as a positive shift in the x-axis (B). $ALDH^+$ cells are defined and quantified (as a percent of total cells) by their registry within a fluorescent region or "gate" that has been generated using the baseline fluorescence of the negative control cells (region outlined in blue).

***Cell Cycle Analsysis***

DNA content was measured from sorted cells to determine cell cycle profile. Cells were fixed in 70% EtOH for 15 min on ice or over night at 4°C, with EtOH added slowly during fixation while vortexing to reduce cell clumping. Cells were incubated in buffered staining solution containing 0.05% Triton X-100, 0.1 mg/ml RNase A, and 50 µg/ml PI in PBS for 30 min at 37°C, briefly spun down then suspended in fresh PBS and analyzed using a FACScan or FACSCalibur flow cytometer. The Watson algorithm was performed in FlowJo to determine the distribution and gating of cells in different states of DNA replication.

***Side Population Assay***

Hoechst 33342 dye excluding, Side Population cells (SP), were identified and analyzed as previously described (Goodell et al., 1996; Ho et al., 2007). Cells briefly trypsinized, resuspended and incubated in RPMI with 2% FBS, 10 mM HEPES buffer (Gibco), and 6 µg/ml Hoechst 33342 dye (Sigma-Aldrich) for one hour at 37°C. After incubation, cells were briefly spin down, resuspended in fresh, ice cold HBSS containing 1 µg/ml PI and passed through a 70 µm cell strainer to ensure a single cell suspension. As a negative control, cellular efflux of Hoechst 33342 dye was inhibited by the addition of 100 µM Verapamil (Sigma-Aldrich). Flow cytometric analysis and sorting for SP cells was performed on a MoFlow.

### *2.2.2 Tumor Cell Growth Assays*

***Colony Formation Assays***

The ability to generate anchorage independent colonies from sorted cells was assayed by soft agar colony formation. Cells were suspended in 0.33% SeaKem agar (FMC Bioproducts) in growth medium supplemented with 20% FBS and plated in quadruplicate over a layer of 0.5% agar base medium in 12-well plates. The plates were incubated at 37°C in a humid atmosphere of 5% $CO_2$ for 2-3 weeks until microscopically visible colonies (>50 cells) stained with 0.05% crystal violet could be counted. To determine anchorage dependent colony formation, limiting dilutions of tumor cells (50-500 cells/ml) were plated on 6 well plates (9.5 $cm^2$ well area) in growth media and after a two week incubation, colonies were stained with 0.5% methylene blue and stained colonies were counted using Image J software (NIH).

***Spheroid Colony Formation Assay***

To promote the growth of floating spheroid colonies in liquid culture, lung cancer cells were grown in ultra-low adhesion flasks (Corning) or on non-tissue culture treated plastic Petri dishes. In addition, cells were either grown in normal serum containing media (RPMI + 5% FBS) or serum free media supplemented with 10 ng/ml EGF and 20 ng/ml FGF2 to encourage the selection of putative cancer stem cells. Within two days tumor cell anoikis was observed and within one week, the formation of floating spheroid colonies could be observed by microscopy. Secondary spheroid formation was assayed by dissagregating spheroid colonies into a single cell suspension, culturing these cells in sphereoid colony growth conditions and counting the number of observable floating colonies after two weeks of growth.

***Xenograft Formation Assays***

The NOD/SCID mouse xenograft model was used to characterize the tumorigenicity of prospective human lung cancer stem cell populations. All *in vivo* experiments were performed in female NOD/SCID mice, bred and purchased from the UTSW Mouse Breeding Core, and the care and treatment of experimental animals were in accordance with institutional guidelines.

To assay the capacity for tumor initiation, limiting dilutions of prospective lung cancer stem cell and prospective non-lung cancer stem cell (1000-100000 cells) were injected into either orthotopic or paratopic sites in the mouse and the incidence of tumor formation was scored in mice by surgical observation and or BLI. Orthotopic implantations of lung cancer cells were performed by directly injecting 50 uL of cells suspended in PBS into the right lateral thorax, at the lateral dorsal auxiliary line, just above the lower rib line (Cui et al., 2006). To avoid potential pneumothorax, a small 30½ guage needle was used and the needle entry sites in the skin and chest wall of the recipient mice were spaced at least 1 mm away from each other. To aid in the detection of xenografts formed from orthotopic injections and from limiting dilutions of tumor cells, bioluminescent imaging (BLI) was used to detect xenograft derived luminescence. Cell lines expressing CMV promoter driven luciferase (designated by the suffix, –luc) were injected into mice and BLI images were taken weekly. Xenograft derived luminescence was captured in anesthetized mice by a charge-couple device camera (Xenogen) with a 5 min exposure, 10 minutes after a subcutaneous injection of 450 mg/kg D-luciferin substrate (Biosynth) in PBS (Paroo et al., 2004). Live Image 2.60.1 software (Xenogen) was used to process and analyze BLI images.

Paratopic implantations of lung cancer cells were carried out by injecting 100 uL of cells suspended in PBS subcutaneously into the right flank. Patient tumor cells were suspended in a 1:2 mixture of PBS and chilled matrigel (BD Biosciences) and injected accordingly. To minimize leakage at the injection site, 27½ or 30½ gauge needles were used and the subcutaneous region was made accessible for injection by lifting the skin at the site of injection to alleviate pressure on the injected volume. Prior to subcutaneous injection, fur on the right flanks of mice was shaved off using an electric razor to improve detection of xenograft formation. To compare the growth rates of tumors formed from prospective lung cancer stem cells and non-lung cancer stem cells, non-limiting cell dilutions (> 100000 cells) were injected paratopically and tumor volume caliper measurements were performed weekly for up to ten weeks. Tumor volumes were calculated as follows: $V_{tumor} = (\pi/6)(d_{large})(d_{small})^2$.

### *2.2.3 Molecular Expression Analysis*

***Microarray Expression Analysis***

Transcript expression data for most lung cancer cell lines has been previously generated in the Minna Lab by both Affymetrix (U133 plus 2.0 and U133AB chips) and Illumina (WG6-V2 and V3 BeadChips) array platforms. The Ilummina WG6-V3 BeadChip kit (> 48000 probes) was used to generate expression array data from putative lung cancer stem cells isolated by FACS. Tumor cell RNA was isolated using Qaigen RNeasy kit and total RNA quality was confirmed by formaldehyde gel and/or capillary electrophoreses on the Experion System (Bio-Rad). Total RNA was labeled, amplified

and re-analyzed for quality prior to hybridization by the UTSW Simmons Comprehensive Cancer Center Genomics Core.

MATRIX (MicroArray Transformation in Microsoft Excel) software 1.41 is a Microsoft Visual Basic program created by Dr. Luc Girard (luc.girard@utsouthwestern.edu) in the Minna Lab, used to import and analyze microarray expression data. Using MATRIX, transcript expression was normalized across samples by the median value, then normalized expression signals were $log_2$-transformed and color coded. For determining expression correlates for a sample property (such as ALDH activity), a Pearson's correlation analysis was performed. For comparison between sample classes (such as $ALDH^+$ and $ALDH^-$ cells), the ratio of log2-transformed signals from sample classes were generated and two-sample t-tests were performed in MATRIX to filter out non-significant differences in expression ($P < 0.05$).

***Quantitative Real Time PCR Analysis***

Expression of stem cell transcripts by quantitative real time PCR (qPCR) was performed using TaqMan Assay probes (Applied Biosystems) (Table 2.1). The reverse transcriptase reaction was performed using the iScript cDNA synthesis kit (Bio-Rad) on RNA isolated either by TRIzol/Chloroform extraction or by an RNeasy kit (Qaigen). iTaq Supermix with Rox (Bio-Rad), a premade formula containing iTaq DNA polymerase, optimized buffers, nucleotides and ROX passive detection dye, was used to perform the qPCR. Reactions were run in duplicate wells on a 96-well plate in a 7300 Real Time PCR System (Applied Biosystems). 7300 System Software (Applied

Biosystems) was used to derive Ct values and ΔCt values were calculated using GAPDH amplification as a control.

To compare expression of a transcript in non-isogenic samples, expression was normalized to expression data derived from a reference RNA sample (Stratagene), which included pooled RNA from normal and timorous tissues. Expression data was displayed as fold expression normalized to the reference RNA sample, which was set to 1.

### *2.2.4 Gene Silencing*

To silence expression of *NOTCH3* in lung cancer cells, a pLKO.1 lentiviral shRNA vector targeted against *NOTCH3* was purchased from Open Biosystems (Source ID: TRCN0000020237) and shRNA against *NOTCH3* was stably expressed in lung cancer cells. An shRNA vector directed against *GFP* and transformed into competent DH5α *E. Coli*. Competent cells were first thawed, placed on ice for 10 min then incubated with 20 ng of vector DNA for 30 min. After incubation, cells were heat shocked at 42°C for 45 seconds, chilled on ice for 2 min then incubated in 800 µl of SOC media at 37°C for one hour prior to being streaked on LB agar containing 100 µg/ml ampicillin. Ampicillin resistant colonies were selected and expanded in culture and plasmid DNA was extracted using a Qiagen HiSpeed Plasmid Midi kit. Restriction digests of plasmid DNA was performed and resolved by gel electrophoresis to confirm the vector fidelity.

Lentiviruses were made by transfection of 293T packaging cells with a three plasmid system. 293T cells were grown in DMEM containing 10% FBS and transiently transfected with shRNA vector together with pMD.G-VSVG and pCMV-ΔR8.91 viral

packaging plasmids using Fugene6 (Roche). Viral supernatant was harvested once a day for up to three days, passed through a 0.45 μm filter and stored as 1 ml aliquots at -80°C. The viral supernatant was used for the transduction of lung cancer cells with 8 μg/ml polybrene (Sigma-Aldrich). Stable shRNA expressing lung cancer cells were generated after two weeks culture in 4 μg/ml puromycin.

### *2.2.5 Immunohistochemistry*

Immunohistochemical (IHC) staining for ALDH1A1 using monoclonal antibodies (Abcam), ALDH3A1 (Santa Cruz Biotechnology) and CD133 (Miltenyi Biotec) was performed on TMA samples as follows: 5 μm thick formalin-fixed and paraffin-embedded tissue sections were deparaffinized, hydrated, heated in a Biocare decloaker for 30 min pretreated with Target Retreval Solution (Dako), and washed in Tris buffer. Peroxide blocking was performed with 3% $H_2O_2$ in methanol at room temp for 15 min, followed by 35 min incubation in Tris-buffered saline containing 10% FBS. Slides were incubated with the primary antibody (1:100 ALDH1A1 and 1:200 ALDH3A1) at room temp for 65 min, washed with Tris-buffered saline, followed by incubation with Envision Dual Link+ Polymer-Labeled system (Dako) for 30 min. Staining was developed with chromogen substate (Dako) for 5 min and then counterstained with hematoxylin, dehydrated, and mounted.

The expression was quantified using light microscopy (total magnification, 200x) and cytoplasmic expression was quantified using a four-value intensity score (0, 1, 2, and 3) and the percent of $IHC^+$ tumor cells (0-100%). Intensity scores were defined as follows: 0 = no appreciable staining; 1 = barely detectable staining in epithelial cells

compared with the stromal cells; 2 = readily appreciable staining; and, 3 = dark brown epithelial cell staining. An expression score was obtained by multiplying the intensity and reactivity extension values (range, 0-300). Expression scores from samples stained with the ALDH1A1 and ALDH3A1 antibodies were dichotomized by their mean values into high or low staining categories. Samples with an ALDH1A1 expression score $\geq 13.3$ were considered $ALDH1A1^{high}$ and samples with an ALDH3A1 expression score $\geq 25$ were considered $ALDH3A1^{high}$. Samples with detectable membranous or cytoplasmic CD133 staining were classified as $CD133^{+}$, whereas samples with no detectable CD133 expression were classified as $CD133^{-}$.

| Gene | RefSeq # | Pathway | Function | TaqMan Assay # |
|---|---|---|---|---|
| *SHH* | NM_000193 | Hedgehog | Ligand | Hs00179843_m1 |
| *DHH* | NM_021044 | Hedgehog | Ligand | Hs00368306_m1 |
| *IHH* | NM_002181 | Hedgehog | Ligand | Hs01081801_m1 |
| *PTCH1* | NM_000264 | Hedgehog | Receptor | Hs00181117_m1 |
| *PTCH2* | NM_003738.3 | Hedgehog | Receptor | Hs00184804_m1 |
| *SMO* | NM_005631 | Hedgehog | Receptor/Signal Transducer | Hs00170665_m1 |
| *GLI1* | NM_005269 | Hedgehog | Transcription Factor | Hs00171790_m1 |
| *GLI2* | NM_005270 | Hedgehog | Transcription Factor | Hs00257977_m1 |
| *GLI3* | NM_000168 | Hedgehog | Transcription Repressor | Hs00609233_m1 |
| *DLL1* | NM_005618 | Notch | Ligand | Hs00194509_m1 |
| *DLL3* | NM_016941 | Notch | Ligand | Hs00213561_m1 |
| *DLL4* | NM_019074 | Notch | Ligand | Hs00184092_m1 |
| *JAG1* | NM_000214 | Notch | Ligand | Hs00164982_m1 |
| *JAG2* | NM_002226 | Notch | Ligand | Hs00171432_m1 |
| *NOTCH1* | NM_017617 | Notch | Receptor | Hs00413187_m1 |
| *NOTCH2* | NM_024408 | Notch | Receptor | Hs00225747_m1 |
| *NOTCH3* | NM_000435 | Notch | Receptor | Hs00166432_m1 |
| *NOTCH4* | NM_004557 | Notch | Receptor | Hs00270200_m1 |
| *HES1* | NM_005524 | Notch | Transcription Factor | Hs00172878_m1 |
| *HEY1* | NM_012258 | Notch | Transcription Factor | Hs00232618_m1 |
| *HEY2* | NM_012259 | Notch | Transcription Factor | Hs00232622_m1 |
| *MAML2* | NM_032427.1 | Notch | Effector | Hs00287205_m1 |
| *ASCL1* | NM_004316.3 | Notch | Target Gene of Hes1 | Hs002699_m1 |
| *WNT1* | NM_005430 | Wnt | Ligand | Hs00180529_m1 |
| *WNT2b (aka WNT13)* | NM_024494.1 | Wnt | Ligand | Hs00257131_m1 |
| *WNT3a* | NM_033131.2 | Wnt | Ligand | Hs00263977_m1 |
| *WNT10b (aka WNT12)* | NM_003394.2 | Wnt | Ligand | Hs00559664_m1 |
| *FZD1* | NM_003505 | Wnt | Receptor | Hs00268943_s1 |
| *DVL2* | NM_004422.2 | Wnt | Regulator | Hs00182901_m1 |
| *DVL3* | NM_004423.3 | Wnt | Regulator | Hs00610263_m1 |
| *APC* | NM_000038 | Wnt | Effector | Hs00181051_m1 |
| *β-CAT* | NM_001904 | Wnt | Effector | Hs00170025_m1 |
| *TCF1* | NM_000545 | Wnt | Transcription Factor | Hs00167041_m1 |
| *OCT4 (aka POU5F1)* | NM_002701 | other | Transcription Factor | Hs01895061_u1 |
| *NANOG* | NM_024865 | other | Transcription Factor | Hs02387400_g1 |
| *SOX2* | NM_003106 | other | Transcription Factor | Hs00602736_s1 |
| *MYC* | NM_002467 | other | Transcription Factor | Hs00153408_m1 |
| *BMI1* | NM_005180 | other | Transcriptional Repressor | Hs00180411_m1 |
| *KLF4* | NM_004235 | other | Transcription Factor | Hs00358836_m1 |
| *GAPDH* | | Glycolysis | | 4352934E |

**Table 2.1: TaqMan probes for qPCR analysis.**

# CHAPTER THREE

# CHARACTERIZATION OF PUTATIVE LUNG CANCER STEM CELLS

## 3.1 Introduction

The cancer stem cell model suggests that in many cancers, tumor initiation and propagation is driven by a population of self-renewing tumor cells known as cancer stem cells. Similar to normal tissue stem cells, cancer stem cells possess the capacity for self-renewal and differentiation through the aberrant retention or reactivation of developmental signaling programs such as the Notch, Wnt and Hedgehog pathways (Reya et al., 2001). In addition, tumor cell heterogeneity, metastasis and recurrence after therapy may also be facilitated by cancer stem cells (Clarke and Fuller, 2006; Visvader and Lindeman, 2008; Wicha et al., 2006). Therefore, the identification and characterization of cancer stem cells may lead to translational breakthroughs, including identification of new diagnostic biomarkers as well as therapeutic targets related to tumor cell self-renewal.

While cancer stem cell populations have been identified in many myeloproliferative and solid tumor malignancies, the identification and characterization of putative lung cancer stem cell populations remains elusive and underdeveloped. As discussed in Chapter One, this is likely due to the complexity of the disease, as well as the paucity of selectable human lung stem cell markers. However, as more putative cancer stem cells are identified in various malignancies, it is becoming apparent that

many cancer stem cell populations from different tumors share identifiable markers and selectable features. For example, CD133 expression reportedly identified many cancer stem cell populations including some leukemic, brain, colon and prostate cancer stem cell populations (Table 1.1). In addition, cancer stem cell populations have also been enriched in many tumors through the selective culturing of cancer cells as floating sphere colonies in serum free, mitogen defined conditions. While some of these markers and selectable features may be unique to certain tumor stem cell populations, it stands to reason that some of these means may enrich for human lung cancer stem cells.

Many reported cancer stem cell associated markers are proteins that are expressed on the surface of stem cells. The transmembrane glycoprotein, CD44, is an adhesion molecule expressed in a variety of hematopoietic and epithelial cell types. Apart from its role in cell attachment, CD44 is also known to function in cell signaling, lymphocyte activation, homing and migration (Ponta et al., 2003). In cancer, CD44 expression has been reported to be upregulated in a variety of CSC populations (Table 1.1). In a 2006 report by Krause et al. elevated CD44 expression was detected in CML stem cells and transgenic murine leukemia stem cells lacking CD44 expression failed to engraft in recipient mice. The failure of to transplant the disease was discovered to be a consequence of the reduced bone marrow homing capabilities of $CD44^{-/-}$ CML stem cells, suggesting that CD44 is a functional marker of CML stem cells (Krause et al., 2006). Other surface markers that may select for prospective lung CSCs include CD151, a tetraspanin expressed on basal lung epithelial stem cells (Hajj et al., 2007), CD117, also known as c-kit receptor expressed on HSCs , and the stem cell marker CD133, which is reportedly expressed in a variety of stem cell and cancer stem cell populations (Table

1.1). In addition to surface markers, the enzymatic activity of specific stem cell proteins may also serve as a potential marker for lung cancer stem cell populations. As described in Chapter One, the ABCG2 transporter mediated exclusion of fluorescent Hoechst 33342 dye as well as the elevated enzymatic activity of ALDH proteins have been demonstrated to identify stem cell and cancer stem cell populations in a variety of tissues.

The goal of this study was to identify putative lung cancer stem cells using markers and selection strategies that have previously enriched for stem cells and cancer stem cells in other tissues. Cells enriched using these methods from established lung cancer cell lines and resected patient lung tumors were assayed for growth features ascribed to cancer stem cells, including enrichment for clonogenicity, tumorigenicity and tumor cell self-renewal.

## 3.2 Results

### *3.2.1. Analysis of Stem Cell Associated Surface Markers in Lung Cancer*

Where a stem cell population has been described in solid tumors and leukemias, cancer stem cells represent only a small subpopulation of the total tumor cell population. Thus, it is hypothesized that lung cancer stem cells represent a rare or uncommon cell population within tumor cell lines and resected lung tumors.

To identify potential tumor stem cell subpopulations, the expression of a panel of stem cell associated surface markers was assayed in eight lung cancer cell lines with different histological subtypes. The detection of fluorescent antibodies specific to these surface markers was performed by flow cytometric analysis. To ensure fluorescent

detection was not due to non-specific binding of the antibody, cells were stained with a fluorescent isotype control for each antibody tested. Cells stained with the isotype control were used to set the baseline fluorescence during flow cytometric analysis.

Surprisingly, nearly all of these surface markers failed to identify a tumor cell subpopulation as they were either not detected or ubiquitously expressed within each cell line (Table 3.1). For example, the stem cell marker CD44 was expressed nearly all tumor cells within the seven cell lines tested. By contrast expression of the stem cell marker CD133 was absent in nearly all cell lines except for H1395, where greater than 80% of the total cells were $CD133^{+}$ (Table 3.1). In cases with near ubiquitous marker expression, the typical distribution of fluorescent marker positive cells was sharply unimodal in most cell lines. Because of this, reproducibly isolating $marker^{+}$ or $marker^{+/high}$ cell subpopulations, for example, would be nearly impossible. Therefore, it was concluded that ubiquitously expressed markers were unfit for selectively isolating lung cancer stem cells in these samples.

Of the panel of markers tested, only CD151 expression was reproducibly detected in a subpopulation of lung tumor cells (Table 3.1). To test if CD151 cells were enriched in clonogenic cells, H358 $CD151^{+}$ and $CD151^{-}$ cells were isolated by FACS and plated in limiting dilutions (Figure 3.1A). After two weeks, no significant difference in colony formation was observed between sorted cells, suggesting CD151 does not select for a highly clonogenic tumor cell subpopulation (Figure 3.1B).

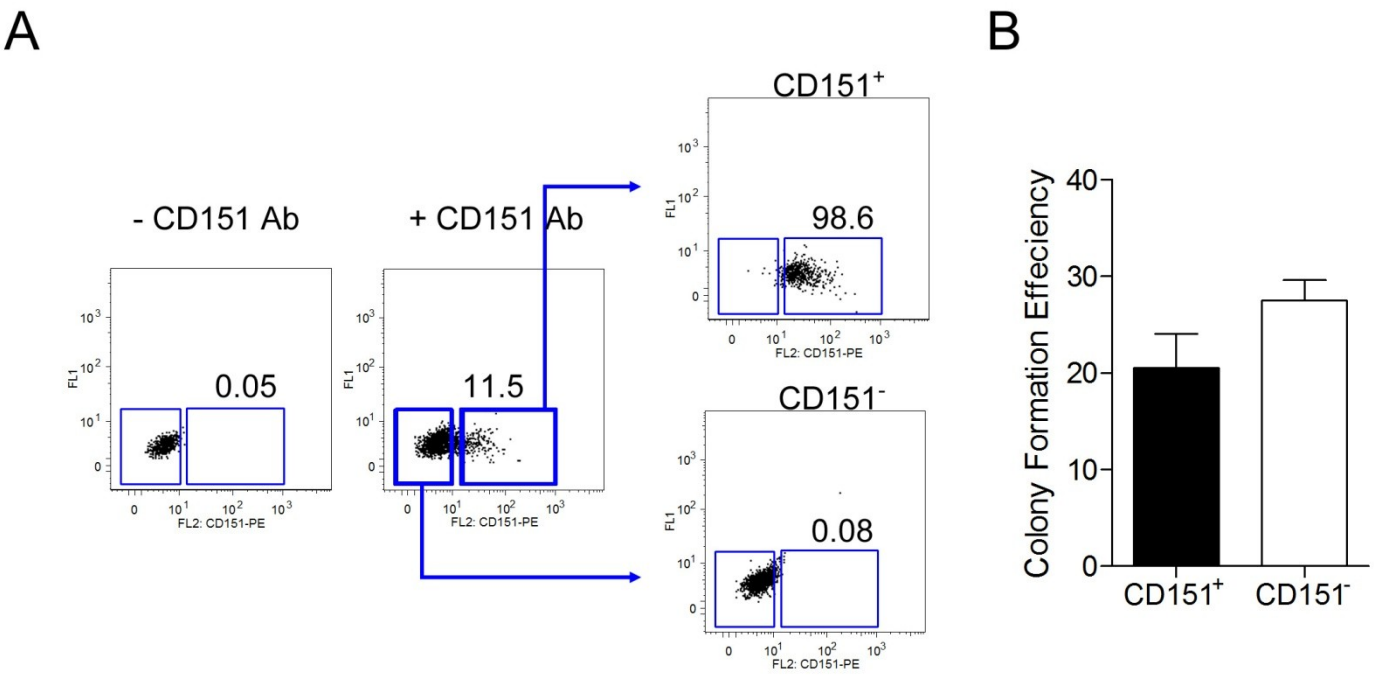



**Figure 3.1: Isolation and characterization of CD151$^+$ lung cancer cells.** Unstained H358 cells served as a negative control to generate gates that define CD151$^+$ (annotated as percent of total cells above the CD151$^+$ gate) and CD151$^-$ cell populations. In the presence of the fluorescent CD151 antibody, 11.5% of the total cell population was CD151$^+$. Post sort analysis of isolated CD151$^+$ and CD151$^-$ cells ensured the purity (>95%) of sorted populations (A). Colony formation analysis revealed no significant enrichment of clonogenicity in sorted H358 CD151$^+$ and CD151$^-$ cells.

| Cell Line: | H460 | H1299 | H2087 | H1395 | A549 | H358 | H292 | H1417 |
|---|---|---|---|---|---|---|---|---|
| Type: | LC | LC | AC | AC | BAC | BAC | SCC | SCLC |
| CD24 | **** | - | - | **** | **** | **** | **** | **** |
| CD30 | - | - | - | - | - | - | - | - |
| CD34 | - | - | - | - | - | - | - | - |
| CD44 | **** | **** | **** | **** | **** | **** | **** | *** |
| CD45 | - | - | - | - | - | - | - | - |
| CD47 | **** | **** | *** | - | **** | - | **** | **** |
| CD87 | - | - | - | - | - | - | - | - |
| CD117 | - | - | **** | - | - | **** | - | **** |
| CD133 | - | - | - | **** | - | - | - | **** |
| CD151 | **** | **** | **** | **** | **** | ** | **** | **** |
| CD184 | - | - | - | - | - | - | - | - |
| CD318 | **** | **** | **** | **** | **** | **** | **** | **** |

*-= not detected, * = <1%, ** = 1-10%, *** = 10-50%, **** = > 50%*

**Table 3.1: Surface marker expression in lung cancer cell lines.** The percent of marker positive cells was determined by flow cytometry.

### *3.2.2 Side Population Cell Analysis in Lung Cancer*

***Detection of Side Population Cells in Lung Cancer Cell Lines***

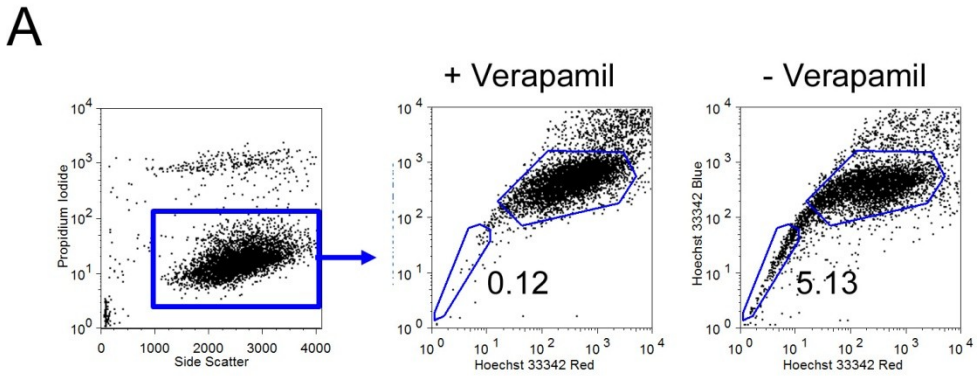
First developed as an assay for identifying hematopoietic stem cells, the Side Population assay, or Hoechst 33342 dye exclusion assay, relies on the differential capacities of stem cells and non-stem cells to efflux the fluorescent Hoechst 33342 dye (Goodell et al., 1996). When incubated in the presence of Hoechst 33342 dye stem cells are identified by flow cytometry as a subpopulation of fluorescently dim cells referred to as SP cells, where as non-stem cells are identified as brightly fluorescent cells referred to as MP cells. The ability of SP cells to remove Hoechst 33342 dye is thought to be due to the expression of efflux pumps such as the ATP-binding cassette transporter ABCG2 (Zhou et al., 2001).

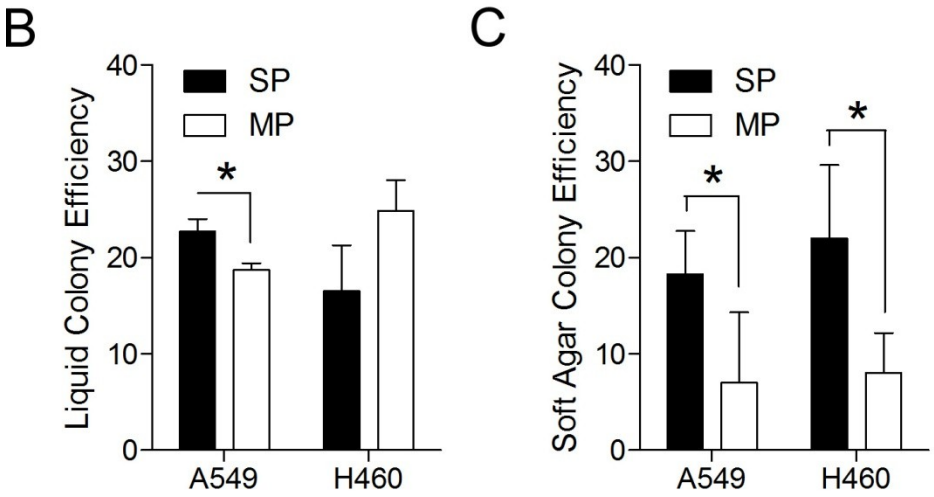
To test if lung cancer possessed subpopulations of SP cells, a panel of NSCLC cell lines were assayed by flow cytometry for Hoechst 33342 dye exclusion, as described in Chapter Two. An aliquot of each cell line tested was incubated with Verapamil to inhibit Hoechst 33342 dye efflux, thus serving as a negative control for SP and MP cell gating. SP cells were detected in all five cell lines tests (A549, H358, H460, H1299 and H2087) with the proportion of SP cells ranging from 0.5% in H1299 cells to 5.6% in H460 cells. However, by decreasing the concentration of Hoechst 33342 from 7.5 to 5 μM the proportion of detected H460 SP cells was increased from 5.6 ± 2.3% to 12.4 ± 4.9%. This suggests that the cellular efflux of Hoechst 33342 as well as the availability of the chromagen during incubation, influence the detection of SP cells and that reducing the amount of the fluorescent dye could contaminate SP cells with non-SP cells that simply haven't accumulated sufficient Hoechst 33342 to shift out of the SP gate.

