UNIVERSITY OF CALIFORNIA

Los Angeles

Slug induces malignant phenotypes in models of human pulmonary premalignancy

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Cellular and Molecular Pathology

by

Nicole Lee Rodriguez

2013

ABSTRACT OF THE DISSERTATION

Slug induces malignant phenotypes
in models of human pulmonary premalignancy

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Doctor of Philosophy in Cellular and Molecular Pathology
University of California, Los Angeles, 2013
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Chronic inflammation is associated with increased lung cancer incidence. The transcription factor Slug is overexpressed in both chronic inflammatory diseases of the lung, such as Chronic Obstructive Pulmonary Disease (COPD) and Idiopathic Pulmonary Fibrosis (IPF), and lung cancer. Both of these pulmonary diseases are associated with increased risk for lung cancer. This study demonstrates that Slug overexpression in a model of pulmonary premalignancy, using Human Bronchial Epithelial Cells (HBEC) and 3D lung organotypic cell culture, is able to induce malignant phenotypes. Overexpression of Slug in HBEC cells induces CXCL8, an angiogenic cytokine often expressed in the lungs of patients suffering from COPD, IPF, and lung cancer. Lung organotypic cell culture of Slug overexpressing HBEC cells indicates that an ability to invade into pulmonary fibroblasts, demonstrating Slug's capability to induce early dissemination during premalignancy. Slug overexpressing cells gain the ability to grow in anchorage independent conditions, indicating a shift towards cellular transformation. Additionally, Slug induces expression of the stem cell genes, Oct4, Sox2, and Klf4, which are associated with tumor initiation. The combination of these malignant phenotypes: invasion,

anchorage independent growth, stemness, and angiogenesis, indicates Slug expression during premalignancy may mediate the initiation of metastatic lung cancer.

The dissertation of Nicole Lee Rodriguez is approved.

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2013

For Mom and Isabella

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LIST OF ABBREVIATIONS

SCC	Small Cell Lung Cancer
NSCLC	Non Small Cell Lung Cancer
LDCT	Low Dose Computed Tomography
COPD	Chronic Obstructive Pulmonary Disorder
IPF	Idiopathic Pulmonary Fibrosis
EMT	Epithelial-to-Mesenchymal Transition
VEGF	Vascular Endothelial Growth Factor
TGF-β	Tumor Growth Factor-β
HGF	Hepatocyte Growth Factor
EGF	Epidermal Growth Factor
IL-1β	Interleukin-1β
MAPK	Mitogen Activated Protein Kinase
Erk	Extracellular signal-regulated Kinase
PI3K	Phosphoinositide-3 Kinase
Akt	Protein Kinase B
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
p38	p38 Mitogen Activated Protein Kinases
CSC	Cancer Stem Cell
CD133	Prominin-1
iPS cells	Induced Pluripotent Stem cells
Oct4	Octamer Binding Transcription Factor 4
Klf4	Krupple Like Factor 4
Sox2	SRY-Box 2 Transcription Factor
EGFR-TKI	Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors
EGFR	Epidermal Growth Factor Receptor also known as ErbB1

Met	also known as Hepatocyte Growth Factor Receptor
p53	Tumor Protein p53
CXCL8	also known as Interleukin 8
TNF-α	Tumor Necrosis Factor Alpha
ASD	Angiogenic Squamous Dysplasia
IL8	Interleukin 8 (gene name)
AP-1	Activating Protein 1
Fra	Fos Related Antigen 1
Jnk	Jun Kinase
C/EBP	CCAAT/ Enhancer-Binding Protein
Dach1	Dachsund 1
Oct1	Octamer Binding Transcription Factor 1
DNA-PK	DNA Dependent Protein Kinase
MORE	More Palindromic Oct Factor Recognition Element
PORE	Palindromic Oct Factor Recognition Element
snRNA	Small Nuclear RNA
POLR2A	RNA Polymerase II, subunit A (gene name)
ALDH1	Aldehyde Dehydrogenase 1
ABCG2	ATP-Binding Cassette, subfamily G, member 2
SCF	Stem Cell Factor
c-Kit	Stem Cell Factor Receptor
Sox9	SRY-Box 9 Transcription Factor
POU2F1	Octamer Binding Transcription Factor 1 (gene name)
miRNA	Micro RNA
NuRD	Nucleosome Remodeling and Deacetylation complex
Jmjd1a	Jumanji Demethylase 1a

bHLH/PAS	basic Helix-Loop-Helix/ Per-Arnt-Sim Transcription Factors
dSim	Single Minded, Drosophila homologue
Sim1	Single Minded 1, mammalian homologue
Sim2	Single Minded 2
AhR	Arylhydrocarbon Receptor
HIF-1α	Hypoxia Inducible Factor 1α
Arnt	Arylhydrocarbon Receptor Nuclear Translocator
HIF-1β	Hypoxia Inducible Factor 1β, alternate name of Arnt
HRE	Hypoxia Regulatory Element
CME	Central Midline Enhancer
miRNA	microRNA
pri-miRNA	Primary microRNA
BIC	a gene, originally found in avian leukosis, without an open reading frame

ACKNOWLEGMENTS

I would first like to thank Dr. Dubinett. You never gave up on me. Thank you for all of your support and encouragement. We did it!

I would also like to thank my committee members, Dr. Denny, Dr. Kelly, and Dr. Effros. Your feedback and suggestions over the years have helped me stay on track and keep focused.

To the Dubinett Lab members, thank you for all your positive attitudes and all your help over the years. It has been a pleasure working with you. Thank you all for letting me bounce ideas off you and talk through my experimental designs. It helped to have someone to talk science with. Extra special thanks to all of you that helped me out those times when Isabella got sent home sick and I had to leave abruptly. Thank you for putting samples in the freezer and media on hungry cells- you are my heroes!

Jeanette and Eileen, I don't think I would have survived graduate school without you. Thank you for bringing up my spirits and supporting me all these years. You are both true friends.

Patty and Wiam, you are my family away from my family. Thank you for your friendship. I love you. Thank you for being you.

Dad, you have supported me your entire life. Thank you for raising me to know that I can do anything I set my mind to. Thank you for your encouragement, you support, and for being the rock of our family. It could have all come crashing down around us, but you held us together.

Isabella, at this point in time you're not old enough to read this, but I just want you to know that I love you. Thank you for being the sweetest and most considerate daughter a mom could have. I couldn't have asked for a better kid.

Research supported in this publication was supported by the National Institutes of Health under Award Number NIH RO1 CA111851. The content is solely the responsibility of the author and does not necessarily represent the official views of the National Institutes of Health.

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PUBLICATIONS AND PRESENTATIONS

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Overview

Lung Cancer Screening Methods and Risk Factors

Lung cancer is the leading cause of cancer death in the United States for both men and women. In 2013, it is estimated that more than 26% of all cancer deaths will be due to lung cancer. The low survival rate is attributed to the fact that the majority of lung cancers are diagnosed in advanced stages of disease, leaving few treatment options. The overall survival rate for lung cancer is 16%, while the survival rate for patients with late stage disease is 4% [1]. The two main types of lung cancer are Small Cell Lung Cancer (SCC) and Non Small Cell Lung Cancer (NSCLC), which are classified based on histological evaluation. The majority of lung cancers are NSCLC, whose subtypes include Adenocarcinoma, Squamous Cell Carcinoma, and Large Cell Carcinoma [2].

Historically lung cancer has been treated based on histological analysis. More recently, a number of driver mutations, somatic gene alterations leading to cellular transformation, have been associated with various cancers including lung cancer. These driver mutations are often in genes that encode signaling proteins that participate in cellular proliferation. Many of these mutations are rare, some occurring in as little as 1% of NSCLC patients [3]. Currently studies are underway to determine unknown somatic mutations that drive tumorigenesis.

Somatic mutations may be invaluable as means of determining methods of targeted therapy. Lung cancer is highly heterogeneous and expresses a diverse combination of mutations. Many specific mutations can be correlated to specific subtypes or clinical variables of lung cancer progression. KRAS mutations are highly expressed in adenocarcinoma, STK11 mutations are mutations common in smokers and EGFR mutations are more likely to be expressed in never smokers [4]. Drugs designed to inhibit these mutations have shown to be effective cancer treatments. An example evidenced in EGFR Tyrosine Kinase Inhibitors (EGFR-

TKIs). It has been shown that patients that harbor EGFR activating mutations have increased probability of response to treatment with EGFR-TKIs [5]. Similar treatments are available for other driver mutations such as EML4-ALK. EML4-AK translocations are caused by chromosomal fusion of the ALK receptor tyrosine kinase, not normally expressed in the lung, and EML4, the nucleophosmin gene. Both genes are located within the short arm of chromosome 2, which undergoes chromosomal rearrangement in both lymphoma and lung cancer [5]. Pharmacological treatments targeting ALK activity are promising therapeutics [3]. Conversely, KRAS mutations, which are present in approximately 30% of NSCLC patients, allow constitutive activation of the Ras signaling pathway and is associated with drug resistance [6,7]. Expression of KRAS in patients also harboring EGFR activating mutations indicates resistance to EGFR-TKI therapies [8]. Continued study of somatic mutations may lead to better targeted therapy and shed light onto mechanisms of drug resistance.

Although treatment strategies are becoming more sophisticated with new targeted therapies a major obstacle to effective lung cancer treatment is detection. Lung cancer is difficult to detect in the early stages, when surgery and other treatments are most effective. Recently, annual screening of high risk patients using Low Dose Computed Tomography (LDCT) scans have proven to reduce lung cancer mortality [9]. Imaging studies that suggest lung cancer are often followed by procedures such as bronchoscopy to make a diagnosis.

Risk factors for lung cancer include tobacco smoke, Chronic Obstructive Pulmonary Disease (COPD), and Idiopathic Pulmonary Fibrosis (IPF). COPD, in the United States, is defined as Chronic Bronchitis or Emphysema. Chronic Bronchitis is caused by constant irritation and inflammation of the airways, which causes airway lining to thicken and increases mucus production, making breathing difficult. Emphysema is the damage or destruction of the air sacs in the lungs, which prevents proper gas exchange. Many people who have COPD have both

bronchitis and emphysema and patients with COPD are at increased risk for developing lung cancer, regardless of smoking history [10]. COPD is commonly found in patients diagnosed with lung cancer with any histologic subtype. Similarly, IPF is common in smokers. Smokers diagnosed with IPF have an increased incidence of lung cancers, predominantly Squamous Cell Carcinoma, than smokers without. The pathogenesis of IPF is unclear, but it is marked by increased incidence of fibroblasts and myofibroblast cells in the lung due to epithelial to mesenchymal transition (EMT), a transdifferentiation program, of alveolar epithelial cells [11].

Chronic inflammation of the lung is a common effect to tobacco smoke, CODP, and IPF. Inflammatory mediators play an important role in tumorigenesis [12]. Growth factors and cytokines that participate in inflammatory responses aid tumorigenesis by activating signaling pathways which contribute to transformation, invasion, angiogenesis, apoptosis resistance, and tumor cell metastasis. Chronic inflammation is associated with tumor progression in many cancer subtypes including gastric cancer, colon cancer, and liver cancer. These cancers are associated with chronic infections: *helicobactor pylori* infection, inflammatory bowel disease, and hepatitis infection respectively [13]. Tobacco smoke and inhaled pollutants can trigger chronic inflammation leading to pathologies such as COPD and IPF. Unfortunately, smoking cessation does not necessarily remove the risk of developing lung cancer, as chronic inflammation persists [10].

Inflammation in Lung Cancer Progression

Inflammation occurs at all stages of tumorigenesis and has been shown to play a role in both tumor initiation and maintenance [14] Chronic infections or exposure to environmental toxins can lead to an inflammatory response, causing immune cells to flood the organ site and produce inflammatory mediators, which premalignant and malignant cells can respond to. Conversely, many solid tumors, including lung tumors, acquire genetic and epigenetic

modifications that allow for the remodeling of the tumor microenvironment. These tumor cells can secrete chemokines to recruit other cell types to the tumor microenvironment [15,16]. Among these chemokines are angiogenic factors such as CXCL8 and Vascular Endothelial Growth Factor (VEGF) which recruit vascular endothelial cells to the tumor site to create tumor vasculature in order to increase blood supply to support growth [17,18]. Tumor secreted inflammatory mediators can also recruit T regulatory cells and prevent anti-tumor immune response while at the same time recruiting pro-tumorigenic immune cells, such as Tumor Associated Macrophages, which secrete cytokines that support angiogenesis, invasion, and metastasis [16,19].

