I, Shailaja Akunuru, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Molecular & Developmental Biology.

It is entitled:
Regulation of cancer stem cell activity and epithelial mesenchymal transition by Rac1 in Human lung adenocarcinoma cells

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Regulation of cancer stem cell activity and epithelial mesenchymal transition by Rac1 in Human lung adenocarcinoma cells

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by

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Abstract

The cancer stem cell (CSC) theory predicts that a small fraction of cancer cells possesses unique self-renewal, expansion and differentiation activities in tumorigenesis. While this theory remains controversial in solid tumor studies, the enriched cancer stem cell population has been characterized by drug-resistant side population (SP), CD133<sup>pos</sup>, ALDH<sup>high</sup> and several other markers in many solid tumors. Using human lung cancer cell lines (A549 and H441) and primary human non-small cell lung adenocarcinoma (NSCLA) cells, we demonstrated that SP cells isolated from NSCLAs have significantly increased migration, invasion and metastatic activities compared to non-SP cells. We show that Rac1, a member of Rho-GTPases, is a critical mediator of the NSCLA SP CSC activity, by regulating SP cell adhesion, migration, invasion, and lung colonization activities. We further established that NSCLAs SP cells represent a distinct population from phenotypic CSC populations marked by CD133<sup>pos</sup>, ALDH<sup>high</sup>, or CD24<sup>low</sup>CD44<sup>high</sup>, with each displaying enriched CSC activity demonstrated by expression of self-renewal genes and tumor-initiating activity in xenografted mice. Non-CSCs and CSCs defined by either absence or presence of single CSC marker respectively (SP, ALDH<sup>high</sup>, CD133<sup>pos</sup>, and CD24<sup>low</sup>CD44<sup>high</sup>) are plastic and dynamic, with a distinct inter-conversion kinetics. Epithelial-mesenchymal transition (EMT) previously proposed as one of the underlying mechanisms of CSC plasticity, abolishes NSCLAs SP cells while increasing other CSC markers such as ALDH<sup>high</sup>, CD133<sup>pos</sup> and CD24<sup>low</sup>CD44<sup>high</sup>. Rac1 activity was significantly increased in cells that have undergone EMT. Inhibition of Rac1 activity by NSC23766, a Rac1 inhibitor, or Rac1 knockdown, partially blocks EMT process and suppresses EMT induced phenotypic non-CSC to CSC conversion. Thus, we propose that there exist multiple, phenotypically distinct cancer cell subpopulations with CSC properties in NSCLA cells and phenotypic non-CSCs and CSCs can be dynamically
regulated through EMT. Importantly, targeting Rac1 GTPase that is intimately involved in EMT process may represent a novel strategy in inhibiting CSC activity and suppressing non-CSC to CSC transition.
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Chapter I: Introduction

Lung cancer, leading cause of cancer deaths

Lung cancer is the leading cause of cancer deaths in the United States and worldwide. The highest known risk factors for lung cancer are smoking and environmental pollutants like asbestos and radon. This disease is broadly classified into two histo-pathological groups, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC represents about 80% of lung cancers. NSCLCs are further classified into three sub-groups: adenocarcinomas (AC), squamous cell carcinomas (SCC), and large cell carcinomas (LCC) [1,2]. Adenocarcinomas represent about 55% of the NSCLC subgroup and 40% of all lung cancers. Despite the continuous development of cancer therapeutics, the overall five year survival rate from the time of diagnosis for all lung cancers is less than 15% [3]. Existing chemo therapeutics, radiation therapy and surgery only partly remove the tumors, leaving behind therapy resistant cells which can rapidly regenerate the tumor at the primary site and potentially can metastasize to secondary tumor sites. It has been proposed that distal metastases can be generated from residual therapy resistant cells.

Cancer initiating cells have stem cell characteristics

The idea of cancer initiating cells was first conceived several decades ago by the discovery of the distinct subpopulation of cells isolated from a lymphoma tumor mass that were capable of driving tumorigenesis [4]. The lack of sophisticated techniques like cell sorting and mouse xenograft models in 1960’s left the identity and functional characteristics of these cells unknown; leaving the existence of cancer stem/initiating cells as a theory. In 1994, the first evidence of the potential validity of this theory came from the discovery of cancer initiating cells identified in Acute Myeloid leukemia (AML) [5]. A rare population of CD34⁺ CD38⁻ cells isolated from AML patients could generate
leukemia in SCID mice while more differentiated cells CD34^+CD38^- and CD34^- cells could not [6]. Later it was proposed that leukemia initiating cells are malignant derivatives of hematopoietic stem cells generated from aberrant hematopoiesis.

Recently there has been a debate about the nomenclature for these rare tumor propagating cells, between two different terms “cancer stem cells” and “cancer initiating cells”. The term “cancer stem cells” does not imply that the cancer cells are derived from the oncogene induced transformation of normal tissue stem cells, but rather refers to a subset of cancer cells that have stem-like self renewal properties and can further divide to generate more differentiated cells that constitute the bulk of tumor [7]. However recently another review suggests the term “cancer initiating cells” or “cells of origin” to describe the cells that have undergone the first event of transformation that eventually lead to cancer [8]. Debate continues even though consensus has been reached to use the term “Cancer stem cells” or “cancer propagating cells” at the 2007 AACR consortium [9].

Another interesting debate about cancer stem cells is about the frequency of these cancer stem cells in individual cancers. First, Hope JK et al described a minute portion of cells (approximately 1/10^6) in human acute myeloid leukemia can generate tumor growth in immune compromised NOD/SCID mice [10]. Later, this was challenged by studies of Kelly NP et al which demonstrated that all pre-B/B lymphoma cells can establish lymphoma in syngeneic recipients, debating that identifying the human cancer stem cells by mice xenograft models is probably selecting for the rare population of cells that adapted to growth in foreign environment [11]. This challenge was further counteracted by the MLL-EL driven leukemia cord blood transplantation study by Kennedy JA et al showing that the frequency of leukemia stem cells is similar between syngeneic and xenogeneic transplants [12] and the frequency of the cancer stem cells is
tumor dependent. Irrespective of these debates on frequency and nomenclature, the
field of CSC has expanded rapidly in recent years.

In the past five years various publications reported the identification of tumor
initiating cells from solid tumors like brain [13], breast [14], colon [15,16], hepatic [17],
pancreatic [18], prostate [19,20] and lung cancers [21,22]. These cancer initiating cells
are shown to be chemotherapy and radiation therapy resistant and are identified by
either enzyme activity (Hoechst dye-low side population or aldehyde dehydrogenase
enzyme activity, ALDH\textsuperscript{high}) or expression of different cell surface stem cell markers
(summarized in Table 1).

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<tr>
<td>CD133⁺/CD44⁺/α2β1\textsuperscript{high}</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>CD133⁺</td>
<td>Colon cancer</td>
</tr>
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<td>CD133⁺</td>
<td>Medulloblastoma, Glialblastoma</td>
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Table I-1. Cancer stem cells identified by different cell surface markers

The tumor initiating nature of these cells is commonly tested by formation of
tumors in immune compromised mice (NOD/SCID or NSG mice) by either subcutaneous
injection or injection to the orthotopic site. In several different cancers, these cancer
stem cells were described to have stem cell like self-renewal capabilities and could
proliferate to generate all differentiated cell types that constitute the bulk of the tumor
[17,23]. From studies of several different systems, the traditional stem cell-specific
genes such as nestin (CD133\textsuperscript{pos} cells from gliomas) [24], Hedgehog (Hh-
CD44\textsuperscript{high}CD24\textsuperscript{low} breast cancer cells) [25], sonic Hh (CD44\textsuperscript{high} CD24\textsuperscript{high} ESA⁺ pancreatic
cancer cells) [18], Oct3/4 and Nanog (Stro-1⁺ CD44⁺ CD105⁺ sarcoma cells) [26], Notch [27] were shown to be up-regulated in these cancer stem cells thereby contributing to their self-renewal properties.

**Lung cancer stem cells have stem cell characteristics**

Currently, the topic of lung cancer stem cell’s research remains relatively unexplored. The first hint of the existence of lung cancer stem cells came from the discovery of elevated expression of the cytosolic enzyme aldehyde dehydrogenase (enzyme implicated in drug resistance, ALDH) associated with chemo-resistant NSCLC [28]. Later, Ho *et al.* identified lung cancer stem cells from six human lung cancer cell lines based on the Hoechst 33342 dye efflux [22]. Hoechst dye low side population (SP) cells were enriched for tumor initiating cells compared to non-side population (NSP) cells. ATP-binding cassette (ABC) transporters actively pump a wide range of cytotoxic drugs out of the stem cells and high expression levels of ABC transporters are associated with drug resistance. SP cells display up-regulation of ABCG2 (implicated in Hoechst dye efflux) and other ABC transporters (MDR1, MRP1, ABCA2) and resistance to multiple chemotherapeutic drugs. Interestingly, SP cells were more invasive than NSP cells in an *in vitro* matrigel invasion assay [22].

CD133, a cell surface glycoprotein, is a marker for hematopoietic and neuronal stem cells and was recently shown to be expressed by cancer stem cells isolated from medulloblastomas [13], glioblastomas [29], prostate [30] and colon [15] carcinomas. Eramo *et al.* identified a rare population of CD133⁺ tumor initiating cells in human SCLC and NSCLC tumors [21]. 10,000 CD133⁺ cells form tumors when implanted subcutaneously in NOD/SCID mice whereas 200,000 CD133⁻ cells are incapable of tumor establishment. Spheres grown from the primary tumors were shown to be enriched for self-renewing CD133⁺ cells. Tumor initiating characteristics of CD133⁺ cells were not directly compared against SP cells in primary human lung tumors.
Lung adenocarcinoma stem cells with higher ALDH activity displayed *in vitro* features of CSCs including, proliferation, self-renewal, resistance to chemotherapeutics and expression of ALDH1 was positively correlated with stage and grade of the lung tumors [31]. Lung adenocarcinoma cells with high ALDH enzyme activity derived from cell lines were more tumorigenetic, and this tumorigenecity was dependent on elevated notch1 signaling [32]. However potential relationship of these three different cancer stem cell markers (SP, CD133<sup>pos</sup>, ALDH<sup>high</sup>) identified in NSCLC, e.g. whether they represent overlapping or hierarchy populations of NSCLC, has not been established.

**Cancer stem cells are potential seeds for metastasis**

Metastasis is defined as spread of cancer cells from the primary tumor site to other distal organs. Metastasis is responsible for about 90% of all cancer mortalities. Cancer metastasis is a very complex and multi-step process involving epithelial-mesenchymal transition (EMT), intravasation into blood or lymphatic vessels, endothelial cell docking at the secondary site, trans-endothelial migration/extravasation and invasion through basal lamina, tissue parenchyma and finally metastatic out growth in the distant organ (Figure 1). Organ-directed metastasis is thought to be driven by secretion of chemokines and cytokines at the secondary metastatic site [33,34]. In NSCLC the CXCL12/CXCR4 axis was shown to be involved in organ-directed metastasis [35]. It is suggested that chemokines and cytokines generated at the secondary site drive the endothelial cell docking and extravasation in the capillaries specifically at that site.

Metastasis in mice, described as an experimental metastasis model that has been extensively studied, involves directly injecting cancer cells into the circulation of immune compromised mice by tail vein injection and analysis of the tumor foci development in the lungs, considered metastasis. This experimental metastasis model eliminates the EMT step because the cancer cells are directly introduced into circulation by intravenous injection. However, this is a valid model to understand the molecular
mechanisms which underlie cellular behaviors of migration, invasion and homing which contribute to metastasis processes.

![Diagram of the metastasis process](image)

**Figure I-1. Cancer stem cells (CSC) are the seed for metastasis**

Interestingly, the concept of secondary metastasis post therapy has been attributed to these cancer stem cells. Cancer stem cells with their tumor initiating characteristics and more migratory, invasive behavior make the ideal candidates for developing tumors at the secondary metastatic site (Figure 1). Breast cancer cell lines enriched for CD44<sup>high</sup>/CD24<sup>low</sup> tumor initiating cells express higher levels of proinvasive genes and display increased invasiveness [36]. In pancreatic cancer, CD133<sup>pos</sup> cancer stem cells are heterogeneous for CXCR4 expression, and CD133<sup>pos</sup>/CXCR4<sup>pos</sup> cells are shown to contribute to metastasis [37]. In human liver cancer, CD45<sup>-</sup>/CD90<sup>+</sup>/CD44<sup>+</sup> cells form metastatic lesions in the lungs of immune compromised mice, and antibody-mediated blockage of CD44 activity abrogates tumor growth and metastasis [38]. Recently another interesting evidence for association of CSC to metastasis came from
the clinical data correlating the presence of CD26\textsuperscript{pos} cells in circulation and primary colon tumor to liver metastasis [39].

**Role of EMT in CSC activity and metastasis**

The epithelial mesenchymal transition (EMT) process is known to be crucial for embryonic development, where cells lose expression of cell-cell contact proteins, induce expression of mesenchymal markers and undergo cytoskeletal changes acquiring fibroblast-like morphology. Because this process results in a more migratory and invasive cell morphology, EMT has been associated with cancer metastasis. Transcription factors like Twist and Snail have been demonstrated to be critical for EMT, resulting in increased migration and metastasis in several cancers including lung cancer [40].

Recently a seminal study from R.A. Weinberg’s lab linked the concepts of EMT and cancer stem cells in breast cancer [41]. This work revealed that EMT promotes cells with stem characteristics in mammary cancer cells, leading to a potential role of EMT to metastasis process mediated by cancer stem cells. The potential contribution of EMT to cancer stem cell maintenance and metastasis in lung cancer remains unclear.

**Rho GTPases regulate metastasis and EMT**

Rho GTPases, small GTPases of the Ras superfamily, function in a variety of signaling pathways upon activation of cell surface receptors. Rho GTPases can be either in active conformation (GTP bound) or inactive confirmation (GDP bound). When in active conformation Rho GTPases are capable of binding to effector proteins to mediate their downstream signaling functions. Rho GTPases regulate critical cellular processes such as gene expression, cell proliferation, membrane trafficking and actin cytoskeleton remodeling (Figure 2, adapted from Bosco E et al Cellular and molecular life sciences, 2009). Most of the knowledge of Rho GTPase functions are derived from studies of Rac1, Cdc42 and Rho A. Rac1 regulates the actin rich protrusions (lamellipodia) and
membrane ruffling, Cdc42 regulates the formation of filopodia and Rho A regulates the formation of actin-myosin bundles (stress fibers) and focal adhesions [42]. Therefore the coordinated regulations of increased Rac1, Cdc42 at the leading end and decreased Rho A at the trailing end of the cell result in motility of cell in the direction of chemo-attractant.

Potential role of Rac1 in tumorigenesis and invasion has been implicated previously by mice genetics studies. Deletion of Tiam1, Rac regulator leads to delayed skin tumor initiation and progression induced by H-ras mutations and DMBA treatment [43]. K-ras induced lung tumorigenesis was partially blocked by Rac1 loss in mice [44]. Rac1 was shown to be involved in invasion and migration of several cancer cells including NSCLC cells [45]. Rho GTPases can regulate metastasis by modulating cell adhesion, migration, invasion and induction of angiogenic factors [46]. RhoA and Rac1 regulate invasion of tumor cells by regulating the levels of matrix metalloproteinases (MMPS), which degrade the extracellular matrix [47]. Additionally Tiam1 (Rac-GEF,
Guanine nucleotide exchange factor) regulate the transcriptional levels of TIMP-1 and TIMP-2 (tissue inhibitors of MMPs) in renal cell carcinomas and the high invasiveness of these tumor cell type was associated with TIMPs expression [48].