***Characterization of the Growth Properties of Lung Cancer Side Population Cells***

To assay the growth characteristics of Hoechst 33342 dye effluxing cells, SP and MP A549 and H460 cells were isolated by FACS and cultured in colony formation conditions. In two-dimensional, liquid media culture conditions, the numbers of colonies generated by isolated SP and MP cells were not substantially different (Figure 3.2B). However in soft agar, the production of three-dimensional colonies was nearly two fold greater than the number of colonies generated by MP cells (Figure 3.2C).

The capacity for isolated lung cancer SP and MP cell xenograft production was assayed in immunocompromised mice. Sorted A549 and H460 cells were injected subcutaneously and the tumor growth was monitored weekly by palpation and caliper measurements. After six weeks no substantial difference in the number or size of xenografts was observed between injected SP and MP cells (Figure 3.2D). This data suggest that the Side Population assay is not an efficient means for enriching for highly tumorigenic and clonogenic lung cancer cells.




D

| Cell Type | Cell Number | Tumor Incidence |
|---|---|---|
| A549 SP | 100000 | 1/3 (33%) |
| A549 MP | 100000 | 1/3 (33%) |
| H460 SP | 100000 | 2/6 (30%) |
| H460 MP | 100000 | 3/6 (50%) |

**Figure 3.2: Detection and growth characteristics of lung cancer side population cells**. Example Hoechst dye 33342 exclusion profile of viable, Propidium Iodide negative A549 cells (left panel). As a negative control, A549 cells are treated with Verapamil to inhibit Hoechst dye efflux (center panel) and an SP gate is generated in the lower left quadrant or "side population," and an MP cell gate is generated in the upper right quadrant. The proportion of Hoechst 33342 dye low cells is determined in the absence of Verapamil (right panel) (A). Sorted SP and MP cells from A549 and H460 were assayed in cloning culture conditions, (*$P < 0.05$) (B). The formation of subcutaneous xenografts was assayed in sorted cells however no difference in tumorigenicity was observed between SP and MP cells.

### *3.2.3 Analysis of Lung Cancer Cell Sphere Colonies*

***Generation of Lung Cancer Sphere Colonies***

The culturing of sphere colonies assay is a technique, first developed for the study of the central nervous system, for establishing and maintaining mitotically capable stem cells *in vitro* (Reynolds and Weiss, 1992). The sphere colony assay is a variation of the two-dimensional colony-forming assay, whereby cells are cultured in limiting dilutions on a non-adhesive substrate in serum-free, mitogen defined media, and are induced to form floating three-dimensional spheroid colonies. In this culture condition, normal tissue stem cells are selected for and enriched in clonally derived floating sphere colonies. The capacity for this assay to select for and maintain stem cell populations is attributed to 1) the lack of serum in the media (which often promotes stem cell differentiation), 2) the addition of stem cell mitogens in the media (such as bFGF and EGF) and 3) an increase in supportive cell-cell interaction that more closely mimics a three-dimensional *in vivo* setting.

Cancer stem cells from a variety of tumor types have also been enriched using the sphere colony assay. For example, the conditions that have been used to culture "neurospheres" and "mammospheres" have also been used to select for cancer stem cell sphere colonies in gliomas and breast cancers (Al-Hajj et al., 2003; Liu et al., 2007; Singh et al., 2003). In addition to the stem cell selective attributes ascribed to the sphere colony assay, the selection of anoikis-resistant (anchorage-dependent cell death) tumor cells may also enrich for cells that are more primed for migration and metastatic behavior. Taken together, these attributes provided strong rationale for the sphere colony assay as a means for selecting and culturing putative lung cancer stem cells.

To generate lung cancer sphere colonies, cells from 3 patient NSCLC tumor samples and 12 NSCLC cell lines were plated on non-adherent bacterial dishes in serum free media containing the stem cell mitogens bFGF, EFG and B27 supplement (referred to as $RPMI^+$ media). However after two weeks in this culture condition, few viable cells remained and no sphere colonies were detected. To optimize the survival and production of sphere colonies in culture, various lung cell culture media such as KSFM, HITES, and ALC4 media with and without 5% FBS was tested in NSCLC cell lines for the production of sphere colonies. As a result, floating lung cancer sphere colonies were observed after one week of culture in $RPMI^+$ with 5% FBS (Figure 3.3A and 3.3B). In this culture condition floating sphere colonies were detected in 15/33 NSCLC cell lines with morphology consistent with previously reported tumor sphere colonies (Eramo et al., 2008; Ghods et al., 2007; Liu et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2003).

***Characterization of the Growth Properties of Lung Cancer Sphere Colony Cells***

Compared to equal numbers of cells plated in normal growth conditions, cells cultured in sphere colony conditions grew much slower (Figure 3.3D). This was thought to be due in part to the selection of rare or uncommon anoikis-resistant cells, however fewer dead cells were observed during the selection of sphere colony cells than was expected. Therefore it was hypothesized that sphere colonies are at least partially formed from cell aggregation rather than from a clonal, single cell origin. To test whether spheres were a product of cell aggregation, HCC827 cells were stained with either a fluorescent red (Mito-red) or green (Mito-green) cell tag and cultured in sphere colony

conditions together in a 1:1 ratio. If sphere colonies are clonally derived than they would fluoresce either red or green. However if sphere colonies are a product of cell aggregation, then the resulting spheres would fluoresce red and green. After one week in sphere culture conditions, HCC827 spheres contained both red and green cells indicating that cell these spheres are at least partially derived from cell aggregation (Figure 3.3C). This would suggest that while these cells may be anoikis-resistant, they are not all derived from a highly clonogenic stem-progenitor cell.

To test if tumor sphere derived anoikis-resistant cells are enriched in clonogenic potential, monolayer derived and sphere derived cells were plated in two-dimensional liquid colony formation conditions and soft agar colony formation conditions. Sphere derived cells failed to produce sizable two-dimensional colonies however they were proficient in generating anchorage-independent colonies in soft agar (Figure 3.3E). This is less likely due to the elevated clonogenic capacity of sphere derived cells but rather, the selective advantage for anchorage independent growth in soft agar.

Finally to test for enrichment in tumorigenicity, groups of five NOD/SCID mice were injected with limiting dilutions of H358 cells derived from normal culture conditions and sphere colony culture conditions. Lung tumor cells were injected orthotopically rather than paratopically to assay for potential metastatic growth and to ensure tumor growth in an environment that more appropriately simulates clinical tumor growth. To aid in the detection of tumor cell growth in the lungs of mice, the signal from H358 cells expressing the firefly luciferase enzyme (H358-luc) was detected weekly using bioluminescence imaging (Figure 3.4A). After eight weeks, mice were sacrificed and tumor growth was confirmed by surgical observation. As a result, sphere derived

H358-luc cells were no more capable of generating tumors in mice from limiting dilutions than H358-luc cells derived from normal culture conditions (Figure 3.4B).

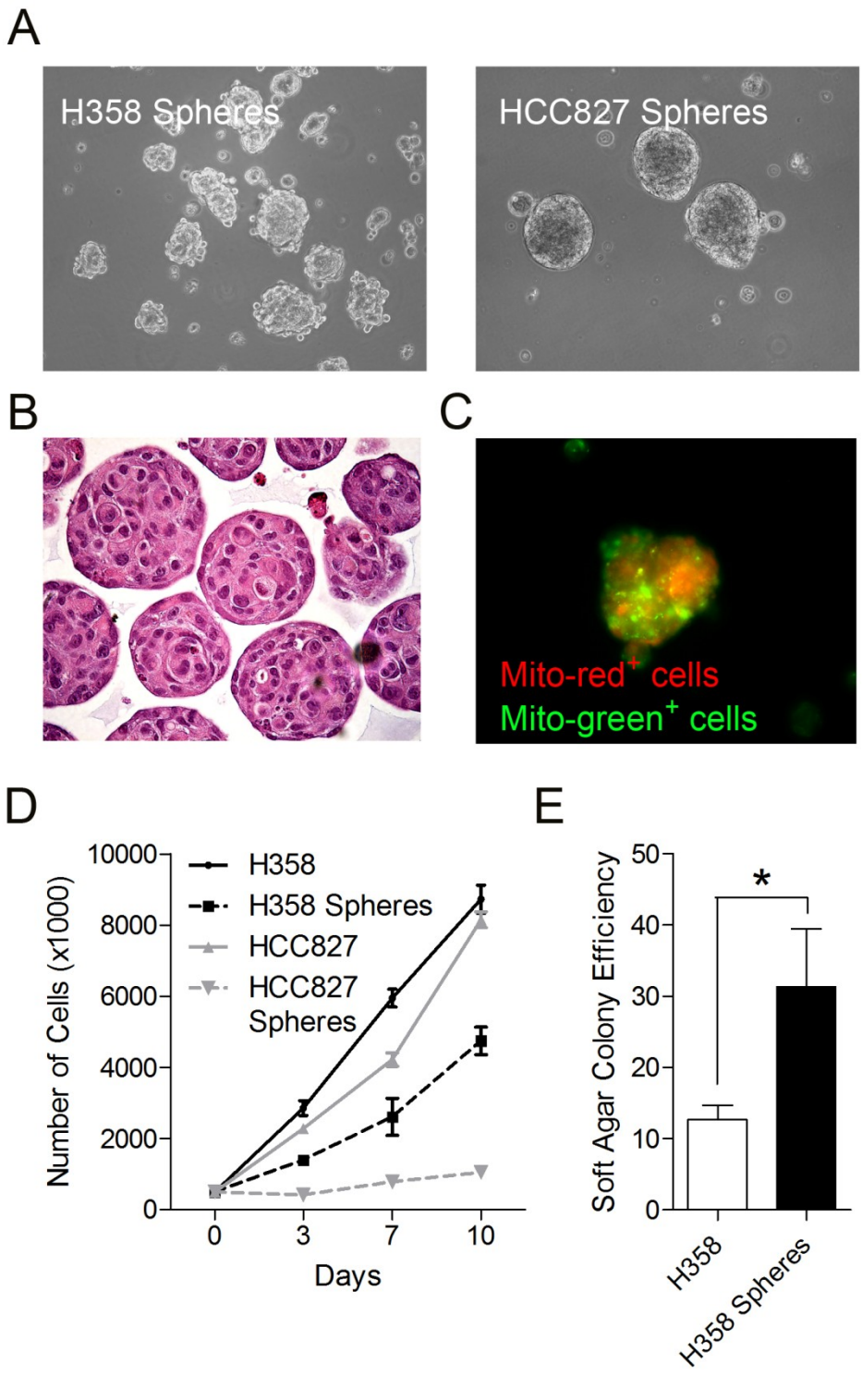
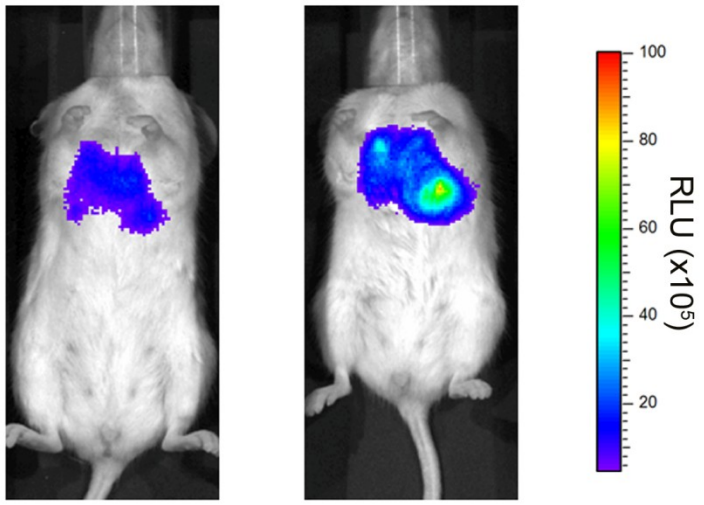

A
H358 Spheres
HCC827 Spheres
B
C
Mito-red+ cells
Mito-green+ cells
D
Number of Cells (x1000)
10000
8000
6000
4000
2000
0
H358
H358 Spheres
HCC827
HCC827 Spheres
0
3
7
10
Days
E
Soft Agar Colony Efficiency
50
40
30
20
10
0
*
H358
H358 Spheres

**Figure 3.3: Formation of lung tumor sphere colonies.** H358 and HCC827 cells cultured in non-adherrent conditions promoted to formation of floating lung tumor sphere colonies after one week (A). Hematoxylin and eosin staining of sectioned HCC827 spheres (B). HCC827 sphere colony generated from a mixture of cells stained with Mito-tracker red and Mito-tracker green demonstrated HCC827 spheres are a product of tumor cell aggregation (C). Cell proliferation in monolayer culture and sphere colony culture conditions was assayed by periodic cell count and found cell proliferation was greatly reduced in sphere colony culture conditions (D). However, sphere derived cells were significantly more clonogenic in soft agar than their monolayer culture derived counterparts (*$P < 0.05$) (E).

A




B

| Cell Type | Cell Number | Tumor Incidence |
|---|---|---|
| H358 Monolayer | 100000 | 3/5 (60%) |
| | 10000 | 2/5 (40%) |
| H358 Sphere | 100000 | 3/5 (60%) |
| | 10000 | 3/5 (60%) |

**Figure 3.4: Tumor initiation from limiting dilutions of H358-luc sphere derived cells.** H358 cell line expressing CMV promoter driven luciferase (H358-luc) were cultured in normal and sphere colony culture conditions and injected in limiting dilutions, into the lower right lung of NOD/SCID mice. Mice were monitored weekly for tumor formation by BLI (A). Tumor derived luminescent signal, quantified as relative light units (RLU), was detected equally in monolayer derived and sphere derived H358-luc cells (B).

### *3.2.4 Analysis of Lung Cancer Cell Aldehyde Dehydrogenase Activity*

***Identification of ALDH$^+$ Tumor Cell Subpopulations in Lung Cancer***

As mentioned in Chapter One, the expression and enzymatic activity of aldehyde dehydrogenases, specifically of ALDH1A1, has been associated with stem cell populations in a variety of organ systems. In addition, ALDH activity has been shown to identify cancer stem cells in a variety of tumor types (Table 1.1) (Cheung et al., 2007; Ginestier et al., 2007; Huang et al., 2009b). Therefore, it was hypothesized that lung cancer cells with elevated ALDH would be enriched in cancer stem cell activity.

To determine if lung tumors harbored a population of ALDH$^+$ cells, the Aldefluor assay was used to detect and quantify the percentage of ALDH$^+$ cells in a panel of 11 patient NSCLC tumor samples, 45 NSCLC lines, and 7 SCLC lines. Using the Aldefluor assay, rare ALDH$^+$ tumor cells could be detected by flow cytometry and heterogeneous expression of lung tumor ALDH could also be observed by microscopy (Figure 3.5A and 3.5B). In some cell lines, the detection of ALDH$^+$ cell subpopulations was effected by culture conditions, primarily culture confluence (Figure 3.6). This could be due to the growth suppressive environment a fully confluent cell culture might create, such as the depletion of media nutrients or a restricted area for growth. Recently, Pine et al. have shown that tumor cell asymmetric division, a process involved in stem cell differentiation, is altered by microenvironmental conditions (Pine et al., 2010). Therefore, to ensure consistency between analyses, flow cytometry was formed on sub-confluent cultures in log growth phase.

A subpopulation of ALDH$^+$ cells was detected by flow cytometry in 42/45 NSCLC cell lines tested (comprising 0.1% to 38% of the total cell population) and in the

epithelial component of all eleven NSCLC patient tumors tested (2% to 30% of the total $EpCam^+$ cell population) (Table 3.2). Because disassociated primary tumor samples may contain $ALDH^+$ mesenchymal or hematopoietic stem cells, reactivity to the epithelial antibody EpCam was used to define $ALDH^+$ epithelial tumor cells by flow cytometry (Figure 3.5C). Interestingly most SCLC cell lines tested were enriched in $ALDH^+$ cells.

To investigate the role of ALDH protein family members in lung tumor cell ALDH activity, Pearson correlation analysis between tumor cell ALDH activity, measured by flow cytometry, and mRNA levels of 18 ALDH isozymes (measured by Affymetrix U133AB microarrays) found the strongest correlation with *ALDH1A1* ($r^2$ = 0.63) (Table 3.2). This result is consistent with previous reports by Moreb et al. that suggest lung cancer ALDH activity detected by flow cytometry is preferentially driven by ALDH1A1 (Moreb et al., 2008; Moreb et al., 2007).

Because markers for lung cancer stem cells have previously been validated in transgenic mouse models of lung cancer, we set out to determine if mouse lung tumors harbored a subpopulation of $ALDH^+$ cells that express mouse cancer stem cell markers. To test this, the Aldefluor assay was performed on early passage Ink4a/$ARF^{-/-}$ oncogenic K-ras-induced mouse lung adenocarcinoma cells. Flow cytometry revealed an $ALDH^+$ subpopulation (2 ± 0.4%) and when co-stained with a fluorescently conjugated antibody for the murine stem cell marker Sca-1, $ALDH^+$ tumor cells were observed to be greatly enriched for Sca-$1^+$ cells (74 ± 1.9%), whereas the proportion of Sca-$1^+$ tumor cells was not enriched in the $ALDH^-$ bulk cell population (25 ± 5.2%) (Figure 3.7A). These results are consistent with the concept that ALDH activity also marks a subpopulation of stem-like cells in transgenic murine K-ras/Ink4/$Arf^{-/-}$ lung tumors. To determine if these

$ALDH^+$ murine tumor cells were BASCs, the expression of the BASC markers SPC and CCSP were assayed by immunocytochemistry. As a result, cytoplasmic SPC expression was observed in nearly all tumor cells however CCSP was not detected, suggesting that these tumor cells may have been derived from a transformed type 2 pneumocyte progenitor cell rather than an oncogenic BASC (Figure 3.7B).

***Characterization of the Growth Properties of $ALDH^+$ Lung Cancer Cells***

To evaluate to growth properties of lung cancer cells with elevated ALDH activity, $ALDH^+$ and $ALDH^-$ cells were sorted and purified by FACS (Figure 3.8). Sorted cells were subsequently assayed for clonogenic growth in soft agar or liquid media conditions. H2009, H1299, and H2087 $ALDH^+$ cells produced significantly more robust anchorage-independent soft agar colonies compared to their more abundant $ALDH^-$ counterparts. Similarly, isolated H2009 and H358 $ALDH^+$ lung cancer cells generated significantly more two dimensional colonies in liquid media compared to their bulk $ALDH^-$ cell counterparts (colony formation efficiencies: $25 \pm 3.6\%$ vs. $9 \pm 2.9\%$ and $40 \pm 3.5\%$ vs. $11 \pm 0.6\%$ respectively, $P < 0.005$) (Figure 3.9A). Interestingly, H2009 $ALDH^+$ lung cancer cells generated more colonies with a densely packed morphology, whereas colonies generated from $ALDH^-$ lung cancer cells were different, forming mostly diffuse and abortive colonies (Figure 3.9B). The morphologic differences of the colonies derived from $ALDH^+$ and $ALDH^-$ cells are reminiscent of cancer stem cell enriched "holoclones" and non enriched "meroclones" and "paraclones" found in prostate cancer (Li et al., 2008).

Upon isolation and culture in normal growth conditions, H358 $ALDH^+$ lung cancer cells were observed to initially proliferate more rapidly than $ALDH^-$ cells, although this proliferative difference was less pronounced after a few cell divisions (Figure 3.10A). This difference in proliferative capacity was confirmed by cell cycle analysis which revealed a greater proportion of $ALDH^+$ cells in replicative S-phase than $ALDH^-$ cells in S-phase. Additionally H358 $ALDH^-$ cells appeared to have a greater accumulation of non cycling G1/G0-phase cells, supporting the observation that $ALDH^-$ cells initially cycle less frequently than ALDH cells (Figure 3.10B).

To assay for self-renewal in sorted cells *in vitro*, $ALDH^+$ and $ALDH^-$ lung tumor cells were cultured in normal growth conditions for up to two weeks. Cultures generated from isolated $ALDH^+$ and $ALDH^-$ H358 cells were assayed for ALDH by flow cytometry. As a result, the cultures derived from $ALDH^+$ lung tumor cells reproduced the parental distribution of $ALDH^+$ and $ALDH^-$ cells, whereas the cultures derived from $ALDH^-$ lung tumor cells primarily generate $ALDH^-$ cells (Figure 3.11A). The proportion of Hoechst 33342 dye effluxing SP cells was also used as a surrogate measure of self-renewing cancer stem cells in sorted $ALDH^+$ and $ALDH^-$ H1299 cells (Figure 3.11B) (Ho et al., 2007). Again, only $ALDH^+$ H1299 derived cultures could generate both SP and non-SP cells whereas cultures derived from $ALDH^-$ H1299 cells were restricted in their ability to generate SP cells (Figure 3.11C). These data indicate that the $ALDH^+$ cell fraction is enriched with cells that can regenerate ALDH and SP tumor cell heterogeneity *in vitro*, a function indicative of tumor self-renewal and differentiation.

To test the tumorigenic capacity of the putative $ALDH^+$ lung cancer stem cell population, one million isolated $ALDH^+$ and $ALDH^-$ cells from H358 and H1299 tumor

cell lines were injected subcutaneously into NOD/SCID mice. The tumors generated from $ALDH^+$ H1299 and H358 cells were significantly larger and grew faster compared to tumors from their $ALDH^-$ counterparts (two-way ANOVA test: $P = 0.016$ and $P = 0.003$ respectively) (Figure 3.12).

As a more stringent measure of tumorigenic capacity, xenograft formation from limiting dilutions of sorted $ALDH^+$ and $ALDH^-$ lung cancer cells was assayed. Groups of four NOD/SCID mice were given a subcutaneous injection of $10^5$, $10^4$, or $10^3$ sorted $ALDH^+$ or $ALDH^-$ H358 cells and tumor formation was scored after eight weeks. To aid in the early detection of small tumors, the luciferase driven luminescence from H358-luc cells was detected weekly using bioluminescence imaging. After eight weeks, mice were sacrificed and tumors were excised. As a result, H358 $ALDH^+$ cells were found to have a greater frequency of tumor formation, particularly at low numbers of injected cells compared to their $ALDH^-$ counterparts, suggesting $ALDH^+$ cells are enriched in tumor initiating capacity (Figure 3.13). To confirm the tumorigenic properties of $ALDH^+$ cells in patient samples, $EpCam^+ALDH^+$ and $EpCam^+ALDH^-$ cells from two patient samples, LT6 and LT7 were isolated by FACS and injected subcutaneously into NOD/SCID mice. Tumor growth was monitored weekly for up to ten weeks by palpation and incidence of tumor formation was finally scored after surgical observation and excision of the xenograft. As a result, only mice injected with $ALDH^+$ patient tumor cells had detectable tumors, whereas equal dilutions of $ALDH^-$ cells failed to generate xenografts (Table 3.4).

To determine if xenografts formed from $ALDH^+$ patient lung cancer cells could recapitulate ALDH heterogeneity, the Aldelfuor assay was performed on disassociated LT7 xenograft cells. Flow cytometric analysis of $EpCam^+$ cells revealed a xenograft

$ALDH^+$ tumor cell population proportional to the $ALDH^+$ cell population found in the original patient tumor (Figure 3.14B). Interestingly, the xenograft was enriched in $EpCam^+$ cells as compared to the patient tumor. In addition, re-analysis of tumors derived from sorted H358 cells revealed $ALDH^+$ tumors contained a proportion of $ALDH^+$ cells similar to their unsorted parental H358 cells, whereas the much smaller tumors derived from $ALDH^-$ cells possessed a very small residual $ALDH^+$ cell population (Figure 3.14A). These data suggest that isolated $ALDH^+$ lung cancer cells can recapitulate ALDH tumor cell heterogeneity *in vivo* and are thus are better capable of self-renewal and differentiation as compared to $ALDH^-$ lung cancer cells.

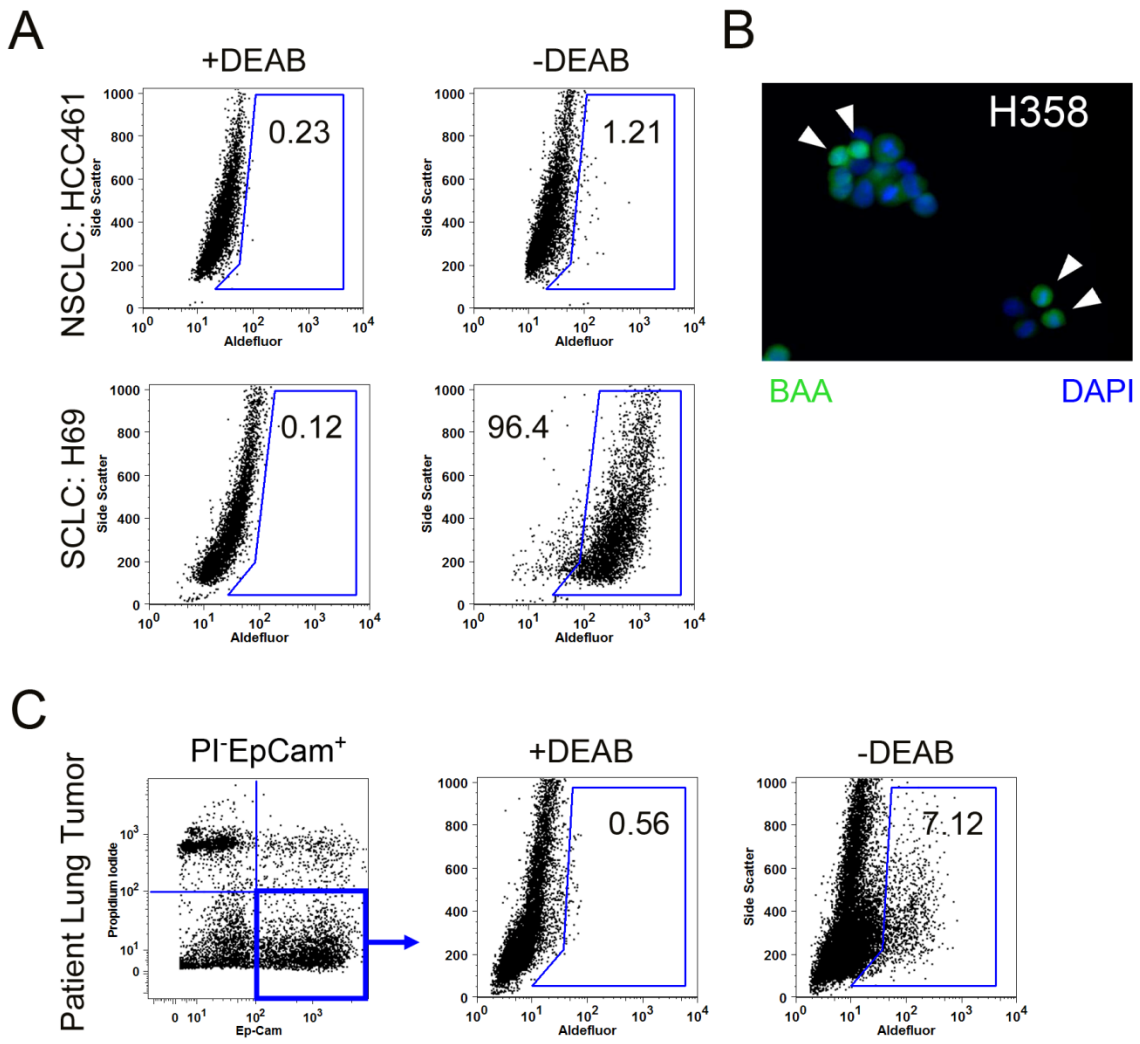



**Figure 3.5: ALDH in lung cancer cell lines and resected NSCLC patient tumors.** Example Aldefluor analysis of a NSCLC and SCLC cell line. Baseline fluorescence was established by inhibiting ALDH activity with DEAB (left panels) and used to generate a gate that will identify $ALDH^+$ cells in lung cancer cells that have not been incubated with DEAB (right panels) (A). H358 $ALDH^+$ that have accumulated the fluorescent BAA product cells could be observed by microscopy (white arrows)(B). Example analysis of a resected lung adenocarcinoma using the Aldefluor assay. Viable epithelial tumor cells were gated as PI negative and Ep-Cam positive and were analyzed for ALDH (C).