Inflammatory mediators common to the lung tumor microenvironment include Transforming Growth Factor-β (TGF-β), Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF), and Interleukin -1β (IL-1β) [20,21,22]. These growth factors and cytokines share an ability to activate downstream pathways leading to phenotypic changes necessary for tumor progression. TGF-β, HGF, EGF, and IL-1β are associated with activation of MAPK/ Erk, PI3K/ Akt, NF-κB, and p38 signaling pathways, which in turn induce angiogenesis, invasion, migration, metastasis, and prevent apoptosis, all of which are hallmarks of cancer progression [23,24].

Epithelial to Mesenchymal Transition

A number of the growth factors and cytokines associated with inflammation are also associated with induction of EMT. Epithelial to mesenchymal transition is a complex process necessary for development and wound healing. Epithelial cells, following molecular cues, trigger a genetic program that allows for plasticity in the epithelial phenotype [25]. Cells that undergo EMT downregulate junctional proteins, such as E-cadherin, Occludin, and claudins, which maintain cell-to-cell contacts and epithelial polarity. Concurrently, these cells begin to express mesenchymal cell associated proteins, such as N-cadherin, Vimentin, matrix

metalloproteinases, and Smooth-Muscle Actin, which allow for a migratory and invasive phenotype [26,27]. It is believed that tumor cells undergo the process of EMT in order to invade the surrounding stroma and metastasize to distant organ sites [24,25]. More recently, studies have shown that cells that have undergone EMT show stem cell phenotypes, meaning EMT may be participating in inducing or maintaining a cancer stem cell phenotype [28,29,30].

The Cancer Stem Cell Phenotype

The concept of Cancer Stem Cells (CSCs) arose to account for the therapeutic resistance and dormant behavior of many cancer types, leading to the recurrence of disease following treatment. CSCs were termed as such due to their phenotypic resemblance to normal stem cells, including the expression of cell surface markers associated with stem cells and genes required for stem cell maintenance [31]. CSCs are thought to arise either through the transformation of normal stem or progenitor cells or from differentiated cells that have acquired self renewing capacity. CSCs are characterized by the ability to efflux dye or drugs, the capacity for self renewal, and their ability to initiate tumors following serial transplantation *in vivo* and reestablish the phenotypic heterogeneity of the primary tumor [30,31].

A number of stem cell associated genes have been shown to be expressed in lung cancer cells, CD133, Sox2, Oct4, *ALDH1*, and *ABCG2*. Lung CSCs are thought to be characterized by the expression of CD133 on the cell surface. CD133 is a cell surface protein first discovered as a characteristic surface protein in hematopoietic stem cells. Its cellular function is still unknown and its validity as a marker of cancer stem cells is under debate as it has been implicated to have wide expression in luminal epithelial organs, including the lung, in a mouse model [32]. Still, CD133 positive cells have been shown to have increased expression in human lung tumors compared to normal human lung tissue [33,34]. Expression of CD133 in lung cancer cells marks them with the ability to grow in sphere assays, in which stem cell

phenotypes are thought to be required for growth. Additionally, an increase in tumorigenicity in xenograft tumor experiments by cells selected for CD133 expression has been shown in both lung and glioblastoma [33,35]. Analysis of lung cancer patient samples showed that the expression of CD133 in patients treated with platinum based chemotherapeutics had a shorter recurrence free survival period than those that were CD133 negative, indicating that CD133 may characterize a more highly chemoresistant population of cells [34].

While CD133 is a cell surface marker of unknown function, a number of transcriptional and epigenetic regulators specific to pluripotent stem cells have been shown to be increased in a subset of cancer cells and may be driving the CSC phenotype. Oct4, when expressed along with Klf4, Sox2, and Myc in somatic cells, has been shown to induce pluripotent stem cells [36,37,38]. In lung cancer, Oct4 expression is increased in cells that stain positive for CD133, along with the drug efflux gene *ABCG2*, also associated with stem cell phenotype. Loss of Oct4 expression abrogated the ability of lung CSCs to resist chemotherapy and radiotherapy and form spheres in culture. Loss of Oct4 also decreased invasion, soft agar colony formation *in vitro,* and reduced the formation of tumors and prolonged survival *in vivo* [39].

Besides Oct4, another pluripotent stem cell maintenance gene, Sox2, has been implicated in human lung tumorigenesis. Sox2 expression is increased in Small Cell Lung Cancer and the Squamous Cell Carcinoma subtype of Non Small Cell Lung Cancer [40,41]. Sox 2 expression is increased in Squamous Cell Carcinoma tumors due to amplification of chromosome segment 3q26.33 as well as a by chromosome independent mechanism. Knockdown of SOX2 in Squamous Cell Carcinoma cell lines harboring chromosome 3q26.33 amplifications significantly reduce anchorage independent growth and proliferation [41]. Additionally, Sox2 was found to be overexpressed in the side population fraction of the A549 adenocarcinoma lung cancer cell line. The side population represents a subset of cells sorted

by increased ability to efflux Hoechst33342 dye. Knockdown of Sox2 in A549 cells reduced tumorigenesis in mouse xenograft experiments. Following RNA-seq analysis of A549 cells expressing shRNA for SOX2, it was found that 246 genes were altered, indicating Sox2 regulates a transcriptional network associated with oncogenesis [42].

While the existence of CSCs are under debate, it is clear that expression of genes associated with stem cells and their ability to self renew and resist drug treatments are present in human cancers. The mechanisms by which stem cell genes are activated and the specific functions associated with them in regards to cancer remains to be determined.

Slug Mediates EMT and Drug Resistance

The transcriptional regulators of EMT, such as Twist and the Snail family of transcription factors, Snail and Slug, have been implicated in participating in all aspects of tumorigenesis, including CSCs and drug resistance [12,17]. Besides EMT and drug resistance, Slug expression is associated with apoptosis resistance, invasion, and metastasis [43,44,45].

Slug is a zinc finger transcription factor that is expressed during vertebrate embryogenesis and participates in the development of the mesoderm and the migration of neural crest cells, where it is critical in the developmental process of EMT [46]. Increased Slug expression has been observed in a number of cancer types including lung, breast, colorectal and pancreatic cancers [44,47,48,49]. Slug is a known repressor of the adherens junction protein, E-cadherin, which is critical for maintenance of cell-to-cell junctions among differentiated epithelial cells [50]. During cancer progression, Slug directly represses the expression of E-cadherin and other epithelial cell-to-cell junction proteins, such as Desmoplakin, Claudin-1, and Occludin, by binding to specific E-box sequences in their promoters [47,51,52,53]. Slug, through its regulation of EMT, has been shown to increase invasion and metastasis in cancer cells, allowing tumor cells to eventually disseminate from the primary

tumor [24,25]. In relation to lung cancer, Slug has been shown to be induced in premalignant, early stage, and late stage lung cancer tissues and is an independent prognostic indicator of worse overall survival. Additionally, increased Slug expression correlates with shorter recurrence-free survival of lung cancer [48,54,55]. The early expression of Slug, as well as its association with poor prognosis, indicates that Slug is an important regulator of tumorigenesis beyond the scope of EMT.

It has become apparent that cells that have undergone EMT are also resistant to multiple drugs, while cells sensitive to chemotherapeutics retain their epithelial markers, specifically E-cadherin [56]. Slug, in particular, has been identified as conferring resistance to chemotherapeutics in ovarian cancer and mesothelioma cells. Knock down of Slug expression sensitized mesothelioma cells to three chemotherapeutic agents: doxorubicin, paclitaxel, and vincristine [57,58]. Slug-conferred drug resistance is not limited to chemotherapeutics, as Slug is also implicated in resistance to targeted therapies, specifically EGFR-TKIs. Cancer patients who have EGFR activating mutations respond well to EGFR-TKI treatments, but ultimately develop acquired resistance to these drugs through acquired mutations and bypass mechanisms using alternate signaling pathways such as HGF/Met [29,59,60,61]. Slug, which has been shown to be a downstream target of HGF/Met induced EMT, has been implicated in EGFR-TKI resistance *in vitro*, and is more highly expressed in patients resistant to EGFR-TKIs [51,62,63]. The exact mechanism by which Slug contributes to multi-drug resistance has yet to be discovered, but it may lie in the ability of Slug to interfere with p53-mediated apoptosis and cellular stress responses to genotoxic insults [64]. Cells overexpressing Slug resist radiation-induced apoptosis through direct transcriptional repression of Puma, a downstream mediator of p53 [65,66].

Slug is implicated in mediating EMT, apoptosis, and drug resistance as a downstream target of a number of cancer associated signaling pathways. Slug expression can be induced by a number of growth factors and cytokines present in the tumor microenvironment and inflamed lungs. Inhibition of cancer associated signaling molecules and their receptors such as TGF-β, HGF/Met amplification, and EGFR are currently being investigated, but as exampled by EGFR-TKI drug resistance, cancer cells may become resistant to these treatment strategies. Slug, as a common downstream effector of these growth factor pathways, may prove a more viable treatment target [63,67,68].

CHAPTER 1: Slug Induces Malignant Phenotypes in a Model of Human Lung Premalignancy

Introduction

Slug involvement in Angiogenesis

Slug has been identified as a molecular mediator of a broad group of carcinogenic phenotypes which include invasion, motility, apoptosis resistance, and drug resistance. A number of studies have implied that Slug induces angiogenesis in tumors. Gene array analysis of expression patterns following modifications of Slug expression indicate that both VEGF and CXCL8, both potent inducers of angiogenesis, are regulated by Slug [69,70]. Furthermore, Slug expression has been reported to increase vascularization *in vivo* in xenograft models of lung adenocarcinoma and glioma [44,45].

Angiogenesis in Malignancy and Angiogenic Squamous Dysplasia

Angiogenesis is the formation of new capillaries from existing blood vessels. Besides the normal development of blood vasculature, most often angiogenesis occurs during the evolution of a tumor. Angiogenesis is necessary for tumors to grow larger than 1-2mm. Growing tumors require the formation of new blood vessels to allow the tumor to acquire the nutrients and oxygen it needs to prevent necrotic death. However, angiogenesis has been recently demonstrated to not be limited to development or late stage tumor growth. Premalignant lesions can also acquire new blood vessels, as demonstrated in the occurrence of Angiogenic Squamous Dysplasia (ASD) [71]. ASD are rare lesions found in the bronchial epithelium consisting of capillaries projecting into metaplastic or dysplastic tissues. These lesions are identified through bronchoscopy, a diagnostic tool that allows for the identification of atypical lesions in the large airway. Neoangiogenesis has been reported to be increased in the bronchial

epithelium of smokers and ASD has been associated with patients with a high risk for lung cancer [72,73].

CXCL8 is regulated by Inflammation Mediated Signaling Pathways

CXCL8 is part of the CXC family of chemokines and is a potent chemoattractant for inflammatory cells and leukocytes [17,74]. CXCL8 is a known mediator of angiogenesis, and has been shown to be upregulated in human lung cancer cell lines as well as in patients with COPD and IPF [75,76,77,78,79].

CXCL8 expression can be rapidly triggered by proinflammatory mediators such as TNF-α, EGF, HGF, IL-1β, by cellular stress, and by bacterial and viral proteins through cellular signaling cascades. The NF-κB, Jnk and ERK signaling cascades regulate the primary transcription of the gene encoding CXCL8, *IL8.* NF-κB transcription factors, such as p65 and p50, and Activating Protein-1 (AP-1), which is a heterodimer of Jun and Fos family transcription factors, have binding sites in the *IL8* promoter. NF-κB transcription factor binding is required for *IL8* transcription, while AP-1 is only necessary for maximal promoter activity [80].