RhoGTPases playing an important role in EMT, a process by which cells undergo profound morphological changes involving cytoskeletal changes, acquiring a migratory phenotype has been implicated previously [49] and will be interesting to explore further in lung cancer. Radisky DC et al have shown Rac1b being the mediator of EMT induced ROS production [50] and more recently Rac1 was shown to regulate TGFβ1 mediated EMT and MMP9 production in transformed keratinocytes [51].

To improve the overall survival rate of NSCLC patients it is critical to understand the biology of the conventional therapy resistant CSCs. Studies characterizing different CSC markers in NSCLC are very preliminary and tumor initiation properties were tested by subcutaneous xenograft models. However the metastatic potential of these CSC populations has not been established. We sought to identify the potential relationship of these different CSC markers and plasticity of the each CSC marker. Further, to help develop novel strategies to target therapy resistant cells, we examined the role of EMT on NSCLC CSC plasticity and the role of Rac1 in EMT mediated CSC plasticity and metastasis.
References


Chapter II: Rac1 targeting suppresses human non-small cell lung adenocarcinoma cancer stem cell activity

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Key words: Non-small cell lung adenocarcinoma, Rho GTPase, Rac1, Targeting, Cancer stem cells.

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Abstract

The cancer stem cell (CSC) theory predicts that a small fraction of cancer cells possess unique self-renewal activity and mediate tumor initiation and propagation. However, recent findings suggest that non-CSCs can convert into CSCs, suggesting that effective targeting of both CSCs and non-CSCs is necessary in cancer therapy. Here we have investigated the hypothesis that Rac1, a Rho GTPase implicated in cancer cell proliferation and invasion, is critical for tumor initiation and metastasis of human non-small cell lung adenocarcinoma (NSCLA). Rac1 knockdown by shRNA suppressed the tumorigenic activities of human NSCLA cell lines and primary patient NSCLA specimens, including effects on invasion, proliferation, anchorage-independent growth, sphere formation and lung colonization. Isolated side population [1] cells representing putative CSCs from human NSCLA cells contained elevated levels of Rac1-GTP, enhanced in vitro migration, invasion, increased in vivo tumor initiating and lung colonizing activities in xenografted mice. However, CSC activity was also detected within the non-SP population, suggesting the importance of therapeutic targeting of all cells within a tumor. Further, pharmacological or shRNA targeting of Rac1 inhibited the tumorigenic activities of both SP and non-SP NSCLA cells. These studies indicate that Rac1 represents a useful target in NSCLA, and its blockade may have therapeutic value in suppressing CSC proliferation and metastasis.
Introduction

Lung cancer remains the leading cause of cancer deaths worldwide. The disease is broadly classified into two histo-pathological groups - small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with the later group representing ~80% of lung cancer cases. Adenocarcinomas occur in about 55% of the NSCLC and 40% of all lung cancers. Despite of continued development of cancer therapeutics, currently the overall five year survival rate for lung cancer patients is less than 15%[2]. Often, existing chemotherapy, radiation therapy or surgery can only partly remove the tumor burden, leaving behind therapy resistant cancer cells that may regenerate the tumor at the primary site and/or metastasize to secondary sites to initiate new tumors.

Recent publications have reported the identification of cancer initiating or cancer stem cells (CSCs) from blood [3], brain [4], breast [5], colon [6,7], hepatic [8], pancreatic [9], prostate [10,11], as well as lung cancers [12,13,14]. The CSCs are identified either by unique cell properties such as Hoechst dye exclusion (Hoechst dye-low side population) or by expression of specific surface markers such as CD133, ALDH, or CD24/CD44, and they are frequently associated with chemotherapy and radiation therapy resistance. CSCs are defined by their stem cell like self-renewal capabilities, their ability to differentiate into cell types that constitute the bulk of the tumor, and to initiate tumors at a significantly reduced dosage in mouse xenograft studies [8,15]. NSCLC initiating cells have been isolated from human lung cancer cell lines based on increased Hoechst 33342 dye efflux activity [13]. The Hoechst dye low side population (SP) cells are enriched for tumor initiating activity compared to non-side population (NSP) cells and express elevated ABCG2 and other multi-drug resistance transporters that may mediate therapeutic resistance.

The advancement of the cancer stem cell theory has led to the proposal that targeting CSC’s can lead to eradication of the residual therapy resistant tumor cells in patients. Recently Gupta et al suggested that inducing differentiation of CSC by using salinomycin, a selective potassium
ionophore can block mammary CSC activity and metastasis [16]. However, several recent reports have shown that CSCs and non-CSCs can be plastic and inter-convertible in nature [17] [18]. For example, JARID1 negative cells were shown to represent a transient slow cycling non-CSC population that can give rise to fast cycling JARID1 positive CSCs in melanoma (13). There is evidence that non-CSCs can convert to CSCs through interaction with extracellular matrix and other environmental cues (14). This raises the possibility that approaches solely targeting CSCs are not sufficient for cancer therapy, because the remaining non-CSCs may be reprogrammed to CSCs to reinitiate tumorigenesis.

Rac1 is an intracellular molecular switch that transduces signals in a variety of oncogenic pathways. It is frequently found to be elevated in expression and/or activity in a variety of tumor cells and regulate important cellular processes relevant to cancer cell behaviors, including gene expression, cell proliferation, actin cytoskeleton remodeling and is essential for cell directional migration and adhesion. Rac1 activity can influence cell cycle progression and survival, and it was shown to be required in K-ras mediated lung tumor growth in a murine spontaneous lung cancer model [19]. However, whether Rac1 contributes to human NSCLA tumor growth and/or metastasis, particularly if Rac1 plays a role in regulating CSCs and/or non-CSCs, requires further investigation. In the current work we determine that Rac1 is critically involved in NSCLA cell migration, invasion and lung metastasis of both SP and non-SP cells, therefore serving as a useful therapeutic target by inhibiting tumor initiation and metastasis of both CSC and non-CSC populations of NSCLA.

Materials and Methods

Cell culture
A549, H23, H1299 and H441 cells were cultured according to the guidelines from ATCC. Human bronchial epithelial cells (HBEC) were generous gift from Dr. Jeffery Whitsett (Cincinnati Children’s Hospital Medical Center). Primary patient lung adenocarcinoma samples were obtained
with written consent from patients under an approved Institution Review Board protocol by University of Cincinnati Scientific Review Committee (IRB# 01-09-27-07), and were used in the experiments according to Cincinnati Children’s Hospital Medical Center Scientific Review Committee (IRB # 07-06-57) that the identity of the patients remains anonymous. Tumors were minced and resuspended in DMEM containing 0.5 mg/ml Liberase (Roche) and 1% penicillin and streptomycin. After 45 minute incubation, slurry of cells was passed through 100 micron filter and total cells were washed, plated in 10% fetal bovine serum containing growth media. Epithelial cancer cells were enriched by growing cells in sphere culture conditions.

A549, H23, H1299, H441, HBEC or primary adenocarcinoma cells were infected with lentivirus expressing YFP tagged scrambled shRNA (scr) or Rac1 shRNA1 or Rac1 shRNA2 described previously [20]. Scrambled shRNA construct was generous gift from Dr. Lee Grimes (Cincinnati Children’s Hospital Medical Center) and Rac1 shRNA constructs were generous gift from Dr. Jim Mulloy (Cincinnati Children's Hospital Medical Center). After 72 hours of infection, YFP positive cells were sorted using FACS and utilized for different experiments.

For Rac1 cDNA mutant rescue experiments, four mismatch point mutations were made in the shRNA binding sites of Rac1 cDNA in the MIEG3 vector using site-directed mutagenesis kit (Stratagene, Agilent technologies) per manufacturer’s directions. A549 cells were infected with Rac1 mutant expressing retrovirus and after 72 hours, GFP positive cells were sorted using FACS. The cells were subsequently infected with lentivirus expressing scr shRNA or Rac1 shRNA. After 72 hours, GFP+ YFP+ cells were used to perform several functional assays.

Cell proliferation assays

Cells were plated (2000 cells/well) on 96 well plate in triplicates. Number of live cells on each day was determined by non-radioactive MTS proliferation assay (Promega).

For BrdU incorporation assay, BrdU (10μg/ml) was added to cells at 60% confluency for 2 hours at 37°C. Cells were collected, fixed, stained and FACS analysis was performed as described previously [21]. To detect BrdU positive cells in CD133+ and CD133− populations, cells were
stained with CD133/2-APC antibody (Miltenyi Biotechnologies Inc.) after BrdU staining. The BrdU positive cells were gated from both CD133⁺ and CD133⁻ cells.

**Soft agar colony formation assay**

Cells were seeded (10,000 cells/well) in 0.3% low melting point agarose made in growth media containing 10% fetal bovine serum and layered on top of 0.6% agarose in growth media. Number of colonies formed after either 2 weeks (A549, H1299) or 3 weeks (H441, H23) was counted under light microscope.

**Sphere formation assay**

Cells (10,000 cells/ml) were plated in suspension culture conditions in serum-free sphere media (DMEM:F12 containing 0.4% BSA, 10μg/ml insulin, 10ng/ml EGF, 10ng/ml FGF) on 6-well plates pre-coated with 1% agarose to prevent cell attachment. Media was replaced every 2-3 days and number of spheres formed in 2 weeks was counted under light microscope.

**Adhesion assay**

Plates were coated with 50μg/ml fibronectin overnight at 4°C and blocked with 2% BSA for 2 hrs at 37°C. After blocking, Cells were plated (10,000 cells/well) and incubated at 37°C for 60 minutes. Non-adherent cells were aspirated and plates were washed three times with PBS. Numbers of cells attached to the wells after washes were determined by using non-radioactive proliferation MTS assay.

**Migration and invasion assays**

For trans-well migration assay, 50,000 cells were added to upper chamber in serum free media and migration at 37°C towards 10% FBS containing growth media was determined either after 24 hours (A549, H1299, H23) or 48 hrs (H441). Cells migrated through the membrane were fixed, stained with Giemsa stain (Sigma) and counted under light microscope.
For invasion assay, lower chambers of matrigel coated invasion plates were coated with 10μg/ml fibronectin overnight at 4°C and cells invading through matrigel were fixed and stained either after 48 hours (A549 cells) or 72 hours (H441 cells) similar to migration assay.

**Immuno-staining**

Cells were plated on fibronectin coated slides and after 18-20 hours cells were fixed using 3.7% Formaldehyde. Cells were stained for actin cytoskeleton (Rhodamine-Phalloidin, Invitrogen), nuclei (DAPI, Invitrogen) using standard immuno-staining methods described previously [22]. Alternatively cells were stained with either phospho-FAK (Focal Adhesion Kinase, Millipore) or vinculin (Sigma) or phospho-paxillin (Cell signaling Technologies).

**Side-population, CD133 cell staining and isolation**

Cells are trypsinized and washed with PBS. Cells are stained with Hoechst 33342 staining buffer as described previously at a final concentration of 5 μg/ml Hoechst 33342 [23] for side population (SP), or with anti-CD133 antibody for CD133 positive cells. The cells were analyzed or sorted for SP/CD133+ cells by flow cytometry.

To obtain Rac1 knockdown in SP or CD133+ cells, cells were infected with lentivirus expressing YFP tagged scr or Rac1 shRNA and after 72 hours cells were stained for side population or CD133. YFP positive SP or non-SP cells were sorted for either Western analysis or functional assays.

**Rac1-GTP pull-down assay**

To perform Rac1 pull down assays, cells were lysed by adding lysis buffer containing 20 mM Tris HCl pH 7.6, 100 mM NaCl, 10 mM MgCl, 1% Triton X-100, 0.2% SDS, protease and phosphatase inhibitors directly to adherent cells. Cell lysates containing equal amounts of protein were incubated with glutathione beads conjugated to GST-PAK1 containing active Rac1 interacting domain and processed further as described previously [24].

**Lung colonization assay**
Use of mice as xenograft hosts was approved by the IACUC committee at Cincinnati Children’s Hospital Medical Center (Protocol# 8D06052). Specified number of cells were suspended in PBS and injected intravenously into immune compromised NOD/SCID/γc −/− (NSG) mice by tail vein injection. At the end of the study, lungs were fixed in Bouin’s solution to count number of tumors.

**Subcutaneous xenograft assay**

Specified number of cells are suspended in 200 µl of PBS:matrigel mix (1:1 volume) and injected subcutaneously into the flanks of immune compromised NOD/SCID/γc −/− (NSG) mice. After 3-4 weeks of injection tumor size was measured weekly using calipers and the tumor volume was determined by using formula $0.52XLXW^2$ cm3.

**Tumor cell homing assay**

Cells expressing YFP were injected intravenously into NSG mice. After 24 hours, lungs were isolated after perfusion with PBS. Total lung cells were isolated by Liberase digestion and total cell count was determined by Hemavet cell counter. Percentage of YFP positive cells was determined by flow-cytometric analysis of total lung cells. Homing index was determined by calculating percentage YFP positive cells homed to lung normalized to control cells.

**Results**

**Rac1 targeting impairs proliferation and colony formation of human NSCLA cells** - To investigate the role of Rac1 GTPase in NSCLA cell growth, A549, H441, H1299, and H23 cells were infected with lentivirus encoding scr or Rac1 shRNA (shRNA1, 2). Western blot analysis revealed efficient knockdown of Rac1 protein with both shRNA constructs (50% and 90% respectively compared to scr) in A549 cells (Fig 1A). Rac1shRNA1 expression partially reduced the proliferation of the lung cancer cells while Rac1shRNA2 more potently inhibited cell growth (Fig 1B) and caused a significant decrease of number of colonies grown in soft agar colony formation assay (Fig 1C). Further, cell cycle analysis performed by BrdU staining and FACS
analysis revealed a decrease in S-phase and a corresponding increase in G0/G1 phase of the cell cycle upon Rac1 targeting (Fig 1D). In H441 cells, Rac1 shRNA2 led to ~75% reduction in Rac1 protein (Supplemental Fig 1A), and a relative minor effect on proliferation (Supplemental Fig 1B). In H1299 and H23 cells, Rac1 knockdown caused a significant reduction in proliferation (Supplemental Fig 1C, 1E) and soft agar colony formation (Supplemental Fig 1D, 1F). To determine if the Rac1 knockdown effects on proliferation and soft agar growth are specific to Rac1, we performed shRNA-resistant Rac1 cDNA mutant rescue experiments in the knockdown cells. Expressing a Rac1 shRNA resistant mutant cDNA could mostly rescue the proliferation of Rac1 knocked down in A549 cells without a detectable effect on the scr shRNA treated control cells (Fig 1E). Similarly we observed a rescue of soft agar colony formation by expressing shRNA-resistant Rac1 cDNA mutant (Fig 1F). Together, these results indicate that Rac1 is required for the proliferative potential of NSCLA cells.

**Rac1 knockdown results in decreased adhesion, migration and invasion of NSCLA cells** - Consistent with the known role of Rac1 in cytoskeleton regulation, Rac1 knockdown in both A549 and H441 cells resulted in altered actin cytoskeleton organization (data not shown). Suppression of Rac1 in A549 cells caused decreased focal adhesion complex formation, with control cells exhibiting robust focal adhesion complexes visualized by immunostaining for the focal adhesion proteins p-FAK, vinculin, and p-paxillin whereas the Rac1shRNA2 infected cells demonstrated reduced focal adhesion complex formation (Fig. 2A). We also observed decreased p-FAK, p-paxillin and p-MLC in Rac1 knockdown cells compared to control cells by Western blot analysis (Supplemental Fig 2A). Consistent with the decreased adhesion complexes in Rac1shRNA infected cells, adhesion to fibronectin was reduced in these cells compared to control cells (Fig 2B). Similar to the effect on proliferation, Rac1 partial knockdown in H441 cells resulted in a relatively minor effect on adhesion to fibronectin compared to A549 cells (Supplemental Fig 2B). In addition, Rac1 knockdown in both A549 and H441 cells resulted in decreased trans-well
migration and invasion activities compared to control cells (Figs 2C, 2D, & Supplemental Fig 2C, 2D). Similarly, Rac1 knockdown in H1299 or H23 cells drastically decreased migration compared to control cells (Supplemental Fig 2E, 2F). Expressing a shRNA-resistant Rac1 cDNA mutant was able to completely rescue the migration phenotype of Rac1 knockdown in A549 cells (Fig 2E). These data demonstrate the importance of Rac1 in NSCLC cancer cell adhesion, migration and invasion.