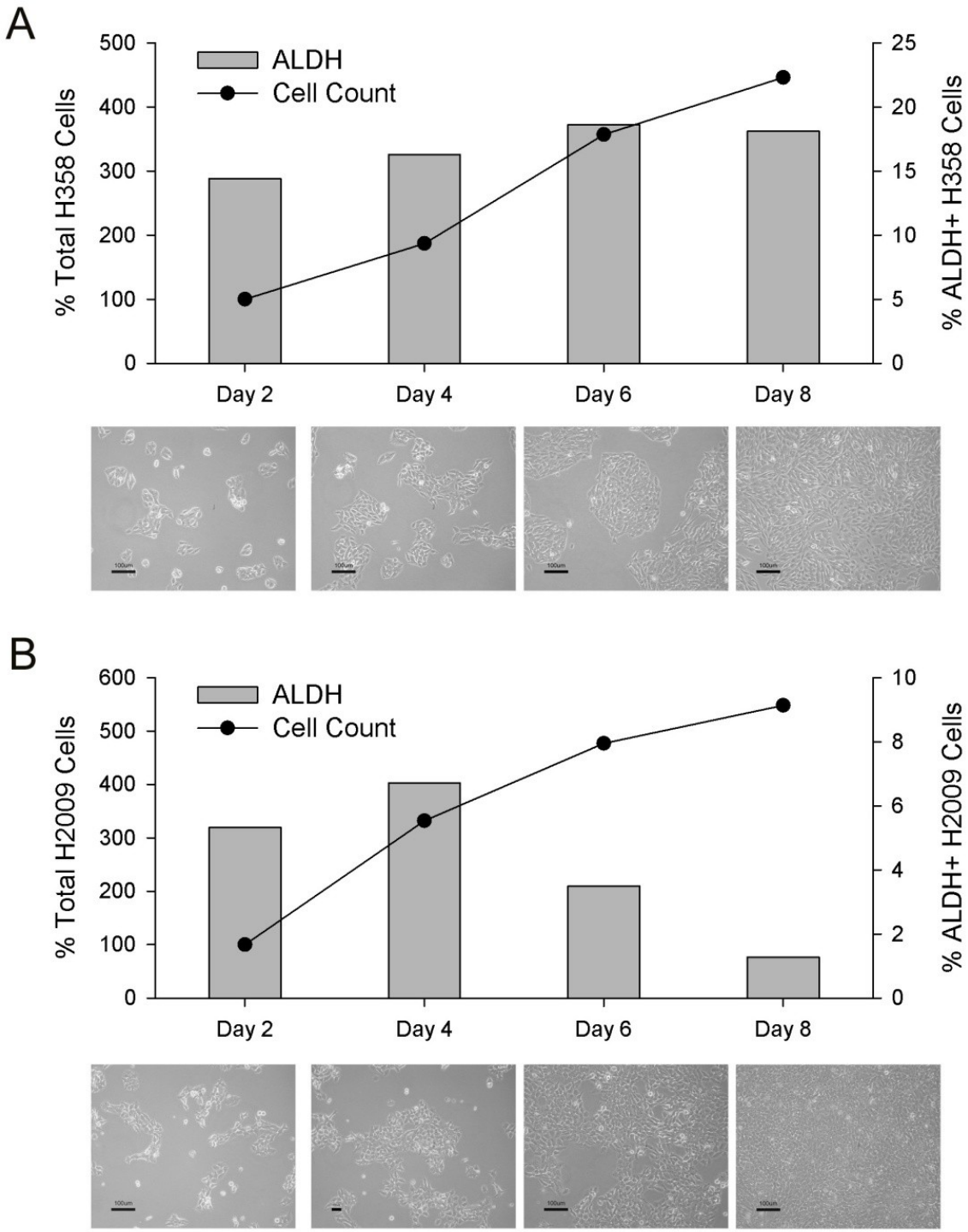



**Figure 3.6: Effect of cell density on H358 and H2009 ALDH$^+$ cell subpopulation.** Cells were counted and assayed for their ALDH$^+$ cell proportion every two days (A and B, upper panels). Cells density was observed prior to harvest by microscopy (A and B, lower panels). Although the proportion of H358 ALDH$^+$ cells was not affected by cell density, a reduction in H2009 ALDH$^+$ cells was commensurate with increased cell density.

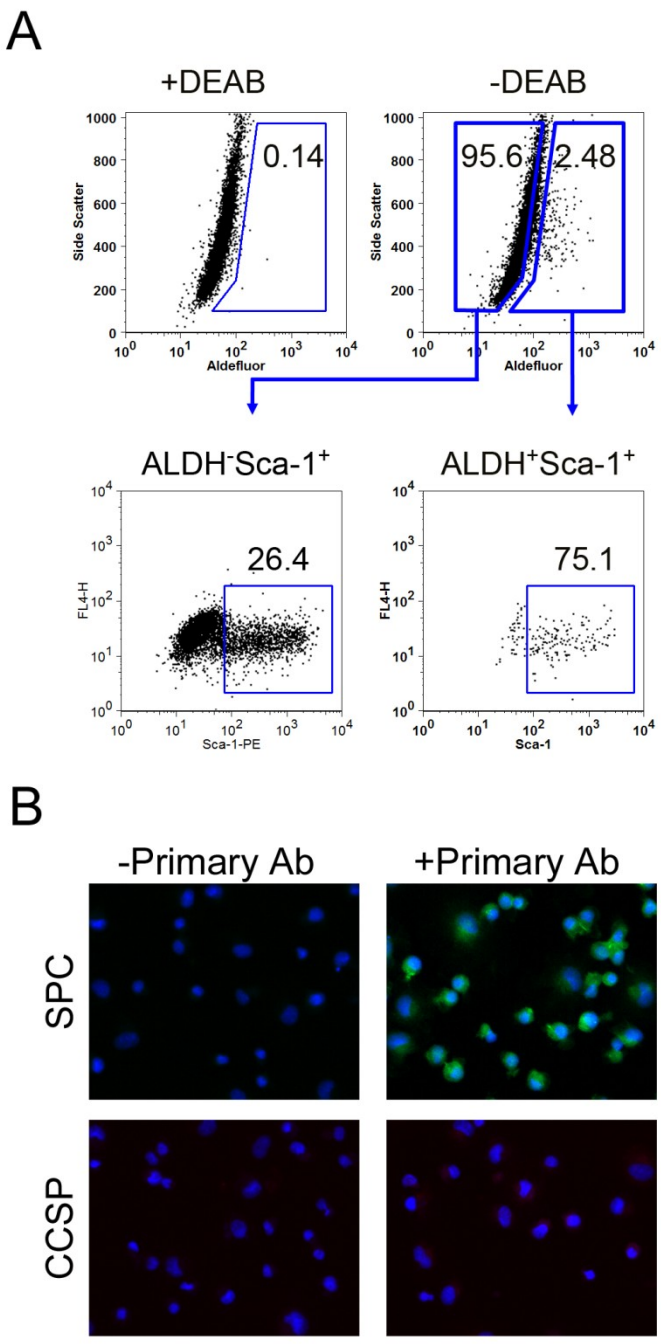



**Figure 3.7: Lung stem cell markers in murine adenocarcinoma cells.** Aldefluor analysis of Ink4a/ARF$^{-/-}$ K-ras induced murine adenocarcinoma cells identified an ALDH$^{+}$ tumor cell population that was enriched in Sca-1 expression, compared to the bulk ALDH$^{-}$ tumor cells (A). Murine adenocarcinoma cells unstained with primary antibody were used as a negative control for IHC. Expression of the type II pneumocyte marker SPC (SPC in green, DAPI in blue) was detected in all tumor cells however the Clara cell marker CCSP was not detected (B).

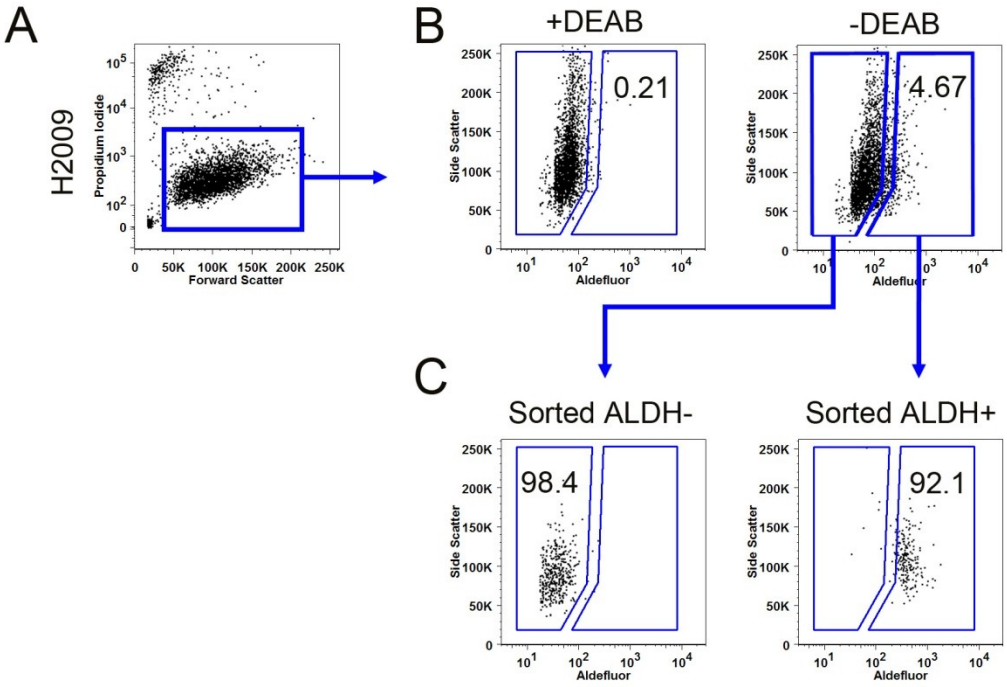



**Figure 3.8: Enrichment of ALDH$^{+/-}$ tumor cell subpopulations by FACS.** Example sort for ALDH$^{+}$ and ALDH$^{-}$ cells from H2009 cells. Viable H2009 cells were gated as Propidium Iodide negative and analyzed for ALDH (A). A sample incubated with DEAB was used to generate the ALDH$^{+}$ and ALDH$^{-}$ gate for sorting (B, left and right panel). After sorting, the purity of the sorted ALDH$^{-}$ and ALDH$^{+}$ H2009 cells was analyzed by flow cytometry using the same gate layout for the initial sort (C, left and right panel). In this example, samples were greatly enriched for ALDH$^{+}$ and ALDH$^{-}$ cells after FACS (> 90% in each case).

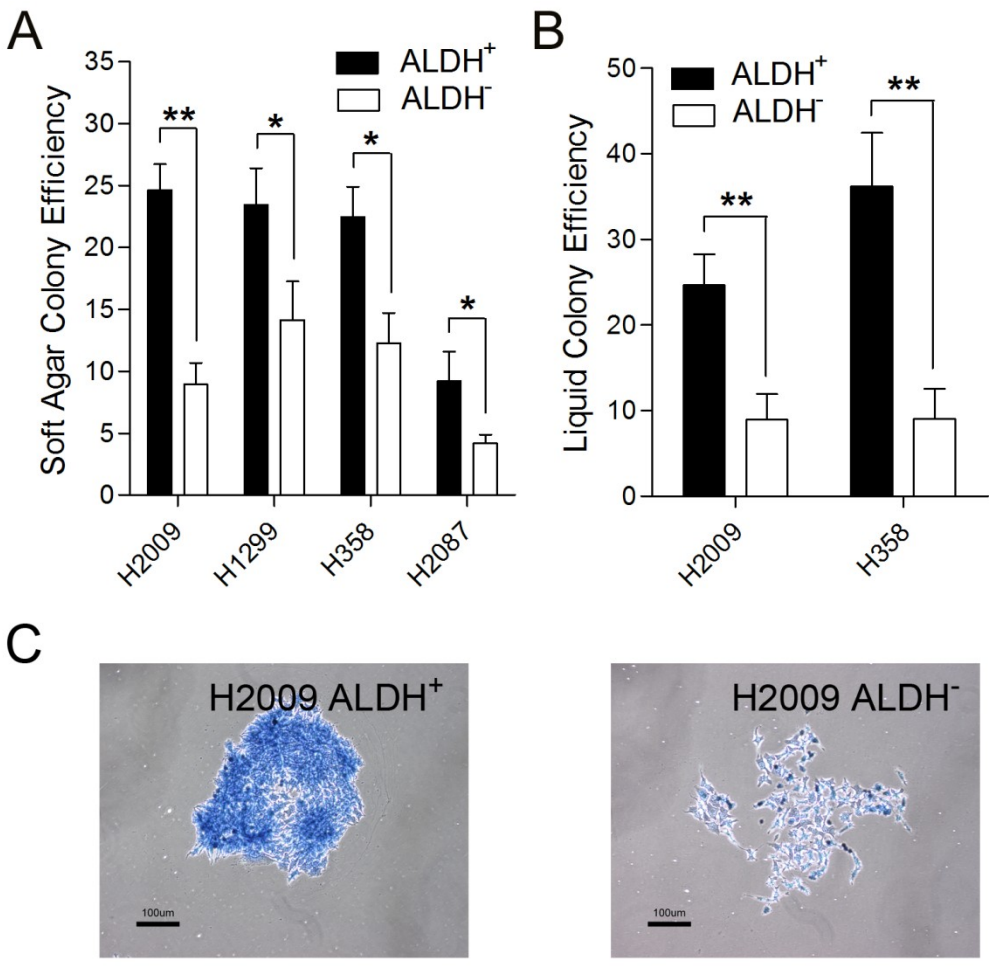



**Figure 3.9: Clonogenicity of ALDH$^+$ lung cancer cells *in vitro*.** ALDH$^+$ and ALDH$^-$ lung cancer cells were isolated by FACS and 500 sorted cells were plated in 0.33% agar in 12 well dishes. After 3 weeks, soft agar colonies were stained with crystal violet dye and colonies with >50 cells counted (A, $n = 4$, $*P < 0.05$, $**P < 0.01$). Colony formation efficiencies of sorted H358 and H2009 cells were determined in liquid culture after two weeks of growth from limiting dilutions of plated cells (500, 1000 and 2000 cells) (B). Microscopy of H2009 ALDH$^+$ and ALDH$^-$ cell derived colonies stained with methylene blue. ALDH$^+$ cells produced dense "holoclone" colonies (left panel) whereas ALDH$^-$ cells generated primarily diffuse "meroclone" colonies (right panel) (C).

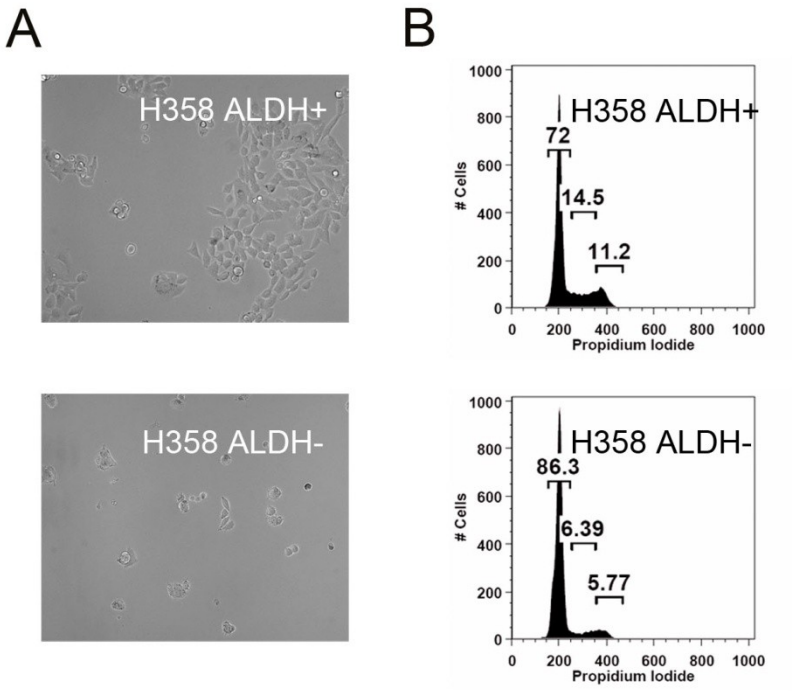



**Figure 3.10: Proliferation of isolated ALDH$^+$ and ALDH$^-$ lung cancer cells *in vitro*.** Sorted H358 ALDH$^+$ and ALDH$^-$ cells were cultured in normal growth conditions for one week and imaged by light microscopy (A). Isolated H358 ALDH$^+$ and ALDH$^-$ cells were fixed after sorting and analyzed for their DNA content by flow cytometry. The percent of total cells in G1/G0, S, and G2/M-phase are displayed above their corresponding peaks on the cell cycle histogram (B). ALDH$^+$ cells contain a greater proportion of cells in S and G2/M-phase division than ALDH$^-$ cells, indicating ALDH$^+$ cells more frequently divide than ALDH$^-$ cells.

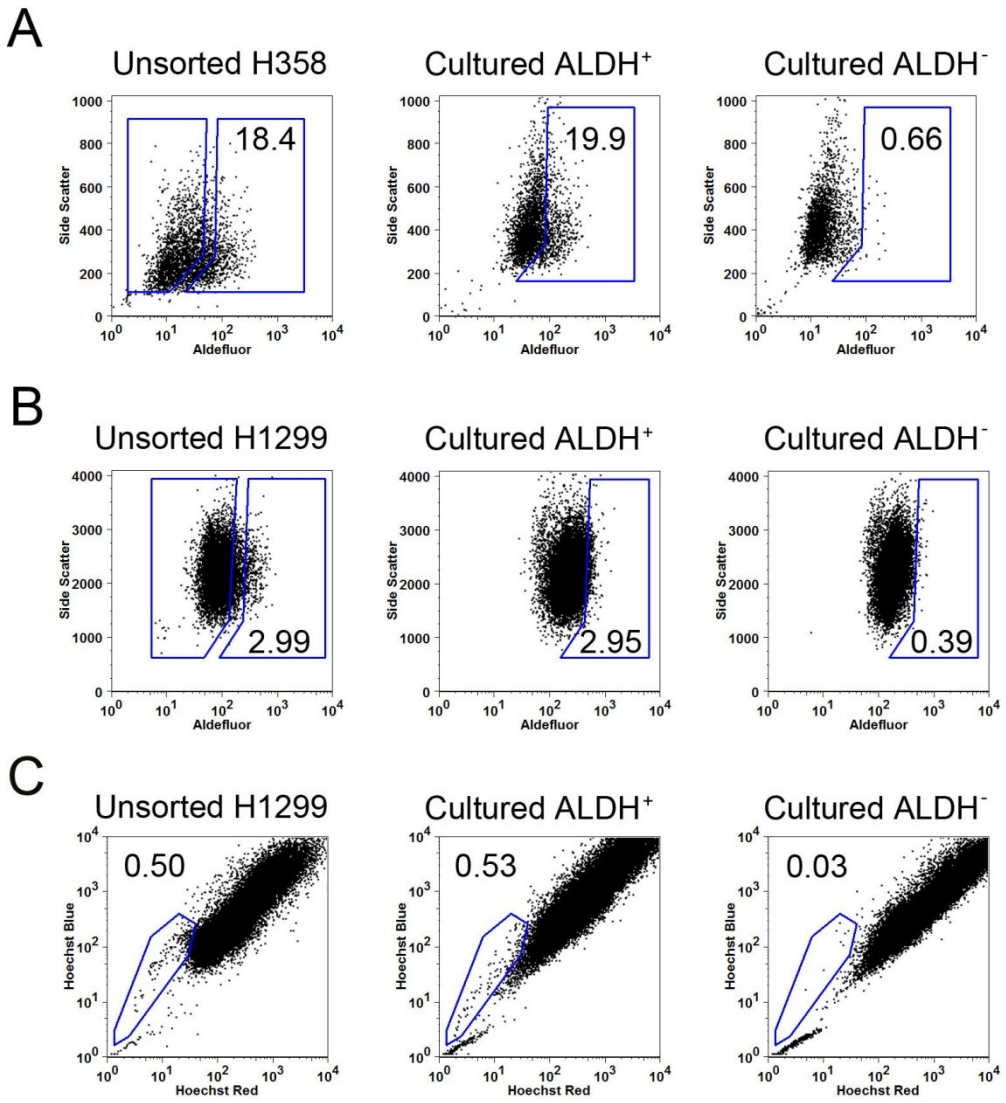



**Figure 3.11: Self-renewal of ALDH$^+$ lung cancer cells *in vitro*.** ALDH$^+$ and ALDH$^-$ cells were isolated by FACS and cultured in normal growth conditions for two weeks then reanalyzed for proportion of ALDH$^+$ cells by flow cytometry (A and B). In each case, sorted ALDH$^+$ cells generated both ALDH$^+$ and ALDH$^-$ cells, whereas cultures derived from ALDH$^-$ cells remained mostly ALDH$^-$. In addition, unsorted H1299 and cultures derived from ALDH$^+$ and ALDH$^-$ H1299 cells were incubated with Hoechst 33342 dye to detect the presence and proportion of SP cells by flow cytometry and ALDH$^+$ cells had ~10 fold higher number of SP cells (C).

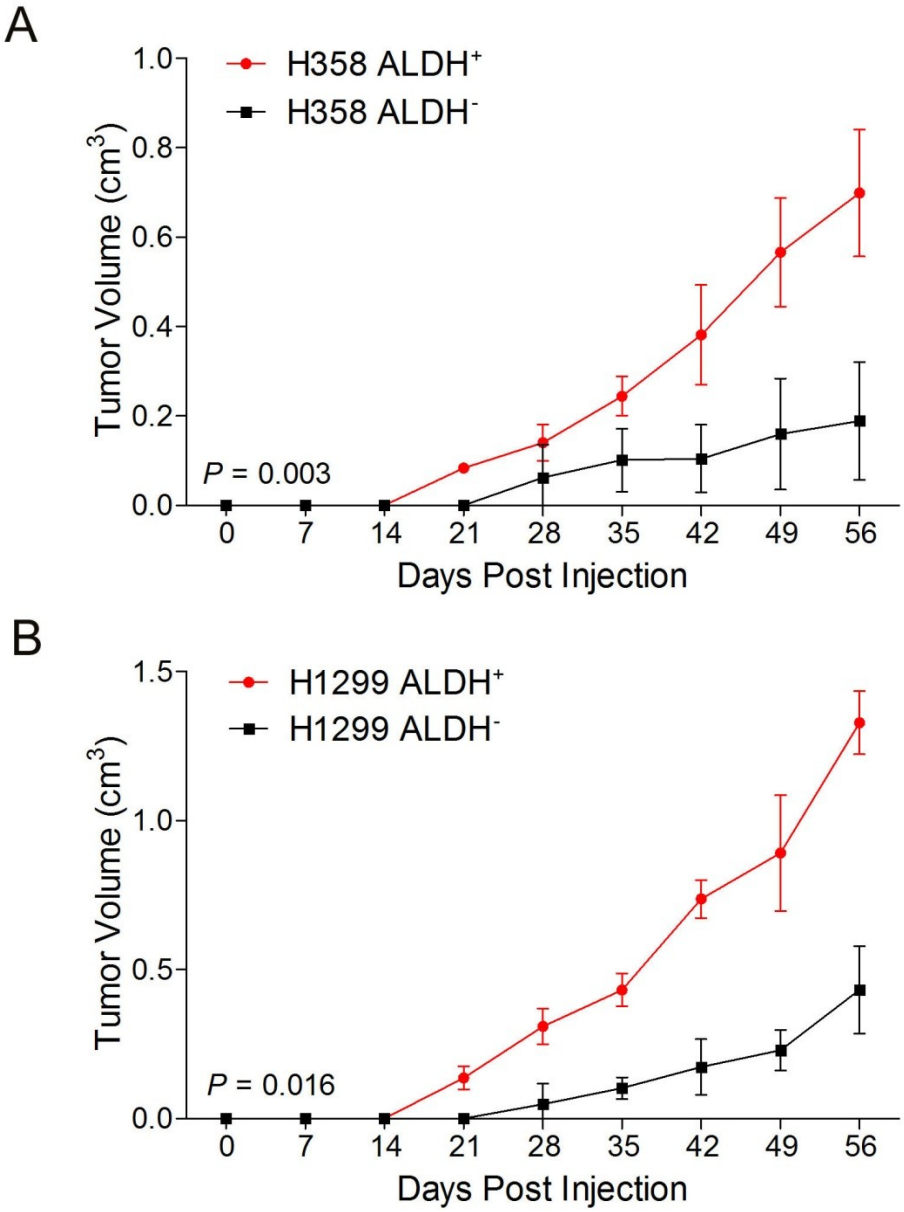



**Figure 3.12: Tumor growth of isolated ALDH$^+$ and ALDH$^-$ lung cancer cells.** Tumor growth curves were generated from $10^6$ isolated ALDH$^+$ and ALDH$^-$ H358 (A) and H1299 cells (B) injected into the flanks of five NOD/SCID mice. In each cell line, ALDH$^+$ cells generated statistically larger xenografts over time compared to xenografts derived from ALDH$^-$ cells.

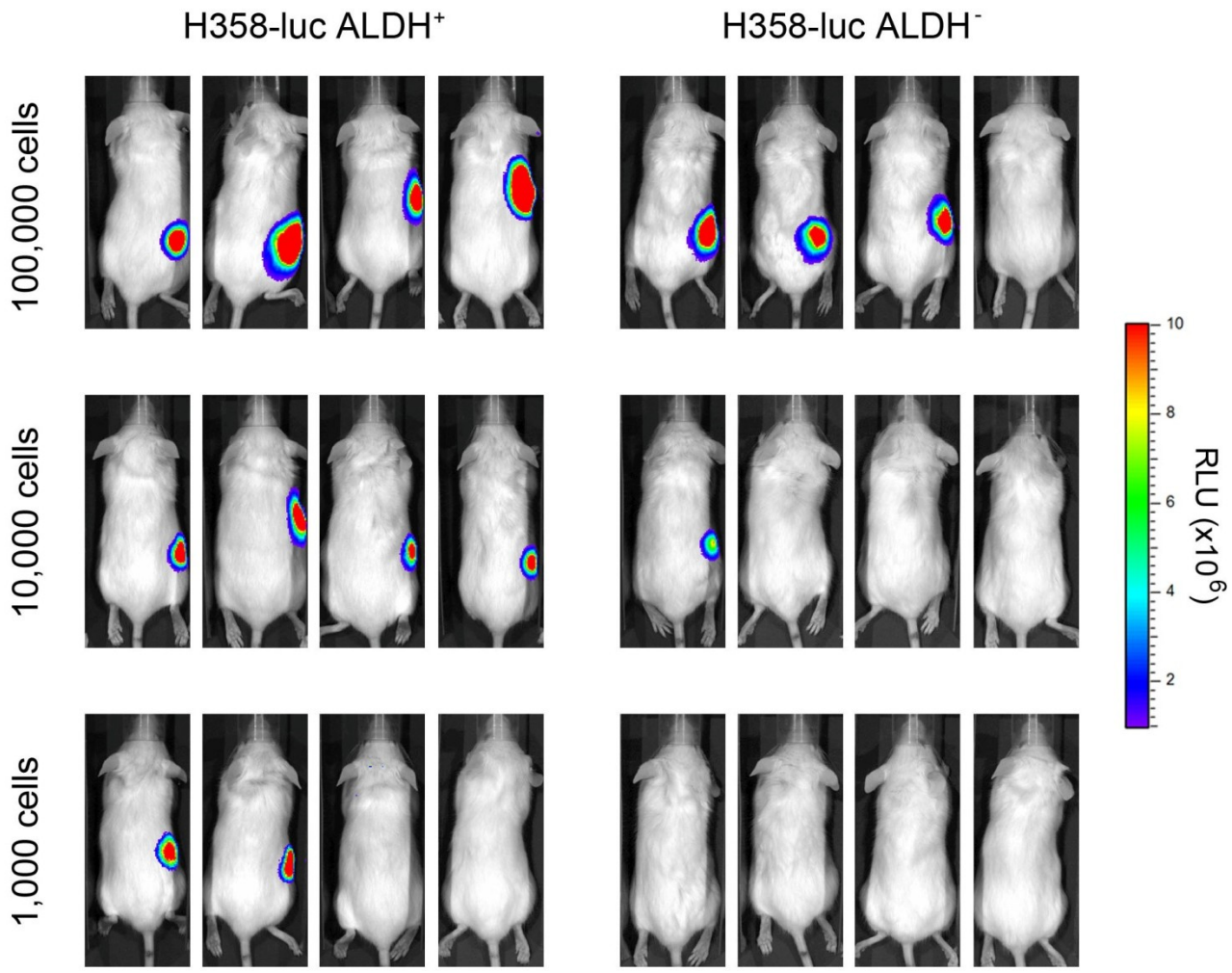



**Figure 3.13: Tumor initiation from limiting dilutions of sorted H358-luc cells.** $ALDH^+$ and $ALDH^-$ cells were sorted from the H358 cell line expressing CMV promoter driven luciferase (H358-luc) and injected, in serially limiting dilutions, subcutaneously into the right flank of female NOD/SCID mice. Mice were monitored weekly for tumor formation by palpation and BLI. Tumor derived luminescent signal, quantified as relative light units (RLU), was detected in most mice injected with H358-luc $ALDH^+$ cells, whereas tumor derived luminescence was detected in less than half of the mice injected with H358-luc $ALDH^-$ cells. The most striking difference in tumor formation between $ALDH^+$ and $ALDH^-$ cells was observed in mice injected with $10^5$ or less cells.