Several other transcription factors have been found to regulate *IL8* in addition to NF-κB and AP-1; CAAT/enhancer-binding protein (C/EBP), Dachshund 1(Dach1), and Octamer 1(Oct1) [81,82] . C/EBP has been shown to work cooperatively with NF-κB at the *IL8* promoter, but is not required for induction of *IL8* by stimuli such as TNF-α [83]. C/EBP binds weakly to the *IL8* promoter unless NF-κB subunits are bound to the promoter as well [84]. Dach1 is better known as a putative tumor suppressor gene. Dach1 can occupy and repress AP-1 sites, preventing c-Jun binding [85]. It has also been shown that Dach1 expression is lost in glioma and breast tumor cells and re-expression of Dach1 can prevent Jun mediated anchorage-independent growth by directly inhibiting transcription of the *Jun* promoter [85,86,87]. Dach1 has been shown to bind the AP-1 site in the *IL8* promoter [81].

The Oct1 Transcription Factor

Oct1 has been shown to bind to an Octamer motif found in the *IL8* promoter and prevent NF-κB binding and transcription of *IL8* [82]. The main function of Oct1 is as a stress sensor, although it has overlapping targets with Oct4. Loss of Oct1 expression in not required for cell survival but its loss causes cells to be sensitive to genotoxic insults such as ionizing radiation, hypoxia, or DNA damage [88,89]. Oct1 is a bipotential regulator which can activate or repress genes based on its binding partners at individual promoters [90]. It regulates a number of housekeeping genes and can prevent apoptosis when activated by DNA Protein Kinase (DNA-PK), a kinase known to induce housekeeping genes following stress signaling [91]. Unphosphorylated Oct1 is capable of binding to the Octamer motif, but once phosphorylated it gains the ability to bind to more complex DNA sequences, termed Palindromic Oct factor Recognition Element (PORE) and More palindromic Octamer Related Element (MORE) sites. This change in binding activity is due to flexibility in its POU DNA binding domain, which changes conformation after Oct1 phosphorylation to allow stronger binding to DNA binding sites [92,93]. MORE and PORE sites are located in metabolic housekeeping genes such as Histone H2B, U2 and U6 snRNA, and *POLR2A*. The steady state levels of these genes do not change in the absence of Oct1, but loss of Oct1 prevents their induction and makes cells hypersensitive to stress agents [94].

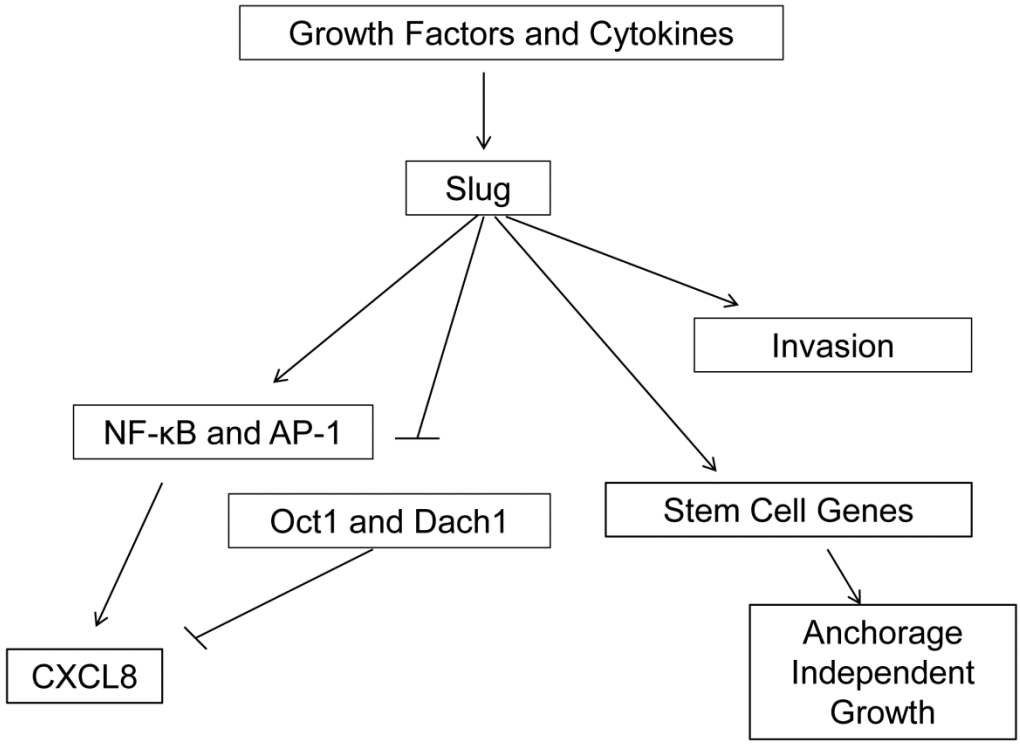
Oct1 is in the same family of transcription factors as Oct4, which is required for the maintenance of induced pluripotent stem (iPS) cells. Oct1 and Oct4 have overlapping target genes, as they are both able to recognize PORE sequences, but Oct1 does not participate in the induction of iPS cells [94]. Still, Oct1 has been identified as potentially participating in the CSC phenotype in lung adenocarcinoma and breast cancer tumor cells. Oct1 was found to bind to the promoters of *ABCG2* and *ALDH1A1*, dye efflux genes associated with the stem cell

phenotype. Additionally, knockdown of Oct1 reduced tumor cell engraftment in a xenograft mouse model [95]. Loss of Oct1 expression is associated with an anti-tumorigenic metabolism shift in cells, meaning when expressed it is potentially participating in cancer growth and proliferation [89].

Slug and the Stem Cell Phenotype

Aside from roles in cell proliferation, invasion, migration, and angiogenesis, Slug can be implicated directly and indirectly as a means of creating a stem cell phenotype. It is indirectly associated with CSCs through its ability to induce EMT, which in turn contributes to the stem cell phenotype, but there is also a direct role for Slug in stem cell phenotype through the promotion of cell survival. Slug is induced by the SCF/c-kit signaling pathway in hematopoietic progenitor cells. Loss of Slug expression in mice prevents robust survival of hematopoietic progenitors through SCF/c-kit signaling and allows for p53-mediated apoptosis following exposure to ionizing radiation [65,66,96]. More recently, it has been shown that Slug cooperates with Sox9 in the regulation of the mammary stem cell state. Neither Slug nor Sox9 alone can induce the formation of mammary stem cells, as shown by their ability to reconstitute mammary glands in mouse mammary fat pads, but when co-expressed they induce a complimentary array of transcription factors associated with mammary basal and luminal cells that can reproduce mammary glands *in vivo* [97].

Slug induces malignant phenotypes in a model of lung premalignancy



Materials and Methods

Antibodies and Reagents

Antibodies were purchased as follows: Slug, pJnk, total Jnk, pErk, total Erk, pEGFR, Oct4, Sox2, Klf4, alpha-tubulin (Cell Signaling), Oct1 (Millipore), IkBα (BD Biosciences), p50, p65, Goat anti-Rabbit HRP conjugate (Santa Cruz), Dach1 (Abcam), Goat anti-Mouse HRP conjugate (BioRad), Alexa Fluor 488 FITC conjugate (Invitrogen). TGF-β, EGF, TNF-α were purchased from Peprotech. Chemical inhibitors BMS-345541, U0126, SP600125 were purchased from Sigma.

Cell Culture

Parental and EGFR Human Bronchial Epithelial Cells (HBEC), a kind gift from Dr. John D. Minna, were created according to procedures previously described [98]. HBEC cells were cultured in KSFM supplemented with 0.2ng/ml rEGF and 30µg/ml Bovine Pituitary Extract (Life Technologies) at 37ºC and 5% CO_2. HBEC cells are trypsinized in 0.25%Trypsin-EDTA and neutralized using Trypsin Neutralization Solution (Lonza). Growth factor and cytokine treatments were carried out in 6 well plates in supplement free KSFM. Cells were serum starved for 4-20 hours prior to incubation with TGF-β, EGF, or BSA vehicle control for 24-72 hours with final cell confluence at 70-85%.

Generation of Slug Overexpressing Cells

Slug overexpressing cell lines were generated using lentiviral overexpression plasmids, pLVX-CMV-SLUG and pLVX-CMV-NEG empty vector (Genecopeia). Briefly, HEK293t kidney cells (ATCC) were transfected with either pLVX-SLUG or pLVX-NEG along with VSVG (Addgene) envelope protein and pDelta vpr (Addgene) packaging protein plasmids using BioT (Bioland Scientific) transfection reagent according to the manufacturer's protocol. Transfection was

carried out in serum free DMEM (Life Technologies) overnight. Following transfection, HEK293t cells were given fresh DMEM media supplemented with 10% Fetal Bovine Serum (Gemini Bioscience) and 1% Penicillin-Streptomycin (Life Technologies) and incubated 6-8 hours in 5ml media or overnight in 8ml media. Viral supernatants were collected over 48-72 hours, pooled and stored at 4ºC. Pooled viral supernatants were then spun down at 300 x g for 5min to and then filtered using a 0.45µm filter to remove any cells. HBEC parental cells were plated at 20% confluence levels in 6well plates and allowed to attach. HBEC cells were then incubated in 1ml of viral supernatant with 10µg/ml polybrene (Sigma) for 6-8 hours. Following incubation transduced HBEC cells were cultured in complete KSFM and allowed to recover for 48 hours. Following recovery, virally transduced HBECs were selected for gene expression using 80-250µg/ml Hygromycin B (Invitrogen) for 7-14 days. Hygromycin B concentrations for selection were determined by incubating individual parental HBEC cell lines in increasing amounts of Hygromycin B for 7-14 days order to determine the lowest effective dose.

Western Blotting

Whole cell protein lysates were collected using Radio-Immunoprecipitation Assay (RIPA) buffer supplemented with 1% Triton-X 100, Complete Protease Inhibitor Cocktail (Roche), Sodium Fluoride, activated Sodium Orthovandidate, and phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined using BCA assay (Pierce). Immunoblotting was carried out on 8-10% SDS-PAGE gels according to and transferred to PVDF membrane (Millipore). Blots were blocked in either 5% milk or 5% Bovine Serum Albumin in Tris Buffered Saline containing 0.05% Tween-20 (Fisher) for 1 hour. Following antibody incubations, blots were developed using either Western Lightning Plus ECL (Perkin Elmer) or SuperSignal Chemiluminescent (Pierce) followed by exposure to film.

ELISA

Cell lines were incubated in supplement free KSFM for 48 hours. Supernatants were collected and spun in microcentrifuge at 2,000 x g to remove any cells. CXCL8 and VEGF ELISAs were performed according to manufacturer's instructions and normalized to whole cell lysate protein concentration as determined by BCA assay.

RNA Isolation and qPCR

Cells were plated in 60mm dishes and serum starved in supplement free KSFM for 24-48 hours. RNA was isolated using RNeasy RNA isolation kit (Qiagen) according to manufacturer's protocol. cDNA was created using High Capacity RNA-to-cDNA kit (Life Technologies) using 2µg RNA according to protocol. *IL8* qPCR was run using self designed primers- IL8 Forward: 5'-GCAGCTCTGTGTGAAGGTGCAGT-3', IL8 Reverse: 5'- CTGTGTTGGCGCAGTGTGGTCC-3', HPRT1 Forward: 5'- GCTGAGGATTTGGAAAGGGTGT-3', HPRT1 Reverse: 5'-CACACAGAGGGCTACAATGTGATG-3' (IDT). qPCR reaction was run using iQ SYBR Green Supermix (BioRad) on iCycler PCR machine (BioRad) and delta delta Ct analysis was performed with iQ5 software (BioRad).