Rac1 knockdown prevents lung colonization of NSCLC cells in mice - Next, we examined the effect of Rac1 knockdown on tumorigenesis and metastatic behavior of lung adenocarcinoma cells. Rac1 knockdown or control cells were injected intravenously into immuno-deficient NSG mice in a lung colonization model. Both A549 (Fig 3A) and H441 cells (Supplemental Fig 3A) expressing Scr (500,000 cells/mouse) caused tumor formation in the lungs while Rac1 knockdown cells (500,000 cells/mouse) failed to form tumors in the lungs 8 weeks post-injection. As expected, Rac1shRNA1 infected cells that showed a partial Rac1 protein knockdown formed reduced number of tumors. To determine if the effect on lung colonization is related to a homing defect of the Rac1 knockdown cells in the lung, we tested the ability of the tumor cells to home to the lung tissue 48 hours after tail vein injection. Flow cytometry analysis revealed that the YFP+ Rac1 knockdown cells displayed significantly decreased lung homing activity compared to the YFP+ Scr cells (Fig 3B). In addition, subcutaneous xenograft of the tumor cells (500,000 cells/mouse) in NSG mice established that Rac1 knockdown cells had delayed tumor development and reduced tumor volume compared with the control cells (Fig 3C). Interestingly, sphere formation of H441 cells, which correlates with tumor initiating potential, was also compromised upon Rac1 knockdown (Supplemental Fig 3B) whereas no effect was observed by Rac1 knockdown on sphere forming activity of the non-transforming HBEC control cells (Supplementary Fig 3C). Thus, it is likely that Rac1 knockdown effects tumor cell lung colonization, growth due to a combined effect on cancer cell homing and proliferation in the lung.
**Side population cells contain elevated Rac1-GTP and increased migration, invasion, and lung colonization activities** - The cancer stem cell theory suggests that a fraction of the cancer cell population is enriched for tumor initiating capability, thus requiring preferential targeting to achieve therapeutic benefits. Side population is one of the stem cell markers that has been used to isolate lung cancer stem cells [13]. We isolated SP cells by flow cytometry from A549 cells (Fig 4A) and confirmed by RT-PCR that they express increased ABCG2 transporter (Supplemental Fig 4A). Interestingly, SP cells contained increased Rac1 activity (Fig 4B) and displayed increased migration compared to non-SP cells or parental cells (Fig 4C). Inhibition of the Rac1 activity by using a small molecule Rac inhibitor, NSC23766, resulted in decreased migration and invasion of SP cells (Supplemental Fig 4B, 4C), suggesting elevated Rac1-GTP in the SP cells contributes to these tumor cell behaviors. Furthermore, the SP cells showed increased lung colonization capability *in vivo* compared to non-SP or parental cells (Fig 4D). Interestingly, despite distinct tumor initiating activities *in vivo*, SP and non-SP cells proliferated at a similar rate as the parental cells *in vitro* (Supplemental Fig 4D). However, SP cells displayed increased colony formation activity when growing in soft agar compared to non-SP and parental cells (Supplemental Fig 4E). The residual tumorigenic activity exhibited by the non-SP cells was not due to impurity of these cells, because parallel FACS analysis found over 99.9% enrichment for non-SP cells from the Hoechst 33342 dye sorting (data not shown). Therefore it is likely that a plasticity of the non-SP cells allows them to give rise to cancer initiating cells resulting in the observed residual tumorigenicity.

SP cells were also detected in H441 cells at a lower percentage than A549 cells (Supplemental Fig 4F; 0.5-2% vs. 4-10%). Similar to A549 SP cells, H441 SP cells displayed increased lung colonization in immune compromised mice compared to non-SP cells (Supplemental Fig 4G), and formed more colonies in soft agar compared with non-SP and parental cells (Supplemental Fig 4H).
These results lead us to conclude that SP cells from NSCLA represent a subpopulation in the bulk tumor cells that contain elevated Rac1 activity, increased migration, invasion, anchorage-independent growth activities, and are enriched for cells that are capable of colonizing lung. They also raise the possibility that non-SP cells could remain tumorigenic, albeit with reduced CSC activity, to give rise to tumors, at least in part due to their ability to convert into SP cells.

**Rac1 knockdown suppresses adhesion, migration, and invasion of both SP and non-SP cells** - To further examine the effect of Rac1 knockdown on SP cells, A549 cells were infected with lentivirus either containing scr or Rac1 shRNA, and SP and non-SP cells were isolated by flow cytometry. Western blot analysis confirmed the effectiveness of Rac1 knockdown in both SP and non-SP cells (Supplemental Fig 5A). In line with our earlier data on parental cells, Rac1 knockdown altered the cytoskeletal organization of both SP and non-SP cells (Supplemental Fig 5B), and reduced focal adhesion complexes as visualized by p-FAK immunostaining of both SP and non-SP cells (Fig 5A). Consistently, the adhesion activity of both SP and non-SP cells to fibronectin was also decreased (Fig 5B). Further, Rac1 knockdown decreased migration and invasion of both SP and non-SP cells (Fig 5C, 5D). Thus, Rac1 targeting can suppress migration and invasion of both SP and non-SP cancer cells.

**Rac1 targeting decreases proliferation and lung colonization of both SP and non-SP cells** - To examine whether the observed inhibition of proliferation of the overall cancer cell population by Rac1 knockdown is due to a specific effect on CSCs, we next tested the growth properties of isolated SP and non-SP cells before and after transduction of Rac1-specific shRNA. The proliferation of SP and NSP cells *in vitro* appeared similar under standard tissue culture conditions, and Rac1 knockdown blocked *in vitro* proliferation of both SP and non-SP cells to the similar extent (Fig 6A). BrdU labeling showed that both SP and non-SP cells were inhibited in S-phase transition with a corresponding increase in G0/G1 phase of cell cycle after Rac1
knockdown (Fig 6B). While scrambled RNA did not affect the increased colony formation activity of SP cells in a soft agar assay compared with non-SP cells in either reduced serum or normal serum conditions (Fig 6C; data not shown), Rac1 shRNA was able to significantly reduce colony formation of both SP and non-SP A549 cells. In tail-vein injected NSG mice, the lung colonization of Rac1 shRNA SP cells was drastically decreased compared to scr cells and the corresponding non-SP cells showed no tumor colonization activity (Fig 6D). These results provide strong evidence that Rac1 targeting is effective in inhibiting proliferation and metastasis of both SP and non-SP cells. To test if the Rac1 knockdown effects on proliferation are applicable to other cancer stem cells marked by CD133, a BrdU incorporation assay was performed in which BrdU positive cells were gated in CD133$^+$ or CD133$^-$ population of both scr and Rac1 shRNA treated cells. We observed a decrease in the BrdU$^+$ cells in both CD133$^+$ and CD133$^-$ subpopulations upon Rac1 knockdown (Fig 6E). Thus, Rac1 is required for proliferation in both SP and CD133$^+$ population cells.

**Rac1 knockdown decreases migration, invasion, sphere formation, and metastatic activities of primary human NSCLA cells** - To further examine the relevance of Rac1 targeting on NSCLA cells, we have determined the functional outcomes of Rac1 knockdown in primary patient NSCLA cells that were TTF-1 positive (data not shown). Primary human tumor cells were infected with the lentivirus expressing Scr or Rac1 shRNA and subsequent Western blot analysis confirmed the effective knockdown of Rac1 protein expression in the isolated cells (Fig 7A). Similar to our observations in NSCLA cell lines, Rac1 knockdown in the primary patient cells showed a decrease in trans-well migration and invasion activities (Fig 7B, 7C). Significantly, the sphere formation capability was also compromised upon Rac1 knockdown (Fig 7D, lung adenocarcinoma sample #1) or NSC23766 treatment in two different lung adenocarcinoma samples (Supplemental Fig 6, lung adenocarcinoma sample #2). Finally, Rac1 shRNA was effective in suppressing the primary tumor cell colonization in lung compared to Scr control (Fig...
These results suggest that Rac1 targeting is beneficial to suppressing patient NSCLA cancer stem cell activity.
Discussion

In the present work we show that Rac1 is required for adhesion, migration and lung colonization of NSCLA cells. SP cells isolated from human adenocarcinoma cell lines have enriched lung colonization activity in immunodeficient mice. We further determined that this is associated with their elevated Rac1 activity and increased migration and invasion, as well as increased anchorage-independent growth ability. Importantly, the lung colonization, migration, and invasion activities of both SP and non-SP cells can be effectively blocked by either a Rac1 inhibitor or Rac1 knockdown using shRNA, and the effect may be applicable to the CD133+ and CD133- cell populations. These beneficial effects on tumor cell suppression also appear to apply to primary patient derived NSCLA cells and are irrespective of the p53 or K-ras mutation status (Supplemental Table 1). We propose that Rac1 plays a crucial role in regulating CSC tumor initiating, metastatic activities and thus represents a novel and useful therapeutic target in NSCLA.

The discovery of a population of self renewing cancer stem cells in multiple types of cancer including lung cancer has led to the proposal that CSCs, not non-CSCs in a given tumor, are responsible for tumor initiation and possibly metastasis [13,14,25]. Clinical observations of secondary metastasis post-surgery or -chemotherapy has added to the concept that a residual population of tumor cells may escape conventional therapy and give rise to heterogeneous tumors at metastatic sites. Thus, CSCs are likely the source cells present in the primary tumors which possess unique proliferative and metastatic advantages. To date, CSCs are mostly identified by using various markers. Breast cancer cell lines enriched for CD44+/CD24- markers express higher levels of pro-invasive genes and display higher invasive potential [26]. In pancreatic cancer, CD133+/CXCR4+ cells are shown to be responsible for metastasis [27]. In human liver cancer, CD45+/CD90+/CD44+ cells form metastatic lesions in the lungs of immune compromised mice, and blockage of CD44 activity by an antagonizing antibody is shown to block tumor growth and metastasis [28]. In line with these observations, SP lung cancer cells have been shown to
possess enriched CSC activity by forming subcutaneous tumors in xenograft mice at a reduced cell number [13]. Here, we found that SP cells isolated from NSCLA cells display increased migration, invasion, homing and lung colonization activities, in addition to enriched tumorigenic capability. These observations are consistent with the notion that CSCs are unique in their metastatic potential as well as tumor initiation ability.

Conventional chemotherapeutic agents mainly target malignant cells by either inducing DNA damage or blocking DNA replication. CSCs may be resistant to the effect of these agents through their elevated drug resistance or relative quiescence [29]. To apply the CSC theory, several innovative therapeutic strategies aimed at eradicating CSCs have been developed. To tackle leukemia CSC, neutralizing antibodies to autocrine signaling mediators important for CSC growth such as CD123 [30], antagonist for leukemia CSC localization in the bone marrow niche such as CXCR4-inhibitor AMD-3100 and CD44 antibodies [31], and inhibitors of signaling pathways specifically upregulated in CSCs that are important for the self renewal such as NFκ-β inhibitor parthenolide [32], have been shown to have efficacy in AML or CML. In solid cancers, IL4 has been shown to be useful in colorectal cancer stem cell suppression [33,34], BMP4 was found to induce glioblastoma CSCs to differentiate into non-CSCs [35], and salinomycin, a selective potassium ionophore, could target breast cancer stem cell proliferation and induce differentiation [16]. Our current study adds to this list of potential CSC targeting approaches by presenting evidence that Rac1 inhibition could be efficacious for suppressing both tumor initiation and metastasis of NSCLA CSCs.

Effective targeting of CSCs for therapeutic benefit requires accurate identification of the CSC population. In lung cancer, CD133+, ALDHhigh, and SP have been used as markers to track CSC activity, and in vitro anchorage-independent growth, sphere formation assays and subcutaneous xenograft models have been employed as readouts for the relative CSC activity [12,13,14]. Although each of these markers may help enrich the CSCs, it is clear that they do not accurately identify the tumor initiating cells in human NSCLA tumors, as this population might be quite
complex and could be represented by an overlap of several different markers. Thus, using SP or CD133 as the sole marker for CSC identification may not include all CSC activity in a bulk culture. To this end, it will be interesting to determine whether SP cells overlap with CD133+ cells so that a more pure population of CSCs can be isolated.

The issue of CSC plasticity has been raised recently by several studies [17,36]. It seems possible that both CSCs and non-CSCs could be dynamic populations – CSCs able to give rise to non-CSCs, and non-CSCs may convert to CSCs in given conditions. A recent review [37] raised an interesting point that the reprogramming from differentiating cancer cells to CSCs, unlike that of reprogramming of fully differentiated cells into iPS cells, might occur readily in cancer cells. Recent evidence from Boiko et al [36] and Roesch et al [17] have shown that non-CSCs can indeed covert to CSCs under suitable conditions. We have found that highly purified non-SP cells still retain residue CSC activity in vitro and in mice, raising the possibility that non-CSCs of NSCLA can produce CSCs or SP marker is insufficient in identifying CSCs. This consideration highlights the difficulty of utilizing the CSC theory to design new strategies against cancer, since it can be inferred that it will be necessary to effectively target both CSCs and non-CSCs or multiple marker populations in order to achieve true therapeutic benefits. Importantly, we show that targeting Rac1 can effectively block the lung metastatic and tumor initiating activities of both SP and non-SP NSCLA cells, and such benefits may apply to the CD133+/CD133- tumor initiating populations. Future stringent evaluation how Rac1 contributes to various aspects of CSC activity in multiple marker positive subpopulations will significantly add to the understanding of Rac1 targeting in lung tumorigenesis.
Acknowledgments

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References

Figure 1. Knocking down Rac1 expression effectively inhibits human non-small cell lung cancer cell proliferation. (A) Cell lysates collected from either scrambled shRNA (Scr) or Rac1 shRNA (shRNA1, shRNA2) A549 cells were subjected to Rac1 western blot analysis. GAPDH was used as loading control. (B) Infected cells were sorted and plated on 96-well plate and proliferation assay was performed using MTS reagent. Assay was performed in triplicates and above is one representative of three independent experiments. (C) Infected cells were sorted,
 plated for soft agar colony assay and colonies per field were counted after 2 weeks. Assay was performed in triplicates and above is one representative of three independent experiments. (D) Infected cells were sorted and incubated with BrdU in log phase of cell growth. Cells are trypsinized, stained with BrdU antibody, 7AAD and cell cycle analysis was performed using flowcytometry. Assay was performed in triplicates and above is one representative of four independent experiments. Error bars represents SD. (E) Control cells or cells expressing shRNA resistant Rac1 mutant were infected with scr or Rac1 shRNAs, sorted and plated on 96-well plate. Proliferation assay was performed using MTS reagent. Assay was performed in triplicates and above is one representative of two independent experiments. (F) Infected cells were sorted, plated for soft agar colony assay and colonies were counted after 10 days. Assay was performed in triplicates and above is one representative of two independent experiments.
Figure 2. Targeting Rac1 blocks non-small cell lung cancer cell adhesion, migration and invasion. (A) Infected A549 cells were sorted, plated on fibronectin coated slides and stained with either p-FAK (top panel) or vinculin (middle panel) or p-Paxillin (lower panel) and DAPI. Images were collected using fluorescent microscope at 40X magnification. Images above are representative of several images obtained from two independent experiments. (B) A549 sorted cells were plated on fibronectin coated 96-well plate for in vitro adhesion assay and cells attached
to plate after 1 hour was determined using MTS reagent. Adhesion assay was performed with five replicates and the data is representative of three independent experiments. (C) Sorted cells were plated on trans-well migration plates and migration of cells toward 10% FBS was measured overnight. Assay was performed in replicates and above data was representative of three independent experiments. (D) Sorted A549 cells were plated on matrigel coated invasion plates and migration of cells toward 10% FBS and 10\mu g/ml fibronectin was measured after 48 hours. Assay was performed in triplicates and the above is a representative of three independent experiments. Error bars represents SD. (E) Control or cells expressing Rac1shRNA resistant mutant were infected with Rac1 shRNA and sorted. Cells were plated on trans-well migration plates and migration of cells toward 10% FBS was measured overnight. Assay was performed in triplicates and above data was representative of two independent experiments.
Figure 3. Knocking down Rac1 expression suppresses lung cancer cell homing and tumor growth in the lung of recipient mice. (A) 5X10^5 A549 cells were injected into tail vein of NSG mice (n=6 per condition) and lungs were dissected after 8 weeks. Lung were stained with Bouins solution and destained in 70% ethanol. Quantification of lung colonization data was shown in the right panel. Error bar represents SE. Depicted is a representative of two independent experiments. (B) Tumor cell homing assay was performed as described in methods (n=6 per condition in each experiment). Homing index was measured as percentage of YFP positive cells detected in lung, normalized to control. Depicted is a representative of three independent experiments. (C) 5X10^5 scr or Rac1 shRNA infected cells were injected subcutaneous into flanks of NOD/SCID mice and tumor volume was measured weekly for 7 weeks. Error bar represents SE.
Figure 4. Side population cells possess elevated Rac1 activity, increased migration, invasion and proliferative activities, and enriched tumor initiating activity in mouse lung.