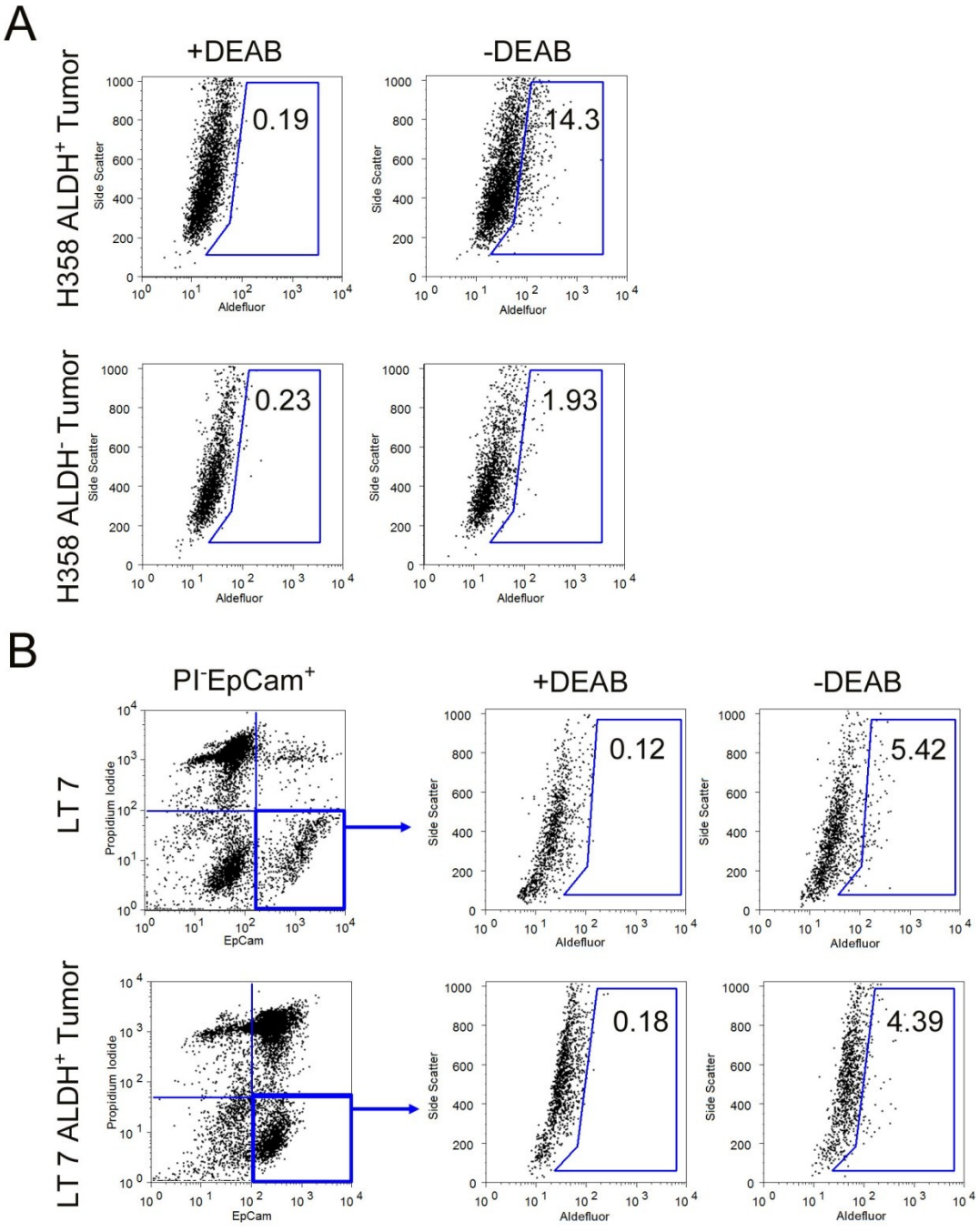



**Figure 3.14: Self-renewal of ALDH$^+$ lung cancer cells *in vivo*.** H358 ALDH$^+$ and ALDH$^-$ cells were isolated by FACS and injected into NOD/SCID mice. The resulting tumors were assayed for ALDH activity (A). Similarly, ALDH$^+$ and ALDH$^-$ cells from EpCam$^+$ patient tumors were injected in mice. The resulting tumors from ALDH$^+$ cells were assayed for the presence of viable EpCam$^+$ALDH$^+$ cells (B).

| Samples | Histotype | %ALDH$^+$ |
|---|---|---|
| LT 1 | SCC | 6.6 |
| LT 2 | AC | 7.1 |
| LT 3 | AC | 9.9 |
| LT 4 | AC | 17 |
| LT 5 | AC | 19 |
| LT 6 | AC | 2.4 |
| LT 7 | SCC | 5.4 |
| LT 8 | SCC | 2.2 |
| LT 9 | AC | 28 |
| LT 10 | SCC | 30 |
| LT 11 | AC | 8.7 |
| Calu-6 | NSCLC-NOS | 0.0 |
| H2126 | LC | 0.0 |
| H2085 | AC | 0.0 |
| H23 | AC | 0.1 |
| H1770 | LCNE | 0.3 |
| HCC15 | SCC | 0.4 |
| HCC461 | AC | 0.5 |
| H1355 | AC | 0.6 |
| H460 | LC | 0.7 |
| H2887 | NSCLC-NOS | 0.8 |
| H157 | SCC | 0.8 |
| H1648 | AC | 0.8 |
| H2106 | LCNE | 0.9 |
| HCC95 | SCC | 1.1 |
| H1975 | AC | 1.2 |
| H2122 | AC | 1.3 |
| H2073 | AC | 1.4 |
| H1373 | AC | 2.1 |
| H1993 | AC | 2.4 |
| HCC2109 | AC | 2.7 |
| HCC1195 | AC | 2.9 |
| H1299 | LC | 2.9 |
| H1155 | LC | 3.0 |
| Calu-1 | NSCLC-NOS | 3.5 |
| HCC515 | AC | 3.7 |
| HCC4006 | AC | 3.9 |
| H441 | AC | 4.4 |
| H2009 | AC | 4.4 |
| H820 | BAC | 5.6 |
| H1666 | BAC | 7.0 |
| HCC827 | BAC | 7.3 |
| H1395 | AC | 7.4 |
| H1792 | AC | 7.7 |
| HCC44 | AC | 8.6 |
| H226 | ME | 8.6 |
| H1869 | SCC | 9.7 |
| H2347 | AC | 11 |
| H2052 | ME | 11 |
| H3255 | AC | 13 |
| H2087 | AC | 14 |
| HCC193 | AC | 16 |
| H358 | BAC | 17 |
| HCC2429 | NSCLC-NOS | 18 |
| H1819 | AC | 38 |
| H1693 | AC | 38 |
| H1184 | SCLC | 2.6 |
| H82 | SCLC | 4.8 |
| H2171 | SCLC | 65 |
| H526 | SCLC | 79 |
| H146 | SCLC | 87 |
| H889 | SCLC | 91 |
| H69 | SCLC | 92 |

*AC: Adenocarcinoma, BAC: Bronchioalveolar Carcinoma, LC: Large Cell Carcinoma, NSCLC-NOS: Non-Small Cell Lung Cancer - Not Other Specified, LCNE: Large Cell Neuroendocrine Carcinoma, SCLC: Small Cell Lung Cancer, SCC: Squamous Cell Carcinoma*

**Table 3.2: Proportion of ALDH$^+$ cells in lung cancer cell lines and primary patient samples.** The percent of ALDH$^+$ cells in patient resected lung tumors LT1-LT11 were determined from viable EpCam$^+$ cells populations.

| ALDH Isozyme | Average Correlation Coefficient ($r^2$) |
|---|---|
| *ALDH1A1* | 0.63 |
| *ALDH1B1* | 0.23 |
| *ALDH3B2* | 0.18 |
| *ALDH5A1* | 0.16 |
| *ALDH8A1* | 0.16 |
| *ALDH6A1* | 0.15 |
| *ALDH9A1* | 0.14 |
| *ALDH2* | 0.13 |
| *ALDH16A1* | 0.1 |
| *ALDH1L1* | 0.1 |
| *ALDH1A2* | -0.03 |
| *ALDH3A1* | -0.19 |
| *ALDH4A1* | -0.22 |
| *ALDH3B1* | -0.26 |
| *ALDH7A1* | -0.27 |
| *ALDH1A3* | -0.32 |
| *ALDH18A1* | -0.32 |
| *ALDH3A2* | -0.65 |

**Table 3.3: Correlation of ALDH activity and ALDH isozyme expression.** Pearson correlation analysis between the percent of $ALDH^+$ cells measured by flow cytometry and ALDH isozyme transcripts measured by microarray indicate expression of *ALDH1A1* was most closely associated with ALDH activity in lung cancer cell lines. Values in red indicate a positive correlation between gene expression and ALDH activity. Green $r^2$ values indicate a negative correlation.

| Cell Type | Cell Number | Tumor Incidence |
|---|---|---|
| H358 ALDH$^+$ | 100000 | 4/4 (100%) |
| | 10000 | 4/4 (100%) |
| | 1000 | 2/4 (50%) |
| H358 ALDH$^-$ | 100000 | 3/4 (75%) |
| | 10000 | 1/4 (25%) |
| | 1000 | 0/4 (0%) |
| LT 6 ALDH$^+$ | 100000 | 1/3 (33%) |
| LT 6 ALDH$^-$ | 100000 | 0/3 (0%) |
| LT 7 ALDH$^+$ | 50000 | 2/3 (66%) |
| LT 7 ALDH$^-$ | 50000 | 0/3 (0%) |

**Table 3.4: Tumor formation from limiting dilutions of sorted ALDH$^+$ and ALDH$^-$ lung cancer cells.**

### 3.3 Discussion

In this study, lung cancer cell lines and patient lung tumors were interrogated for tumor cell subpopulations with enhanced clonogenic, tumorigenic and stem-like growth features. To aid in the detection and isolation of prospective lung cancer stem cells, markers and growth conditions previously described to select for cancer stem cells in other tumors, were employed to identify and isolate putative lung cancer stem cells.

The first strategy for isolating lung cancer stem cells described in this chapter was the detection of surface stem cell associated markers expressed in a panel of lung cancer cell lines. It is hypothesized that cancer stem cells only make up a small fraction of the total tumor cell population. According to some estimates in leukemia, cancer stem cells may comprise less than one percent of the total cancer cell population (Bonnet and Dick, 1997). The capacity for flow cytometric analysis to analyze the size, shape and fluorescent emissions of bound antibodies on thousands of cells per second provide an appropriate means to search for rare tumor cell populations. After profiling for the expression 12 putative cancer stem cell markers in eight different lung cancer cell lines, only one marker in one cell line clearly selected for a tumor cell subpopulation. H358 cells possessed an isolatable $CD151^+$ subpopulation; however these tumor cells were not enriched in clonogenic capacity, and thus unlikely to be enriched in lung cancer stem cells.

During the course of this study, Eramo et al. reported that lung tumors possessed rare, highly tumorigenic, stem-like $CD133^+$ tumor cells (Eramo et al., 2008). However in a panel of lung cancer cell lines CD133 expression did not select for a tumor cell subpopulation. This would suggest that either a cancer stem cell population does not

exist in these cell lines, or that CD133 is not a relevant cancer stem cell marker in these samples. Yet the results described in this chapter, as well as the limitations of CD133 as a pan-lung cancer stem cell marker (described in Chapter One) would support the later conclusion.

In addition to claiming CD133 as a lung cancer stem cell marker, Eramo et al. also reported that lung cancer stem cells could be cultured in serum free, mitogen defined media as tumor sphere colonies (Eramo et al., 2008). However the same culture conditions reported by Eramo and others to select for cancer stem cell sphere colony growth failed to select for viable colonies in a panel of tested lung cancer cell lines and patient samples. This is likely due to the fact that current culture conditions for lung cancer stem cells have not been fully optimized to include all the necessary growth factors to sustain undifferentiated cell growth in suspension. Indeed, less than one third of the tumors tested by Eramo et al were able to culture as spheres in the conditions described (Eramo et al., 2008). Nevertheless, the addition of serum to the sphere colony culture media enabled to the production of tumor spheres in nearly half of the cell lines tested. Like some neurospheres, these lung cancer sphere colonies were partially formed from cell aggregation, suggesting these spheres are not necessarily cloned from a single stem-like progenitor (Mori et al., 2006). Clonogenic analysis of sphere derived cells indicated these cells were highly clonogenic in soft agar compared to their two-dimensional culture derived counterparts. However the selection or adaptation for anchorage independent growth during sphere colony formation confounded the interpretation of the soft agar colony as it might reflect more the capacity for cell survival

in an anchorage independent culture condition (anoikis-resistance) rather than a measure of clonogenicity.

To clarify the biological differences in culture selected cells, the capacity to initiate the formation orthotopic xenografts was assayed. Although more technically challenging to perform and monitor, an orthotpic xenograft assay was chosen for study over the more popular subcutaneous xenograft model because of its greater physiologic relevance. However, because of the difficulty in reproducibly seeding tumor cells into the pleural space, many tumors formed outside the lung parenchyma and onto the chest wall, thus limiting "orthotopic-ness" of this assay. Nevertheless, no great difference in the ability to initiate xenograft growth was observed in sphere colony cultures and monolayer cultured cells tumor cells, suggesting that sphere colonies were not enriched in tumorigenic cancer stem cells.

The search for putative lung cancer stem cells also lead to the identification of tumor cell subpopulations with differential stem cell associated enzymatic activities. The first enzyme based assayed tested was the Side Population assay. According to prior reports, stem cells as well as some cancer stem cells were identified by their preferential capacity for effluxing a fluorescent dye (Feuring-Buske and Hogge, 2001; Kondo et al., 2004; Patrawala et al., 2005; Szotek et al., 2006; Wulf et al., 2001). In the glioma cell line C6, SP cells constitute approximately half a percent of the total tumor cell population, however these rare tumor cells were highly clonogenic and capable of greater tumorigenic growth in mice compared to majority of MP cells (Kondo et al., 2004). As further evidence of their "stemness," SP cells could be increased as sphere colonies cultured in serum free stem cell mitogen defined media, and in differentiation inducing

media, produced progeny that exhibited neuronal and glial cell lineage markers (Kondo et al., 2004). However the SP phenotype failed to select for highly tumorigenic and clonogenic cells from lung cancer cell lines. This is in contrast to the study by Ho et al. in 2007 that claimed Hoechst 33342 dye effluxing lung cancer cells are cancer stem cells (Ho et al., 2007). In support of the data presented in this chapter however, more recent studies have been published questioning the viability of the Side Population assay as a method for isolating cancer stem cells (Platet et al., 2007; Wu and Alman, 2008; Zhong et al., 2007). Of particular concern is the variability of SP cell detection. Because factors such as incubation time and concentration of Hoechst 33342 dye have such a dramatic effect on the proportion of SP cells detected, the detection of SP cells becomes an artifact of the assay parameters. In addition, because Hoechst 33342 is a DNA binding dye, MP cells that cannot efflux cellular Hoechst 33342 are more susceptible to cell death or growth arrest from DNA-damage response programs (Zhong et al., 2007). This would suggest that while the SP phenotype might be associated with stem-ness in some tumor cells, its utility as a method for isolating tumor cell subpopulations remains limited.

As described in the last results section in this chapter, lung cancer cell lines and tumor cells were assayed for putative lung cancer stem cells with elevated ALDH enzymatic activity. Flow cytometric analysis of tumor cell lines and patient samples found that most NSCLCs possessed a readily detectable $ALDH^+$ cell population, enriched in clonogenic and tumorigenic capacity and capable of stem cell-like self-renewal.

During the course of this study, a report by Jiang et al. also indicated that $ALDH^+$ cells from H358 and H125 cell lines were enriched in cancer stem cell activity (Jiang et al., 2009a). While these conclusions would seem to support some observations in the

Jiang et al study, discrepancies in the detection and isolation of $ALDH^+$ cells in the Jiang et al study raise some concerns. In contrast to a report by Jiang et al. ALDH activity was not detected as a bimodal distribution in lung cancer cell lines. Furthermore, $ALDH^+$ cell population were conspicuously larger and brighter than their $ALDH^-$ cell counterparts in the Jiang et al. study suggesting that cells gated as $ALDH^-$ were in fact greatly contaminated with cellular debris (Jiang et al., 2009a). Therefore the results of functional studies of sorted cells in the Jiang et al. report become difficult to interpret (Jiang et al., 2009a). To avoid this problem, a gating strategy was employed in this study that excluded cell debris and non viable cells from the assay.

Flow cytometric analysis found that most SCLC cell lines were greatly enriched in ALDH, suggesting putative cancer stem cells are abundant in SCLCs. SCLC is a highly aggressive and lethal disease, characterized by primitive neuroendocrine features and a propensity for rapid recurrence and metastasis after therapy. In culture, SCLCs often grow in serum free media as floating stem-cell like colonies and generate rapid xenograft production when implanted in mice. Recently, SCLC ALDH protein expression was found to be controlled by the neuronal lineage factor ASCL1 and knockdown of this gene transcript lead to a reduction in ALDH activity and xenograft production (Jiang et al., 2009c). These observations suggest ALDH is associated with SCLC "stemness" but further characterization of SCLCs with an isolatable $ALDH^+$ and $ALDH^-$ cell populations will be necessary to directly address the utility of ALDH as a SCLC stem cell maker.

In mice Sca-1 has been described as a stem cell marker in a variety of murine tissues including normal mammary glands and prostate, as well as in normal and

cancerous lung tissue (Kim et al., 2005; Lawson et al., 2007; Welm et al., 2002). By analyzing adenocarcinoma cells from K-ras induced Ink4a/ARF$^{-/-}$ mice, a high degree of overlap in murine tumor cells with Sca-1 expression and ALDH activity was detected, suggesting ALDH might be a associated with murine lung cancer stem cells. Unlike BASCs however, these cells do not express CCSP and future experiments will determine whether these ALDH$^{+}$Sca-1$^{+}$ cells possess the capacity for self-renewal and tumorigenicity.

To determine the functional characteristics of sorted ALDH$^{+}$ and ALDH$^{-}$ cells the growth of sorted cells in xenograft formation assays and *in vitro* clonogenic assays was compared. Isolated ALDH$^{+}$ cells were significantly more tumorigenic and clonogenic than their ALDH$^{-}$ counterparts. In addition, isolated ALDH$^{+}$ cells were determined to regenerate tumor heterogeneity for ALDH$^{+}$ and ALDH$^{-}$ cells, whereas this capacity was diminished in isolated ALDH$^{-}$ cells. Nevertheless, sorted ALDH$^{-}$ cells from H358 and H1299 cell lines were capable of forming tumors in mice (albeit with reduced size and frequency than their ALDH$^{+}$ counterparts), suggesting residual tumor propagating activity persists in ALDH$^{-}$ cells. In contrast, ALDH$^{-}$ cells from two primary patient lung cancers were unable to generate tumors in mice. This could be due to the existence of multiple cancer stem cell populations (that ALDH may not identify), cell plasticity with in tumor cell lines or the contamination of ALDH$^{-}$ cells with residual ALDH$^{+}$ cells.

# CHAPTER FOUR

# TARGETING LUNG CANCER STEM CELL SIGNALING

## 4.1 Introduction

The notion that tumor initiation and progression are driven by a subpopulation of phenotypically stem-like cancer cells provides strong rationale for identifying and targeting these cells therapeutically. Indeed the prospect of cancer stem cells as the source for disease recurrence after therapy indicates complete remission would require the eradication or inactivation of cancer stem cells. To achieve cancer stem cell targeted therapy, the factors that facilitate cancer stem cell function must first be identified. In this regard the characterization of the molecular pathways that are required for the viability and maintenance of self-renewing tumor initiation cells may ultimately lead to improved therapies for cancer.

As discussed in Chapter One, many cancers including lung cancer are initiated and propagated through the aberrant self-renewal and differentiation of adult stem cells. As a key function of both stem cells and cancer stem cells, the process of self-renewal functions to ensure the propagation of a cohort of primitive cell progeny with equal replicative potency. However in carcinogenesis, the signaling mechanisms that control stem cell self-renewal are often dysregulated, leading to aberrant tumor stem cell self-renewal. Therefore, modulating aberrant cell signaling involved in tumor cell self-renewal is believed to be a potential strategy for achieving targeted cancer stem cell

therapy (Al-Hajj and Clarke, 2004; Clarke and Fuller, 2006; Misaghian et al., 2009; Reya et al., 2001; Sullivan et al., 2010).

Self-renewal of normal and malignant stem cells involves a diverse network of regulatory mechanisms including, but not limited to, signaling of Wnt, Hedgehog, Notch, Bmi1, PI3K/Akt, EGFR/Neu pathways. These signaling pathways have become increasingly studied as potential targets for managing cancer stem cell populations in a variety of tumor types. For example, molecular expression analysis of prostate cancer stem cell enriched sphere colonies revealed enrichment in genes associated with PI3K signaling. Through Western blot analysis of prostate cancer spheres, Dubrovska et al. showed significant levels of phosphorylation of downstream effectors of the PI3K signaling pathway, including Akt, GSK3β, and FoxO proteins (Dubrovska et al., 2009). Deletion of PTEN, which negatively regulates PI3K signaling, has been shown to promote tumor initiation in murine models of prostate cancer through the aberrant expansion of prostate progenitor cells, therefore it was hypothesized that PI3K signaling was an important pathway for prostate cancer stem cell signaling (Wang et al., 2006). To test this, Dubrovska and colleges suppressed PI3K signaling by shRNA knockdown of FoxO3 or pathway inhibition by chemical inhibitors in prostate cancer cells. As a result of PI3K pathway suppression, substantial growth inhibition of $CD133^{+}CD44^{+}$ prostate cancer stem cells was observed (Dubrovska et al., 2009). The role of PI3K/Akt signaling in tumor stem cell self-renewal has also been demonstrated in breast cancer, where suppression of HER2 regulated PI3K/Akt signaling by chemical inhibitors, trastuzumab and LY294002, resulted in the reduction of $ALDH^{+}$ breast cancer stem cell abundance, self-renewal and tumorigenic growth (Korkaya et al., 2008).

Evidence for self-renewal signaling pathway cross-talk in cancer has become increasingly evident. Recently, the inhibition of Notch signaling in EGFR driven breast cancer by gamma-secretase inhibition was found to not only reduce the expression of downstream Notch signaling targets such as *HES1*, but also dramatically reduce Akt phosphorylation (Efferson et al., 2010). As a result tumor initiation and growth was substantially reduced in preclinical mouse models (Efferson et al., 2010). Similarly in gliomas and embryonal brain cancers, treatment with a gamma-secretase inhibitor resulted in the dose dependent suppression of tumoral Notch and Akt signaling. Inhibition of these signaling pathways resulted in a dramatic reduction in $CD133^+$ and SP brain cancer stem cells, leading to the disruption of neurosphere colony production *in vitro* and prevention of tumor initiation in mouse xenograft models (Fan et al., 2010; Fan et al., 2006). Recently $CD133^+$ and $ALDH^+$ colon cancer stem cells have also been shown to require Notch signaling for self-renewal. As such, inhibition of Notch signaling by gamma-secretase inhibition resulted in colon cancer stem cell apoptosis and differentiation (Sikandar et al., 2010).

As discussed in Chapter One, self-renewal pathways involved in lung stem cell homeostasis have only recently been described and further characterization of lung stem cell and cancer stem cell populations will be necessary to properly elucidate the roles these pathways play in carcinogenesis (Sullivan et al., 2010). In the past few years the role of Notch pathway signaling has become increasingly studied in normal and malignant lung development. Notch signaling, which is hypothesized to promote the maintenance of a primitive, less differentiated cell state in the lung epithelia, has also been hypothesized to play an oncogenic role in lung cancer.

Recently the discovery of frequent alterations in NSCLC Notch signaling has been associated with poor patient prognosis (Westhoff et al., 2009). In a study of 49 patient tumor samples, a strong correlation was observed between activated Notch1 expression by IHC and the expression of the Notch target gene *HES1*. Sequencing analysis revealed Notch1 activation and *HES1* expression was correlated with mutations in the *NOTCH1* gene and that these gain of function mutations were associated with a reduction in NSCLC patient survival (Westhoff et al., 2009). These data would suggest that Notch signaling is promoting an aggressive tumor cell phenotype in some NSCLCs. Furthermore, these data support the oncogenic role of Notch in the natural history of lung cancer and present a strong rationale for the targeting of activated Notch signaling in lung cancer (Westhoff et al., 2009).

From a pharmacodynamic perspective, Notch signaling presents an appealing therapeutic target because of the high degree of cross-talk with many other relevant oncogenic signaling pathways. In NSCLC cell lines, the suppression of activated Notch receptor signaling by gamma-secretase inhibition has lead to xenograft growth inhibition, reduction in tumor cell clonogenicity, apoptosis and suppression of MAPK/ERK signaling (Haruki et al., 2005; Konishi et al., 2007; Konishi et al., 2010; Lin et al., 2010). While these studies provide evidence for the potential therapeutic utility of targeting Notch signaling in lung cancer, it has yet to be determined if targeting Notch effects the tumor stem cell subpopulation driving the disease.

To date, very little is known about the molecular characteristics of lung cancer stem cells, in part because prospective lung cancer stem cell populations have only recently been described. Consequently, it is unclear if and how lung cancer stem cells

might signal to achieve their unique stem-like phenotype, and how lung cancer stem cell signaling might be exploited for new targeted therapies. The aim of this study was to characterize the activity of known stem cell associated signaling pathways in isolated $ALDH^+$ lung cancer stem cells (described in Chapter Three) with the translational goal of identifying therapeutically targetable signaling pathways essential for cancer stem cell function.

## 4.2 Results

### *4.2.1 Analysis of Notch Signaling in Lung Cancer Stem Cells*

The expression of known stem cell signaling associated transcripts were assayed by qPCR expression analysis in HBECs, which retain some normal lung stem/progenitor cell function, NSCLC and SCLC cell lines (Ramirez et al., 2004; Vaughan et al., 2006). Expression analysis revealed the overexpression of many Notch signaling transcripts in NSCLC cell lines, including the Notch receptor genes *NOTCH2* and *NOTCH3*, and the Notch target gene *HES1*, indicating active pathway signaling in many tumor cell lines (Figure 4.1). Interestingly some elements of the Notch signaling pathway, such as *DLL1*, *JAG1*, *JAG2* and *NOTCH1* were predominantly elevated in HBECs, suggesting a context for Notch signaling in normal lung epithelial stem cells (Figure 4.1).

Previous studies have indicated Notch signaling promotes normal and tumor stem cell homeostasis, therefore it was hypothesized that elevated Notch signaling in lung cancer may function to sustain lung cancer stem cells (Androutsellis-Theotokis et al., 2006; Fan et al., 2006; Sansone et al., 2007). To test this, $ALDH^+$ lung cancer stem cells

and $ALDH^-$ non-cancer stem cells were isolated from H358, HCC2429 and H2009 cell lines by FACS. Reverse transcription was performed on isolated RNA from sorted cells and qPCR analysis was performed to assay for the expression of key Notch signaling transcripts. As a result, transcript expression was detected in each sorted cell population; however the abundance of Notch signaling transcripts was greater in the $ALDH^+$ cell population of each cell line compared to bulk $ALDH^-$ cell populations (Figure 4.2A). The elevated expression of both pathway receptors such as *NOTCH1*, *NOTCH2* and *NOTCH3* as well as transcriptional effectors such as *HES1* in $ALDH^+$ cells indicated that Notch pathway activity was enhanced in the lung cancer stem cell stem cell population.

To test for Notch pathway activity, lung cancer cell lines were treated with DAPT, a gamma-secretase inhibitor that blocks the cleavage and release of intracellular Notch, thus suppressing canonical Notch signaling. A substantial reduction in expression of downstream Notch target genes *HES1*, *HEY1* and *HEY2* was observed by qPCR in lung cancer cells treated with DAPT, confirming Notch pathway suppression (Figure 4.2B). Previous studies report gamma-secretase treatment causes tumor cell cycle arrest and apoptosis (Konishi et al., 2007; Rao et al., 2009; Rasul et al., 2009). In lung cancer cell lines, DAPT mediated suppression of the Notch pathway in was also associated with cell cycle growth arrest. In H358 and HCC2429 cells, DAPT treatment caused an accumulation of cells in G1/G0-phase and a reduction in replicative S-phase compared to DMSO control treated cells, indicating cell growth arrest (Figure 4.3A). Compared to DMSO treatment, DAPT treatment in H2009 cells resulted in an accumulation of cells in G2/M-phase as well as a reduction in replicative S-phase (Figure 4.3A).

Because $ALDH^+$ lung cancer cells expressed elevated Notch signaling transcripts, it was hypothesized that $ALDH^+$ cells may be sensitive to DAPT treatment. To test this, the percent of $ALDH^+$ cells was compared in cell lines treated with DMSO and DAPT by flow cytometry. As a result, DAPT appeared to reduce the proportion of $ALDH^+$ cells in H358, HCC2429 and H2009 cells in a dose dependent manner. A significant reduction in $ALDH^+$ cells was observed in cells treated with 10 μM DAPT compared to DMSO treatment ($P$ = 0.035, 0.034, 0.019 in H358, HCC2429 and H2009 respectively) (Figure 4.2C). These data would suggest that the elevated Notch signaling detected in $ALDH^+$ lung cancer cells resulted in sensitivity to DAPT treatment, and that Notch signaling is likely important for $ALDH^+$ cell maintenance.

To determine if the growth inhibitory effects observed by cell cycle analysis in unsorted cell lines were occurring in the lung cancer cell population, H358 $ALDH^+$ and $ALDH^-$ cell treated with DMSO and DAPT were fixed and DNA content was assayed by flow cytometry. In accordance with data presented in Chapter Three, $ALDH^+$ cells were relatively more proliferative than $ALDH^-$ cells, as a greater proportion of cells were engages in replicative S-phase. Upon treatment with DAPT, a G1/G0-phase growth arrest was observed in both sorted cell populations however a greater cell cycle arrest phenotype was observed in $ALDH^+$ cells, indicating that the reduction of lung cancer stem cells by DAPT treatment is due to preferential growth arrest of $ALDH^+$ lung cancer cells (Figure 4.3B and 4.3C).