Inhibitor Studies and Immunofluorescence

HBEC cells expressing SLUG or NEG empty vector control were plated in 6 well plate and serum starved for 4 hours in supplement free KSFM. Cells were next incubated with either BMS-345541, U0126, or SP600125 for 45 minutes or 48 hours. Supernatants for ELISA and whole cell protein lysates for normalization and immunoblotting were collected as previously described. HBEC cells expressing SLUG or NEG empty vector control and were simultaneously plated in 8 chamber slides treated for cell culture and were treated with BMS-345541 for 45 minutes. Following pre-treatment with inhibitor, control wells were treated with TNF-α for 25

minutes. All 8 chamber wells were then washed with PBS, fixed with 4% Parafomaldehyde (Electron Microscopy Science), and permiabilized using 0.25% Triton-X 100 solution. Primary antibodies p65 and p50 were incubated overnight at 4ºC, secondary antibody Alexa Fluor 488 (Invitrogen) was incubated at room temperature for 45 minutes, followed by DAPI stain (Sigma) at 5µg/ml for 5 minutes. Cells were mounted using Prolong Gold antifade reagent (Invitrogen). Fluorescent microscopy was carried out using Nikon Eclipse Live Cell Scope.

Co-Immunoprecipitation

$1.2x10^6$ HBEC cells expressing SLUG or NEG empty vector control were plated in 150mm dishes. Cells were serum starved in supplement free KSFM for 48 hours. For TNF-α treated control dishes, cells were treated with TNF-α or 0.1% BSA vehicle control for 25 minute prior to cell lysate collection. Whole cell lysates were collected in RIPA buffer supplemented with Complete Protease Inhibitor Cocktail, Sodium Fluoride, activated Sodium Orthovanidate, and PMSF. Protein concentration was determined using BCA assay. Protein lysates were pre-cleared using Pure Proteome Magnetic Protein A beads (Millipore) for 2 hours at 4ºC. Following preclear step, 500µg lysates were incubated with 2µg p65 or p50 primary rabbit antibodies (Santa Cruz), or normal rabbit IgG (Cell Signaling) overnight at 4ºC on a rotator. Antibody/ Antigen mixtures were then incubated with 50µl Pure Proteome Magnetic Protein A beads for 3 hours at 4ºC on a rotator and washed with Phosphate Buffered Saline (PBS) supplemented with 0.1% Tween-20. Antibody-protein complexes were eluted from magnetic beads using 0.2M Glycine, pH 2.0 and neutralized with 1.0M Tris pH 8.5. Elutes were immunoblotted for Oct1 following protocol previously described.

siRNA

Parental HBEC cell lines were plated in 6 well plates at 30% confluence. Cells were washed with PBS and incubated with supplement free KSFM for 1 hour. siRNA sequences specific for Slug mRNA (Invitrogen) or control non-silencing siRNA (ThermoFisher) were incubated with RNAi Max Lipofectamine (Invitrogen) in Opti-MEM media (Invitrogen) for 20 minutes. Transfection complexes were incubated with HBEC cells for 5 hours. Complete KSFM media was replaced and cells were allowed to recover overnight. Following recovery, transfected cells were serum starved for 20 hours followed by EGF of 0.1% BSA vehicle control treatment for 48 hours. Supernatants were collected for analysis by ELISA. Whole cell protein lysates were collected as previously described for ELISA normalization and for immunoblot confirmation of Slug knock down.

Anchorage Independent Cell Growth Assay

96 well plates were coated with 1.2% Noble Agar (Sigma) diluted with 2x DMEM (Sigma) to a final concentration of 0.6% and allowed to solidify at room temperature. A549 cells were purchased from ATCC and maintained in RPMI 1640 (Cellgro) supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin. HBEC cells expressing either SLUG or NEG vector control were trypsinized and re-suspended in 500µl complete KSFM. 500µl of 2x DMEM were then added to single cell suspensions which were then incubated in a 37ºC water bath for 30 minutes. 500µl of 1.2% Noble Agar incubated at 58ºC is then added to the cell suspension which is immediately plated into previously coated 96 well plate and allowed to solidify for 10 minutes at room temperature. Embedded HBEC cells are allowed to incubate overnight at 37ºC before being fed complete KSFM media. Images of each well are taken at serial sections while cells are kept at 37ºC and 5% CO_2 using the Nikon Eclipse Live Cell Scope. Images are then

stacked into a single focused for each well and condensed image is used to identify colony growth.

Lung Organotypic Three Dimensional Cell Culture

HBEC cells expressing SLUG or NEG vector control were plated into Lung Organotypic Three Dimensional Cell Culture system was performed according to protocol described previously[99]. After 12 days, three dimensional cell cultures were then rinsed in PBS and fixed for 6 hours or overnight in 10% Neutral Buffered Formalin (ThermoFisher). Following fixation, cultures were next rinsed with double distilled water, and soaked in 70% ethanol. Samples were next paraffin embedded, sliced, and processed for Hematoxylin and eosin staining by Pathology Core Facility.

Results

Slug overexpression induces CXCL8 in HBECs

In order to determine the role of the Slug transcription factor in lung premalignancy, HBEC cells overexpressing Slug were generated (Fig. 1A). HBEC cells are derived from normal lung epithelium from the large airways of smokers and ex-smokers. They were immortalized through the stable expression of Cdk4 and hTERT, and are used here as a model of lung premalignancy [98]. Slug expressing cell lines were labeled SLUG and empty vector controls NEG. Published data based on microarray studies indicated that Slug may regulate two molecules which mediate angiogenesis in lung cancer, VEGF and CXCL8. In order to validate these reports, ELISA assays were performed. HBEC SLUG cells did not show a consistent pattern in VEGF expression compared to NEG (Fig. 1B), but CXCL8 was consistently increased in SLUG lines compared to respective NEG vector controls (Fig. 1C). CXCL8 is normally controlled by inflammatory signaling pathways at the mRNA level by regulating transcription of the CXCL8 gene, *IL8*. In order to determine whether Slug overexpression modulated levels of *IL8* mRNA, qRT-PCR was performed. qPCR results indicate that the CXCL8 gene, *IL8*, is more highly expressed in SLUG cells versus NEG controls (Fig. 1D).

NF-κB signaling regulates CXCL8 expression following Slug overexpression

A number of signaling pathways regulate the *IL8* gene by regulating nuclear localization or expression levels of transcription factors that can bind the *IL8* promoter. Among these transcription factors, the strongest regulators of *IL8* transcription are NF-κB and AP-1. Although, AP-1 is important for basal *IL8* expression, NF-κB signaling is necessary for maximum gene induction. In order to block NF-κB signaling, SLUG and NEG cells were incubated for 48 hours with BMS-345541, a specific chemical inhibitor of NF-κB signaling (Fig. 2A). BMS-345541 is capable of binding to an allosteric site of IκB, a protein which binds NF-κB transcription factors

and holds them in the cytoplasm. By binding to IκB, BMS-345541 prevents its phosphorylation and degradation, thus preventing NF-κB dependent gene transcription. This inhibition is demonstrated in NEG cells treated with TNF-α, a well known inducer of NF-κB signaling. DMSO vehicle control treated cells that were also treated with TNF-α have clear nuclear localization of p65, while cells pretreated with BMS-345541 have p65 localized to the cytoplasm following TNF-α treatment (Fig. 2B). Blocking NF-κB signaling prevents CXCL8 expression, indicating that NF-κB is a major pathway through which Slug induces *IL8* expression (Fig. 2A).

Cellular localization of p65 and p50 are not different between NEG and SLUG cells

Surprisingly, when NEG and SLUG cells stained for p65 and p50 were compared, there was no clear difference between their cellular localization patterns (Fig. 3-5). In both HBEC3 and HBEC2 cells, p65 cellular localization appeared to be mostly cytoplasmic regardless of Slug expression (Fig. 3-4). The same was true of p50 expression in HBEC2 SLUG and NEG cells (Fig. 5). Additionally, BMS-345541 treatment appeared to have little effect on the cellular localization on p65 or p50 in the SLUG and NEG expressing HBECs, although it was capable of dramatically decreasing CXCL8 expression in SLUG expressing cells (Fig. 2A). These observations are supported by the overall lack of IκBα degradation, a subunit of the IκB complex, in the SLUG cell lines versus the NEG cells (Fig. 6A).

Slug does not change expression patterns of NF-κB pathway components

The downregulation of CXCL8 following BMS-345541 treatment but the lack of nuclear localization of p65 and p50 in SLUG cells is paradoxical. There has been some literature that implicates Slug as indirectly regulating the expression of NF-κB pathway constituents. RelA (another name for p65) has been described as being upregulated as an indirect result of Slug expression during mesoderm development [46]. To this end, p65 and p50 protein levels were compared between NEG and SLUG cell lines to determine if Slug was inducing CXCL8 through

increased expression of p65 and p50 subunits. No change in p65 or p50 expression was found between NEG and SLUG cell line pairs (Fig. 6A).

Oct1 protein expression is not regulated by Slug

This unusual circumstance, where there is no increase of nuclear NF-κB yet BMS-345541 is capable of inhibiting CXCL8 indicates that NF-κB may not be the only means by which Slug induces CXCL8 expression. It may be that basal NF-κB activation is sufficient, but the difference in SLUG cells is the absence of an *IL8* transcriptional repressor. Because Slug itself is a transcriptional repressor, it is possible that it is repressing an *IL8* transcriptional repressor, such as Oct1 or Dach1. Oct1 is thought regulate *IL8* by binding to the complementary strand of the NF-κB consensus site, displacing NF-κB transcription factors and preventing *IL8* promoter activation [82]. The promoter of the Oct1 gene, *POU2F1*, was analyzed for Slug consensus sequences, 5'-CAGGTG-3' and 5'-CACCTG-3', also known as E-boxes. The 5'CAGGTG-3' sequence was found to be approximately 700bp upstream of the *POU2F1* promoter start site (Fig. 6B). The *DACH1* promoter was also analyzed, but no Slug DNA binding sequences were found (data not shown). Western blot analysis for Oct1 and Dach1 were performed to determine if the repressors of *IL8* were being downregulated in the context of Slug overexpression. Oct1 expression did not change between NEG and SLUG cell lines, nor did Dach1; with the exception of the HBEC2 cell line (Fig. 6C).

Oct1 does not interact with p65

It is clear that inhibition of IκB degradation is somehow affecting CXCL8 expression downstream of Slug (Fig. 2A). While there is not a general increase of IκB degradation in SLUG cells versus NEG cells (Fig. 6A), chemically preventing its degradation with BMS-345541 dramatically affects CXCL8 in SLUG cells. While there is no difference in p65 or p50 cellular localization between NEG and SLUG cells (Fig. 3-5), it may be that the p65 or p50 that lies

within the nucleus without induction is being prevented entry due to BMS-345541 treatment. What may be the key difference between the NEG and SLUG cell lines' ability to induce CXCL8 are the different cofactors that are located at the *IL8* promoter and interact with p65 or p50. Loss of Oct1 has been shown to allow for amplified NF-κB target gene expression in mice and Oct1 has been reported to interact directly with p65 and prevent its transcriptional activity [100]. This led to the hypothesis that Oct1 binding to NF-κB subunits may be modulating gene transcription at the *IL8* promoter. To determine if Oct1 was binding NF-κB subunits differentially between NEG and SLUG cells, p65 and p50 were immunoprecipitated and the eluates were blotted for Oct1 (Fig. 7). No Oct1 binding was found in either the p65 or p50 immunoprecipitation samples. NEG cells treated with TNF-α were meant to serve as a positive control, ensuring that p65 was present in the nucleus to bind with Oct1. The absence of Oct1 binding p65 in HBEC cells following TNF-α treatment may indicate that this interaction may be cell type specific, as the original publication used endothelial cells, or requires Oct1 overexpression, which is not necessary for CXCL8 repression in our system. In the context of Slug overexpression, Oct1 is not interacting with NF-κB subunits in HBEC cells although Oct1 is present in both NEG and SLUG cell lines. Input protein indicates that there was Oct1 protein present in all protein lysates used for immunoprecipitation (Fig. 7).