(A) A549 cells were stained with Hoechst 33342 dye and analyzed by flowcytometry for side population (left panel). Cells were treated with 10μM Fumitremorgen for inhibitor control (right panel). Depicted is a representative of several SP analyses. (B) Cell lysates collected from sorted SP and non-SP cells were subjected to GST-PAK pull down assay and processed for Rac1 western blot analysis to determine the Rac1 activity. Total Rac1 blot was used as a control. Depicted is a representative of three independent Rac1 activity pull-down assays. (C) Sorted A549 cells were plated for trans-well migration assay and cells migrated overnight towards 10% FBS were stained and counted. Above is a representative of three independent experiments and
error bars represents SD. (D) 5X10^4 sorted SP and non-SP cells were injected into tail vein of NSG mice (n=4 per condition). Lungs were dissected out at the end of 12 weeks. Right panel shows quantification of lung colonization data. Error bar represents SE. Above is a representative of three independent experiments.
Figure 5. Targeting Rac1 effectively suppresses the adhesion, migration and invasion activities of SP cells as well as non-SP cells. (A) Sorted cells were plated on fibronectin coated slides, fixed and subjected to immunostaining with p-FAK antibody. Cell images were collected at 40X magnification using Fluorescent microscope. Above depicted are representative of multiple images collected. (B, C, D) A549 sorted cells were either plated on fibronectin coated 96-well plate for *in vitro* adhesion assay (B), on trans-well plates for migration assay (C) or matrigel coated invasion plates for invasion assay (D). All assays were performed in triplicates and error bars represent SD. Depicted are representative of three independent experiments.
Figure 6. Rac1 knockdown inhibits the proliferation and tumor seeding activities of SP cells as well as non-SP cells *in vitro* and *in vivo*. (A) Sorted cells were plated in 96-well plate and proliferation assay was performed using MTS reagent. Assay was performed in triplicates and error bars represents SD. Depicted is a representative of three independent experiments. (B) A549 sorted cells were incubated with BrdU and cell cycle analysis was performed by BrdU staining and flowcytometric analysis. Assay was performed in triplicates and error bars represent SD. Above is a representative of two independent experiments. (C) Sorted cell were directly plated for soft agar colony formation assay and the number of colonies formed were counted after 2-3 weeks. Assay was performed in triplicates and error bars represent SD. Above is a
representative of three independent experiments. (D) 50,000 sorted cells were injected into tail
vein of NSG mice and the lungs were isolated for analysis after 12 weeks. Number of lung tumors
was counted under light microscope and error bar represents SE. Results are representative of
three independent experiments. (E) Cells were infected with either scr or Rac1 shRNA and
incubated with BrdU. Cells were stained with CD133 antibody during the BrdU staining described
in methods. BrdU positive cells gated from CD133⁺ and CD133⁻ cells were analyzed by FACS.
Figure 7. Rac1 targeting inhibits primary non-small cell lung cancer cell migration, invasion, sphere forming and lung colonization activities. (A) Cells isolated from primary tumor sample were infected with either scr or Rac1 shRNA and sorted cells were subjected to Rac1 western blot analysis. GAPDH was used as loading control. Above depicted is a representative of multiple western blots. (B, C, D) Primary human adenocarcinoma cells were plated for either trans-well migration assay (B) or invasion assay (C) or sphere assay (D). For sphere assay, sorted cells were directed plated in sphere growth media described in methods section. Number of spheres formed after 10 days were counted using light microscope. All the assays were performed in triplicates and error bar represents SD. Results are representative of three independent experiments. (E) For lung colonization assay, 5X10^5 sorted cells were injected
into tail vein of NSG mice (n=4 per condition) and mice were sacked after 6 weeks. Number of lung tumors was counted under dissecting microscope. Error bar represents SD.
Supplemental Table 1: Summary of Rac1 knockdown data on NSCLC cell lines

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<tr>
<th>Cell line</th>
<th>K-ras status*</th>
<th>P53 status*</th>
<th>Rac1KD effects</th>
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<td>A549 (primary site)</td>
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<td>P53 wild type</td>
<td>1. Proliferation (+++)&lt;br&gt;2. Soft agar (+++)&lt;br&gt;3. Migration (+++)&lt;br&gt;</td>
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<td>P53 mutant</td>
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<td>P53 mutation</td>
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+ low; ++ Moderate; +++ high, * ATCC
Supplemental figure 1. Effects of stable Rac1 suppression on H441 cell proliferation. (A) Cell lysates collected from either scrambled shRNA (Scr) or Rac1 shRNA (shRNA1) H441 cells were subjected to Rac1 western blot analysis. GAPDH was used as loading control. (B) Sorted cells were plated and incubated with BrdU in log phase of cell growth. Cells are trypsinized and stained with BrdU antibody, 7AAD to perform cell cycle analysis. Assay was performed in triplicates and a representative experiment is shown. Error bars represent SD. (C, E) H1299 (C) and H23 cells (E) were transduced with lentivirus expressing either scr shRNA or Rac1 shRNA. 72 hours after the transduction, cells were plated for a proliferation assay. The number of cells was determined by MTS measurements at different time points. Assays were performed in triplicates and error bars represent SD. (D, F) The cells were plated for soft agar colony formation.
assay. Number of colonies formed was counted under light microscope after 2-3 weeks. Assays were performed in triplicates and error bar represents SD.
Supplemental figure 2. Effect of Rac1 suppression on focal adhesion complexes, adhesion, migration and invasion. (A) A549 cells expressing either scr or Rac1 shRNAs were sorted and plated. Cell lysates collected from adherent cells were processed for p-MLC, p-FAK, p-Paxillin, Rac1 Western blot analysis. GAPDH was probed as a loading control. Data is representative of three experiments. (B, C, D) H441 cells expressing either scr or Rac1 shRNAs were sorted and were plated on fibronectin coated plates for adhesion assay (B), on trans-well migration plate for migration assay (C) or matrigel coated invasion plates for invasion assay (D). All assays were performed in triplicates and data are representative of three independent experiments. Error bars represent SD. (E, F) H1299 (E) and H23 cells (F) were transduced with either scr shRNA or Rac1 shRNA and 72 hours later cells were plated for the trans-well migration assay. Cells migrated overnight were stained and counted. The assay was performed in triplicates and error bar represents SD.
Supplemental figure 3. Suppression of Rac1 expression in H441 cells inhibits lung colonization in mice. (A) Lung colonization assay was performed with scr or Rac1shRNA expressing H441 cells. Number of tumors formed after 12 weeks were counted and error bar represents SD. Data is representative of three independent experiments. (B) H441 cells expressing either scr or Rac1 shRNA were plated for sphere assay as described in Methods. Number of spheres formed after 10 days were counted under light microscope. Assay was performed in triplicates and error bars represent SD. Depicted is a representative of two independent experiments. (C) HBEC cells expressing either scr or Rac1 shRNA were plated for the sphere assay. Number of spheres formed after 10 days were counted under light microscope. The assay was performed in triplicates and error bars represent SD.
Supplemental figure 4. Properties of H441 SP and non-SP cells under Rac1 suppression.

(A) Quantitative RT-PCR for ABCG2 transporter gene was performed using RNA collected from sorted cells. ABCG2 expression was normalized to GAPDH and the relative expression was represented as fold-change from control cells. RT-PCR experiment was performed in triplicates and depicted is a representative of four independent experiments. Error bars represents SD. (B) H441 SP cells were plated for migration assay either in the presence of a vehicle or a Rac inhibitor, NSC23766 (50μM). Cells migrated overnight were quantified as described in Methods. Error bars represent SD and the depicted is representative of two independent experiments. (C) H441 SP cells were plated for invasion assays in the presence or absence of the Rac inhibitor
NSC23766 (50μM). Cells invading through matrigel were quantified as described in Methods. Error bars represent SD and the depicted is representative of two independent experiments. (D) SP, NSP, or non-sorted cells were plated in 96-well plates and the proliferation was measured by the MTS assay. Assays were performed in triplicates and error bars represents SD. Depicted is a representative of three independent experiments. (E) SP, non-SP, or non-sorted cells were plated for soft agar colony assay and the colonies were quantified after 2 weeks. Assays were performed in triplicates and error bar represents SD. Data is representative of two independent experiments. (F) H441 cells were stained with Heochst dye and analyzed by flow cytometry. Depicted is representative of three independent analyses. (G) SP or NSP H441 cells (3X10^4) were sorted and injected into tail vein of NSG mice (n=3 per condition). Lungs were collected after 10-12 weeks and number of tumors formed in each lung was counted. Error bar represents SD. Depicted is representative of three independent experiments. (H) H441 SP, NSP, or non-sorted cells were plated for soft agar assay and colonies formed after 21 days were counted. Assays were performed in triplicates and error bar represents SD. Data is representative of two independent experiments.
**Figure S5**

(A) Cell lysates collected from FACS sorted A549 cells were subjected to Rac1 western blot analysis. GAPDH was used as loading control. Shown is a representative of three Western blots.

(B) FACS sorted A549 cells were plated onto fibronectin coated slides and stained for actin cytoskeleton and nuclei by rhodamine-phalloidin and DAPI, respectively. Shown is a representative of several images obtained in two independent experiments.

**Supplemental figure 5. Effect of Rac1 suppression on cell actin organization.** (A) Cell lysates collected from FACS sorted A549 cells were subjected to Rac1 western blot analysis. GAPDH was used as loading control. Shown is a representative of three Western blots. (B) FACS sorted A549 cells were plated onto fibronectin coated slides and stained for actin cytoskeleton and nuclei by rhodamine-phalloidin and DAPI, respectively. Shown is a representative of several images obtained in two independent experiments.
Supplemental figure 6. Effect of Rac1 inhibition on sphere formation. Cells isolated from primary human lung adenocarcinoma were plated for sphere assay either in the presence of vehicle or NSC23766 (50μM). The number of spheres formed after 10 days were counted under microscope. Assays were performed in triplicates and error bars represent SD. Depicted is a representative of two independent experiments.
Chapter III: Lung cancer stem cells and non-cancer stem cells can be enriched in multiple distinct phenotypic populations and are plastic

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Key words: Non-small cell lung adenocarcinoma, Cancer stem cells, SP, CD133\textsuperscript{pos}, ALDH\textsuperscript{high}, EMT, Rac1.

Highlights

- CSCs defined by expression of different markers are distinct phenotypic populations with minimal overlap in human lung adenocarcinomas.
- Distinct marker-defined CSC and non-CSC populations are plastic.
- EMT regulates the plasticity of distinct CSC populations differently.
- Rac1 GTPase modulates EMT and CSC generation.
Summary

The cancer stem cells (CSCs) represent a population of cancer cells that possesses unique self-renewal, expansion and differentiation activities required for tumorigenesis. While the CSC theory remains controversial in solid tumor studies, cancer stem cell enriched populations have been characterized by drug-resistant side population (SP), CD133<sup>pos</sup>, ALDH<sup>high</sup> and several other markers. Using human lung cancer cell lines and human patient primary NSCLA cells, we show here that (1) SP cells represent a distinct population from phenotypic CSC populations marked by CD133<sup>pos</sup>, ALDH<sup>high</sup>, with each displaying enriched tumor-initiating activity in xenografted mice; (2) non-CSCs and CSCs isolated based on either unique enzymatic activities (SP, ALDH<sup>high</sup>) or cell surface markers (CD133<sup>pos</sup>, CD24<sup>low</sup> CD44<sup>high</sup>) are interconvertable and plastic, with unique kinetics; (3) the epithelial-mesenchymal transition (EMT) process, previously proposed as one of the potential mechanisms of conversion between CSCs and non-CSCs, abolishes SP cells, and increases CD133<sup>pos</sup>, ALDH<sup>high</sup> CSCs populations; (4) Rac1 activity is significantly increased in cells that have undergone EMT and Rac1 suppression can block the EMT process and inhibit tumor initiating activity.

Significance

Our studies indicate that multiple interconvertible cell subpopulations with CSC properties exist in NSCLAs. Hence elimination of one population CSCs may not be an efficacious strategy for cancer therapy because other CSC populations, as well as plastic conversion from non-CSCs, can potentially propagate tumors. Our current work suggests that targeting Rac1 GTPase represents a novel strategy that inhibits both CSC activity and the dynamic transition of the non-CSCs to CSCs.
Introduction

Lung cancer is the leading cause of cancer deaths worldwide. This disease is broadly classified into two subtypes, i.e. small cell lung cancer and non-small cell lung cancer, based on histo-pathological characteristics. Adenocarcinomas constitute about 80% of NSCLC and about 40% of total lung cancers. Despite of the advancement in therapies, lung cancer continues to be a major killer because of existence of chemo and radiation therapy resistant tumor cells.

With the recent advent of cancer stem cell theory, several sub-populations of cells have been identified by cell surface markers or enzyme activities as cancer stem cells (CSC) in solid tumors. These CSCs have been shown to display the characteristics of self renewal and tumor initiating properties in xenograft mouse models (Al-Hajj et al., 2003; Chiba et al., 2006; Lapidot et al., 1994; O'Brien et al., 2007; Singh et al., 2004). In Lung cancer, three different CSC populations have been identified to date: drug resistant side population (SP) (Akunuru et al., 2011; Ho et al., 2007), CD133^{pos} cells (Eramo et al., 2008; Levina et al., 2008), and ALDH^{high} cells (Deng et al., 2010; Sullivan et al., 2010). SP cells isolated from several NSCLC cell lines were shown to have higher expression of ABC transporters and were resistant to chemotherapeutic drug treatments (Ho et al., 2007). We previously have shown NSCLAC SP cells were more metastatic in xenograft models and the more invasive behavior of SP cells was driven by high Rac1-GTP activity (Akunuru et al., 2011). Drug resistant cells derived from NSCLC cell lines were enriched for CD133^{pos} cells and displayed elevated cytokine expression (Levina et al., 2008). Lung adenocarcinoma stem cells with higher ALDH activity were more tumorigenic, and the tumorigenecity was dependent on elevated notch1 signaling (Sullivan et al., 2010). However, the potential relationship of these different marker-defined CSCs, e.g. whether they represent overlapping or hierarchy populations of NSCLC, has not been established.
The advancement of the cancer stem cell theory has led to the proposal that targeting CSCs can eradicate residual chemo-radiation therapy resistant tumor cells in patients. In line with this theory Gupta et al recently suggested that differentiation of CSCs by salinomycin, a selective potassium ionophore, can block mammary CSC activity and metastasis (Gupta et al., 2009b). The efficacy of such reagents has yet to be tested in pre-clinical and clinical models of solid tumors. However, multiple recent reports have indicated that CSCs and non-CSCs can potentially be plastic and inter-convertible in nature (Roesch et al., 2010) (Gupta et al., 2009a). EMT was proposed to be one of the potential mechanisms for CSC generation (Mani et al., 2008). Identifying potential mechanisms and molecular pathways involved in EMT and CSC plasticity may help in developing novel, more effective cancer therapies.