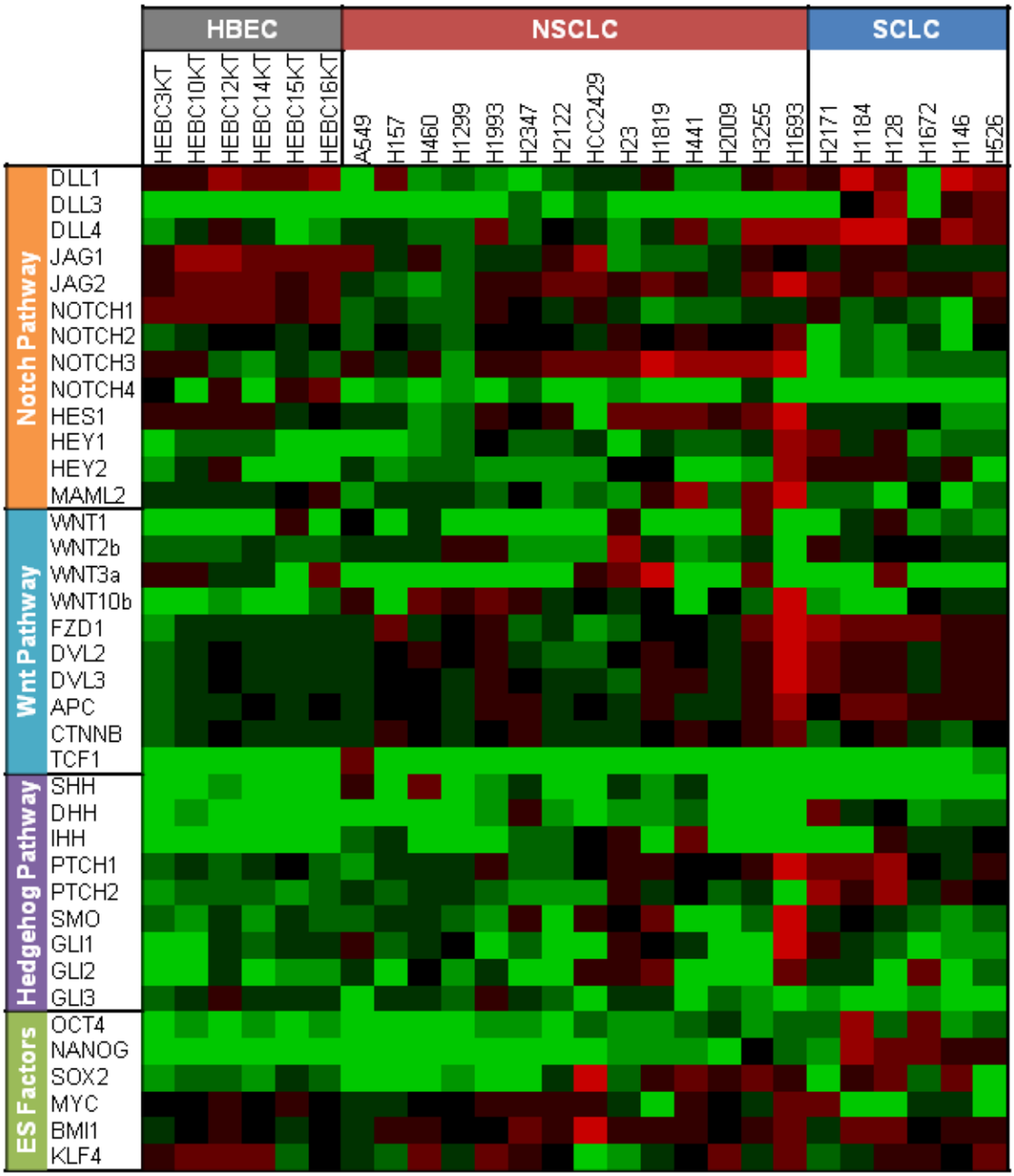



**Figure 4.1: Expression of stem cell associated factors in HBEC, NSCLC and SCLC cell lines.** A heatmap representing expression of a panel of known stem cell signaling transcripts in lung cell lines, normalized to a reference sample. Bright red indicates transcript overexpression compared to expression in reference sample, whereas bright green indicates relatively low transcript expression.

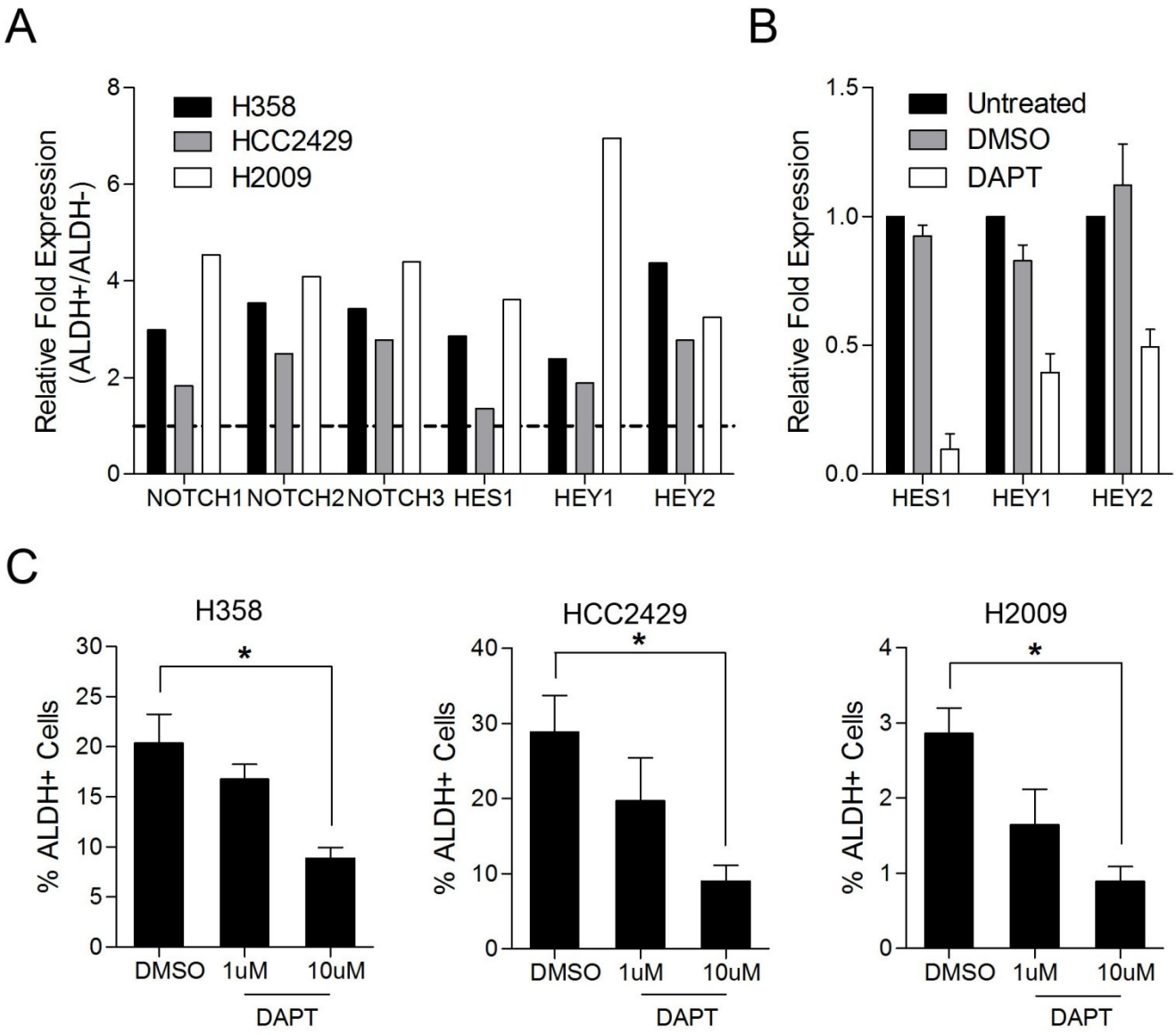



**Figure 4.2: Elevated Notch transcript expression in ALDH$^+$ lung cancer cells is suppressed by DAPT.** Notch signaling transcript levels were analyzed in duplicate by qPCR in sorted ALDH$^+$ and ALDH$^-$ lung cancer cells. Notch transcripts (shown as a ratio of ALDH$^+$ / ALDH$^-$ mRNA levels) were expressed in greater abundance in ALDH$^+$ lung cancer cells compared to ALDH$^-$ lung cancer cells (A). qPCR analysis revealed a decrease in Notch signaling target genes in unsorted lung cancer cells treated with 10 μM DAPT for 24 hrs, indicating pathway suppression (B). Cell lines treated with 1 μM or 10 μM DAPT for five days had reduced ALDH$^+$ cell populations compared to control DMSO treated cells ($n = 3$, $^*P < 0.05$) (C).

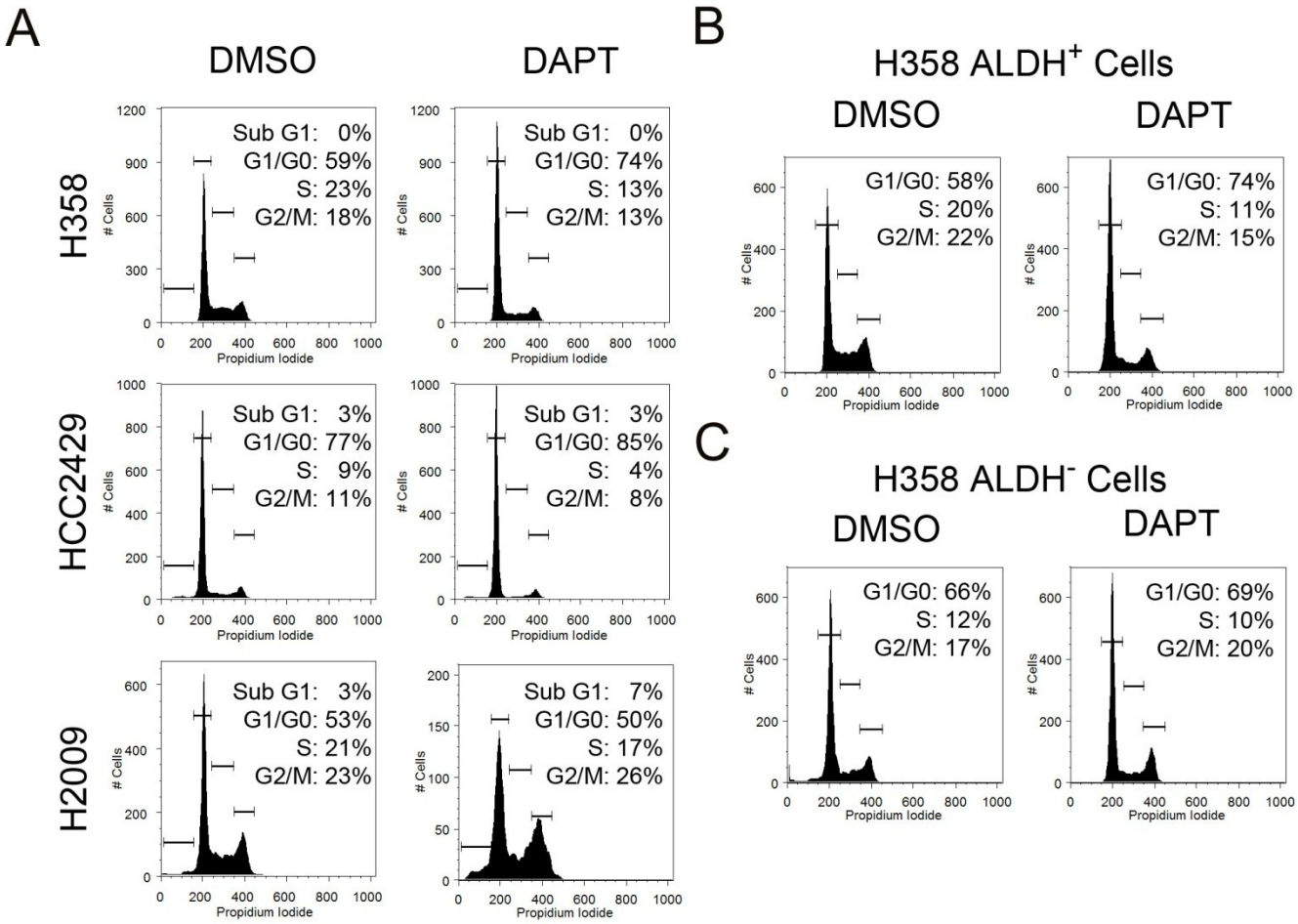



**Figure 4.3: Suppression of Notch signaling by DAPT arrests ALDH$^+$ lung cancer cell growth**. Cell cycle analysis was performed on DMSO control treated and 10 μM DAPT treated H358, HCC2429 and H2009 cells. DAPT caused an accumulation of cells in G1/G0-phase in H358 and HCC2429 cells, and an accumulation in G2/M-phase cells in H2009 cells. DAPT treatment also caused a reduction in replicative S-phase cells in H358, HCC2429 and H2009 cells (A). H358 cells sorted for ALDH were treated with DMSO and DAPT. Cell cycle analysis of H358 ALDH$^+$ cells treated with DAPT revealed cells arrested at G0/G1-phase, and reduction in S-phase cells (B). Cell cycle analysis of H358 ALDH$^-$ cells treated with DAPT revealed a similar accumulation in G0/G1-phase cells and reduction in S-phase cells although the effects were less pronounced (C).

### *4.2.2 Suppression of Oncogenic NOTCH3 Expression in Lung Cancer by shRNA*

All four Notch receptors have been implicated in human cancers, however depending on the cellular context Notch family members can act as tumor suppressors or oncogenes (Radtke and Raj, 2003). According to microarray expression, many cell lines express *NOTCH1*, *NOTCH2*, and *NOTCH3*, but not *NOTCH4*. Furthermore, expression of these three Notch receptor genes tended to be enriched in $ALDH^+$ lung cancer cell subpopulations.

While the role of Notch1 and Notch2 signaling are less clear in lung cancer, several reports have identified the importance of Notch3 signaling in lung development and lung cancer proliferation, suggesting Notch3 may play a proto-oconcogenic role (Dang et al., 2003; Haruki et al., 2005; Konishi et al., 2007). To investigate the contextual expression of Notch receptor signaling in lung tissues, qPCR expression analysis of Notch signaling transcripts was performed on HBEC, NSCLC, and SCLC cell lines. Transcript expression was normalized to a reference sample consisting of RNA pooled from normal and cancerous tissues, as described in Chapter Two. Expression analysis revealed a heterogeneous pattern of transcript expression across bronchial epithelial and lung tumor cell lines (Figure 4.4A). For example, *NOTCH1* expression was greatly elevated in HBEC lines compared to tumor cell lines, suggesting *NOTCH1* may function as a lung tumor suppressor, or a gene important for normal lung stem cell function (Figure 4.4A). In addition *HES1* expression was readily detectable in all HBEC lines indicating active canonical pathway signaling in normal lung epithelial cells. Expression of Notch signaling transcripts was much less abundant in SCLC compared to HBECs and NSCLC, suggesting reduced Notch signaling activity in SCLC. In contrast,

most NSCLC cell lines tested overexpressed NOTCH3 and *HES1* (Figure 4.4A). This expression patter in NSCLC would appear to support the oncogenic context of Notch3 signaling in some lung cancers.

To examine if Notch transcript expression was associated with lung tumor "stemness," correlation analyses were performed on transcript expression and the percent of ALDH activity in NSCLC cell lines. Interestingly expression of *NOTCH3* was most strongly, positively correlated with ALDH activity in NSCLC cell lines ($r^2 = 0.68$) (Figure 4.4B). In contrast, expression of *NOTCH1*, which is more abundant in normal bronchial epithelial cells, was not correlated with ALDH activity in NSCLC cell lines ($r^2 = 0.03$) (Figure 4.4B). Thus, because *NOTCH3* expression was positively correlated with ALDH activity and was elevated in isolated $ALDH^+$ lung tumor cells, it was hypothesized that *NOTCH3* was important for lung cancer stem cell maintenance. To test this hypothesis, NOTCH3 expression was targeted by stably expressing shRNA against *NOTCH3* in lung cancer cells. The expression of *NOTCH3* transcripts as well as downstream Notch targets *HEY1* and *HEY2* were reduced by ~2-fold in H358 and H2009 cells expressing sh*NOTCH3* compared to H358 and H2009 cells expressing control vector sh*GFP* (Figure 4.5A). Knockdown of *NOTCH3* did not appear to effect *HES1* expression, supporting previous evidence that *HES1* is not a target of Notch3 signaling (Dang et al., 2003). *HEY1* and *HEY2* transcripts were decreased in shNOTCH3 expressing cells indicating pathway suppression. A strong growth suppressive phenotype was observed in cells expressing shNOTCH3. As such, the clonogenic capacity of shNOTCH3 expressing cells was significantly decreased by ~5 fold *in vitro* compared to shGFP control cells ($P < 0.001$) (Figure 4.5B). To determine if the loss of clonogenicity

was related to a reduction in lung cancer stem cells, the proportion of $ALDH^+$ cells was assayed by flow cytometry in H358 and H2009 cells stably expressing shGFP and shNOTCH3. H358 and H2009 cells expressing the non targeting shRNA against *GFP* possessed proportions of $ALDH^+$ cells similar to their uninfected parental lines (15 ± 2.2% and 5 ± 0.3% respectively), however the $ALDH^+$ population was found to be greatly reduced in H358 (8 ± 0.9%) and H2009 (0.5 ± 0.1%) cells expressing shNOTCH3 ($P$ = 0.029 and $P$ = 0.003 respectively) (Figure 4.5C), suggesting that shNOTCH3 mediated suppression of clonogenicity was a result of diminished clonogenic $ALDH^+$ lung cancer cells numbers. Taken together, these findings indicate that Notch signaling is important for the maintenance of $ALDH^+$ lung cancer stem cells.

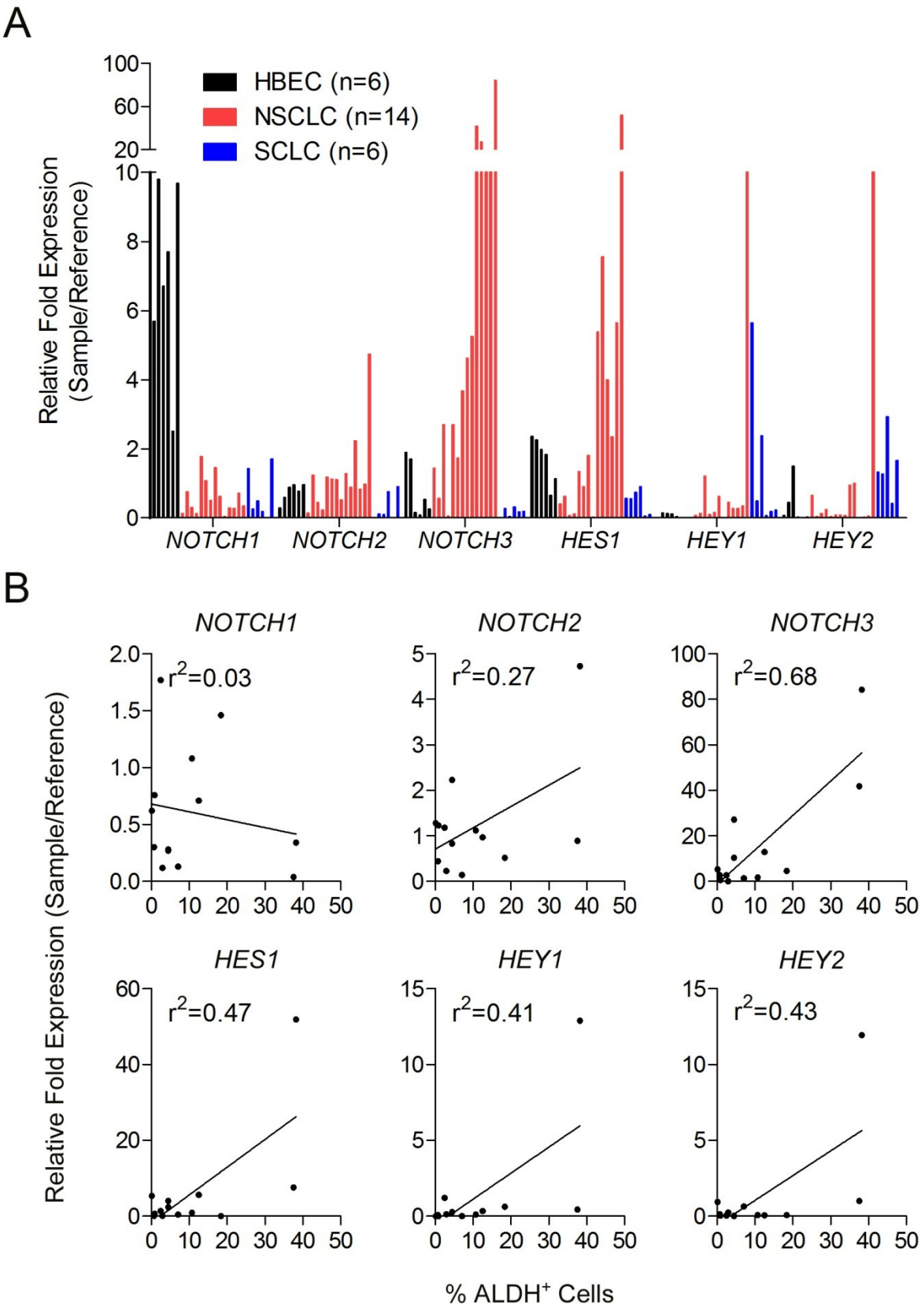

A
HBEC (n=6)
NSCLC (n=14)
SCLC (n=6)
Relative Fold Expression (Sample/Reference)
NOTCH1
NOTCH2
NOTCH3
HES1
HEY1
HEY2
B
NOTCH1
NOTCH2
NOTCH3
HES1
HEY1
HEY2
r2=0.03
r2=0.27
r2=0.68
r2=0.47
r2=0.41
r2=0.43
Relative Fold Expression (Sample/Reference)
% ALDH+ Cells

**Figure 4.4: Notch transcript expression and ALDH correlation in lung cancer.** qPCR expression analysis of Notch transcripts in normal lung (HBEC) and lung cancer (NSCLC and SCLC) cell lines normalized to a commercial reference RNA sample (A). Linear correlation analysis of Notch signaling transcripts and the percent of $ALDH^+$ cell in NSCLC cell lines revealed the strongest positive correlation between *NOTCH3* expression and ALDH abundance (B).

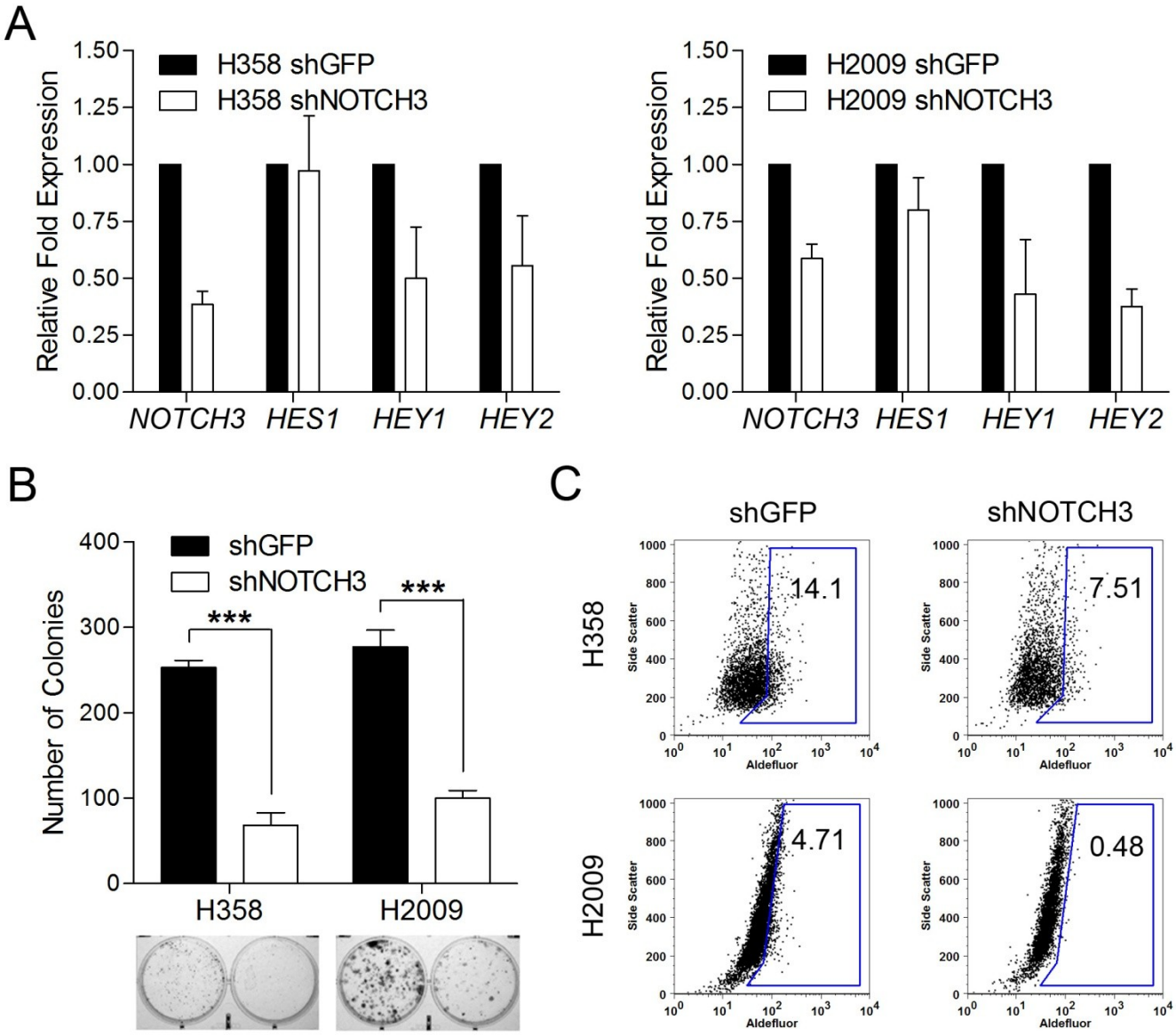



**Figure 4.5: Clonogenicity and ALDH activity of lung cancer cells expressing shRNA against *NOTCH3*.** qPCR expression analysis of *NOTCH3* and downstream Notch signaling targets *HES1*, *HEY1* and *HEY2* were analyzed in H358 (left panel) and H2009 cells (right panel) expressing shRNA targeted against *NOTCH3* and *GFP* as a control (A). Expression of shNOTCH3 resulted in a reduction in *NOTCH3*, *HEY1* and *HEY2* expression. Colony formation analysis revealed a significant reduction in cells expressing shNOTCH3 compared to cells expressing the control vector shGFP (***$P <$ 0.01) (B). Aldefluor analysis of shGFP cells detected an ALDH population similar in abundance to non-infected cells. However, H358 and H2009 cells expressing shNOTCH3 had reduced $ALDH^+$ cell subpopulation (C).

## 4.3 Discussion

The discovery of a stem-like tumor cell population with the preferential capacity to reconstitute heterogeneous tumor growth and dissemination has important implications for cancer therapy. Specifically, if current conventional therapy fails to eliminate the most tumorigenic cancer cell subpopulation, the source of tumor recurrence, then the goal of therapeutics should be to eradicate the cancer stem cell population. One approach to targeting cancer stem cells has been to inhibit signaling pathways that promote stem cell self-renewal. In this study, lung cancer cells were assayed for the expression of known stem cell associated self-renewal pathways. Microarray expression analysis revealed broad expression of Notch signaling molecules in NSCLCs as well as in stem/progenitor enriched HBEC lines. Further molecular characterization of isolated $ALDH^+$ lung cancer cells revealed elevated expression of Notch signaling transcripts in lung cancer stem cells, suggesting enhanced pathway activity in these cells. Inhibition of Notch activity by molecular and chemical means resulted in a reduction in clonogenic $ALDH^+$ cell populations, indicating the potential therapeutic potential of targeting Notch signaling to eradicate or inactivate lung cancer stem cells.

The reduction of lung cancer stem cell numbers by gamma-secretase inhibition appeared to be due primarily to the preferential growth arrest of $ALDH^+$ lung cancer cells in response to treatment. While eradication of $ALDH^+$ would be preferred, the reduction of $ALDH^+$ cells is a start. Improvements in the development of Notch inhibitors, such as more specific and potent gamma-secretase inhibitors, or Notch signaling effector specific blocking antibodies may improve the efficacy of this treatment strategy. Recently Merck has developed a potent gamma-scetretase inhibitor that has been shown to promote

apoptosis in lung cancer cells; however this reagent was not available for this study (Konishi et al., 2007; Konishi et al., 2010).

Judging by these preliminary results, anti-Notch therapies may not be effective stand alone therapies in lung cancer. However there is evidence to suggest that inhibition of Notch signaling in cancer may also potentiate sensitivity to other forms of therapy. For example, gamma-secretase inhibition in colon cancer resulted in the sensitization of colon cancer cell lines to chemotherapy by blocking Notch signaling activation of the pro-survival PI3K/Akt pathway (Meng et al., 2009a). Because of the high degree of cross-talk between Notch signaling and other important signaling pathways, it might be reasonable to assume similar results can be achieved in lung cancer. Further experiments will need to be done to determine how to exploit pathway cross-talk when targeting Notch signaling in lung cancer. Worthy future experiments might include combining current Notch signaling inhibitors with inhibitors of PI3K/Akt, mTOR, or EGFR signaling in lung cancer. Future experiments would also benefit from the use of specific signaling reporter assays rather than just relying on transcript expression as the sole pathway readout.

The most limiting factor facing current Notch inhibition therapy is the high toxicity associated with gamma-secretase inhibitor treatment. This is due in part to the relatively sloppy specificity of current gamma-secretase inhibitors, as current inhibitors not only inhibit signaling through all Notch receptors, but also through some other signaling cascades that require gamma-secretase activity. To improve anti-Notch therapy, it will be necessary to develop specific inhibitors that are selective in blocking receptor signaling activity, especially considering the fact that in some cellular contexts,

certain receptors function as tumor suppressors (Radtke and Raj, 2003). In addition, knowing how anti-Notch therapy might affect normal stem cell populations would be important.

In this study, molecular characterization of HBECs, NSCLC and SCLC cell lines revealed divergent expression of Notch signaling factors between normal and malignant lung cells. Specifically, *NOTCH1* expression was elevated in HBECs, which are enriched in normal lung stem/progenitor cells, compared to tumor cells and *NOTCH3* was over expressed in NSCLC cell lines. Furthermore, selective knockdown of *NOTCH3* expression by shRNA lead to a dramatic reduction in clonogenic $ALDH^+$ lung cancer cells. These data would suggest that anti-Notch therapy in lung cancer would benefit for specifically targeting Notch3 signaling, which is important for lung cancer stem cell homeostasis. In addition, the differential expression of Notch signaling receptors in normal and malignant lung cells may provide a therapeutic window for new and more specific Notch inhibitors.