Erk and Jnk pathways are involved in CXCL8 regulation

CXCL8 expression downstream of Slug may not be exclusively regulated by NF-κB signaling. Erk signaling regulates expression of the Fos family of proteins, while Jnk signaling regulates transcription of Jun proteins. Members of these two transcription factor families form heterodimers which make up AP-1, which binds and activates the *IL8* promoter. In order to determine if AP-1 may play a role in Slug mediated CXCL8 expression, Erk and Jnk signaling were chemically inhibited with U0126 and SP600125 respectively. Erk signaling is activated in

SLUG cells compared to NEG cells (Fig. 8B) as indicated by increased phosphorylation. Activated pJnk also appears to also be increased in SLUG cells versus NEG cells, but is not as robust as seen with pErk. Inhibiting either signaling pathways reduces the amount of CXCL8 when compared to DMSO vehicle control in SLUG cells (Fig. 8A). This indicates that Jnk and Erk may be increasing Jun and Fos family members respectively. It should also be noted that Dach1, which has been shown to directly inhibit c-Jun expression by binding to its promoter, does not change among NEG and SLUG expressing HBECs (Fig. 6C).

TGF-β and EGF induce Slug expression in HBECs

A number of inflammatory mediators are capable of inducing Slug expression in lung tumor cells, but it has yet to be determined if any of these growth factors or cytokines are capable of this effect during premalignancy. TGF-β and EGF, but not HGF are able to induce Slug expression in HBEC cells (Fig. 9A-B, data not shown). Because Slug overexpression has been shown to induce CXCL8, EGF and TGF-β treated HBEC cells were assayed for increased CXCL8 expression. TGF-β did not induce CXCL8 (data not shown), but EGF treatment did (Fig. 9C-D).

EGFR activating mutations do not induce Slug expression

Slug is implicated in EGFR-TKI resistance and EGF signaling is capable of inducing Slug. HBEC cells harboring EGFR activating mutations were evaluated for Slug expression. Slug was not increased in HBECs expressing EGFR with either the exon 19 deletion or L858R activating mutations compared to wild type EGFR expressing HBECs (Fig. 9E). These results do not conflict with the previous finding of EGF induced Slug expression (Fig. 9A-B), as ligand mediated signaling responses among EGFR (also known as ErbB1) and its family members ErbB2, ErbB3, and ErbB4 are highly diverse. Binding of EGF and EGF-like ligands, which may also bind to other ErbB receptor family members, activate specific signaling cascades

depending on ligand identity and ErbB heterodimerization [101]. EGFR signaling has been shown not only to induce different signaling cascades depending on ligand and receptor dimerization partners, but EGFR mutants have shown differential signaling activation amongst themselves as well [102]. There is most likely a difference in the signal potentiated by EGF activation versus the ligand independent activation of EGFR mutants. This difference in receptor activation may lead to activation of different downstream signaling cascades, which can account for the difference in Slug induction.

Slug is not required for EGF-induced CXCL8 expression

Because Slug overexpression in HBECs leads to increased CXCL8 expression (Fig. 1C) and EGF can induce both Slug and CXCL8 expression (Fig. 9B-D), it is possible that Slug is required for EGF induction of CXCL8. To determine its role, siRNA knockdown of Slug was performed in order to determine if Slug was required for EGF-mediated CXCL8 induction. siRNA sequences for Slug completely abrogated Slug expression (Fig. 10C-D) but loss of Slug did not prevent increased CXCL8 following EGF treatment (Fig. 10A-B). Slug knockdown appears to have enhanced EGF induction of CXCL8. This result indicates that Slug may be participating in a negative feedback loop and may be repressing negative regulators of CXCL8 in this particular situation.

Slug induces invasion in 3D Organotypic Cell Culture

Slug is best known for mediating EMT and invasion during cancer progression. To determine if Slug expression induced invasion in a premalignant model, SLUG and NEG cells were cultured in three dimensional conditions. The 3D cell organotypic culture method has been proven to allow for 3D growth of HBEC cells in an environment that mimics the lung and allows for their differentiation into ciliated, columnar epithelial cells [99]. Following culture for 12 days in 3D organotypic culture, SLUG cells clearly invade into the layer of primary lung fibroblasts (Fig.

11B) and grow several cell layers thick while the NEG cells form a layer of epithelial cells 3-4 cell layers deep and remain as an intact layer on top of the fibroblasts (Fig. 11A).

Slug overexpression induces Anchorage Independent Growth

It was shown previously that HBECs, without oncogenic changes such as p53 loss or KRAS mutations, are unable to form colonies in soft agar [103]. Slug expression in HBECs allow for anchorage independent growth. HBEC2 and HBEC3 cells expressing NEG or SLUG were culture for 21 days in soft agar. HBEC3 cells expressing SLUG showed colony growth as early as 6 days after plating and had a large number of colonies by day 21 (Fig.12D). HBEC2 cells expressing SLUG were not as robust in their anchorage independent growth as the HBEC3 SLUG cells, but colonies were seen by 21 days in culture (Fig. 13D). This ability for SLUG expressing HBEC cells to grow in anchorage independent conditions indicates a shift towards transformation which is dependent on Slug expression.

Slug overexpression induces Stem Cell associated Transcription Factors

Slug's ability to induce anchorage independent growth indicates that Slug may be playing an important role in transformation. Additionally, CSC populations are thought to contain tumor initiating cells and Slug has recently been implicated in regulating a mammary stem cell phenotype. Expression of stem cell associated genes may explain Slug induce anchorage independent growth. Oct4, Klf4, and Sox2 are known to regulate induction of iPS cells from somatic cells and Oct4 expression has been found to promote lung tumor growth in xenograft mouse studies. HBEC3 Slug expressing cells have increased levels of Oct4, Klf4, and Sox2, while HBEC2 Slug expressing cells mildly induce Klf4 (Fig. 14). Knockdown of individual genes Oct4, Klf4, and Sox2, would be required to determine if they are required for anchorage independent growth. If they prove to be involved, ability of HBEC3 SLUG cells to induce all

three of the stem cell associated transcription factors may explain why it is better able to grow in soft agar compared to HBEC2 SLUG cells.

Discussion

Cancer deaths in the United States peaked during 1990-91 and have been decreasing since. Decreased death rates for prostate, colon, and lung cancer in men, and colon and breast cancer in women accounted for the overall improvement in cancer statistics since 1990. The shift toward fewer U.S. cancer deaths is due to better early detection methods and treatments, at least for breast, colon, and prostate cancer. Lung cancer, on the other hand, has a decreased death rate in men primarily due to a reduction in smoking and tobacco use [1,104]. Lung cancer has proven difficult to detect, even using LDCT scanning to screen high risk patients- patients who are current smokers or have COPD. Current screening methods have high false positive rates which lead to invasive testing to diagnose, such as bronchoscopy and biopsy [9]. Early detection of lung cancer would dramatically increase survival rates, as those who are diagnosed with early stage, localized disease have a 52% survival rate, while the majority of those diagnosed with late stage disease and distant metastasis have a 4% survival rate. Currently 56% of lung cancers are diagnosed at late stage and have already metastasized [1].

Slug expression may be used as a biomarker for increased cancer risk, as well a molecular marker to help determine treatment strategies as Slug is associated with drug resistance to chemotherapeutics, radiotherapies, and EGFR-TKIs. Here it has been shown that Slug may contribute to angiogenesis in the premalignant stage. Slug may be compared with LDCT scans and cytology tests which may aid in determining if there is a premalignant or malignant lesion, or if chemoprevention is applicable. Because Slug can regulate CXCL8 expression, it could be used to help identify patients who may benefit from inhibition of CXCR2, the CXCL8 receptor, to prevent angiogenesis.

Understanding exactly how Slug regulates CXCL8 may indicate other biomarkers or treatment targets. Increased expression of Fos or Jun family proteins, differential microRNA

(miRNA) expression, or modulation of Oct1 DNA binding activity through phosphorylation could all be mechanisms by which Slug induces CXCL8.

Although AP-1 is not required for *IL8* promoter activation, it is possible that it is participating in Slug mediated CXCL8 expression, as shown by pErk and pJnk inhibition (Fig. 8). Further testing as to whether there is an increase of the Jun or Fos families of proteins following Slug overexpression would give insight as to whether AP-1 is participating in CXCL8 regulation. Chromatin Immunoprecipitation experiments in the context of Slug overexpression as well as in knockdown of candidate AP-1 subunits would be required to prove this. Still, Jun or Fos family transcription factor induction following Slug overexpression does not explain BMS-345541 inhibition of CXCL8.

CXCL8 inhibition by BMS-345541 without a change in IκB degradation in the NEG and SLUG expressing HBEC cell lines, suggest that there are either BMS-345541 off-target affects occurring or that CXCL8 is being affected by a cofactor that is coupled to basal NF-κB transcription. Oct1 was not shown to interact with p65 through co-immunoprecipitation experiments, but it does not rule out Oct1 binding to the *IL8* promoter and displacement of NF-κB subunits. Slug is known to regulate cellular stress pathways through modification of genes that participate in apoptosis and genotoxic stress [57,64,65,66]. It is possible that Slug induction of CXCL8 is an extension of its ability to protect cells from stress events such as irradiation and chemical insult. Oct1 is known to participate in cellular stress pathways as well, and loss of Oct1 sensitizes cells to hypoxia, radiation, and chemical toxicity [88,93]. Although the *IL8* promoter does not contain MORE and PORE sites (data not shown) which are the motifs normally associated with pOct1 binding, it is possible that in the context of Slug, there is a decrease of pOct1 binding and repression of the Octamer site in the *IL8* promoter.

Alternatively, Oct1 may be acting as a bipotential transcription factor, and Slug may be modulating its binding partners and thus modulating Oct1 activity. Oct1 is known to associate with Jmjd1a, a histone demethylase associated with hypoxia signaling pathways, and induce gene transcription. There is no evidence for Slug regulation of Jmjd1a, but it is possible that it is participating in Oct1 *IL8* regulation. Another possibility is that Slug expression is altering Oct1 interactions with the Nucleosome Remodeling and Deacetylase (NuRD) complex. It has been shown that Oct1 interacts with NuRD to repress Oct1 target genes [90].

Independent of Oct1 and other transcription factors known to modulate *IL8*, there is the possibility that *IL8* mRNA is being regulated by a microRNA dependent mechanism. MicroRNAs are small 22nt inhibitory RNA sequences that can bind to the 3'UTR sequences of mRNA and inhibit protein translation or promote mRNA degradation. MiR-146a/b has been associated indirectly with CXCL8 regulation and poor prognosis in lung cancer [105,106]. There is evidence that NF-κB signaling can activate miR-146a/b transcription during innate immune responses through direct transcriptional activation at NF-κB sites in the miR-146a/b promoter. In this context, upregulation of miR-146a/b could participate in CXCL8 regulation through their shared pathway [105]. Conversely, in separate studies conducted in metastatic breast cancer cells, expression of miR-146a/b prevented metastasis and repressed NF-κB signaling, which indirectly repressed IL-6 and CXCL8 expression [107,108]. There are no reports of Slug regulating miR-146a/b and no other miRNAs are known to affect CXCL8 expression. RNAseq or array analysis would need to be performed in order to determine any possible Slug regulated miRNAs.

Regardless of unanswered questions pertaining to Slug regulation of CXCL8, it is clear that Slug participates in a wide variety of pro-tumorigenic events early in lung cancer development. Besides regulating CXCL8, this study has shown that Slug is capable of inducing invasion, anchorage independent growth, and expression of stem cell associated transcription

factors. This may indicate a more important role for Slug in tumorigenesis than previously thought. This study has shown that Slug is capable of inducing both dissemination and possibly tumor initiation, indicating that Slug may mediate tumorigenesis following the parallel progression model of carcinogenesis.

Current dogma designates a linear progression of carcinogenesis; tumors are initiated at a primary organ and gain a number of somatic mutations as the tumor grows and progresses, allowing for eventual invasion, migration, and finally metastasis to a distant site. The parallel progression model postulates that premalignant and transformed lesions develop the ability to invade and migrate early in tumorigenesis, allowing cells to disseminate to distant sites, before the primary tumor is fully malignant, and develop into a metastatic tumor in parallel with the primary tumor. The two models are not mutually exclusive and there is ample evidence for both clinically [109].