In the current study we show that the SP, CD133\textsuperscript{pos} and ALDH\textsuperscript{high} lung adenocarcinomas cells are phenotypically distinct subpopulations. Non-SP, CD133\textsuperscript{neg} or ALDH\textsuperscript{low} cells can convert into SP, CD133\textsuperscript{pos} and ALDH\textsuperscript{high} cells, respectively, under normal culture conditions. TGFβ1 induced EMT enriches for the CD133\textsuperscript{pos} subpopulation while reducing the SP subpopulation. Rac1, one of the Rho GTPase family members, that was known to be involved in metastasis and EMT, is upregulated in the mesenchymal state. Thus, Rac1 inhibition blocked the EMT transition and the dynamic plasticity between CSCs and non-CSCs, underscoring its potential as a novel therapeutic strategy.

**Materials and methods**

**Cell culture**

A549 and H441 cells were cultured according to the guidelines from ATCC. Primary patient lung adenocarcinoma samples were obtained with written consent from patients under an approved Institution Review Board protocol by University of Cincinnati Scientific Review Committee (IRB# 01-09-27-07), and were used in the experiments
according to Cincinnati Children’s Hospital Medical Center Scientific Review Committee (IRB # 07-06-57) that the identity of the patients remains anonymous. Tumors were washed, minced and dissociated in DMEM containing 0.5 mg/ml Liberase (Roche)C and 1% penicillin and streptomycin. After 45 minute incubation, the slurry of cells was passed through a 100 micron filter and cells were washed and plated in growth media. Epithelial cancer cells were enriched by growing cells in sphere culture conditions.

To induce EMT, A549 or H441 or primary adenocarcinoma cells were either treated with 2.5ng/ml TGFβ1 for either 12 (A549 and primary adenocarcinoma cells) or 21 days (H441). A549 cells were infected with lentivirus expressing Twist ER or Snail ER vectors described previously (Dr. R.A. Weinberg’s lab, White head institute, Cambridge, MA). A549 cells expressing Snail/Twist ER were treated with different concentrations of 4-OH tamoxifen for 10 days to induce EMT.

A549, H441, or primary adenocarcinoma cells were infected with lentivirus expressing YFP tagged control shRNA or Rac1 shRNA described previously. Control shRNA construct was a generous gift from Dr. Leighton Grimes (Cincinnati Children’s Hospital Medical Center) and Rac1 shRNA constructs were generous gift from Dr. James Mulloy (Cincinnati Children’s Hospital Medical Center). After 72 hours of infection, YFP positive cells were sorted using FACS and subsequently utilized.

Side-population, ALDH and CD133 cell staining

Cells were either stained with Hoechst 33342 staining buffer for side-population (SP) as described previously at a final concentration of 5 µg/ml Hoechst 33342, or Aldeflor reagent (Stem cell technologies) according to manufacturers guidelines for ALDH\textsuperscript{high} cells or anti-CD133/1 antibody (Miltenyi) for CD133\textsuperscript{pos} cells. The cells were analyzed or sorted for SP/ALDH\textsuperscript{high}/CD133\textsuperscript{pos} cells by FACS with either FACS Canto bench top or FACS Ariel sorter (BD).

Sphere formation assay
Cells (10,000 cells/ml) were plated in suspension culture conditions in serum-free sphere media (DMEM:F12 containing 0.4% BSA, 10µg/ml insulin, 10ng/ml EGF, 10ng/ml FGF) on 6-well plates pre-coated with 1% agarose to prevent cell attachment. Media was replaced every 2-3 days and the number of spheres formed in 2 weeks was counted under light microscope.

**Gene expression analysis**

Sorted cells were washed in PBS and RNA was isolated using RNA micro kit (Qiagen). Concentration and the quality of the RNA were measured by using Nanodrop (Thermo Scientific). cDNA was generated using cDNA synthesis kit (ABI) as per manufacturer’s guidelines. Pre-made Taqman PCR primers/probes for self-renewal genes and “metastasis genes” were utilized (ABI). SYBR-PCR primers for EMT markers described previously were used. Quantitative PCR reaction was performed using qPCR master mix (ABI) with either SYBR or Taqman and ABI700 instrument.

**Immuno-staining**

Cells were plated on fibronectin coated slides and after 18-20 hours cells were fixed using 3.7% Formaldehyde. Cells were stained for actin cytoskeleton (Rhodamine-Phalloidin, Invitrogen), nuclei (DAPI, Invitrogen) using standard immuno-staining methods described previously. Alternatively, cells were stained with either Fibronectin (Sigma) or vimentin (Sigma) or E-cadherin (SantaCruz).

**Rac1-GTP pull-down assay**

To perform Rac1 pull down assays, cells were lysed by adding lysis buffer (20 mM Tris HCl pH 7.6, 100 mM NaCl, 10 mM MgCl, 1% Triton X-100, 0.2% SDS) with protease and phosphatase inhibitors directly to adherent cells. Cell lysates containing equal amounts of protein were incubated with glutathione beads conjugated to GST-PAK1 containing active Rac1 interacting domain and processed further as described previously (Guo et al., 2003).
**Lung colonization assay**

Use of mice as xenograft hosts was approved by the IACUC committee at Cincinnati Children’s Hospital Medical Center (Protocol# 8D06052). Specified number of cells were suspended in PBS and injected intravenously into immune compromised NOD/SCID/γc -/- (NSG) mice by tail vein injection. At the end of the study, lungs were fixed in Bouin’s solution to count number of tumors.
Results

NSCLACs contain multiple, phenotypically distinct subpopulations of CSC activity

To dissect the relative expression of different CSC markers in lung adenocarcinoma cell lines, we probed for SP, CD133\textsuperscript{pos}, ALDH\textsuperscript{high} cells. A549 and H441 cells were stained with either Hoechst dye or an anti-CD133 antibody or an Aldeflour reagent to determine the distribution of SP, CD133\textsuperscript{pos} or ALDH\textsuperscript{high} cells, respectively, in the cells. A549 cells expressed 4-10% SP cells, 0.3-1% CD133\textsuperscript{pos} cells, and 2-8% ALDH\textsuperscript{high} cells, while H441 cells contained 0.5-3% SP cells, 0.1-0.5% CD133\textsuperscript{pos} cells, and 0.5-2% ALDH\textsuperscript{high} cells (Fig1). A549 cells also expressed about 10-15% CD24\textsuperscript{low}CD44\textsuperscript{high}, a set of markers that has been associated with CSC activity in breast cancer (supplemental Fig 1A) while H441 cells expressed 18-30% CD24\textsuperscript{low} with little detectable CD44. We isolated cells from primary NSCLAC tumor samples and cultured them in sphere-forming culture conditions. These cells had a cobble stone epithelial morphology in adherent culture conditions and were TTF-1\textsuperscript{pos} (data not shown). After culturing for 1 week, the primary NSCLAC contain 80% CD133\textsuperscript{pos} cells and 2% SP cells (Fig 1D). Interestingly, when cells were cultured on adherent tissue culture conditions for additional 1 and 3 weeks, the CD133\textsuperscript{pos} population decreased to 20% and 2%, respectively, and the SP population increased to 8-10% and 15-18%, respectively (Fig 1E).

Based on these observations, we sought to determine the potential overlap of these different CSC markers in adenocarcinoma cells. We isolated SP and non-SP cells, stained for CD133 or ALDH marker, and performed flow cytometric analysis of the cells. We observed a minimal overlap between SP and CD133\textsuperscript{pos} cells (Fig 1F) and between SP and ALDH\textsuperscript{high} cells in A549 cells (Fig 1F, 1G left panel) or primary lung adenocarcinoma cells (Fig 1F, 1G right panels). We also observed similar distribution of CD24\textsuperscript{low}CD44\textsuperscript{high} population in SP and non-SP populations of primary adenocarcinoma.
cells; however, A549 SP cells displayed an increased percentage of CD24<sup>low</sup>CD44<sup>high</sup> cells compared to non-SP population (supplemental Fig 1B). Similar observations with regard of the overlap between different CSC marker expressions were made in H441 cells (data not shown). It appears that enriching for CSCs by one set of markers does not correlate with an enrichment of CSCs carrying another set of markers. Since there is minimal overlap of different CSC marker-defined populations, we further examined if different subpopulations of CSCs display distinct patterns of gene expression. We isolated SP, NSP, CD133<sup>pos</sup>, CD133<sup>neg</sup>, ALDH<sup>low</sup>, ALDH<sup>high</sup> populations of A549 and primary lung adenocarcinoma cells and compared the expression profiles of self renewal genes. SP cells displayed increased Oct4 and Nanog expression compared to non-SP cells in both A549 and lung adenocarcinoma cells (Fig 2A; left and right panel respectively). CD133<sup>pos</sup> cells had increased Oct4 and Nanog expression compared to CD133<sup>neg</sup> in primary adenocarcinoma cells but the increase was subtle in A549 cells (Fig 2B). Consistent with previous observations by Sullivan et al, we observed increased Notch1 expression in ALDH<sup>high</sup> cells compared to ALDH<sup>low</sup> A549 cells. Additionally, we observed increased Hes1 and Shh expression in ALDH<sup>high</sup> cells (Fig 2C, left panel). Interestingly, in primary adenocarcinoma cells the ALDH<sup>high</sup> population had increased Oct4 and Nanog expression, but not Notch1, Hes1 and Shh gene expression, compared to ALDH<sup>low</sup> cells. Consistent with the increase in expression of self renewal genes in the different CSC populations, SP, CD133<sup>pos</sup> and ALDH<sup>high</sup> cells displayed significantly increased sphere forming activities compared to non-SP, CD133<sup>neg</sup> and ALDH<sup>low</sup> cells (Fig 2D, 2E, 2F), respectively. These results suggest that distinct CSC populations express different set of self renewal genes for tumor initiation cell propagation.

Recent studies suggest that CSCs may serve as the “seeds” for tumor metastasis at the secondary site. We examined the expressions of genes previously shown to be
associated with metastasis in the different marker-enriched CSCs. SP cells from primary lung adenocarcinoma cells exhibited an increased expression of CXCR4, HoxB9, TGFβ1, VEGFA, IL-6 (Fig 2G, left panel) compared to non-SP cells. ALDH^{high} cells yielded a distinct pattern of expression of the metastasis associated genes including TNFα, HoxB9, VEGFA, IL-6 compared to ALDH^{low} cells. To further examine whether the expression of such metastasis associated genes are reflective of the in vivo metastasic capacity of these CSC populations, we performed the lung colonization assay in NSG mice by tail vein injection of the isolated cell populations. Consistent with our previous observations, SP cells had increased lung colonization activity compared to non-SP cells, and CD133^{pos} cells demonstrated an increased lung colonization compared to CD133^{neg} cells (Fig 2H, left panel). However, no difference between ALDH^{low} and ALDH^{high} cells in a lung colonization activity was detected (Fig 2H, right panel). But ALDH^{high} cell-injected mice had numerous liver tumors (7 mice out of 8 mice injected had liver tumors), while mice injected with ALDH^{low} cells injected did not develop liver tumors. Therefore, multiple populations of marker-enriched CSCs have increased metastatic gene expressions and tumor metastasis activities.

**Marker defined NSCLAC CSC and non-CSC subpopulations are inter-convertible**

Although SP cells display significantly higher tumorigenic activity than the non-SP cells in lung colonization, the non-SP cells could still form tumors in the xenotransplanted mice. This phenomenon can either be explained by the existence of other tumor initiating cells that are non-SP, or a possible conversion of non-SP cells to SP cells to give rise to tumors. To examine the later possibility, i.e. non-CSCs may convert to CSCs, we isolated non-SP cells by FACS and confirmed their high purity (>99.8%) by FACS. When cultured in adherent culture condition, these sorted non-SP cells were able to generate SP cells to the extent of parental cells in 7 days (Supplemental Fig 2A, Fig 3A). To
perform a more stringent analysis, we sorted GFP\textsuperscript{pos} SP cells and GFP\textsuperscript{neg} non-SP cells to 100% purity. When the two populations of cells were mixed at 1:1 ratio and cultured in adherent culture conditions, GFP\textsuperscript{neg} non-SP cells gave rise to GFP\textsuperscript{neg} SP cells after several cell doublings (Fig 3B). In parallel, the frequency of GFP\textsuperscript{pos} SP cells decreased with time, generating GFP\textsuperscript{pos} non-SP cells (data not shown). When single non-SP cells were isolated and grown in 96-well plates, the clones generated from both A549 and primary adenocarcinoma cells contained a significant fraction of SP cells (2-30% for A549 cells, Fig 3C, left panel; 5-38% for primary adenocarcinoma cells, Fig 3C, right panel). Importantly, non-SP cells could generate SP cells \textit{in vivo} (Fig 3D). Such a dynamic conversion between marker defined non-CSCs and CSCs was also observed in non-CSC/CSC populations including CD133\textsuperscript{neg}/CD133\textsuperscript{pos}, ALDH\textsubscript{low}/ALDH\textsubscript{low} and CD24\textsuperscript{high}CD44\textsuperscript{high}/CD24\textsuperscript{low}CD44\textsuperscript{high} cells (Fig 3E, 3F, Supplemental Fig 2B). Interestingly, the kinetics of conversion of different CSC subpopulations designated by different markers varied (Fig 3E, 3F, Supplemental Fig 2B). These results indicate that the non-CSC and CSC populations are plastic and inter-convertible in both tumor cell lines and primary lung adenocarcinoma cells.

**EMT has divergent effects on different CSC subpopulations**

To probe the role of EMT in the plasticity of CSC/non-CSC, we treated A549, H441 and primary adenocarcinoma cells with TGF\textbeta1 to induce EMT. TGF\textbeta1 induces clear morphological changes in all cells consistent with a mensenchymal transition (Supplemental Fig 3A), which is accompanied by a decrease in expression of epithelial markers such as E-cadherin and an increase in expression of mesenchymal genes like Foxc2, N-cadherin, twist, snail and fibronectin (Supplemental Fig 3B,C). The expression changes in E-cadherin, fibronectin and vimentin were also confirmed by immunostaining (Supplemental Fig 3D). The TGF\textbeta1-induced EMT was transient and reversible, since the
withdrawal of TGFβ1 resulted in a reversal of the gene expression profile (Supplemental Fig 3B).