While this study focused on the importance of Notch signaling in lung cancer stem cells, further characterization of lung cancer stem cell populations will inevitably yield additional information on targetable signaling pathways necessary for cancer stem cell function. Lung cancer is a phenotypic and histologically heterogeneous disease and this heterogeneity is also reflected in the different signaling between lung tumor types. For example, Notch signaling transcripts were only rarely detected in abundance in SCLC cell lines, suggesting limited Notch signaling in SCLCs compared to NSCLCs. In 2003, Watkins et al. discovered that SCLC cells as well as the stem cell precursors to SCLC often engaged in signaling through the Hedgehog pathway. When treated with

cyclopamine, a chemical inhibitor of Hedgehog signaling, SCLC expressing Hedgehog signaling components underwent growth arrest and apoptosis (Watkins et al., 2003). This would suggest that the contextual activation of self-renewal signaling pathways in lung cancer is associated with the biology of the tumor initiating cell and the type of lung cancer. It is also likely that heterogeneity exists within lung cancer stem cell populations and that different lung cancer stem cell populations may rely on different self-renewal signaling pathways.

In addition to targeting cancer stem cell pathways of self-renewal, several other approaches, such as cancer stem cell activated immune cell therapy, reversal of cancer stem cell multi-drug resistance, disruption of cancer stem cell niche, or cancer stem cell directed differentiation therapy might also prove to be effective anti cancer stem cell strategies. In a proof of principle study, Schatton and colleges demonstrated that melanoma stem cells could be selectively killed though targeting their prospective identifying marker ABCB5, which is also a mediator of melanoma chemosensitivity (Schatton et al., 2008). Using monoclonal antibodies raised against isolated melanoma stem cells and directed against ABCB5, Schatton found systematic treatment was capable of eliciting antibody-dependent cell-mediated cytotoxicity in melanoma stem cells, resulting in significant tumor growth reduction *in vivo* (Schatton et al., 2008). To achieve similar results in lung cancer will require further characterization of lung cancer stem cell biology as well as the identification of specific, and therapeutically exploitable cellular targets. Accordingly the discovery of lung cancer stem cell sensitivity to *NOTCH3* suppression provides strong rationale for further investigation of anti-Notch therapy in managing lung cancer stem cells.

# CHAPTER FIVE

# CLINICAL RELEVANCE OF LUNG CANCER STEM CELLS

## 5.1 Introduction

Since the mid 1900s lung cancer has remained the most common lethal form of cancer, killing more people in the US than colon, breast, pancreatic and prostate cancer combined (Jemal et al., 2009). These grim statistics are exemplified by fact that only about one in three patients with NSCLC survive past their first year of diagnosis, as the estimated median survival for NSCLC hovers around four to five months (Jemal et al., 2009; Sun et al., 2007). For the 15% of lung cancer patients with SCLC, a disease characterized by rapid growth and dissemination, the prognosis is worse, with only about 5% surviving past their second year of diagnosis (Govindan et al., 2006).

The poor prognosis associated with lung cancer is primarily attributed to the advanced stage at which it is usually detected. Clinical progression of the disease is defined by tumor size (T), lymph node (N) and distal metastasis (M), and summarized by stage (I to IV) according to the TNM system (Mountain and Dresler, 1997). Early disease, often defined as stages I to IIIA is usually asymptomatic and therefore difficult to diagnose. Consequently, over 75% of all lung cancers detected are diagnosed as advanced disease; associated with poor prognosis (Table 5.1) (Hirsch et al., 2001; Spira and Ettinger, 2004). In addition, the recalcitrance of late stage lung cancer to current therapeutic options also contributes to the poor prognosis of this disease.

Unlike breast or prostate cancer which have benefited from advancements in chemotherapy and chemoprevention, similar gains in lung cancer therapy have yet to be achieved. Treatment for lung cancer depends on a variety of factors, most importantly the histopathalogical type and stage of the disease. Surgery remains the dominant form of treatment for NSCLC, particularly for those with early stage NSCLC. Often surgery is followed by adjuvant chemo- or radiotherapy. For patients with unresectable advanced NSCLC, curative treatment options are limited and often patient care is palliative. Unfortunately for most patients with NSCLC, current treatments do not cure the cancer. Therefore, improving lung cancer prognosis hinges on the development of new tools and methods for detection of early stage lung cancer, as well as the development of new therapies that will better manage recurrent disease.

Advances in high-throughput, genome-wide profile technologies have allowed for new investigation into the molecular underpinnings of lung cancer. Utilizing these new genetic profiling tools, the emerging fields of molecular pathology and epidemiology have endeavored to identify genetic and epigenetic factors that are associated with disease behavior. Often these factors include genetic mutations, allelic status, gene copy number, gene promoter methylation, histone modifications and micro-RNA expression associated with a malignant cell phenotype. For example, through large scale linkage analysis of high risk families, a heritable lung cancer susceptibility locus has been identified on chromosome 6q (Bailey-Wilson et al., 2004; You et al., 2009). The potential translational value of this finding could be the detection of a common gene aberration in this locus as a means to screen for patients predisposed to lung cancer.

Patients with this genetic predisposition would be ideal candidates for early detection and prevention programs.

Molecular markers may also have a translational benefit in determining patient prognosis (e.g. patient survival or tumor recurrence). For instance, point mutations in the well studies *KRAS* proto-oncogene have been shown to be associated with elevated oncogenic potential and in some studies, impaired NSCLC patient survival (Kosaka et al., 2009; Slebos et al., 1990). Proteins that are associated with tumor spread, vascularization and metastasis are also good candidate prognostic markers. For example, the abundance of tumor cells expressing the proliferation molecule Ki-67 has been associated with patient survival and clinical course of many cancer types including lung, breast and prostate cancer (Halvorsen et al., 2003; Hommura et al., 2000; Sahin et al., 1991). Predictive markers include genes and proteins that are associated with patient response to therapy (e.g. tumor shrinkage, patient survival benefit or tolerance to therapy). For example, *EGFR* is overexpressed in about 30% of all NSCLCs, but only a subset of these tumors harboring an *EGFR* mutation in the tyrosine kinase domain are sensitive to the tyrosine kinase inhibitors gefitinib (Iressa) and erlotinib (Tarceva) (Lynch et al., 2004; Sharma et al., 2007).

The genomic analyses of lung tumors have, and continue to, provide valuable insight into lung cancer pathogenesis and therapy. Until recently most of these studies have uncovered general molecular characteristics of tumors which are prevalent in the bulk tumor cell population. By using bulk material from tumor samples, genome-wide molecular analyses may overlook factors that are heterogeneously expressed within tumor cell subpopulations. Therefore, there has been a push to study clinically relevant

tumor cell subpopulations. For example, nearly 40% of patients with completely resected stage I NSCLC will experience a recurrence of their disease, suggesting that a subpopulation of tumor cells prone to micrometastatic behavior may be driving the recurrent phenotype (van Klaveren et al., 2009). Therefore, tumor cell subpopulations that drive prognosis are prime candidates deriving useful translational information.

One approach to improving outcome in lung cancer has been to identify and characterize a unique, phenotypically defined lung cancer stem cell population. As described in Chapter One, many cancers, including lung, are hypothesized to be initiated by transformed stem or progenitor cells. Tumor progression, metastasis and recurrence are also believed to be driven by a subpopulation of tumor cells that have retained or reacquired the capacity for stem cell-like differentiation and self-renewal. Therefore, it is hypothesized that the identification and characterization of factors that define and facilitate lung cancer stem cell biology may provide valuable tools for improving the early detection, diagnosis and therapy of lung cancer.

Already, several lines of evidence have been reported that support translational value of applying the cancer stem cell model to clinical lung cancer research. In 2009, Potti and colleges at Duke demonstrated the utility of applying the expression patterns of normal stem cells to predict lung cancer behavior. NSCLCs that overexpressed a set of genes reported to be highly related to embryonic stem cell biology were found to have a poor clinical outcome. In addition, the embryonic stem cell gene signature also predicted for poorly differentiated and late stage lung adenocarcinomas (Stevenson et al., 2009). Finally, the stem cell signature also predicted for cisplatin resistance in a panel of NSCLC cell lines, suggesting that stem cell signaling programs, and by proxy, the

abundance of stem cells in lung tumors, was correlated with prognosis and response to therapy (Stevenson et al., 2009).

Recently, Ooi et al. have reported that the abundance of lung epithelial cells expressing the basal stem cell marker cytokeratin 14 (CK14, encoded by *KRT14*) was increased during lung epithelium repair and that the abundance of $CK14^+$ tumor cells was correlated with NSCLC metastasis and impaired patient survival (Ooi et al., 2010). The abundance of $CK14^+$ tumor cells also was predictive of smoking and tumor histology, supporting the prospect of CK14 as a potentially useful prognostic marker.

As reported previously, lung cancer stem cells have been identified and isolated by their expression of the surface marker CD133 (Bertolini et al., 2009; Eramo et al., 2008). In addition, putative lung cancer stem cells have also been defined by their elevated aldehyde dehydrogenase activity as described in Chapter Three (Jiang et al., 2009a). This enzymatic activity is thought to be primarily driven by ALDH1A1, although it is likely that other ALDH enzymes (such as ALDH3A1, which is overexpressed in basal lung stem cells) may also contribute to the ALDH activity readout (Moreb et al., 2008; Moreb et al., 2007; Patel et al., 2008). The goal of this study was to evaluate the clinical relevance of three putative lung cancer stem cell markers, ALDH1A1, ALDH3A1 and CD133. To achieve this, the expression of these proteins was assayed in a large panel of clinically annotated NSCLC patient samples and expression patterns were investigated for prognostic associations.

| Stage | Tumor | Node | Metastasis | General Description | Survival Rate | |
|---|---|---|---|---|---|---|
| | | | | | 1 Year | 5 Year |
| Local | | | | | | |
| **IA** | T1 | N0 | M0 | **T1 tumor**: ≤ 3 cm, surrounded by lung or pleura; no tumor more proximal than lobe brochus | 94 | 67 |
| **IB** | T2 | N0 | M0 | **T2 tumor**: > 3cm, involving main bronchus ≥ 2 cm distal to carina, invading pleura; atelectasis or pneumonitis extending to the hilum but not entire lung | 87 | 57 |
| **IIA** | T1 | N1 | M0 | **N1**: involvment of ipsilateral peribronchial or hilar nodes and intrapulmonary nodes by direct extension | 89 | 55 |
| Locally advanced | | | | | | |
| **IIB** | T2 | N1 | M0 | | | |
| **IIB** | T3 | N0 | M0 | **T3 tumor**: invasion of chest wall, diaphram, mediastinal pleura, pericardium, main brochus < 2 cm distal to carina; atelectasis or pneumonitis of entire lung | 73 | 39 |
| **IIIA** | T1 | N2 | M0 | **N2**: involvment of ipsilateral mediastinal or subcarinal nodes | 64 | 23 |
| **IIIA** | T2 | N2 | M0 | | | |
| **IIIA** | T3 | N1 | M0 | | | |
| **IIIA** | T3 | N2 | M0 | | | |
| **IIIB** | Any T | N3 | M0 | **N3**: involvment of contralateral (lung) nodes or any supraclavicular node | 32 | 3 |
| Advanced | | | | | | |
| **IIIB** | T4 | Any N | M0 | **T4** tumor: invasion of mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina, separate tumor nodules; malignant pleural effusion | 37 | 7 |
| **IV** | Any T | Any N | M1 | **M1**: Distant metastasis | 20 | 1 |

**Table 5.1: Survival Rates of NSCLC TNM Stages.** The TNM staging as defined by the American Joint Commission on Cancer, denotes T for tumor, N for node, and M for metastasis. This table was adapted from Table 2 (Spira and Ettinger, 2004) and data from (Mountain and Dresler, 1997).

## 5.2 Results

### *5.2.1 Analysis of ALDH1A1 Tumor Expression and Patient Prognosis*

To assay for ALDH1A1 expression in NSCLC, IHC was performed on 282 clinically annotated NSCLC core sections deposited on a tissue microarray. Staining was then evaluated by a pathologist and sample ALDH1A1 expression scores were determined as described in Chapter Two. Expression scores were derived as a function of the abundance of tumor cells stained for ALDH1A1, and the staining intensity. NSCLC ALDH1A1 expression scores ranged from 0 to 250, with a median score of 13.

To compare the clinical features of NSCLC with low and abundant $ALDH1A1^{+}$ tumor cells, stained samples were dichotomized based on the median expression score into $ALDH1A1^{high}$ and $ALDH1A1^{low}$ expression categories (Figure 5.1A). In this way samples were categorized objectively and each expression category contained roughly equal numbers of samples ($ALDH1A1^{high}$ n = 142, $ALDH1A1^{low}$ n = 140). Tumoral ALDH1A1 expression was detected in the cytoplasm whereas very little staining was observed in the surrounding stroma, suggesting some tumor specificity of ALDH1A1 staining (Figure 5.1B).

Chi-square tests of the distribution of high and low ALDH1A1 expressing NSCLCs across patient gender, tumor histology, stage, lymph node spread and smoking history revealed a significant association between ALDH1A1 overexpression and a SCC histology ($P < 0.0001$) (Table 5.2). To test whether ALDH1A1 expression was associated with patient survival, Kaplan-Meier analysis was performed on samples with high and low ALDH1A1 expression. As a result ALDH1A1 overexpression was

associated with impaired patient survival (HR: 1.46, 95%CI: 1.05 to 2.01, $P$ = 0.025) (Figure 5.2A). To test if lung tumor ALDH1A1 expression predicted for poor prognosis in early stage disease, Kaplan-Meier analysis was performed on stage I tumors and tumors with no detectable lymph node spread (N0) with high and low ALDH1A1 expression. In both patient cohorts, ALDH1A1 overexpression was associated with poor prognosis (HR for stage I tumors: 1.65, 95%CI: 1.05 to 2.59, $P$ = 0.029 and HR for N0 tumors: 1.91, 95%CI: 1.26 to 2.88, $P$ = 0.0023) (Figure 5.2A and 5.2B).

The histological and oncogenic heterogeneity found in lung cancer presumes heterogeneity in lung cancer stem cells (Sullivan and Minna, 2010). In support of this, Curtis et al. demonstrated in mice that lung cancer stem cells from mutant K-ras and p53-floxed tumors do not share the same identifiable markers as lung cancer stem cells from mutant *EGFR* tumors (Curtis et al., 2010). Therefore, if ALDH1A1 marked lung cancer stem cells in histological or oncogenic subset of NSCLCs, then the prognostic relevance of ALDH1A1 would be more pronounced in the given subset of tumors. To test for this, Kaplan-Meier analysis was performed on ACs and SCCs with high and low ALDH1A1 expression. In each case ALDH1A1 tended to predict for impaired patient survival, however the associations were not significant (AC $P$ = 0.37 and SCC $P$ = 0.22) (Figure 5.3A and 5.3B). In addition, the distribution ALDH1A1 overexpressing tumors with *EGFR* and *KRAS* mutations were assayed by Fisher's exact test, however no significant association between ALDH1A1 staining and tumor oncogenotype was observed (Table 5.2). Interestingly survival analysis of *EGFR* mutant NSCLCs revealed patients with ALDH1A1 overexpressing tumors tended to have a more favorable prognosis than patients with ALDH1A1$^{low}$ tumors (HR: 0.22, 95%CI: 0.04 to 1.10, $P$ = 0.06) (Figure

5.3C). However in mutant *KRAS* tumors ALDH1A1 expression was not associated with patient survival (Figure 5.3D).

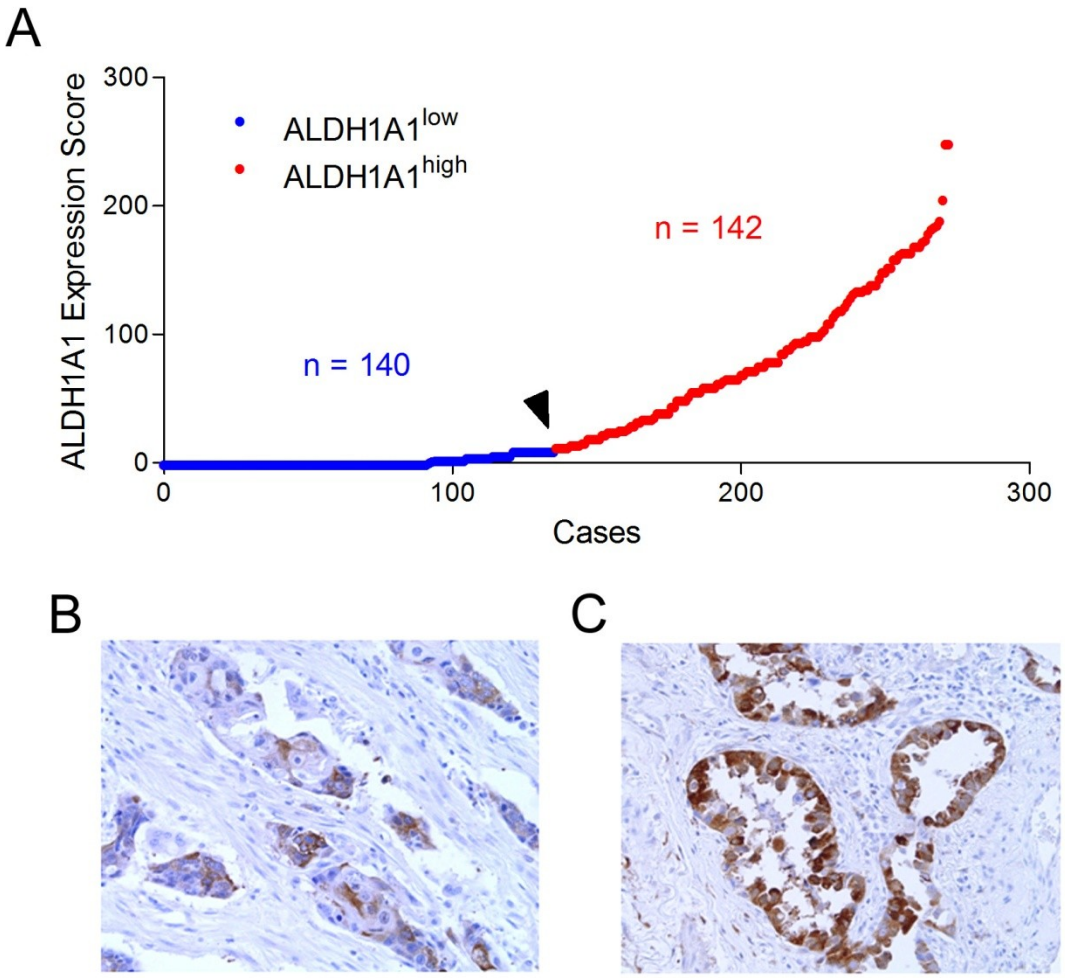



**Figure 5.1: Detection of ALDH1A1 expression in NSCLC.** ALDH1A1 protein expression was detected in NSCLC TMA samples and scored as described in Chapter Two. Tumor samples were dichotomized by the median ALDH1A1 expression score (black arrow), into high (red) and low (blue) expression categories (A). Example IHC of tumor cells with low ALDH1A1 expression (B). Example IHC of ALDH1A1$^{high}$ cytoplasmic tumor cell staining (C).

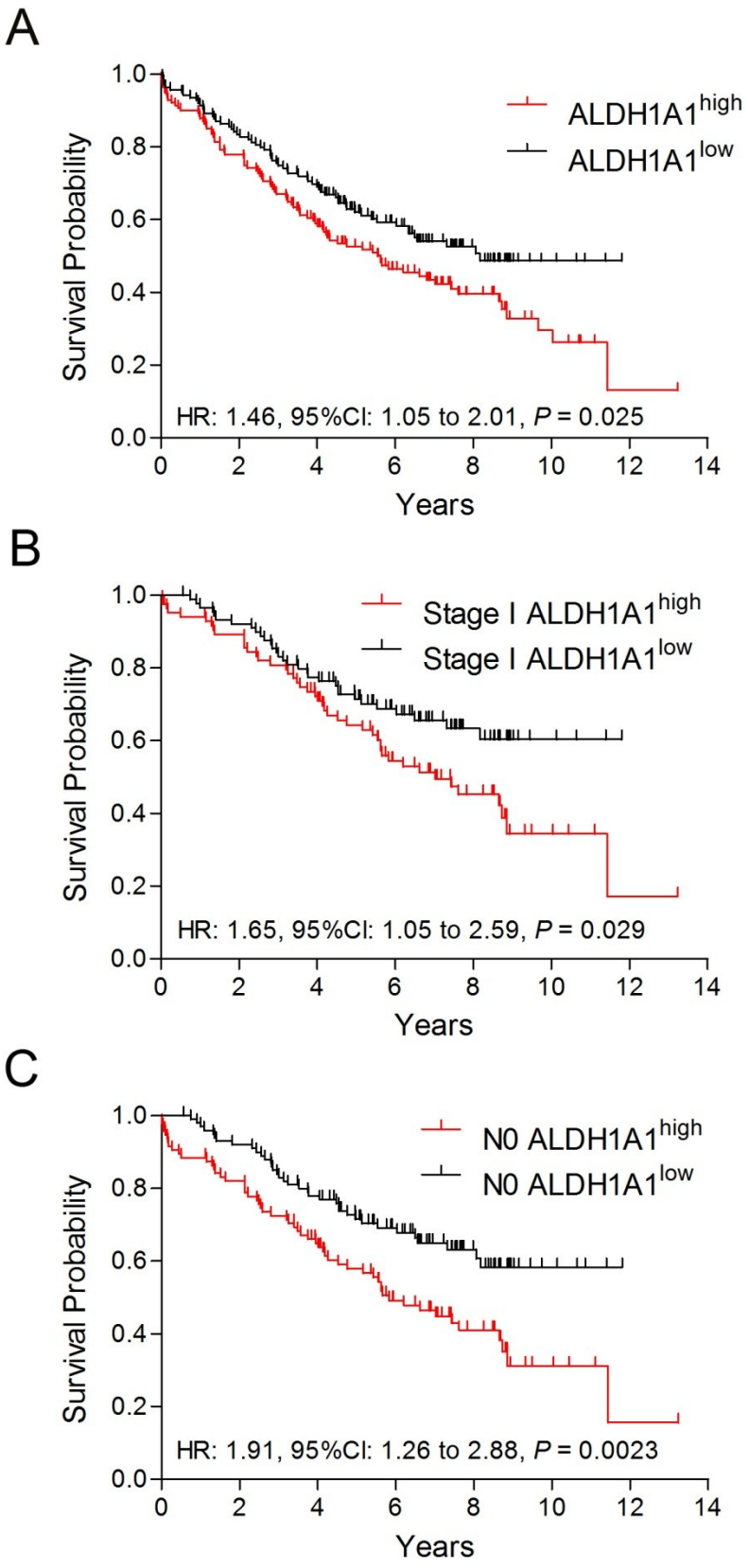

A
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
ALDH1A1high
ALDH1A1low
HR: 1.46, 95%CI: 1.05 to 2.01, P = 0.025
0
2
4
6
8
10
12
14
Years
B
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
Stage I ALDH1A1high
Stage I ALDH1A1low
HR: 1.65, 95%CI: 1.05 to 2.59, P = 0.029
0
2
4
6
8
10
12
14
Years
C
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
N0 ALDH1A1high
N0 ALDH1A1low
HR: 1.91, 95%CI: 1.26 to 2.88, P = 0.0023
0
2
4
6
8
10
12
14
Years

**Figure 5.2: Survival analysis of NSCLC based on ALDH1A1 expression.** Kaplan-Meier plot of overall patient survival based on tumors expressing high (red: n = 142) and low (black: n = 140) ALDH1A1 showed high ALDH1A1 scores were associated with reduced overall survival (A). Kaplan-Meier analysis of Stage I patient samples indicated high ALDH1A1 expression was associated with poor prognosis (red: $n$ = 85, black: $n$ = 90) (B). Similar analysis of patients with no lymph node metastases (N0) revealed ALDH1A1 expression was associated with poor prognosis in this patient group (red: $n$ = 96, black: $n$ = 101) (C).

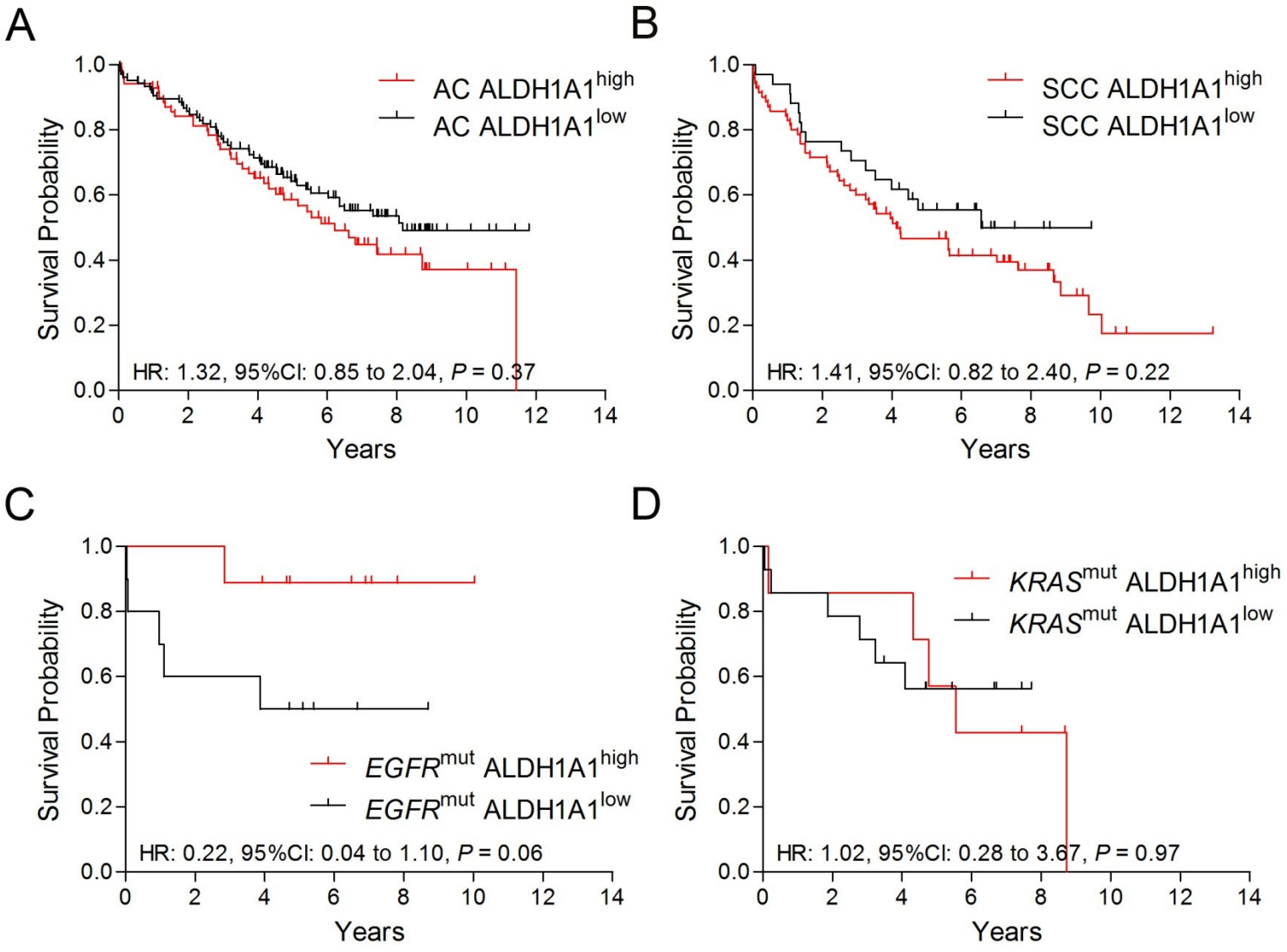



**Figure 5.3: Survival analysis of NSCLC histotypes and oncogenotypes based on ALDH1A1 expression.** Kaplan-Meier analysis of lung adenocarcinoma patient tumors with high and low (red: n = 106, black: n = 71) and low ALDH1A1 expression revealed a trend for elevated ALDH1A1 expression to predict with impaired survival (A). A similar trend was observed in lung squamous cell carcinoma patient tumors (red: n = 71, black: n = 34) (B). Survival analysis of a small cohort of *EGFR* mutant tumors revealed a trend improved survival in patients with elevated ALDH1A1 expression (red: n = 10, black: n = 10) (C). However no association was observed between survival and ALDH1A1 expression in mutant *KRAS* tumors (red: n = 7; black: n = 14) (D).

| Variables | *n* | ALDH1A1$^{high}$ | *P* | ALDH3A1$^{high}$ | *P* |
|---|---|---|---|---|---|
| **Gender** | | | 0.19 | | 0.63 |
| Male | 138 | 75 | | 71 | |
| Female | 144 | 67 | | 70 | |
| **Histologic Type** | | | < 0.0001 | | < 0.0002 |
| AC | 177 | 71 | | 73 | |
| SCC | 105 | 71 | | 68 | |
| **Disease Stage** | | | 0.20 | | 0.04 |
| Stage I | 175 | 85 | | 77 | |
| Stage II | 55 | 33 | | 35 | |
| Stage III | 42 | 19 | | 24 | |
| Stage IV | 7 | 5 | | 5 | |
| **Node Involvment** | | | 0.07 | | 0.02 |
| N0 | 197 | 96 | | 89 | |
| N1 | 54 | 34 | | 36 | |
| N2 | 31 | 12 | | 16 | |
| **Smoking Status** | | | 0.76 | | 0.94 |
| Never | 46 | 20 | | 24 | |
| Former | 134 | 71 | | 67 | |
| Current | 92 | 46 | | 45 | |
| **EGFR Status** | | | 0.63 | | 0.87 |
| Wild Type | 174 | 77 | | 75 | |
| Mutant | 20 | 10 | | 9 | |
| **KRAS Status** | | | 0.35 | | 0.93 |
| Wild Type | 173 | 80 | | 76 | |
| Mutant | 21 | 7 | | 9 | |

**Table 5.2: Correlation between tumor ALDH protein expression and clinical covariates.**

### *5.2.2 Analysis of ALDH3A1 Tumor Expression and Patient Prognosis*

ALDH3A1 expression was assayed in 282 clinically annotated NSCLC samples by IHC, and an expression score was derived based on the percent of cells stained and staining intensity, as described in Chapter Two. ALDH3A1 expression scores ranged from 0 to 223.3, with a median score of 25 (Figure 5.4A). Using this median value as a cut-off, NSCLC samples were categorized by ALDH3A1 expression scores as either $ALDH3A1^{high}$ (n = 141) or $ALDH3A1^{low}$ (n = 141). Similar to ALDH1A1 expression, ALDH3A1 expression was primarily found in the cytoplasm of lung tumor cells, and only rarely observed in surrounding stromal cells (Figure 5.4B). To determine if ALDH3A1 and ALDH1A1 marked for the same tumor cell populations, a linear correlation analysis was performed using sample protein expression scores. Expression of ALDH1A1 and ALDH3A1 appeared to only weakly be correlated in NSCLC samples ($r^2 = 0.21$), indicating little overlap in tumor cell staining (Figure 5.7A).