The differences in these models are important to treatment strategies because the parallel progression model predicts a metastatic tumor that is far more different from the primary tumor that the linear model of progression would. Metastases grown under the parallel progression model may be resistant to drugs that the primary tumor is sensitive to, leading to recurrence and resistance to therapy. Previous studies indicate that Slug expression allows for multiple drug resistance, a phenotype commonly associated with its antagonism of p53 mediated apoptosis and regulation of EMT. This study has demonstrated that Slug expression enables non-transformed cells to gain the ability to invade, as seen in the 3D cell culture, and perhaps the ability to survive and initiate a tumor at a distant organ site, as suggested by Slug mediated anchorage independent growth and stem cell gene expression. Slug may be allowing for early dissemination and tumor initiation, indicating it may be a good target for treatment or for use as a biomarker as its expression would may likely be shared by the primary tumor and

any metastasis. Further study into Slug as it pertains to transformation and CSCs may gain insights into the molecular mechanisms of tumor initiation and drug resistance.

Figures and Tables

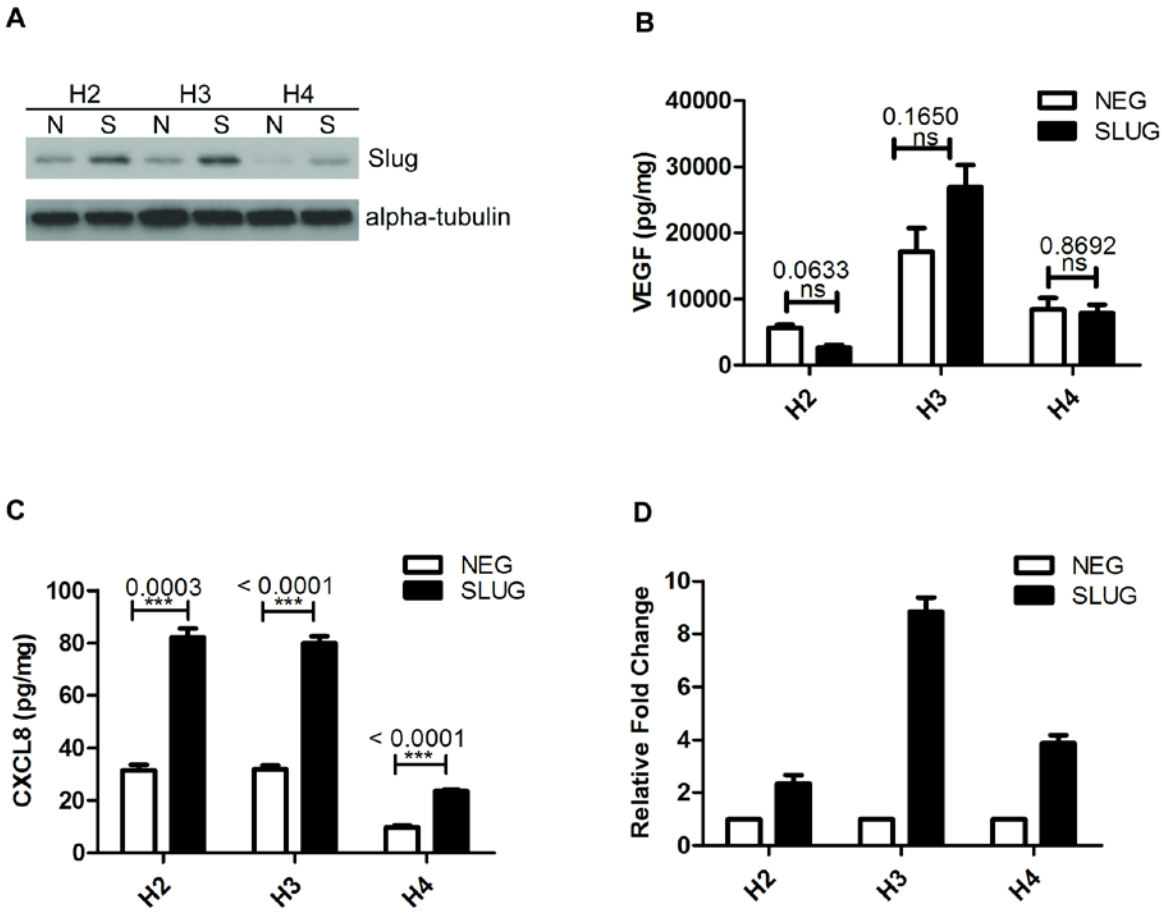


Figure 1. Expression of CXCL8 in Slug overexpressing HBEC cell lines. HBEC2, HBEC3 and HBEC4, designated H2, H3, and H4 respectively. **A)** Western blot of HBEC cell lines generated to overexpress NEG empty vector control, N, or SLUG overexpression plasmids, S. **B)** ELISA of Slug overexpressing cells for VEGF **C)** ELISA of H2, H3, and H4 Slug overexpressing cells for CXCL8 **D)** qPCR of H2, H3 and H4 Slug overexpressing cells for *IL8* gene. Values shown as relative fold change normalized to NEG vector control. **B-C)** p-values calculated using t-test analysis.

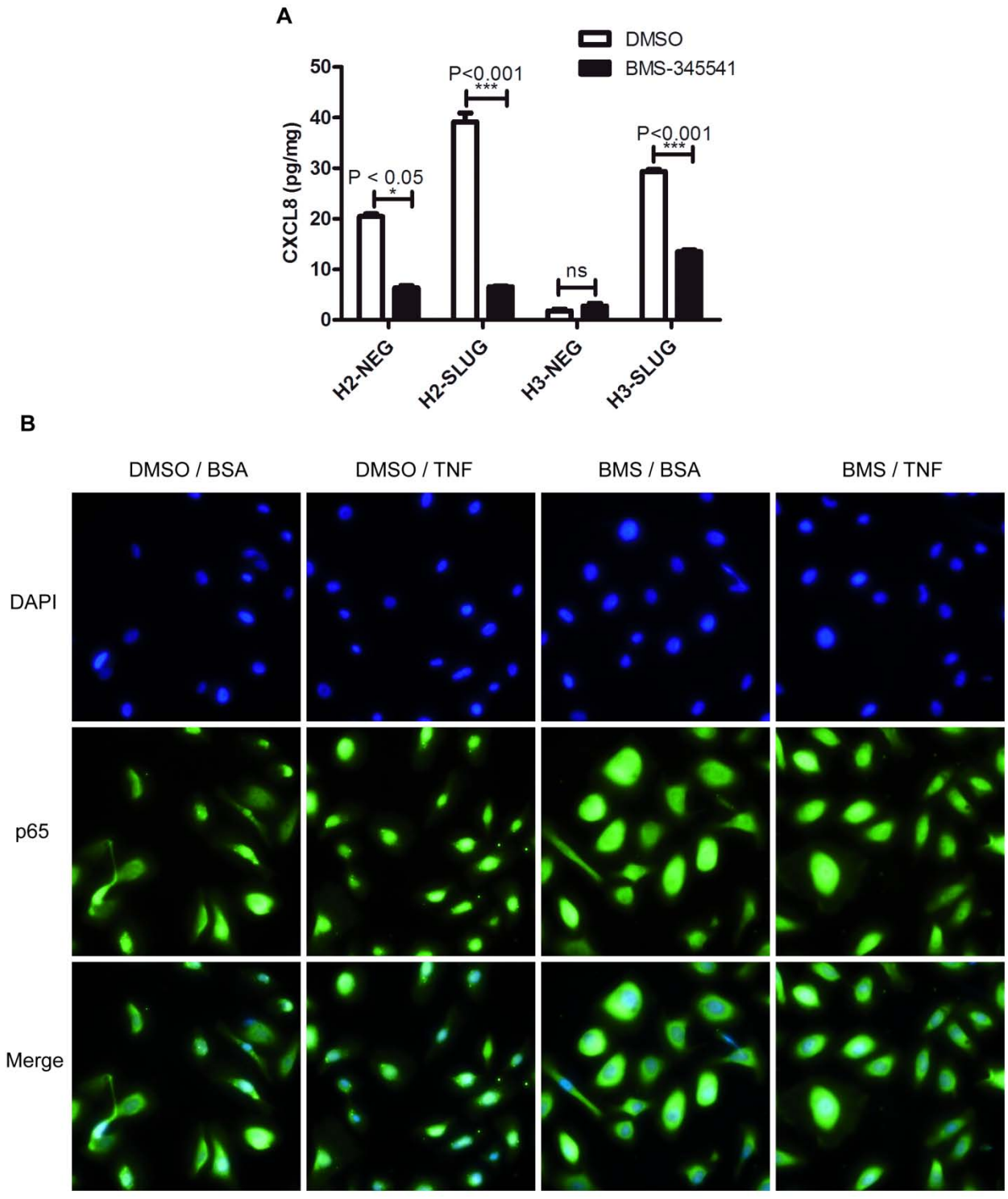


Figure 2. CXCL8 expression is inhibited by the specific NF-κB inhibitor, BMS-345541. A) ELISA of Slug overexpressing cells were treated with 10μM BMS-345541 or DMSO vehicle for 48 hours. p-values calculated using 2-way ANOVA and Bonferroni post-tests. **B)** NEG cell line pre-treated with 10μM BMS-345541 for 45 minutes and treated with 50ng/ml TNF-α for 25 minutes prior to fluorescent staining for p65.

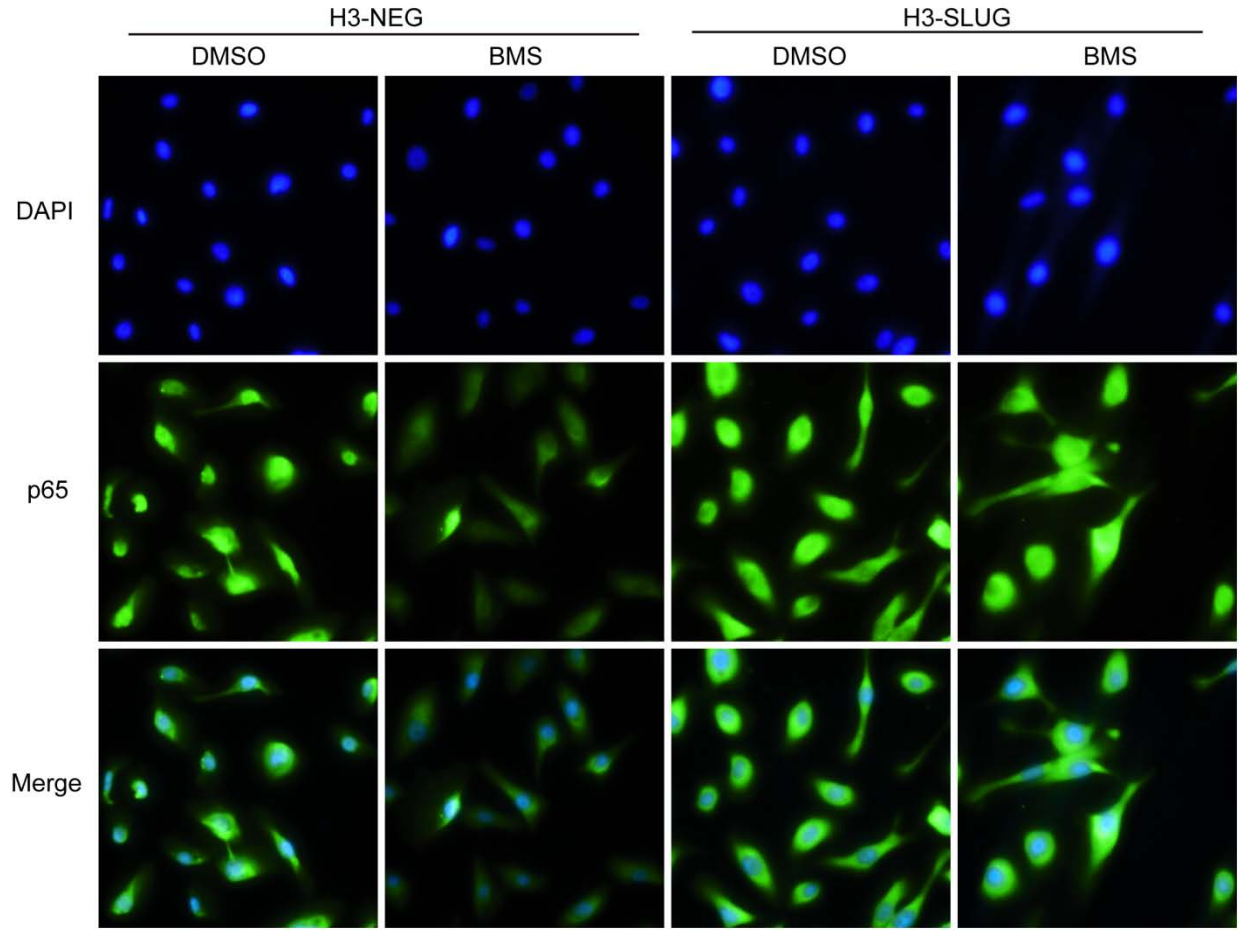


Figure 3. Cellular localization of p65 does not vary between HBEC3 NEG and SLUG cells. HBEC3 Slug overexpressing cells treated with either DMSO vehicle control or 10μM of BMS-345541 for 65 minutes before staining for p65.