Next we examined the effect of EMT on the CSC marker expressions. Surprisingly, TGFβ1 treatment abolished SP cells in A549 (Fig 4A, left and middle panel), H441 (supplementary Fig 4A, left panel) and primary lung adenocarcinoma cells (Fig 4A, right panel). Consistent with the dye-exclusion assay results, we observed a decrease in ABCG2 transporter gene expression in these cells (Fig 4B, left panel, Supplemental Fig 4A, right panel; Fig 4B, right panel). Because TGFβ1-induced EMT was reversible, dye-exclusion activity and ABCG2 expression were restored to that prior to the treatment upon withdrawal of TGFβ1. To rule out that the effect of TGFβ1 on SP cells is not due to non-specific effects of TGFβ1 on ABC transporter expression, we further tested the effect of inducible Twist or Snail activity on SP cell population. 4-OH tamoxifen induced an EMT phenotype in A549 cells expressing Snail-ER (Supplemental Fig 4B) or Twist-ER (data not shown) as evidenced by reduced E-cadherin expression and increased FoxC2 and fibronectin expression. The Twist or Snail induced EMT also decreased both SP population cells and ABCG2 expression (supplemental Fig 4C and data not shown).

We conclude that EMT decreases SP cells in lung adenocarcinoma cells.

Next, we tested the effect of EMT on other CSC marker populations. TGFβ1 treatment increased CD133pos and ALDHhigh populations in A549 cells (Fig 4D, 4E) and this effect was reversible as mesenchymal-epithelial transition, MET brought by TGFβ1 withdrawal reduced the CD133pos and ALDHhigh cells back to the percentage comparable to control cells. Similar to observations of Mani et al in HMEC cells, we showed that TGFβ1 treatment increased CD24lowCD44high cells in A549 cells (Supplemental Fig 4D) and further TGFβ1 withdrawal decreased the population of CD24lowCD44high cells. These results reaffirm that SP cells represent a distinct CSC population from that of CD133pos, ALDHhigh or CD24lowCD44high CSC population, and indicate that EMT is a potential
mechanism involved in altering the plasticity of various CSC marker populations in lung adenocarcinoma cells.

To further determine the effects of EMT on the CSC activity with its differential role in CSC marker expression, we assessed the expression pattern of a number of self renewal genes after TGFβ1 induced EMT. TGFβ1 treatment increased the expression of Oct4, Nanog, and Notch1 compared to the untreated control cells, while the increase in expression of self renewal genes was reduced to control levels upon the withdrawal of TGFβ1 (Fig 4E). Consistent with this effect, we observed a significant increase in sphere forming activity in TGFβ1 treated H441 and primary adenocarcinoma cells (Fig 4F). Concomitantly, TGFβ1 treatment also increased the expression of several metastatic genes including HoxB9, TGFβ1, VEGFA, IL-1β, IL-6 (Fig 4G), and enhanced lung colonization activity (Fig 4H). These results indicate that EMT can serve as a cellular mechanism of plasticity in defined subpopulations of CSCs promoting tumor initiation and metastasis.

Sphere formation induces self-renewal and CSC markers expression similar to TGFβ1 treated cells

Since TGFβ1 treatment increased the sphere formation activity and promoted EMT of lung adenocarcinoma cells, we next evaluated if cells grown in sphere culture conditions resemble mesenchymal cells. A549 cells grown in sphere culture condition displayed increased mesenchymal gene expressions including twist, snail and vimentin compared to adherent cells (Fig 5E). Similar to TGFβ1 treated cells, the sphere forming cells showed increased Oct4, Nanog and Notch1 expression compared to adherent cells (Fig 5A). Consistent with their mesenchymal state, sphere forming cells displayed dramatic decrease in SP cell population (Fig 5B) and the associated decrease in ABCG2 transporter expression (Fig 5C). However these sphere cells were enriched for CD133<sup>POS</sup>
(Fig 5D), ALDH\textsuperscript{high} and CD24\textsuperscript{low}CD44\textsuperscript{high} (data not shown) marked populations. When the lung colonization abilities of the sphere cells were examined, we observed an increased expression of several metastasis associated genes, and increased lung colonization by primary adenocarcinoma sphere cells compared to adherent cells. Thus, the sphere-forming lung cancer cells selectively enrich several CSC subpopulations while suppressing the SP cells, mimicking that of EMT-induced CSC conversion dynamics.

**Rac1 targeting blocks EMT and inhibits CSC activities**

Rac1 is a critical modifier of actin cytoskeletal organization and transcriptional regulation. Since EMT involves a distinct morphological change and transcription alterations, we wanted to determine whether Rac1 is involved in the lung cancer EMT process. TGF\(\beta\)1 treatment increased Rac1-GTP levels in A549 cells and it returned to the untreated control level after removal of TGF\(\beta\)1, consistent with the transient nature of TGF\(\beta\)1 on EMT (Fig 6A). Similarly, we observed increased Rac1-GTP activity in the Twist overexpressing A549 cells that have undergone EMT (data not shown). Interestingly, H441 sphere-forming cells contained an increased Rac1-GTP compared to adherent cells (Fig 6B). ShRNA mediated Rac1 knockdown partially blocked the actin cytoskeletal restructuring associated with TGF\(\beta\)1 induced EMT comparing with the control shRNA treated cells (Fig 6C). Rac1 knockdown was able to rescue the decrease in E-cadherin expression and the increase in Snail expression associated with EMT (Fig 6D). Consistent with a blockade of the mesenchymal state of cells by Rac1 knockdown, we observed a significant decrease in sphere-forming activity (data not shown) and the associated decrease in lung colonization of H441 cells and primary lung adenocarcinoma cells (Fig 6I). NSC23766, a specific Rac-GTP inhibitor, inhibited Rac1 activity in A549 cells (Fig 6E) and partially blocked TGF\(\beta\)1-induced EMT as shown by changes in gene expression of epithelial and mesenchymal markers (Fig 6F). Finally,
NSC23766 could also inhibit the TGFβ1-induced changes in marker defined CSC populations, i.e. a decrease in SP cells (Fig 6G) and an increase in ALDH\textsuperscript{high} cells (Fig 6H). Therefore, Rac1 constitutes a potentially useful target in suppressing EMT and EMT-associated CSC transition.

**Discussion**

Overall, we determined that SP is a phenotypic CSC marker distinct from several other CSC markers including CD133\textsuperscript{pos}, ALDH\textsuperscript{high} and CD24\textsuperscript{low}CD44\textsuperscript{high} populations in lung adenocarcinoma cells. CSC marker expression is plastic and non-CSCs can potentially convert to CSCs. EMT was shown to have a differential effect on CSC marker expression, yet results in increased CSC activity *in vitro* and *in vivo*. Lastly, we found that Rac1 activity is increased in mesenchymal state and Rac1 targeting blocks EMT and EMT associated CSC marker expression.

Clinical observations of the development of secondary metastasis post-surgery or chemotherapy has spawned the hypothesis that these metastases are derived from a small portion of tumor cells that persist despite therapy. Breast cancer cell lines enriched for CD44\textsuperscript{high}CD24\textsuperscript{low} cells reveal high expression of pro-invasive genes and display increased invasive and metastatic potential (Sheridan et al., 2006). In pancreatic cancer, CD133\textsuperscript{pos} cells with higher CXCR4 expression were shown to be responsible for metastasis (Hermann et al., 2008). In human liver cancer, CD45\textsuperscript{−}/CD90\textsuperscript{+}/CD44\textsuperscript{+} cells were determined to form metastatic lesions in the lungs of immune compromised mice, and blockage of CD44 activity by an antagonizing antibody was shown to block tumor growth and metastasis (Yang et al., 2008). In support of this idea, a recent clinical study correlated the presence of CD26\textsuperscript{pos} cells in circulation and primary colon tumor to liver metastasis (Pang et al., 2010). Increased lung colonization and expression of metastasis associated genes in different CSC populations isolated from NSCLA cells in our current
study corresponds with these observations of CSCs displaying higher metastatic potential.

Cancer stem cell theory (reviewed in (Shackleton et al., 2009)) suggests that irrespective of cell of origin, in many cancers especially hematopoietic derived cancers, cancer cells are hierarchically organized similar to normal stem cells. According to this model less differentiated, more tumorigenic cancer stem cells irreversibly undergo epigenetic changes to generate less tumorigenic and more differentiated bulk cancer cells. In contrast, clonal selection theory states that genetic and epigenetic changes occur in individual cancer cells over a long period and cumulative effect derives the selective advantage for the individual clone during cancer progression. In an organ like the lung, where the normal stem and progenitor cell hierarchy remains unclear, it becomes very difficult to determine whether lung adenocarcinomas follow the cancer stem cell model or clonal selection model of tumor progression. In our current study, because of the lack of established lung stem cell markers, we utilized the CSC markers that have been established in other cancers that might or might not have similar hierarchical placement during lung development. Because we observed minimal overlap between the CSC markers in lung adenocarcinomas, this observation raises two possibilities. First, the CSC markers we examined mostly represented a plastic pool of cancer progenitor cells and not a less plastic cancer stem cell population. Second, CSCs are represented by expression of more than one CSC marker and combination of CSC markers that represents less plastic CSCs is yet to be determined.

The concept of potential CSC plasticity has been raised recently by several studies (Boiko et al., 2010; Roesch et al., 2010). CSCs and non-CSCs defined by single marker expression could be dynamic populations, with CSCs able to generate non-CSCs, and vice versa. A recent review (Dirks, 2010) postulated that the unstable genomic environment in cancer cells might readily facilitate reprogramming from
differentiating cancer cells to CSCs, unlike the reprogramming of fully differentiated cells into iPS cells. Recently Boiko et al (Boiko et al., 2010) established CD271$^+$ as a CSC marker in Human melanoma, with CD271$^-$ cells displaying slower tumor growth in human skin grafts on NSG mice. However, the CD271$^-$ cells derived from in vitro or in vivo cultured patient cells yielded increased tumorigenic potential. CSC plasticity was also evidenced by studies of Roesch et al (Roesch et al., 2010) in melanoma cells, where JARID 1B$^{\text{neg}}$ cells could readily convert to JARID 1B$^{\text{pos}}$ cells, establishing that cell heterogeneity for tumor growth is a dynamic process mediated by a temporary pool of CSCs. Similarly Iliopoulos et al (Iliopoulos et al., 2011) have shown that in both breast and prostate cancer cell lines and primary tumors, non-CSC pools (either defined by CD24$^{\text{high}}$CD44$^{\text{high}}$ cells in breast cancer or CD133$^{\text{low}}$CD44$^{\text{low}}$ in prostate cancer) convert to CSCs (CD24$^{\text{low}}$CD44$^{\text{high}}$ and CD133$^{\text{high}}$CD44$^{\text{high}}$ respectively) in an IL6 dependent manner. We have shown previously that highly purified non-SP cells still retain CSC activity in vitro and in vivo, suggesting that non-CSCs of NSCLA can generate CSCs (Akunuru et al., 2011). Indeed simple mindingly, we determined that non-CSCs defined by lack of expression of a single CSC marker (non-SP, CD133$^{\text{neg}}$, ALDH$^{\text{low}}$ cells) can generate cells with specific CSC marker expression (SP, CD133$^{\text{pos}}$, ALDH$^{\text{high}}$ cells respectively). This could be explained by two potential possibilities. First, that true CSCs are part of a larger hierarchy of CSC marker expression, and may contain many markers concurrently. This hierarchy has been partly established in leukemia and glioblastomas but still must be elucidated in other solid tumors. Second, indeed CSC’s may be plastic in their marker expression, rendering CSC-marker based therapeutics a difficult strategy to pursue.

EMT has been proposed as the potential mechanism involved in CSC plasticity based on a recent study in the breast cancer model. EMT promotes cells with stem characteristics in mammary cancer cells which combine both the concepts of EMT...
contributing to metastasis and CSC’s generating metastasis (Mani et al., 2008). Concept of potential role of EMT on CSC plasticity is also recently been reviewed (Gupta et al., 2009a). To expand upon this seminal work, our current study demonstrates that EMT promotes CSC activity in NSCLC confirming Mani et al observations in breast cancer. However we extend their observation of one CSC marker, CD24<sub>low</sub>CD44<sub>high</sub>, to several different CSC markers and demonstrate that EMT differentially regulates different CSC markers that individually have been identified as metastasis enriching populations. Interestingly, in our current study EMT enriched for the expression of self renewal genes like oct4 and nanog corollary to a recent observation of oct4 and nanog over-expression inducing EMT in lung adenocarcinoma cells (Chiou et al., 2010). Contradictory to the increase in other CSC marker expression, we observed a decrease in SP cells and ABCG2 expression in the mesenchymal state of lung adenocarcinoma cells. This is similar to the inhibitory effect of TGFβ1 on SP cells and ABCG2 expression reported previously in MCF7 cells (Yin et al., 2008). However in another recent report, TGFβ1 was shown to induce EGFR inhibitor resistance in H1650 lung adenocarcinoma cells mediated by IL6 (Yao et al., 2010). Interestingly we observed a similar increase in IL6 by TGFβ1 treatment in NSCLA cells, leading to the possibility of potential EGFR inhibitor resistance, which needs to be determined. Based on our current study, we infer that EMT has divergent effects on different CSC populations yet increases self renewal and metastatic properties.

Identifying potential molecular pathways mediating EMT and EMT mediated CSC plasticity will help derive novel strategies to target conventional therapy resistant cells and relapse of the disease. The Rac proteins, members of small RhoGTPases family are critical mediators of cell signaling which can impact tumorigenesis. Because Rac1 is a modifier of actin dynamics, it has been shown to contribute to migration and invasion of various tumour types including NSCLC cells (Akunuru et al., 2011). RhoGTPases
playing an important role in a process of EMT by which cells undergo profound morphological changes involving cytoskeletal changes and acquiring a migratory phenotype is not a surprise and has been implicated previously (Kardassis et al., 2009). Radinsky et al have shown Rac1b being the mediator of EMT induced ROS production (Radisky et al., 2005) and more recently Rac1 was shown to regulate TGFβ1 mediated EMT and MMP9 production in transformed keratinocytes (Santibanez et al., 2010). In our current study, we establish that Rac1 inhibition blocks the EMT and EMT mediated changes in CSC marker expression, highlighting the idea that targeting the Rac1 signaling axis may present a new therapeutic strategy for reducing CSC plasticity.

Our current study demonstrates the potential plasticity of CSC marker expression. Consideration of CSC plasticity highlights the difficulty of utilizing the CSC theory to design new strategies against cancer. Based on this notion it can be inferred that it will be necessary to effectively target CSCs, non-CSCs and the potential mechanism that is involved in non-CSC plasticity (EMT) in order to achieve true therapeutic benefits. Importantly, we show that targeting Rac1 can effectively block the EMT, lung metastatic activities in NSCLA cells.

Acknowledgments

We thank James F. Johnson, Victoria Summey, and Jeff Bailey for assistance in xenograft experiments.