The Chi-square test was used to assess the association between ALDH3A1 expression and clinical covariables including gender, tumor histology, lymph node spread, and smoking history. Like ALDH1A1 expression in NSCLC samples, ALDH3A1 expression was associated with a SCC ($P < 0.0002$). However unlike ALDH1A1 expression, Chi-square contingency analysis of ALDH3A1 expression revealed an association with disease stage and lymph node spread (Table 5.2). The proportion of ALDH3A1high samples was lowest in each clinical covariate corresponding to early stage disease. Despite this association with disease stage and nodal spread, ALDH3A1 expression was not associated with overall patient survival (HR: 1.11, 95%CI: 0.80 to 1.53, $P = 0.53$) (Figure 5.5A). In addition, ALDH3A1

expression did not predict patient prognosis in stage I (HR: 1.33, 95%CI: 0.77 to 2.30, $P = 0.30$) or N0 disease (1.16, 0.76 to 1.76, $P = 0.49$) (Figure 5.5B and 5.5C).

To determine if ALDH3A1 expression conferred any prognostic prediction within certain NSCLC histotype or oncogenotype, Kaplan-Meier survival analysis was performed on AC and SCC samples, as well as mutant *EGFR* and mutant *KRAS* samples. In each patient cohort, no trend or significant difference in survival curves were observed between ALDH3A1$^{high}$ and ALDH3A1$^{low}$ patient samples (Figure 5.6).

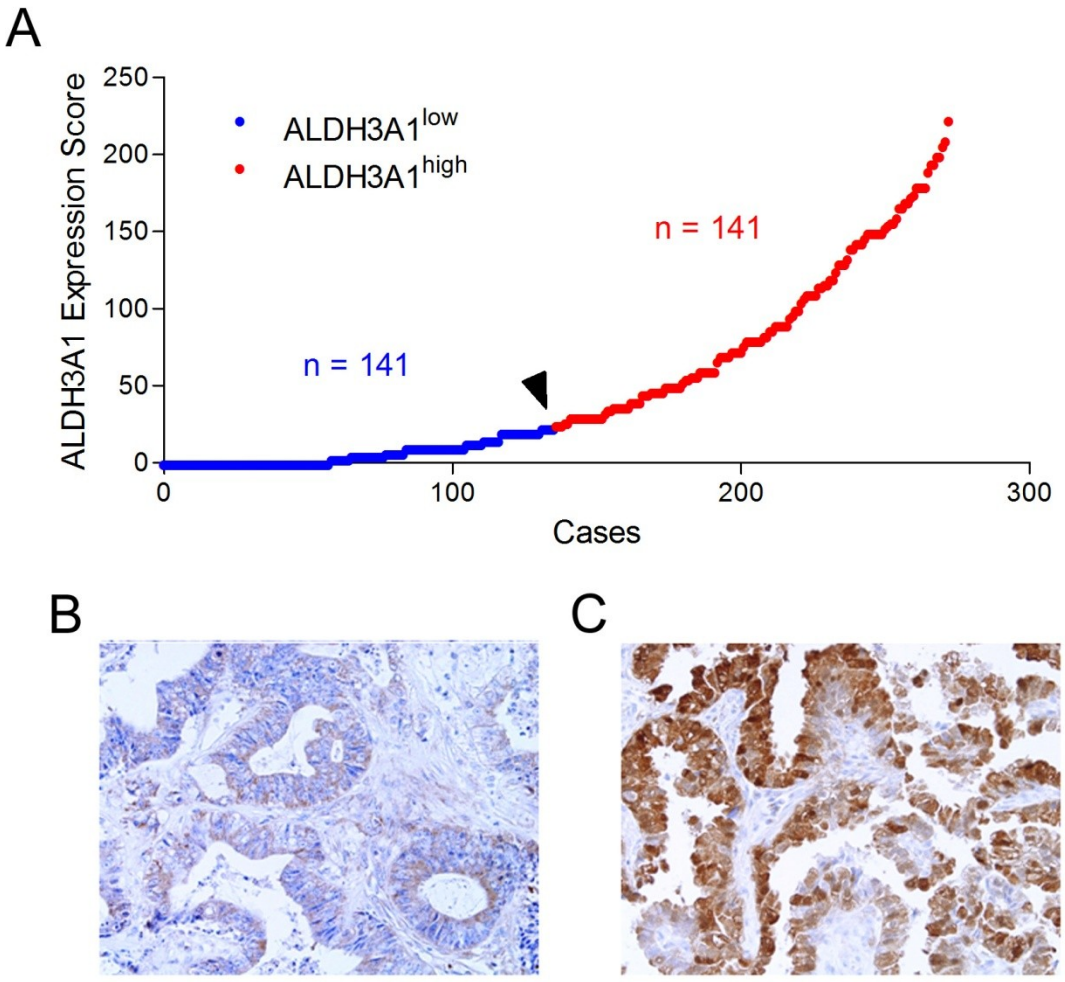



**Figure 5.4: Detection of ALDH3A1 expression in NSCLC.** Tumoral ALDH3A1 protein expression was detected in NSCLC TMA samples and scored as described in Chapter Two. Tumor samples were dichotomized by the median ALDH3A1 expression score (black arrow), into high (red) and low (blue) expression categories (A). Example IHC of tumor cells with low ALDH3A1 expression (B). Example of high ALDH3A1 expression reveals a cytoplasmic staining pattern (C).

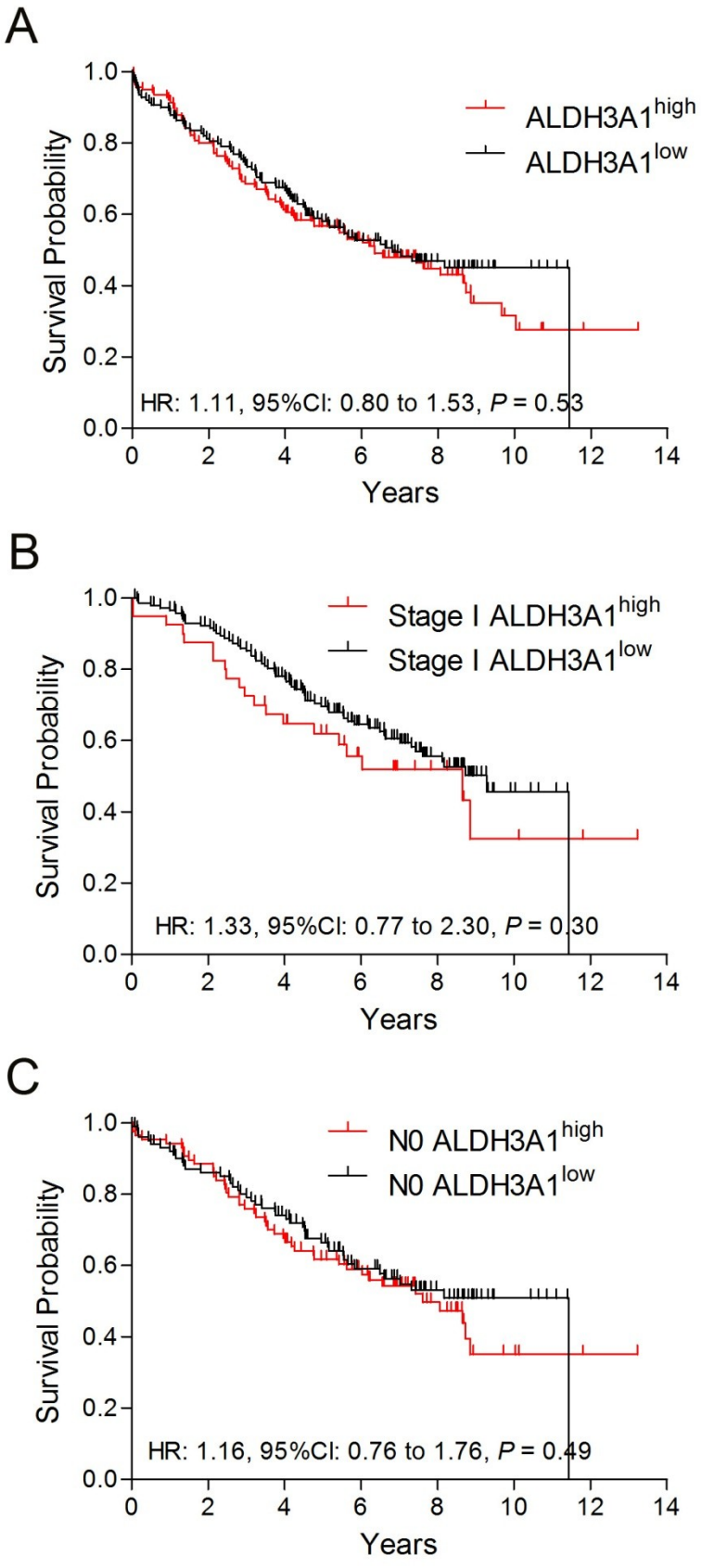

A
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
ALDH3A1high
ALDH3A1low
HR: 1.11, 95%CI: 0.80 to 1.53, P = 0.53
0
2
4
6
8
10
12
14
Years
B
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
Stage I ALDH3A1high
Stage I ALDH3A1low
HR: 1.33, 95%CI: 0.77 to 2.30, P = 0.30
0
2
4
6
8
10
12
14
Years
C
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
N0 ALDH3A1high
N0 ALDH3A1low
HR: 1.16, 95%CI: 0.76 to 1.76, P = 0.49
0
2
4
6
8
10
12
14
Years

**Figure 5.5: Survival analysis of NSCLC based on ALDH3A1 expression.** Survival analysis of patients with tumors expressing high (red: n = 141) and low (black: n = 141) ALDH3A1 indicated no association between ALDH3A1 expression and overall patient survival (A). Kaplan-Meier analysis of Stage I patients (red: $n$ = 45, black: $n$ = 145) found no association between ALDH3A1 expression and patient prognosis (B). Similarly, analysis of tumor cell ALDH3A1 expression in N0 patients (red: $n$ = 87, black: $n$ = 103) found no association between protein expression and survival (C).

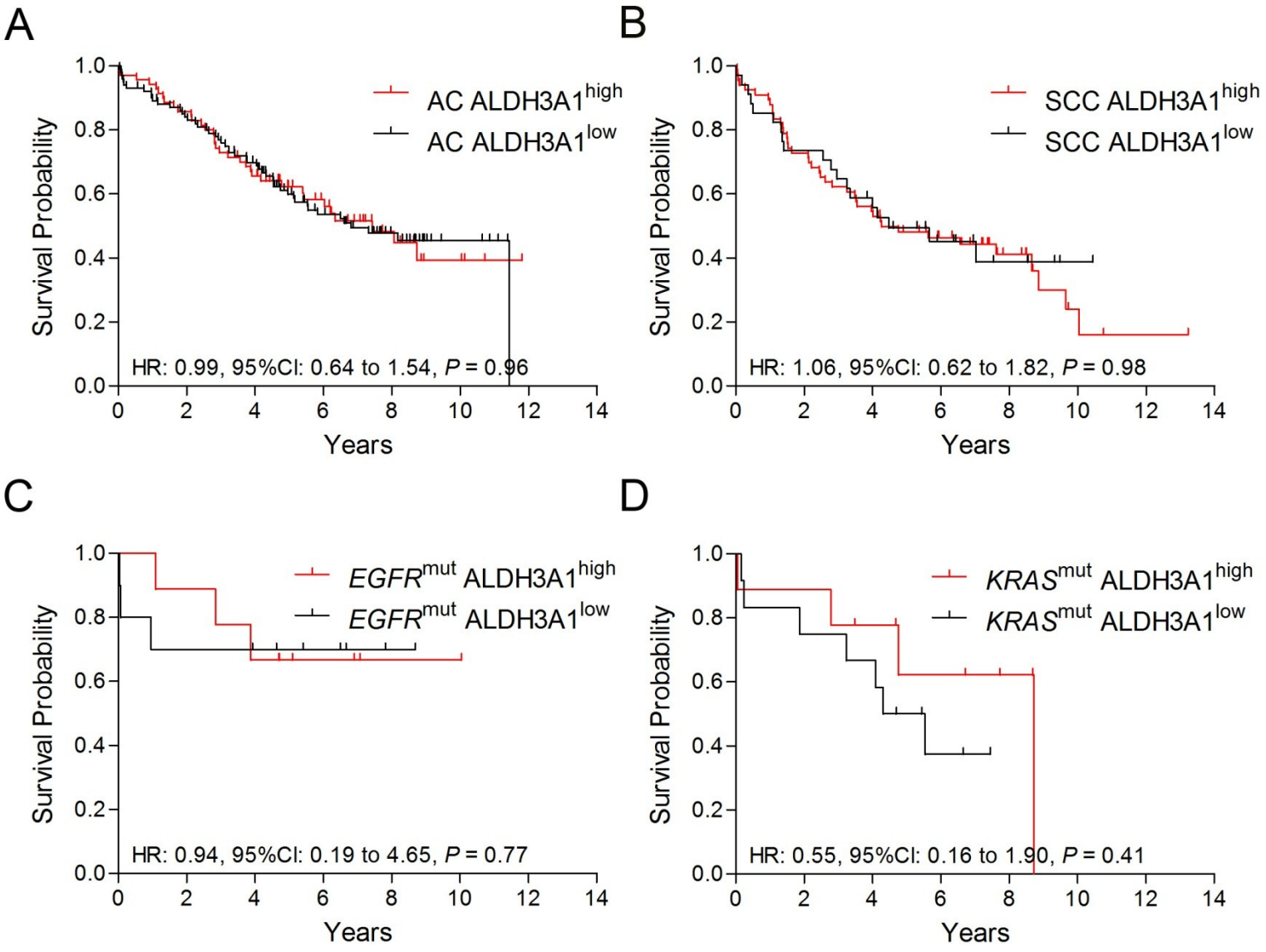



**Figure 5.6: Survival analysis of NSCLC histotypes and oncogenotypes based on ALDH3A1 expression.** Kaplan-Meier analysis of lung adenocarcinoma patient tumors with high and low (red: n = 70, black: n = 102) revealed no association between ALDH3A1 protein expression and patient survival (A). Similarly no association was observed in lung squamous cell carcinoma patient tumors (red: n = 67, black: n = 34) (B). In *EGFR* mutant tumors, ALDH3A1 expression did not predict for patient survival (red: n = 9, black: n = 12) (C). Survival curves from a small cohort of *KRAS* mutant tumors also did not reveal an association between patient survival and ALDH3A1 expression (red: n = 9; black: n = 10) (D).

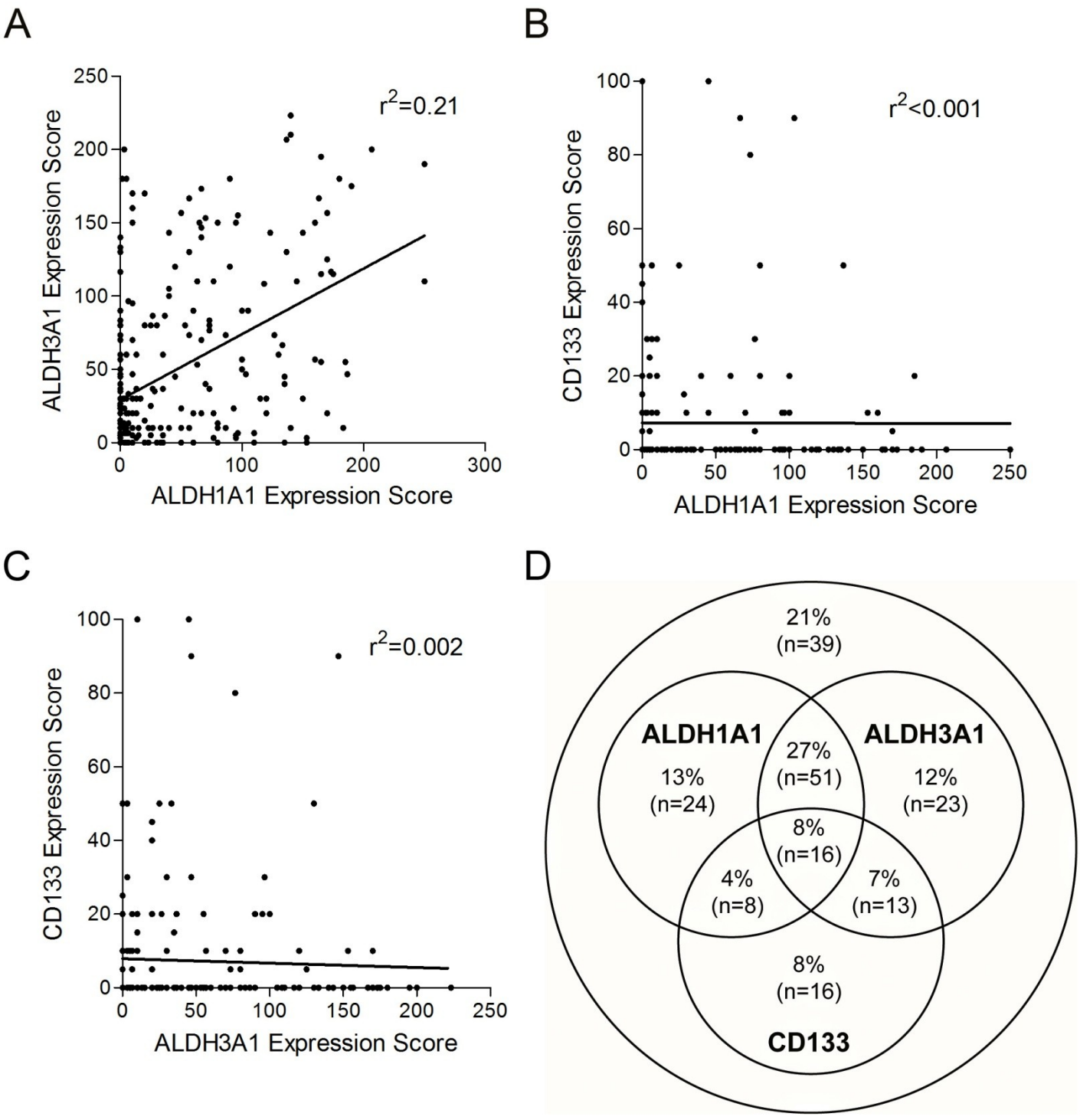

A
ALDH3A1 Expression Score
ALDH1A1 Expression Score
r2=0.21
B
CD133 Expression Score
ALDH1A1 Expression Score
r2<0.001
C
CD133 Expression Score
ALDH3A1 Expression Score
r2=0.002
D
21%
(n=39)
ALDH1A1
ALDH3A1
13%
(n=24)
27%
(n=51)
12%
(n=23)
8%
(n=16)
4%
(n=8)
7%
(n=13)
8%
(n=16)
CD133

**Figure 5.7: Correlation of ALDH1A1, ALDH3A1 and CD133 expression in NSCLC.** Linear regresion analysis between ALDH1A1 and ALDH3A1 revealed a weakly positive linear correlation (A). Linear regression analysis between CD133 and ALDH1A1 expression scores revealed no correlation between staining (insignificant slope deviation from zero, $P = 0.97$) (B). Similar analysis between CD133 and ALDH3A1 expression scores showed no correlation (insignificant slope deviation from zero, $P = 0.61$) (C). Venn diagram of ALDH1A1, ALDH3A1 and CD133 expression demonstrated substantial expression heterogeneity within NSCLC samples.

### *5.2.3 Analysis of CD133 Tumor Expression and Patient Prognosis*

The expression of CD133 in 207 clinically annotated NSCLC samples was assayed by IHC as described in Chapter Two. Expression scores for CD133 in tumor samples were derived as previously described and ranged from 0 to 90, with the median value of 0 (Figure 5.8A). Therefore, samples were dichotomized into sample categories with and without detectable tumoral CD133 (referred to as $CD133^{+}$ and $CD133^{-}$ samples). Unlike ALDH1A1 and ALDH3A1, CD133 expression was primarily detected on the apical/luminal surface of tumor cells (Figure 5.8B). In a few cases CD133 staining was observed in the cytoplasm of tumor cells and/or in the luminal space, however staining was usually weak. Because CD133 is a transmembrane protein, any NSCLC samples that did not have tumor cells with detectable membranous CD133 were considered $CD133^{-}$. Overall, tumoral CD133 expression was less abundant than tumoral ALDH protein expression assayed by IHC. In addition, tumor cells that stained for CD133 usually represented a small fraction of the total tumor cell population, thus contributing to the much smaller range in CD133 expression scores. Linear regression analysis found no correlation between CD133 and ALDH protein expression in NSCLC samples (Figure 5.7B and 5.7C), although CD133 and ALDH protein expression was not mutually exclusive (Figure 5.7D). For example, of the TMA samples stained for all three prospective cancer stem cell proteins, only 8% were $ALDH1A1^{high}ALDH1A1^{high}CD133^{+}$.

To assay for an association between CD133 expression and clinical co-factors, Chi-square tests were performed. Interestingly CD133 expression was more commonly detected in ACs ($P = 0.004$). CD133 expression was also more prevalent in NSCLCs

from patients with no history of smoking ($P$ = 0.009) and in mutant *EGFR* tumors ($P$ = 0.002) (Table 5.3).

To test whether the detection of CD133 expression in NSCLC was associated with patient survival, Kaplan-Meier analysis was performed on $CD133^{+}$ and $CD133^{-}$ TMA samples. As a result CD133 expression was not associated with overall patient survival (HR: 1.05, 95%CI: 0.67 to 1.60, $P$ = 0.81) (Figure 5.9A). Similarly CD133 expression failed to predict patient prognosis in stage I disease and in N0 disease (Figure 5.9B and 5.9C). Further analysis of AC and SCC histotypes found no difference in $CD133^{+}$ and $CD133^{-}$ tumor prognoses. However analysis of tumor oncogenotypes revealed the detection of CD133 was associated with a favorable prognosis in mutant *EGFR* tumors (HR: 0.10 95%CI: 0.01 to 0.99, $P$ = 0.05), but not predictive of survival in mutant *KRAS* lung tumors (Figure 5.9C and 5.9D).

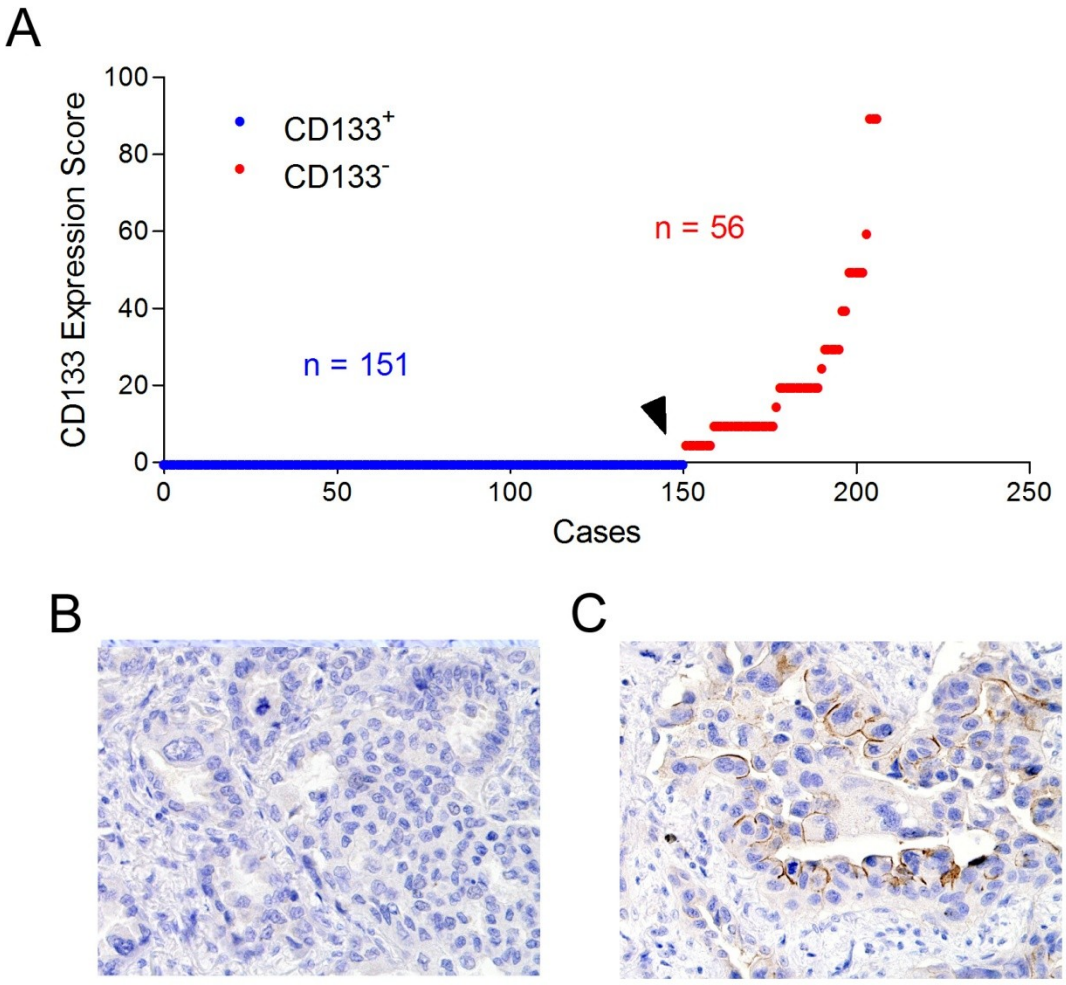



**Figure 5.8: Detection of CD133 expression in NSCLC.** CD133 protein expression was detected in NSCLC TMA samples and scored as described in Chapter Two. Tumor samples were dichotomized by the presence of CD133 expression (black arrow), into positive (red) and negative/absent (blue) expression categories (A). Example IHC of tumor cells with negative/absent CD133 expression (B). Example of membranous $CD133^{+}$ tumor cells by IHC (C).

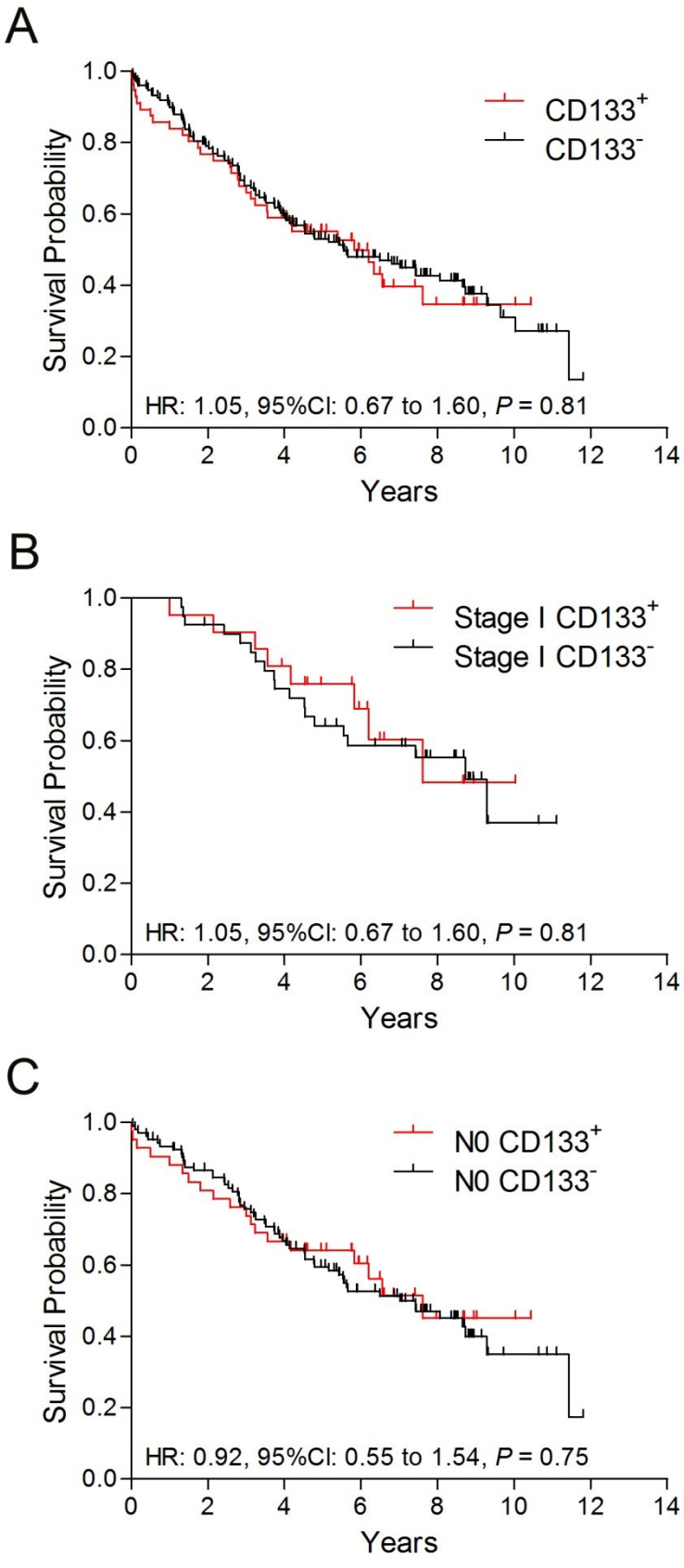

A
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
CD133+
CD133-
HR: 1.05, 95%CI: 0.67 to 1.60, P = 0.81
0
2
4
6
8
10
12
14
Years
B
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
Stage I CD133+
Stage I CD133-
HR: 1.05, 95%CI: 0.67 to 1.60, P = 0.81
0
2
4
6
8
10
12
14
Years
C
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
N0 CD133+
N0 CD133-
HR: 0.92, 95%CI: 0.55 to 1.54, P = 0.75
0
2
4
6
8
10
12
14
Years

**Figure 5.9: Survival analysis of NSCLC based on CD133 expression.** Kaplan-Meier analysis of patient samples expressing detectable CD133 (red: n = 56) and undetectable CD133 expression (black: n = 151) indicated no association between CD133 expression and overall patient survival (A). Survival curves of Stage I patient tumors expressing detectable CD133 (red: $n$ = 21) and undetectable CD133 (black: $n$ = 40) revealed no association between protein expression and survival (B). Similarly no association between CD133 and prognosis was found in N0 patients (red: $n$ = 42, black: $n$ = 105) (C).