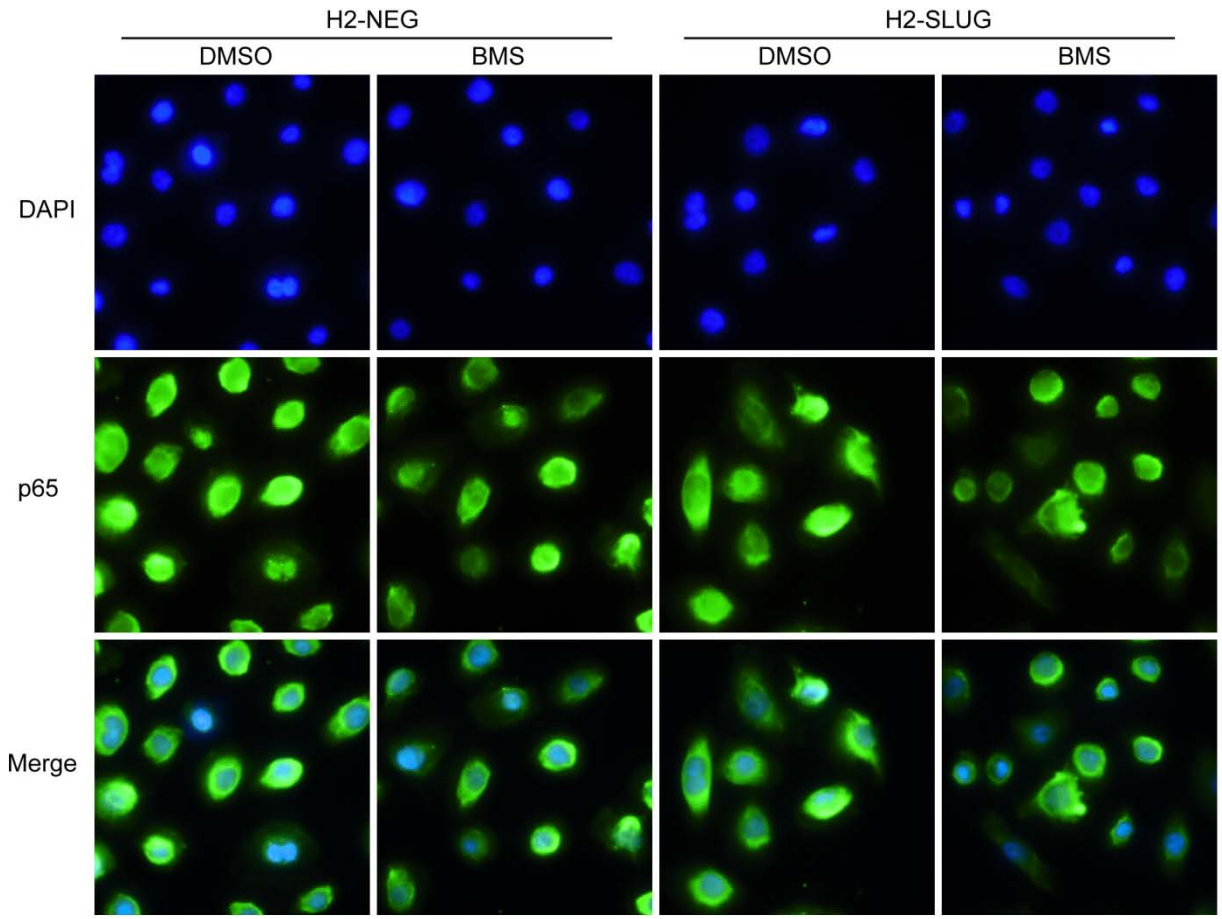


Figure 4. Cellular localization of p65 does not vary between HBEC2 NEG and SLUG cells. HBEC2 Slug overexpressing cells treated with either DMSO vehicle control or 10μM of BMS-345541 for 65 minutes before staining for p65.

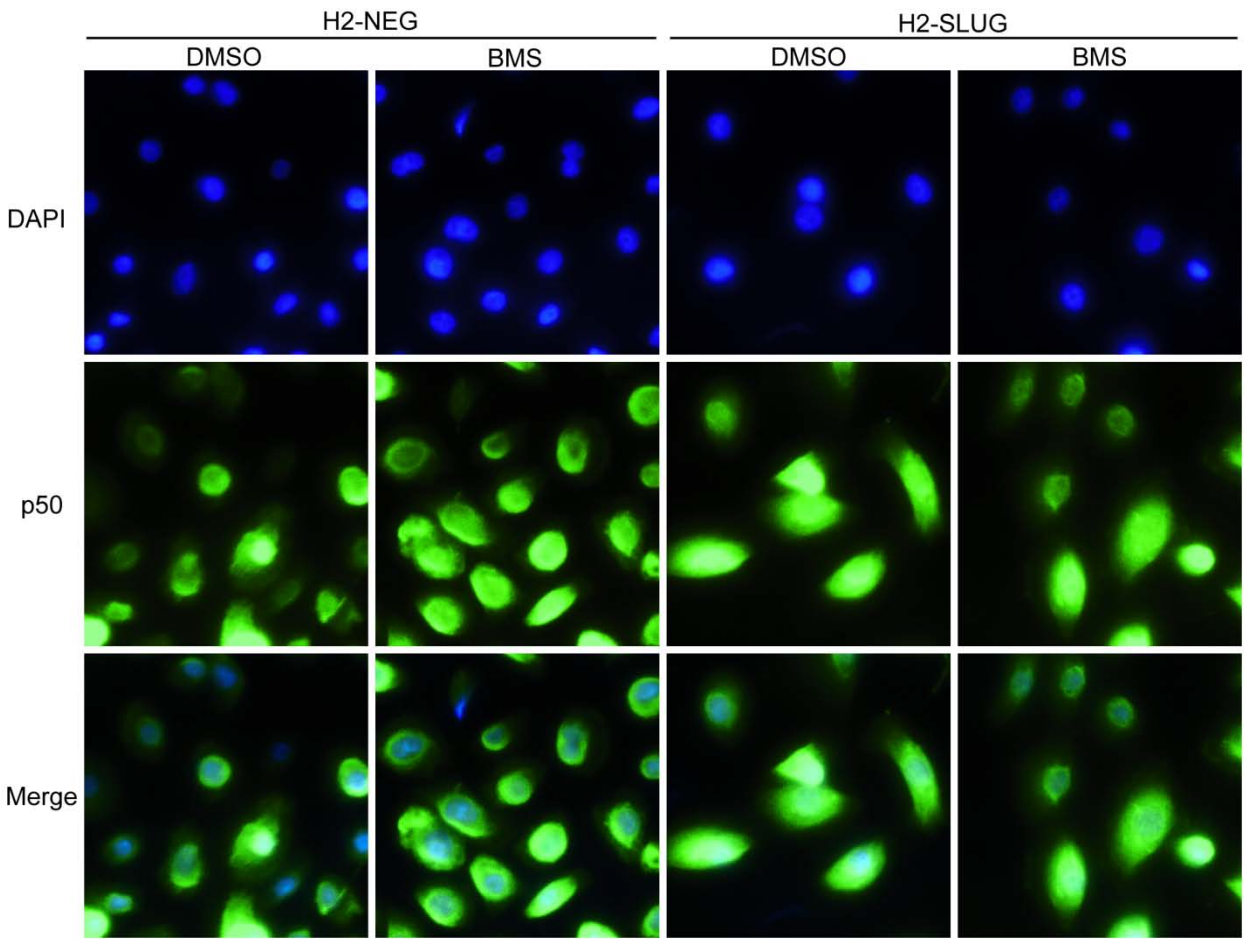


Figure 5. Cellular localization of p50 does not vary between HBEC2 NEG and SLUG cells. HBEC2 Slug overexpressing cells treated with either DMSO vehicle control or 10μM of BMS-345541 for 65 minutes before staining for p50.

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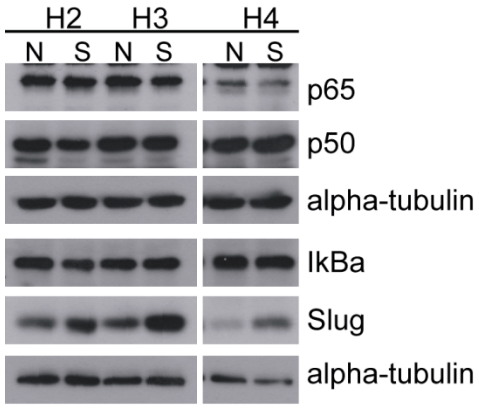

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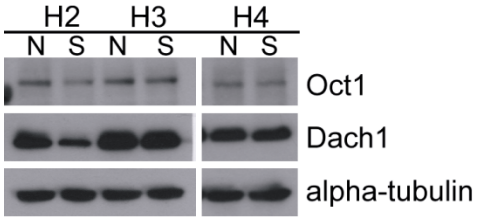

B

>POU2F1:chr1:163886155 [-700..299](+) [human, Homo sapiens]

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Figure 6. Slug does not regulate NF-κB pathway components or negative regulators of *IL8* transcription. A) Western blot for NF-κB pathway components between NEG and SLUG cell lines. **B)** Analysis of *POU2F1* and promoter sequence for Slug DNA binding consensus sequence. Promoter sequence analyzed using Cold Spring Harbor TRED program and validated with NCBI Blast. **C)** Western blot of comparing transcriptional regulators of *IL8*.

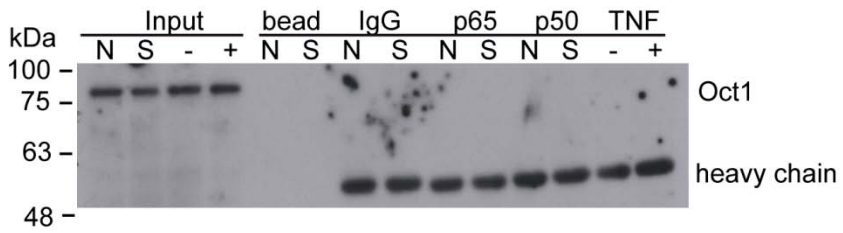

Figure 7. Oct1 does not interact with p65 or p50 subunits. Co-immunoprecipitation assay of HBEC3 SLUG and NEG cell lines. Immunoprecipitation: p65 and p50 as indicated, Western Blot: Oct1, Input protein: whole cell lysates, Controls: bead control samples were immunoprecipitated without antibody, TNF-α control were H3-NEG cells were treated with 50ng/ml TNF-α for 25 minutes in order induce localization of p65 to the nucleus.