References


Figure 1

A. Side population (SP) vs. Fumitremorgin (10µM)

B. CD133pos

C. ALDHhigh vs. DEAB

D. CD133

E. % cells

F. %CD133pos cells

G. %ALDHhigh cells
Figure 1. NSCLACs express multiple, minimally overlapping subpopulations of CSC markers. (A) A549 cells were stained with Hoechst dye for side population (left panel). Cells were stained in the presence of 10µM Fumitremorgin as a negative control (right panel). (B) A549 cells were stained with CD133 antibody and analyzed by FACS. (C) A549 cells were stained with ALDeflour reagent either in the absence or presence of DEAB (for negative control) and FACS analysis was performed. (D) Primary lung adenocarcinoma cells were obtained from patient tumor samples. Percentage of SP, CD133\textsuperscript{pos} and ALDH\textsuperscript{high} was determined by FACS following 1 week of adherent culture. (E) Percentage of SP, CD133\textsuperscript{pos} and ALDH\textsuperscript{high} was determined by FACS after culturing in adherent culture condition for either 2 weeks (left panel) or 4 weeks (right panel). (F) Either A549 cells (left panel) or primary adenocarcinoma cells (right) were co-stained with Hoechst dye and CD133 antibody (right panel) and percentage of CD133\textsuperscript{pos} cells in SP and non-SP cells was determined by FACS. (G) Either A549 cells (left panel) or primary adenocarcinoma cells (right panel) SP, non-SP cells were sorted and stained with ALDeflor reagent. Percentage of ALDH\textsuperscript{high} cells in SP and non-SP cells were determined by FACS.
Figure 2

A

Fold change in gene expression normalized to GAPDH

B

Fold change in gene expression normalized to GAPDH

C

Fold change in gene expression normalized to GAPDH

D

Number of spheres per 2,000 cells

E

Number of spheres per 2,500 cells

F

Number of tumors

G

Fold change in gene expression normalized to GAPDH

H

Number of lung tumors
Figure 2. Different subpopulations of CSC marker expressing cells display distinct patterns of self-renewal, metastatic gene expression and cancer stem cell activities. (A) Either A549 (left panel) or primary adenocarcinoma cells (right panel) SP, non-SP cells were sorted for RNA isolation. Panel of self-renewal gene expression was assessed by qPCR analysis. Relative gene expression was determined by calculating fold change in gene expression from control sample, normalized to GAPDH expression. (B) CD133<sup>pos</sup> and CD133<sup>neg</sup> cells were sorted either from A549 (left panel) or primary adenocarcinoma cells (right panel) and expression of a panel of self-renewal genes was determined by qPCR. (C) ALDH<sup>high</sup> and ALDH<sup>low</sup> cells were sorted either from A549 (left panel) or primary adenocarcinoma cells (right panel) and expression of a panel of self-renewal genes was determined by qPCR. (D, E, F) Primary adenocarcinoma cells were sorted for either SP and non-SP cells (D) or CD133<sup>pos</sup> and CD133<sup>neg</sup> cells (E) or ALDH<sup>high</sup> and ALDH<sup>low</sup> cells (F) and cultured in non-adherent sphere culture conditions. Number of spheres formed after 15 days were determined by counting spheres under light microscope. (G) Primary adenocarcinoma cells were sorted for either SP and non-SP cells (left panel) or CD133<sup>pos</sup> and CD133<sup>neg</sup> cells (middle panel) or ALDH<sup>high</sup> and ALDH<sup>low</sup> cells (right panel) and relative gene expression of a panel of “metastatic genes” was determined by qPCR. (H) 10,000 A549 SP, non-SP, CD133<sup>pos</sup> and CD133<sup>neg</sup> cells (left panel) or ALDH<sup>high</sup>, ALDH<sup>low</sup> cells (right panel) were sorted and injected intravenously into NSG mice. After 12 weeks lungs were dissected and the number of lung tumors was counted to determine the relative lung colonization activity.
Figure 3

A

% SP cells

Day 0  Day 3  Day 7

B

% SP cells derived from non-SP YFP+g cells

Day 0  Day 4  Day 7  Day 10

C

% SP cells

NSP clone number

D

% SP cells

NSP clone number

E

% CD133+ pos. cells

Pre-sort  Post-sort  Day 3 post-sort

F

% ALDH+high cells

Day 7  Day 14
Figure 3. Cancer stem and non-stem cell marker expression transition kinetics are different between various marker-defined states. (A) A549 non-SP cells were sorted using FACS. Sorted cells were plated in adherent culture condition and on days 0, 3, and 7, cells were trypsinized and stained for SP analysis. (B) A549 cells were infected with mieg3-EGFP vector and after 4 days, cells were stained with Hoechst dye. Equal numbers of GFPpos SP cells sorted to 100% purity were mixed with GFPneg non-SP cells. SP cells derived from GFPneg non-SP cells was determined at different time points (day 0, 4, 7,10) by FACS analysis. (C) Either A549 cells (left panel) or primary adenocarcinoma cells (right panel) were stained with Hoechst dye and non-SP cells were sorted into a 96-well plate with single cell per well. Non-SP clones grown from single cells were analyzed by FACS to determine the percentage of SP cells. (D) A549 non-SP sorted cells were injected subcutaneously into immune-compromised NSG mice and cells were isolated from tumors. % SP cells derived from non-SP cells in vivo were determined by FACS analysis. (E) A549 cells were stained with CD133 antibody and CD133neg cells were sorted by FACS. Post-sort purity of the cells was determined by restaining cells with CD133 antibody and FACS analysis. Sorted cells were cultured in a 2D culture condition. On day 3, cells were stained with CD133 antibody and analyzed by FACS. (F) A549 cells were stained with ALDeflor reagent and ALDHlow cells were sorted by FACS. Post-sort purity of the cells was determined by FACS analysis and sorted cells were cultured in a 2D culture condition. On days 7 and 14, cells were stained with ALDeflor reagent and analyzed by FACS to determine the percentage of ALDHhigh cells.
Figure 4

A. Graph showing SP expression in Control and TGFβ1 (12 days) conditions. SP = 5.3% in Control and SP = 0.07% in TGFβ1 (12 days).

B. Graph showing ABCG2 gene expression normalized to GAPDH over time (0 days, 7 days, 12 days, 5 days).

C. Graph showing % CD133^+ cells in Control and TGFβ1 conditions.

D. Graph showing ALDH^high cells over time (0 days, 7 days, 12 days, 5 days).

E. Graph showing fold change in gene expression (Nanog, Oct-4, Shh, Hes1, Notch1) for control and TGFβ1 conditions.

F. Graph showing number of spheres for Control and TGFβ1 conditions with p = 0.036.

G. Graph showing fold change in gene expression (CXCR4, TNFα, HoxB9, TGFβ1, VEGFA, IL1β, IL6) normalized to GAPDH for Control and TGFβ1 conditions.
Figure 4. TGFβ1 induced EMT has divergent effects on different CSC population frequency and tumorigenicity. (A) SP analysis was performed on either control or TGFβ1 treated A549 cells by FACS (left panel). Quantification of SP analysis from TGFβ1 treated A549 cells (middle panel) and primary adenocarcinoma cells (right panel). (B) RNA was isolated from TGFβ1 treated cells and ABCG2 gene expression was determined by qPCR from either A549 cells (left panel) or primary adenocarcinoma cells (right panel). (C) Cells were stained with CD133 antibody and % CD133<sup>pos</sup> cells were determined by FACS analysis from both control and TGFβ1 treated cells. (D) Cells treated with TGFβ1 for different time points were stained and analyzed by FACS to determine % ALDH<sup>high</sup> cells. (E) qPCR was performed on RNA isolated from either control cells, TGFβ1 treated cells or TGFβ1 withdrawn cells for determining self-renewal gene expression. (F) H441 (left panel) and primary human lung adenocarcinoma cells (right panel) were treated with either vehicle or TGFβ1 for 12 days and plated for sphere culture in either presence or absence of TGFβ1. Number of spheres formed after 15 days were counted under light microscope. (G) qPCR was performed on RNA isolated from either control cells, TGFβ1 treated cells or TGFβ1 withdrawn cells for determining potential differences in the expression of metastatic genes. (H) Either control or TGFβ1 treated cells were injected intravenously into NSG mice. Number of tumors formed in the lungs was determined after 10-12 weeks.
Figure 5. Cells grown in sphere culture are enriched for self-renewal gene expression and CSC markers similar to TGFβ1 treated cells. (A) qPCR analysis was performed from cDNAs generated from either H441 cells grown in adherent or sphere
culture conditions to determine the potential difference in expression of self renewal genes. Relative gene expression was determined by normalizing the target gene expression to GAPDH. (B) H441 cells grown in adherent and spheres culture conditions were stained with Hoechst dye to perform SP analysis by FACS. (C) cDNA generated from H441 adherent and spheres cells was used to perform qPCR analysis to determine the ABCG2 gene expression. Target gene expression was normalized to GAPDH. (D) H441 adherent and spheres cells were stained with CD133 antibody and the % CD133\textsuperscript{pos} cells were determined by FACS analysis. (E) cDNA generated from H441 adherent and spheres cells was used to perform qPCR analysis to determine the expression of epithelial and mesenchymal genes. Target gene expression was normalized to GAPDH.
Figure 6

A. Days of TGFβ1 treatment and withdrawal

<table>
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<th>0</th>
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<th>12</th>
<th>5 days</th>
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<tr>
<td>Rac1-GTP</td>
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<td></td>
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</tr>
<tr>
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<tr>
<td>GAPDH</td>
<td></td>
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</tr>
</tbody>
</table>

B. Adherent Spheres

- Rac1-GTP
- Total Rac1
- GAPDH

C. Control vs. 2.5ng/ml TGFβ1

D. Relative gene expression for E-cadherin and Snail

E. NSC23766 (µM)

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<th>100</th>
<th>200</th>
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</thead>
<tbody>
<tr>
<td>Rac1-GTP</td>
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<tr>
<td>Total Rac1</td>
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<td>GAPDH</td>
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</table>

F. Fold change in gene expression

G. % SP cells

H. % ALDH high cells

I. Number of spheres
Figure 6. TGFβ1 induced EMT is mediated by Rac1. (A) Cell lysates were collected from either A549 control or TGFβ1 treated or TGFβ1 withdrawn cells and Rac1-GTP pull down assay was performed as described in methods. Total Rac1 and GAPDH were used as loading controls. (B) Cell lysates were collected from either H441 adherent or sphere cells and Rac1-GTP pull down assay was performed. Total Rac1 and GAPDH were used as loading controls. (C, D) A549 cells infected with lentivirus expressing control or Rac1 shRNAs were treated with TGFβ1 for 7 days and either fixed for immunostaining for actin and nuclei(C) or lysed for RNA isolation for qPCR analysis of either E-cadherin (top panel) or snail (bottom panel) (D). (E) A549 cells were treated with specified concentrations of Rac inhibitor, NSC23766 for 12 hours at 37°C and Rac1-GTP activity assay was performed. (F, G, H) A549 cells were treated with TGFβ1 in the presence of different concentrations of Rac inhibitor, NSC23766 for 12 days. Cells were either used to isolate RNA to determine changes in epithelial and mesenchymal markers determined by qPCR analysis (F) or stained for SP analysis (G) or stained for ALDH analysis (H).
Supplemental figure 1. SP and CD24^{low}CD44^{high} cells are minimally overlapping CSC populations. (A) A549 cells were stained with CD24 and CD44 antibodies and the FACS analysis was performed to determine the percentage of CD24^{low}CD44^{high} population. (B) Quantification of CD24^{low}CD44^{high} cells in A549 (left panel) and primary adenocarcinoma cells (right panel).
Supplemental figure 2. Non-SP and CD24<sup>high</sup>CD44<sup>high</sup> population in NSCLA cells are plastic. (A) A549 SP and non-SP cells were sorted using FACS. Post-sort purity of each of the population was determined by FACS analysis. Sorted cells were plated. On day 7, cells were trypsinized and stained for SP analysis. (B) A549 cells were stained with CD24, CD44 antibodies and CD24<sup>high</sup>CD44<sup>high</sup> cells were sorted by FACS. Post-sort purity of the cells was determined by FACS analysis and sorted cells were cultured in a...
2D culture condition. On different days, cells were stained to determine the % CD24\textsuperscript{low}CD44\textsuperscript{high} cells by FACS.
Supplemental figure 3. TGFβ1 induces EMT in NSCLC cells. (A) Either A549 (left panel) or primary adenocarcinoma cells (right panel) were treated with 2.5ng/ml TGFβ1 for 10-12 days. Cell images were obtained under bright field. (B, C) Changes in epithelial and mesenchymal gene expression were determined by performing qPCR analysis on A549 (left panel) or H441 (right panel) cells or primary adenocarcinoma cells (C). (D) Either A549 (left panel) or H441 (right panel) cells were treated with TGFβ1 and fixed for immunostaining to detect either fibronectin (top left panel) or vimentin (bottom left panel) or E-cadherin (right panel).
Supplemental Figure 4

A

% SP cells

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7

Control TGFβ1

Fold change in ABCG2 gene expression

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5

Control 20 nM 4-OH T 100 nM 4-OH T 1000 nM 4-OH T

B

Fold change in gene expression

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5

FoxC2 E-cadhn N-cadhn Twist Snail FN1

Concentration of 4-OH Tamoxifen

C

% SP cells

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5

Control 20 nM 100 nM 1 μM

Fold change in ABCG2 gene expression

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

Control 20 nM 100 nM 1000 nM

Concentration of 4-OH Tamoxifen

D

CD24low CD44high cells

0 10 20 30 40

0 days 7 days 12 days 5 days

TGFβ treatment withdrawal

-97-
Supplemental figure 4. SNAIL induced EMT reduces SP cells and EMT increases CD24\text{low}CD44\text{high} population in NSCLA cells. (A) H441 control or TGF\beta1 treated cells were either stained for SP analysis (left panel) or collected for RNA isolation for ABCG2 gene expression analysis by qPCR (right panel). (B, C) After treatment with specified concentrations of 4-OH tamoxifen, A549 cells expressing Snail-ER construct were either used to collect RNA for detecting the changes in epithelial and mesenchymal markers (B) or stained for SP analysis (C, left panel), or used to determine the expression of ABCG2 transporter (C, right panel). (D) A549 cells were stained with CD24 and CD44 antibodies and the % CD24\text{low}CD44\text{high} cells were determined by FACS analysis.
Chapter IV: Summary and perspectives

In our initial study we investigated the hypothesis that Rac1, a member of Rho GTPase family, was both implicated in cancer cell proliferation and invasion, and is critical for tumor initiation and metastasis of human non-small cell lung adenocarcinoma (NSCLA). Rac1 knockdown by shRNA suppressed the tumorigenic activities of human NSCLA cell lines and primary patient NSCLA cells through effects on adhesion, invasion, proliferation, anchorage-independent growth, sphere formation and lung colonization. One of the CSC markers enriched for drug efflux channels, side population (SP) was isolated from NSCLA cells. This population of cells displayed elevated levels of Rac1-GTP, enhanced in vitro migration, invasion, increased in vivo tumor initiating and lung colonizing activities in immune compromised mice as compared to non-SP cells. Further, blocking Rac1 activity inhibited the invasive and tumorigenic activities of both SP and non-SP NSCLA cells.

In our subsequent study, we established that NSCLAs expressed at least three other CSC populations (ALDH\textsuperscript{high}, CD133\textsuperscript{pos}, and CD24\textsuperscript{low}CD44\textsuperscript{high}) along with SP, all enriched for tumor initiation and metastatic abilities. Interestingly, these specific marker-defined populations have a minimal overlap. CSCs and non-CSCs defined by expression or lack of expression of a single marker respectively are inter-convertable and plastic, with distinct marker specific kinetics. We further established that this CSC plasticity can be mediated by EMT. Interestingly, while EMT increased the “stemness” of NSCLA cells assessed by levels of conventional self-renewal genes, its effect on different cancer stem cell markers was divergent. Rac1 activity was shown to be significantly increased in cells that have undergone EMT. Therefore, Rac1 suppression blocked the EMT process and inhibited tumor initiating activity. Together, this thesis work highlights the therapeutic utility of targeting the Rac1 signaling axis in NSCLA, since its blockade could potentially suppress CSC plasticity and metastasis.
Potential hierarchy of different CSC markers in NSCLAs

We first established that the drug resistant SP cells in NSCLAs are enriched for tumor initiating and metastatic cells. Our observation is consistent with the several studies describing SP cells as being enriched for drug resistant and tumor initiating properties in several different cancers [1,2,3]. Later, we identified three other CSC markers (CD133<sup>pos</sup>, ALDH<sup>high</sup>, CD24<sup>low</sup>CD44<sup>high</sup>) expressed in NSCLAs, all enriched for expression self renewal genes and exhibiting higher tumorigenecity than their corresponding non-CSCs (CD133<sup>neg</sup>, ALDH<sup>low</sup>, CD24<sup>high</sup>CD44<sup>high</sup> respectively). Consistent with the recent hypothesis that metastases are derived from a small portion of therapy resistant tumor cells, we observed increased lung colonization in all four CSC populations isolated from NSCLAs. Similarly, we observed an increase in expression of metastatic genes in all four CSC populations; however, all CSC populations exhibited interestingly distinct expression patterns. Recently in breast cancer cell lines (CD44<sup>high</sup>CD24<sup>low</sup>) [4], pancreatic cancer (CD133<sup>pos</sup>CXCR4<sup>high</sup>) [5], and liver cancer (CD45<sup>-</sup>/CD90<sup>+</sup>/CD44<sup>+</sup>) [6] different CSC populations were shown to be responsible for metastasis. In support of this idea, a clinical study correlated the presence of CD26<sup>pos</sup> cells in circulation and primary colon tumor to liver metastasis [7]. Correspondingly, increased lung colonization and expression of metastasis associated genes in different CSC populations isolated from NSCLA cells in our current study correlates with these observations of CSCs displaying higher metastatic potential.