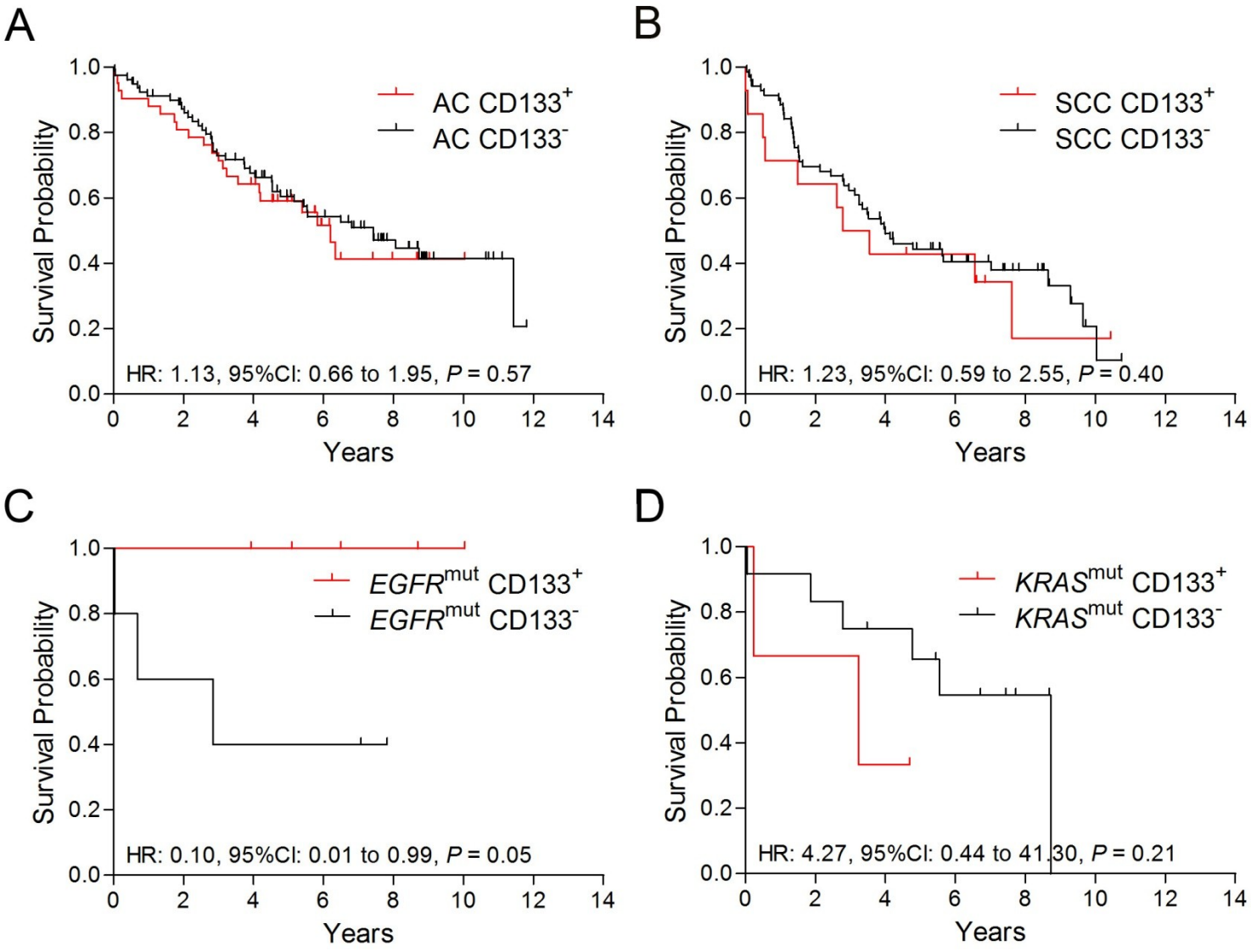



**Figure 5.10: Survival analysis of NSCLC histotypes and oncogenotypes based on CD133 expression.** Kaplan-Meier analysis of lung adenocarcinoma patient tumors with high and low (red: n = 42, black: n = 180) revealed no association between CD133 protein expression and patient survival (A). Similarly no association was observed in lung squamous cell carcinoma patient tumors (red: n = 14, black: n = 71) (B). However, in a small cohort of *EGFR* mutant tumors, CD133 expression was associated with improved survival (red: n = 5, black: n = 5) (C). Survival curves from a small cohort of *KRAS* mutant tumors did not reveal an association between patient survival and CD133 expression (red: n = 3; black: n = 12) (D).

| Variables | *n* | $CD133^+$ | *P* |
|---|---|---|---|
| **Gender** | | | 0.09 |
| Male | 105 | 23 | |
| Female | 102 | 33 | |
| **Histologic Type** | | | 0.004 |
| AC | 122 | 42 | |
| SCC | 85 | 14 | |
| **Disease Stage** | | | 0.12 |
| Stage I | 130 | 36 | |
| Stage II | 41 | 8 | |
| Stage III | 32 | 9 | |
| Stage IV | 4 | 3 | |
| **Node Involvment** | | | 0.65 |
| N0 | 147 | 42 | |
| N1 | 38 | 8 | |
| N2 | 22 | 6 | |
| **Smoking Status** | | | 0.009 |
| Never | 28 | 14 | |
| Former | 103 | 27 | |
| Current | 75 | 15 | |
| **EGFR Status** | | | |
| Wild type | 124 | 37 | 0.002 |
| Mutant | 10 | 8 | |
| **KRAS Status** | | | |
| Wild type | 119 | 40 | 0.38 |
| Mutant | 15 | 3 | |

**Table 5.3: Correlation between tumor CD133 expression and clinical covariates.**

## 5.3 Discussion

In this chapter the clinical relevance of putative lung cancer stem cell markers ALDH1A1, ALDH3A1 and CD133 were investigated in a retrospective study of resected and clinically annotated NSCLC tumor samples. These samples, assembled on a TMA and stained for the expression of cancer stem cell markers by IHC, contained both normal and tumor tissue, allowing for immediate comparison of staining between healthy and malignant tissues. For each marker tested the intensity and abundance of tumor cells stained positive varied greatly between samples, indicating heterogeneous expression between tumors samples. By quantifying these staining properties, NSCLCs could be clustered into categories with absent or abundant marker$^{+}$ tumor cells, allowing for comparative analyses of clinical parameters.

Of the three putative lung cancer stem cell markers assayed by IHC, only ALDH1A1 was consistently associated with patient prognosis. In summary, the abundance of ALDH1A1$^{+}$ tumor cells was associated with impaired patient survival. This would suggest that ALDH1A1 expression is associated with an aggressive, possibly stem cell-like tumor cell phenotype. This conclusion is strengthened by the fact that ALDH activity, previously demonstrated to select for highly tumorigenic, stem cell-like lung cancer cells, is correlated with ALDH1A1 expression in lung cancer cell lines (as described in Chapter Three). These findings are also consistent with data from other tumor types that report ALDH as a cancer stem cell marker. For example in the same study that identified ALDH as a marker for normal and malignant stem cells in breast tissue, Ginestier and colleges found the expression of ALDH1A1 to be a strong predictor for poor patient prognosis in a large collection of 577 clinically annotated breast

carcinomas (Ginestier et al., 2007). In a follow up study published earlier this year, the same research group reported ALDH1A1 expression to be strongly associated with metastasis and poor patient survival in inflammatory breast cancer (Charafe-Jauffret et al., 2010). Interestingly, while elevated ALDH1A1 expression was associated with poor prognosis, it was not associated with the $CD24^{-}CD44^{+}$ breast cancer stem cell phenotype in inflammatory breast cancer, suggesting the existence of multiple cancer stem cell phenotypes in breast cancer (Charafe-Jauffret et al., 2010).

During the course of this study, Jiang et al. reported that ALDH1A1 expression was associated with poor prognosis in a small panel of stage I NSCLCs (Jiang et al., 2009a). In this chapter the analysis of a larger panel of stage I patient tumors as well as patients with no lymph node spread, confirmed the association between ALDH1A1 expression and poor prognosis in early stage and locally advanced NSCLC. These studies promote ALDH1A1 as a potentially useful prognostic biomarker in early stage disease, where clinical intervention has the best chance to improve prognosis. Survival analysis of late stage disease (stage III and IV) with high and low ALDH1A1 expression did not reveal a significant trend patient survival, however this is likely due to the relatively low amount of late stage samples available for analysis. Five out of seven stage IV NSCLCs possessed abundant ALDH1A1 expression; it would be of interest to study more stage IV tumors to confirm the abundance of $ALDH1A1^{+}$ lung cancer cells in late stage disease.

Although univariate analysis of ALDH1A1 expression and patient survival revealed an association with ALDH1A1 overexpression and poor prognosis, multivariate analysis adjusting for clinical factors such as age, stage, histology, nodal involvement and

smoking status found ALDH1A1 expression was not an independent marker of prognosis. The failure to reach statistical significance was due to the influence of the squamous histotype, which was strongly associated with ALDH1A1 overexpression. Separate analyses of ACs and SCCs revealed a trend for ALDH1A1 expression to predict for impaired survival although these associations lacked statistical significance.

The analysis of the lung basal cell marker ALDH3A1 was also performed on the same TMA that was stained for ALDH1A1. Like ALDH1A1, ALDH3A1 expression was detected in the cytoplasm of tumor cells, and IHC reactive tumor cell subpopulations could be detected in many NSCLC samples. In accordance with previous findings, ALDH3A1 expression was associated with a squamous histotype in NSCLC (Patel et al., 2008). Unlike ALDH1A1, ALDH3A1 over expression was not associated with over all patient survival, or survival of stage I or N0 patients; suggesting that ALDH3A1 expression does not identify aggressive NSCLC cells.

In previous reports, ALDH1A1 and ALDH3A1 have been implicated in facilitating lung cancer ALDH activity (Moreb et al., 2008; Moreb et al., 2007). However, analysis of lung cancer cell lines found only ALDH1A1 and not ALDH3A1 expression to be positively correlated with ALDH activity (Chapter Three). Analysis of ALDH1A1 and ALDH3A1 protein expression in 282 NSCLCs found limited overlap in expression. Taken together these data would suggest that ALDH1A1 and not ALDH3A1 expression is more closely associated with an aggressive lung cancer cell population with enhanced ALDH activity.

As reported previously, CD133 expression has been widely used to identify cancer stem cells in a variety of tumor types (Bertolini et al., 2009; Eramo et al., 2008;

Ma et al., 2008; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2003). For example, highly tumorigenic glioma stem cells are identified by their expression of CD133. $CD133^+$ glioma cells are reportedly proficient in transferring the disease to NOD/SCID mice, recapitulating tumor cell heterogeneity, maintain a perivascular niche and are resistant to radiotherapy (Calabrese et al., 2007; Singh et al., 2003; Sunaga et al., 2004). In 2008, Zeppernick et al. investigated the link between CD133 expression in brain cancer and clinical outcome and found though retrospective IHC analysis of 95 glioma samples that the abundance of $CD133^+$ tumor cells was associated with reduced patient survival, tumor recurrence and metastasis (Zeppernick et al., 2008). Recently CD133 has been described as a cancer stem cell marker in lung cancer, however the relationship between CD133 expression and clinical outcome in NSCLC has not been fully explored. To this end, the CD133 expression was assayed by IHC in the same NSCLC TMAs that were stained for ALDH1A1 and ALDH3A1 expression. However by the time the IHC analysis for CD133 expression was performed on TMA samples many of the cores used in the arrays had been exhausted by prior use and thus only 207 out of the original 282 samples remained to be tested. Nevertheless, the remaining samples retained roughly the same distribution of tumors of different histotypes, stages and oncogenotypes.

Similar to findings in NSCLC cell lines (Chapter Three), most NSCLC tumors did not have any detectable CD133 expression. In samples that did have detectable CD133 expression, IHC staining was primarily membranous and appeared on the apical/luminal surface of tumor cells. Approximately one third of the 207 samples had a detectable subpopulation of $CD133^+$ tumor cells; however the presence of $CD133^+$ tumor cells in

lung cancer was not associated with disease progression or patient survival. Furthermore, CD133 expression was not correlated with ALDH1A1 or ALDH3A1 expression, indicating little overlap between the expression of these markers in NSCLC.

Interestingly the expression of CD133 was more prevalent in ACs, mutant *EGFR* tumors and tumors from never smokers. Although tobacco smoke remains the leading cause for most lung cancers, nearly a fifth of all lung cancer cases are from non-smokers (Parkin et al., 2005). The underlying pathology of lung cancers in never-smokers has become an increasingly studied topic, in part because mutations in *EGFR* associated with non-smoker NSCLC effectuate dramatic sensitivity to tyrosine kinase inhibitor therapy (Lynch et al., 2004; Sharma et al., 2007). The fact that CD133 is associated with these clinical variables suggests that CD133 might be useful biomarker for identifying and differentiating between smoker and non-smoker lung cancers. Because lung cancers from non-smokers might represent a distinct and molecularly different disease group in lung cancer, the possibilities exist that these tumors may have arisen from or retain a unique population of malignant lung stem cells. By this same concept, the identity and biology of lung cancer stem cells may also be dependent upon the driving oncogenotype of the tumor (Curtis et al., 2010; Sullivan and Minna, 2010). Therefore, the capacity for the expression of ALDH1A1, ALDH3A1 and CD133 to predict for patient survival (and by proxy an aggressive tumor cell phenotype) was assayed in a small panel of mutant *EGFR* and mutant *KRAS* tumors. In short, neither marker was predictive of patient prognosis in mutant *KRAS* tumors. However, overexpression of ALDH1A1 and detection of CD133 expression in NSCLC was associated with improved patient survival. These observations ran counter to the expectation that as putative lung cancer stem cell

markers, overexpression of ALDH1A1 and CD133 would predict for poor patient prognosis. However with the opposite being true in this study, these finding become reminiscent of the recent studied in murine models of lung cancer, where expression of the cancer stem cell marker Sca-1 was inversely related to lung cancer stem cell activity in mutant *EGFR* ACs (Curtis et al., 2010; Sullivan and Minna, 2010).

The relationship between CD133 and prognosis in mutant *EGFR* lung cancers was totally unexpected and not fully understood. While this study does not support CD133 as a pan-lung cancer stem cell or prognostic marker, it may have potential as a marker for predicting sensitivity to tyrosine kinase inhibitor therapy, or as a biomarker for lung cancer in never smokers. To address these points, confirmation in a larger collection of mutant *EGFR* NSCLCs will be necessary.

Recently another group interrogated the clinical relevance of CD133 expression in NSCLC. Through similar studies of a much smaller cohort of 88 patient tumor samples, and in accordance with findings described in this chapter, Salnikov et al. reported that CD133 expression was not associated with disease progression or clinical outcome (Salnikov et al., 2009). In contrast to previous findings CD133 was reportedly not associated with an AC histotype. This is possibly due to the small numbers of AC cases studied, the different criterion in which CD133 expression was determined, and the fact that Salnikov et al. used a different and relatively new antibody for CD133 not otherwise used in most cancer stem cell studies (Salnikov et al., 2009).

# CHAPTER SIX

# CONCLUSION AND ONGOING STUDIES

## 6.1 Conclusion

The cancer stem cell model proposes that tumor progression, metastasis and relapse after therapy may be driven by a subset of tumor cells that possess stem cell capacity to self-renew. Where and how tumor cells acquire the ability to self-renew has many researchers investigating the possibility of stem cells as a likely origin of some cancers. In lung cancer this premise has been demonstrated in the initiation and progression of lung tumors in transgenic mice. Furthermore, mounting evidence now supports the existence of a subpopulation of self-renewing tumor cells in human lung cancer. These and other reports have begun to build a rationale for targeting pathways of aberrant self-renewal in the treatment of many cancer types. However in the case of lung cancer, translating these findings into new therapies and diagnostic tools can only be accomplished if human lung cancer stem cells can be accurately identified and characterized.

The objective of this thesis was to identify and characterize putative lung cancer stem cells from human lung cancer cell lines and patient tumors and to identify therapeutically targetable factors that are important for lung cancer stem cell function. To this end, the most salient findings include:

1. Aldehyde dehydrogenase activity in lung cancer identifies a tumor cell subpopulation in NSCLC cell lines and patient samples with growth properties consistent with a putative cancer stem cell population including aggressive tumorigenic growth, elevated clonogenicity and self-renewal (Chapter Three).

2. Notch signaling supports lung cancer stem cell homeostasis and suppression of Notch signaling, particularly through Notch3, results in the reduction of lung cancer stem cells (Chapter Four).

3. The surrogate lung cancer stem cell marker ALDH1A1, but not ALDH3A1 or CD133 is a marker of poor prognosis in NSCLC (Chapter Five).

### 6.2 Ongoing and Unpublished Studies

In the previous chapters, expression and activity of ALDH proteins have identified NSCLC cell subpopulations with growth properties consistent with those of a population of lung cancer stem cells. To begin to understand the molecular underpinnings of lung cancer stem cell biology, preliminary molecular analysis of three $ALDH^+$ lung cancer cell populations was performed by qPCR for the expression of a panel of stem cell associated factors. Through this study it was found that Notch signaling, particularly through the Notch3 receptor, was important for the homeostasis of clonogenic $ALDH^+$ lung cancer cell. Importantly, this study was proof-of-principle that the molecular characterization of $ALDH^+$ lung cancer cell populations could provide targetable factors for lung cancer stem cell directed therapy. To broaden this analysis, microarray analysis was recently performed on isolated $ALDH^+$ and $ALDH^-$ cell

populations from five NSCLC cell lines. The immediate goal of this study is to identify genetic factors important for $ALDH^+$ cell function, potential targets for therapy and/or refined markers of lung cancer "stemness". Additionally, microarray analysis of $ALDH^+$ lung cancer cells may provide useful translational information, such as the identification of predictive and/or prognostic biomarkers.

To determine whether $ALDH^+$ lung cancer cells isolated from different lung cancer cell lines expressed a common set of cancer stem cell associated genes, gene expression analysis was performed on $ALDH^+$ and $ALDH^-$ cells were isolated from Calu-1, H358, H2009, H1819, and H2087 cell lines using Illumina WG6-V3 whole-genome microarrays. These lines were chosen for analysis because they possessed readily isolatable $ALDH^+$ cell subpopulations (of different frequencies) that had been demonstrated in previous studies to be enriched for clonogenic and/or tumorigenic cancer cells.

Unsupervised hierarchical clustering applied to the ten samples and the 28,387 filtered genes (standard MATRIX filter settings) did not separate $ALDH^+$ and $ALDH^-$ cell populations. Instead, $ALDH^+$ and $ALDH^-$ cell populations from the same cell lines clustered together, indicating that the differences in mRNA transcripts between clonal cell lines supersede differences between $ALDH^+$ and $ALDH^-$ cells (Figure 6.2A). This is in line with previous data in $ALDH^+$ and $ALDH^-$ breast cancer cells from established breast cancer cell lines (Charafe-Jauffret et al., 2009). Less than 100 genes were commonly differentially expressed (> twofold difference) across $ALDH^+$ and $ALDH^-$ cells from the five cell lines tested and after filtering for significance (Mann-Whitney *U* test, $P < 0.05$) only four genes remained (Figure 6.2B). Nevertheless many interesting

genes were differentially expressed between $ALDH^+$ and $ALDH^-$ cells from the same parental cell line. For example in sorted Calu-1, H2009, H358 and H2087 cells, *ALDH1A3* (a close family member and possible functional homologue of *ALDH1A1*) transcripts were twofold more abundant in the $ALDH^+$ cell populations over the $ALDH^-$ cell populations.

Differential gene expression between $ALDH^+$ and $ALDH^-$ lung cancer cells appears to be mostly cell line idiosyncratic however in H2009, Calu-1 and H2087, many genes differentially expressed in $ALDH^+$ and $ALDH^-$ cells are shared (Figure 6.2C). Likewise, $ALDH^+$ cells from H358 and H1819 also appear to share many differentially expressed genes, suggesting there may be molecularly distinct $ALDH^+$ lung cancer cell subpopulations (Figure 6.2C). Further investigation is underway to confirm interesting gene changes by qPCR, as well as to expand the molecular analysis to other sorted lung cancer cell lines.

In addition to identifying lung cancer stem cell associated mRNA expression patterns, analysis of non-coding microRNA (miRNA) expression in sorted cells is also underway. Mature miRNAs (~22 nucleotides) are post-translational regulators that bind to complementary sequences on protein coding mRNA, usually resulting in gene silencing. Through this gene regulatory mechanism, miRNAs have been reported to play a wide role in development, modulating cell proliferation, apoptosis and stem cell self-renewal.

Alterations in the expression of certain miRNAs have been reported in many human cancers and have been associated with both oncogenic and tumor suppressive functions (Du and Pertsemlidis; Shenouda and Alahari, 2009). Recently miRNAs have

also been implicated in regulating cancer stem cell self-renewal and homeostasis (Yu et al., 2007). To determine the role of miRNAs in lung cancer stem cell biology, expression analysis of mature miRNAs in sorted $ALDH^+$ and $ALDH^-$ lung cancer cells is underway. Preliminary analysis using qNPA (quantitative nuclease protection assay) miRNA microarrays (High Throughput Genomics) has already revealed expression of potentially interesting miRNAs in sorted lung cancer cells that are known to regulate cell proliferation and Notch signaling. Further analysis will determine whether these or other miRNAs are potentially useful markers for lung cancer stem cell activity, or potential molecular targets for therapy.

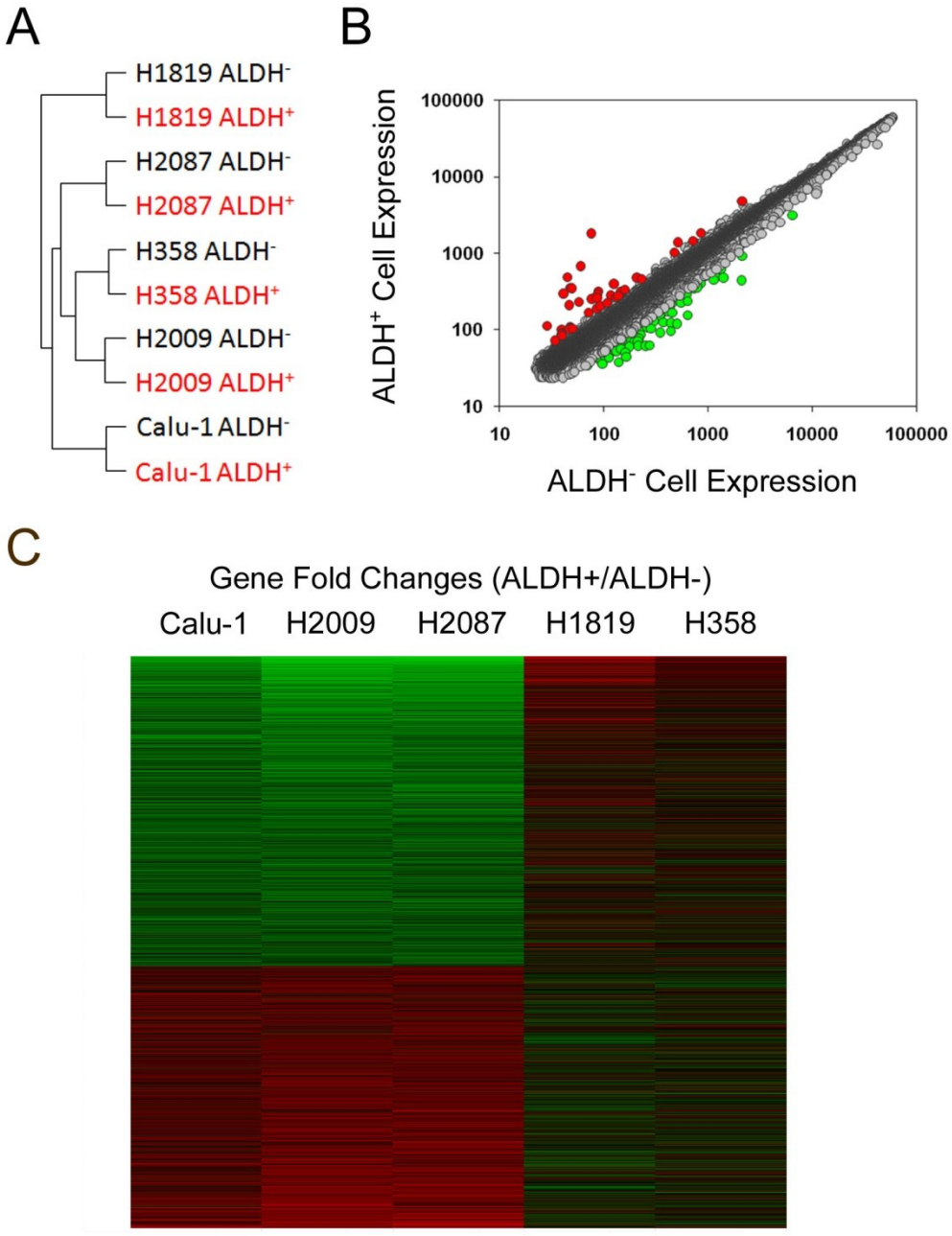



**Figure 6.2: Microarray expression analysis of lung cancer stem cells.** Unsupervised clustering analysis of sorted cell gene expression data (A). Scatter plot of average gene intensities from isolated $ALDH^+$ and $ALDH^-$ cell populations reveal genes commonly expressed twofold of greater in $ALDH^+$ (red dot) and $ALDH^-$ (green dot) cells (B). A heatmap of 2660 differentially expressed genes in sorted cells, represented as fold expression of genes in $ALDH^+$ cells/$ALDH^-$ cells (C).

## 6.3 Perspective on the Challenges to the Cancer Stem Cell Model

As the cancer stem cell model has gained acceptance, debate as to the properties that define *bona fide* cancer stem cells has intensified. Indeed, some have argued that inconsistencies in putative cancer stem cell population phenotypes suggest the current cancer stem cell model is a result of misinterpreting experimental results (Hill, 2006; Kern and Shibata, 2007). The most stringent functional assay for a cancer stem cell has been the generation of a tumor from a very low dilution (1-1,000 cells) of isolated tumor cells; the idea being that clonal expansion of a tumor is driven solely through rare cancer stem cells. As discussed in Chapter Three, this typically entails the injection of small numbers of isolated human tumor cells into immunocompromised mice and assaying for the presentation of tumorigenic outgrowth. In 2007, experiments published by Kelly and coworkers challenged the credibility of this assay, when as few as 10 unsorted mouse lymphoma cells were reported to transplant the disease in congenic recipient mice (Kelly et al., 2007). These findings led Kelly and colleagues to conclude that infrequent tumor cell engraftment in previously reported xenogeneic models may be a result of selective microenvironment pressures and therefore this phenomenon may be underestimating the population of tumorigenic cells in some cancers (Kelly et al., 2007).

The validity of these conclusions was extended to the study of solid tumors the following year when Quintana and colleagues demonstrated that modified xenotransplantation assay conditions, such as the use of a more highly immunocompromised variety of NOD/SCID mice lacking the interleukin-2 gamma receptor (NOD/SCID$^{Il2rg-/-}$), resulted in the efficient generation of melanoma tumors from a single human melanoma cell (Quintana et al., 2008). While it could be argued that in

some cancers, such as lymphoma and melanoma, tumorigenic cancer stem cells are not rare, the potential for extrinsic factors to influence tumor stemness in other cancer types remains a possibility. Other factors such as tumor hypoxia, stromal derived cytokines and tumor vasculature have also been proposed to alter the presence of cancer stem cells (Heddleston et al., 2009; Li and Neaves, 2006; Li et al., 2009; Visvader and Lindeman, 2008). In addition, the process of tumor cell epithelial-to-mesenchymal transition has been suggested as a mechanism for non cancer stem cells to acquire cancer stem cell properties (Gupta et al., 2009; Mani et al., 2008). A better understanding of how these factors contribute to tumorigenesis, tumor progression and tumor heterogeneity will ultimately advance or marginalize the current cancer stem cell model.

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