With the body of knowledge generated by the discovery of various CSC markers in several different cancers, the potential relationship between these different CSC markers in specific cancer types is not clear. According to the cancer stem cell theory (reviewed in [8]) in many cancers, cancer cells are hierarchically organized similar to normal stem cells. The term cancer stem cell does not define the cell of origin of the cancer but rather a less differentiated, more tumorigenic cancer cells that irreversibly undergo genetic, epigenetic
changes to generate less tumorigenic and more differentiated bulk cancer cells. In contrast, the clonal selection theory states that genetic and epigenetic changes occur in individual cells over a long period during tumorigenesis and the cumulative effect contributes to the selective advantage of an individual clone. In the hematopoietic system, where the normal stem cell hierarchy is well established, the hierarchy of cancer stem cells can be deciphered more clearly. However in an organ like the lung, where the normal stem and progenitor cell hierarchy remains unclear, it becomes very difficult to determine whether lung adenocarcinomas follow the cancer stem cell model or clonal selection model of tumor progression. In our current study of NSCLAs, the lack of established lung stem cell markers required the characterization of CSC markers that have been established in other cancers that might or might not have similar hierarchical placement during lung development. The relevance of these markers either during normal lung development and/or lung cancer progression is important to establish. We determined that the overlap between these different CSC markers (SP, CD133\textsuperscript{pos}, ALDH\textsuperscript{high}, CD24\textsuperscript{low}CD44\textsuperscript{high}) is minimal in NSCLA. Because of this minimal overlap between these different marker-defined CSC populations in lung adenocarcinomas, we proposed that the CSC markers we examined in our current study possibly represent a plastic pool of cancer progenitor cells and the CSCs are represented by expression of more than one CSC marker, and combinations of CSC markers that represent less plastic CSCs must be determined.

Establishing whether these different CSC marker-defined populations are arranged in a potential hierarchy during tumorigenesis is critical for identifying appropriate target for developing novel therapies. Patrawala L \textit{et al} established CD44 as a marker enriching for tumorigenic and metastatic cells in prostrate cancer [9]. In their subsequent studies they identified hierarchical organization of prostrate cancer cells by overlap of another proposed normal prostrate stem cell marker, \(\alpha_2\beta_1\textsuperscript{high}\), revealing that CD44\textsuperscript{+} \(\alpha_2\beta_1\textsuperscript{high}\) cells are more tumorigenic than both single positive or double negative population [10]. Similarly a
hierarchical organization of CSC in glioblastoma was established by Chen R *et al.*, based on not only CD133 cell-surface expression but also CD133 transcript expression [11]. Even though, our current study demonstrates that the overlap between different CSC markers is minimal in NSCLAs, we propose that a small fraction of cells defined by overlap of more than one CSC marker might possess high tumorigenicity. Interestingly, Curtis JS *et al.* proposed a role of the genotype of the primary tumor for determining the identity of CSCs in a mouse lung cancer model [12]. Their studies demonstrated Sca1 as a CSC marker in primary tumors with both K-ras activation and P53 loss but not in tumors with K-ras activation alone [12]. This raises the issue of the importance of understanding the mutational status of the individual patient tumors obtained for analysis and also highlights the potential role of genotype on CSC marker overlap. Another layer of complexity was added to the idea of CSC marker overlap by a recent study on BCR-ABL1 lymphoblastic leukemia which demonstrated branching multiple-clonal evolution, rather than linear succession during leukaemogenesis [13]. This study demonstrated multiple genetic sub-clones evolving in parallel leading to development of different CSC populations [13]. With this study in mind, it becomes critical to determine the potential genotypic differences among different marker defined-CSCs (such as SP, CD133$^{\text{pos}}$, ALDH$^{\text{high}}$ populations) isolated from non-cultured primary patient NSCLAs. Alternatively, HBEC cells transformed with different oncogenes can potentially be used in parallel to primary NSCLAs to identify the potential hierarchy of NSCLA CSCs. Understanding potential hierarchy or multiple clonal development of different CSC populations is not only important for understanding lung adenocarcinoma tumorigenesis, but also critical for identifying appropriate targets for developing novel therapeutics.

**CSC and non-CSCs inter-convertibility may be mediated by inflammatory cytokines**

Conventional chemotherapeutic agents mainly target malignant cells by either inducing DNA damage or blocking DNA replication. CSCs may be resistant to the effect of these agents through
their elevated drug resistance or relative quiescence [14]. Recently with the awareness of CSC theory, several innovative therapeutic strategies have been developed to eradicate CSCs. To therapeutically impede leukemia CSCs, neutralizing antibodies to autocrine signaling mediators important for CSC growth such as CD123 [15], antagonists of leukemia CSC localization in the bone marrow niche such as CXCR4-inhibitor, AMD-3100, and CD44 antibodies [16], and inhibitors of signaling pathways specifically upregulated in CSCs that are important for the self renewal such as NFκ-β inhibitor parthenolide [17], have been shown to have efficacy in AML or CML. In solid cancers, IL4 has been shown to be useful in colorectal cancer stem cell suppression [18,19], BMP4 was found to induce glioblastoma CSCs to differentiate into non-CSCs [20], and salinomycin, a selective potassium ionophore, could target breast cancer stem cell proliferation and induce differentiation [21]. However, the efficacy of all these agents in pre-clinical and clinical models is yet to be determined and will be challenged by the concept of potential CSC plasticity raised recently by several studies [22,23]. In our current studies, we demonstrate that CSCs and non-CSCs defined by single marker expression in NSCLAs are dynamic populations, with CSCs able to generate non-CSCs, and vice versa.

Several recent studies have indicated a potential plastic relationship between CSCs and non-CSCs. A review by Dirks P [24] postulated that the unstable genomic environment in cancer cells might readily facilitate reprogramming from differentiating cancer cells to CSCs, unlike the reprogramming of fully differentiated cells into iPS cells. Recently Boiko et al [23] established CD271 as a CSC marker in Human melanoma, with CD271- cells displaying slower tumor growth in human skin grafts on NSG mice. However, the CD271- cells derived from in vitro or in vivo cultured patient cells yielded increased tumorigenic potential. Studies of Roesch et al [22] in melanoma cells, where JARID 1B neg cells could readily convert to JARID 1B pos cells, establish that cell heterogeneity for tumor growth is a dynamic process mediated by a temporary pool of CSCs. Indeed in line with these studies, we determined that non-CSCs defined by lack of expression of a single CSC marker (non-SP, CD133 neg, ALDH low cells) can generate cells with specific CSC
marker expression (SP, CD133\textsuperscript{pos}, ALDH\textsuperscript{high} cells respectively). This could be explained by two potential possibilities. First, that true CSCs are part of a larger hierarchy of CSC marker expression, and may contain many markers concurrently. Second, indeed CSC's may be plastic in their marker expression, rendering CSC-marker based therapeutics a difficult strategy to pursue. Therefore we propose that eradication of all cancer cells, including the drug resistant multiple sub-populations, is necessary for the development of efficacious therapeutic strategies.

Understanding the potential mechanisms and the mediators of this CSC plasticity is critical for design of novel therapeutics. Iliopoulos \textit{et al} [25] have demonstrated that non-CSC pools (either defined by CD24\textsuperscript{high}CD44\textsuperscript{high} cells in breast cancer or CD133\textsuperscript{low}CD44\textsuperscript{low} in prostrate cancer) convert to CSCs (CD24\textsuperscript{low}CD44\textsuperscript{high} and CD133\textsuperscript{high}CD44\textsuperscript{high} respectively) in cancer cell lines and primary tumors. This transition was shown to be mediated by IL6 secretion [25]. Another independent study has established TGF\beta1 mediated IL6 secretion to result in resistance to EGFR inhibitor treatment in NSCLA cell lines [26]. In our current study, we demonstrate that TGF\beta1 treatment results in increase in IL6 expression and a concomitant increase in CSC markers such as CD133\textsuperscript{pos}, ALDH\textsuperscript{high}, and CD24\textsuperscript{low}CD44\textsuperscript{high}. It will be interesting to determine whether the TGF\beta1 mediated increase in CSC markers in NSCLAs is dependent on IL6 expression by using a neutralizing antibody to IL6. Similarly, an IL-6 neutralizing antibody could potentially be used to examine the growth of the tumor from the non-CSC pool \textit{in vivo}. Additionally, several inflammatory cytokines such as TNF\alpha, IL1\beta, IFNy were shown to augment TGF\beta1 induced EMT in NSCLA cell lines [27]. In line with these studies, it will be interesting to determine whether other cytokines have similar effects as TGF\beta1 on plasticity of CSCs or even interestingly, different cytokines has divergent effects on plasticity of different marker-defined CSC populations. Based on these observations, we propose that cytokines may mediate CSC plasticity, and cytokine blocking agents or anti-inflammatory agents in combination with chemo-therapy might be beneficial in
preventing secondary relapse in NSCLAs. Thus understanding the clinical relevance of inter-conversion of CSCs will be critical for therapeutic exploration of CSC markers.

**EMT promotes cells with stem cell characteristics and enhanced metastasis**

EMT promotes cells with stem characteristics in mammary cancer cells which combines both the concepts of EMT contributing to metastasis and CSC’s generating metastasis [28]. To expand upon this seminal work, our current study demonstrates that EMT promotes CSC activity in NSCLC confirming these previous observations in breast cancer. However we extend their observations of one CSC marker, CD24\(^{\text{low}}\)CD44\(^{\text{high}}\), to several different CSC markers and demonstrate that EMT differentially regulates different CSC markers that individually have been identified as metastasis enriching populations. Interestingly, in our current study EMT enriched for the expression of self renewal genes like *oct4* and *nanog* corollary to a recent observation of *oct4* and *nanog* over-expression inducing EMT in lung adenocarcinoma cells [29]. Contradictory to the increase in other CSC marker expression, we observed a decrease in SP cells and ABCG2 expression in the mesenchymal state of lung adenocarcinoma cells. This is similar to the inhibitory effect of TGF\(\beta\)1 on SP cells and ABCG2 expression reported previously in MCF7 cells [30]. However in another recent report, TGF\(\beta\)1 was shown to induce EGFR inhibitor resistance in H1650 lung adenocarcinoma cells mediated by IL6 [26]. Interestingly we observed a similar increase in IL6 by TGF\(\beta\)1 treatment in NSCLA cells, leading to the possibility of potential EGFR inhibitor resistance, which needs to be determined. It will be interesting to determine if CSC populations (such as CD133\(^{\text{pos}}\), ALDH\(^{\text{high}}\), CD24\(^{\text{low}}\)CD44\(^{\text{high}}\)), increased with TGF\(\beta\)1 treatment are more resistant to EGFR inhibitor treatment than SP cells, CSC marker that decreases with EMT.

Irrespective of the divergent effects of EMT on different CSC populations in NSCLA, we observed a consistent increase in expression of self-renewal genes in both TGF\(\beta\)1 induced EMT and sphere cells, establishing the fact that EMT promotes cells with stem cell
characteristics. However, all our observations were based on cancer cell lines and the cultured primary adenocarcinoma cells. It will be interesting to determine if gain of mesenchymal markers or loss of epithelial markers correlate with the expression of “stem genes” such as oct4 and nanog by immuno-histological analysis of NSCLA tumor sections. Based on our current study we propose that loss of epithelial markers will correlate with expression of “stemness genes” and could predict poor prognosis or secondary metastasis in retrospective clinical database. These studies could be instructive in developing prognostic markers and devising CSC based therapies.

There have been numerous studies delineating the effects of EMT on initiation of tumor metastasis in several cancers irrespective of its effects on CSC activity [31,32,33,34,35]. Functional loss of E-cadherin in an epithelial cell has been established as a hallmark of EMT. Over a decade ago, forced expression of E-cadherin in invasive human carcinoma cells was shown to inhibit their ability to metastasize [32]; conversely, loss of E-cadherin was shown to result in transition from adenoma to more invasive carcinoma [31]. Several zinc-finger transcription factors including Twist, Snail, ZEB are capable of directly binding to the E-cadherin promoter [33,36,37,38]. Induction of EMT leads to invasion of epithelial cancer cells to surrounding stroma, intravasation and colonization at the distal site. According to this theory, in the metastatic site mesenchymal cells revert back to their epithelial state to regain the proliferative activities essential for transition from micro metastasis to macro metastasis. This EMT-MET dynamic transition was proposed to be critical for metastasis. However, cells undergoing this dynamic process of EMT-MET have remained elusive in vivo. We propose to use NSCLA cells expressing tamoxifen inducible Twist-ER and Snail–ER to delineate the role of epithelial or mesencymal states in initiation of metastasis and colonization at the distal site. Alternatively, we can use constructs driving either luciferase or GFP expression under Twist or Snail promoter to visualize in vivo EMT-MET dynamics using IVIS technology and cellular behaviors in a time course experiment. Understanding the role
of dynamic conversion of EMT-MET during metastasis independent of CSC activity regulation is critical for developing strategies to block metastasis in early stage cancers.

**Potential molecular mechanisms supporting the link between Rac1 and EMT**

The Rac proteins, members of small RhoGTPases family are critical mediators of cell signaling and were previously demonstrated to be involved in tumorigenesis in skin [39]. K-ras induced lung tumorigenesis was partially blocked by Rac1 loss in mice [40]. Rac1 was shown to be involved in invasion and migration of several cancer cells including NSCLA cells [41]. RhoGTPases playing an important role in a process of EMT by which cells undergo profound morphological changes involving cytoskeletal changes and acquiring a migratory phenotype is not a surprise and has been implicated previously [42]. Recently Rac1 was shown to regulate TGFβ1 mediated EMT and MMP9 production in transformed keratinocytes [43]. In our current study, we establish that Rac1 inhibition blocks the EMT and EMT mediated changes in CSC marker expression. However, we need to establish the molecular mechanism by which Rac1 is regulating TGFβ1 mediated EMT. Radinsky et al have shown Rac1b being the mediator of EMT induced reactive oxygen species (ROS) production [44] and ROS production is elevated during EMT process (our unpublished data). Interestingly, increase in ROS levels was associated with radio-resistance of cancer stem cells [45]. It will be interesting to explore the potential role of ROS in EMT mediated cancer stem cell regulation. Understanding the potential molecular pathway linking Rac1-ROS-EMT-CSC plasticity might help the design of potential therapeutics to target CSC plasticity by inhibiting Rac1 mediated ROS production. Design of such inhibitors is been currently pursued in our laboratory (unpublished data from our lab).

In conclusion, our studies indicate that multiple inter-convertible CSC subpopulations with tumorigenic and metastatic properties exist in NSCLAs. Therefore elimination of one CSC population may not be an efficacious strategy for cancer therapy because of existence of other CSC populations and possibility of plastic conversion from
non-CSCs mediated by EMT that can ultimately lead to propagation and relapse of tumors. Our current work suggests that targeting Rac1 GTPase represents a novel strategy that inhibits both CSC activity and the dynamic transition of the non-CSCs to CSCs.

References