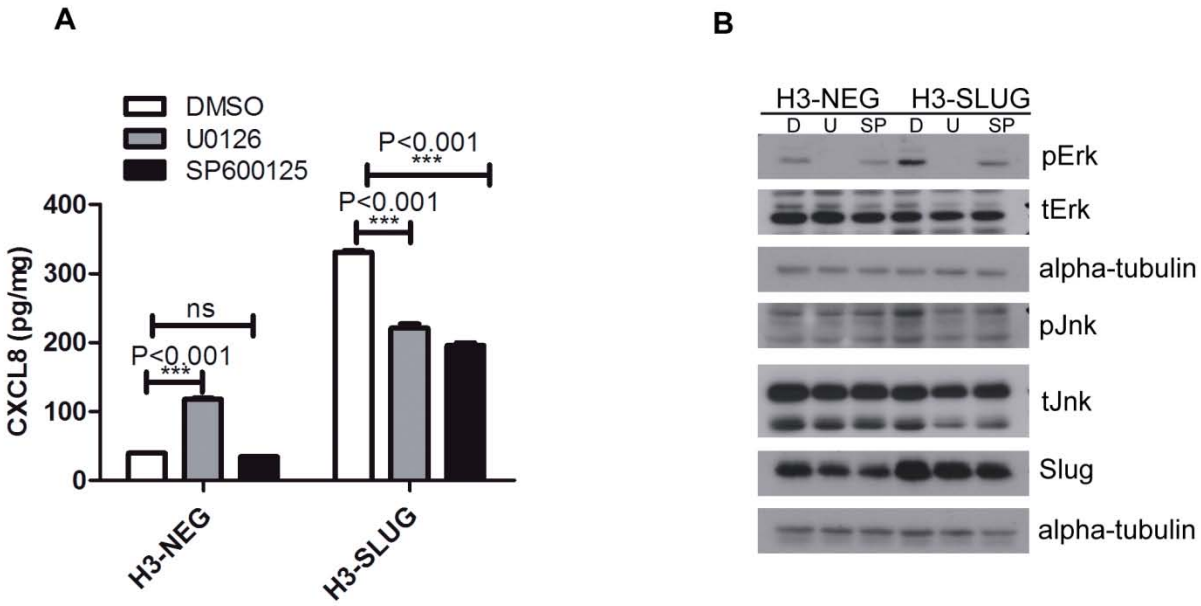

Figure 8. Erk and Jnk inhibition decrease Slug mediated CXCL8 expression. A) ELISA of HBEC3 Slug expressing cells treated with 15μM U0126 or 10μM SP600125 for 48 hours. p-values calculated using 2-way ANOVA and Bonferroni post-tests. **B)** Western blot of H3 Slug expressing cells treated with 15μM U0126 or 10μM SP600125 for 60 minutes.

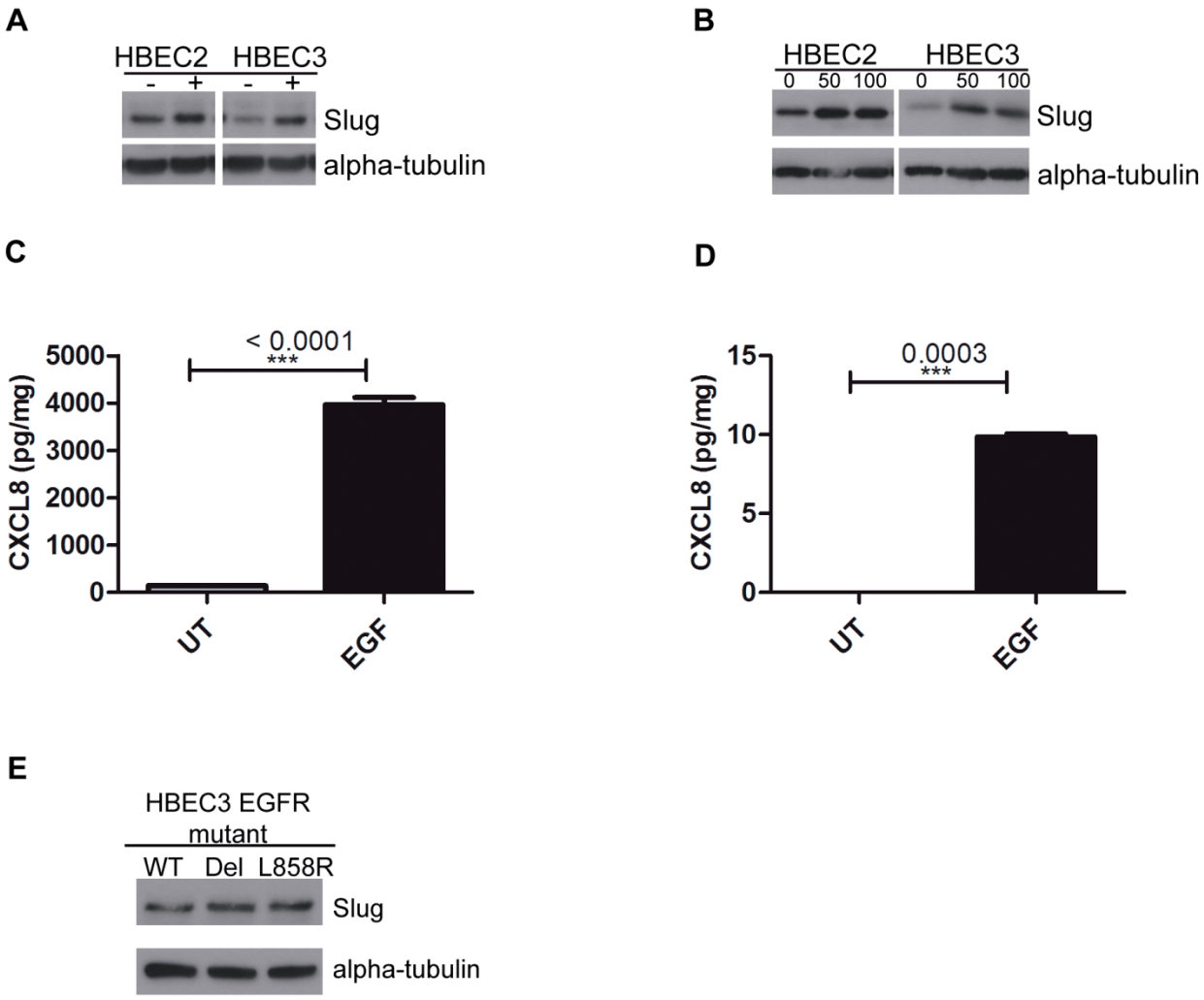

Figure 9. EGF induces both Slug and CXCL8 expression HBEC3 cells but EGFR activating mutations do not. **A)** Western blot of HBEC2 and HBEC3 cells treated with 5ng/ml TGF-β for 24 hours. **B)** Western blot of HBEC2 and HBEC3 cells treated with 0, 50, or 100ng/ml EGF for 48 hours. **C-D)** ELISA of HBEC2 and HBEC3 cells treated with 50ng/ml EGF for 48 hours. **E)** Western blot of HBEC3 cells overexpressing WT, Exon 19 deletion, or L858R EGFR mutations.

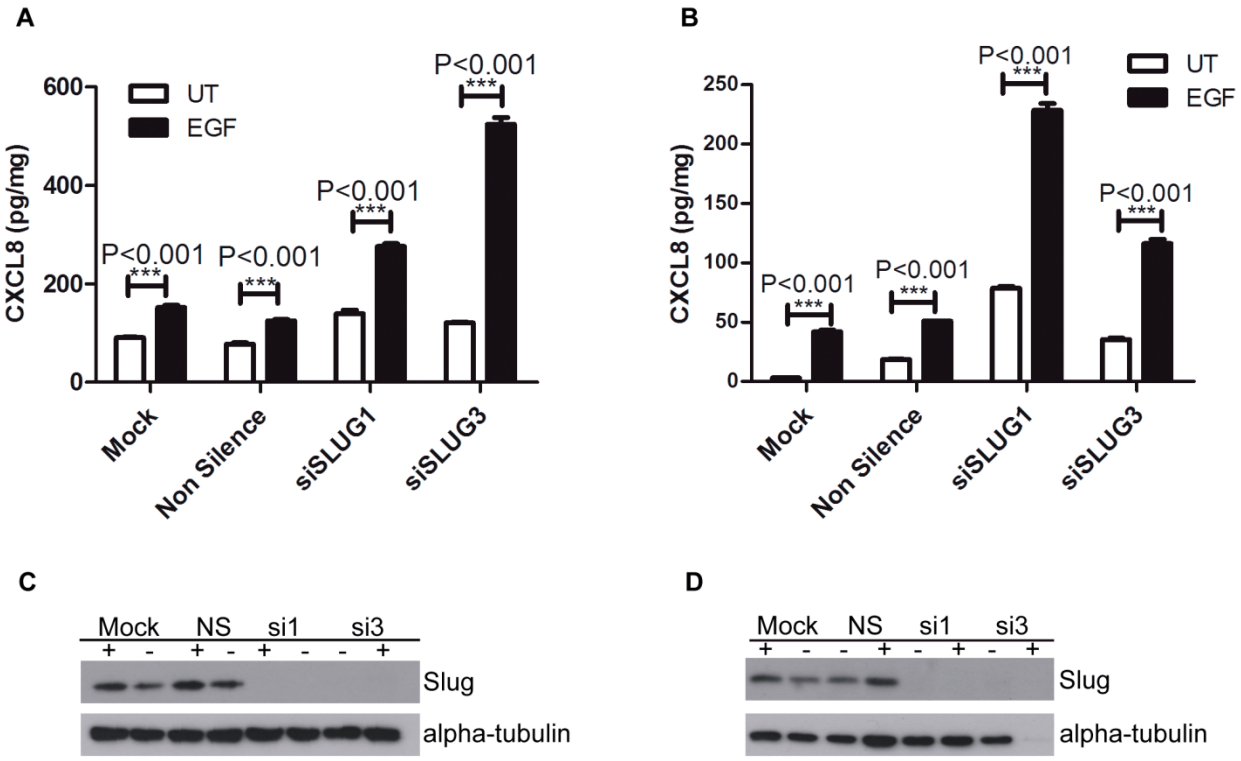

Figure 10. Knock down of Slug expression does not prevent EGF mediated CXCL8 expression. A and C) HBEC2 cells treated with siRNA sequences specific to Slug and treated with 50ng/ml EGF for 48 hours. **B and D)** HBEC3 cells treated with siRNA sequences specific to Slug and treated with 50ng/ml EGF for 48 hours. **A and B)** p-values calculated using 2-way ANOVA.

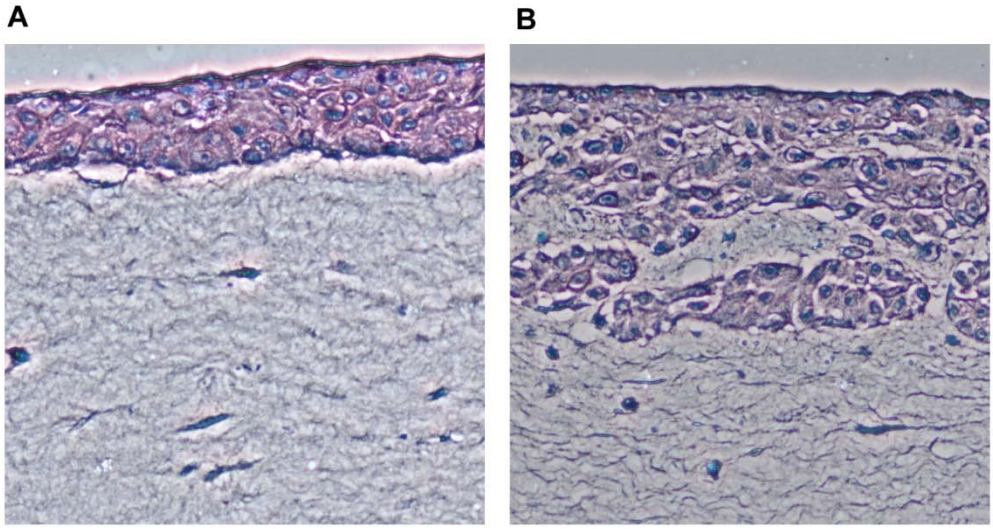

Figure 11. Slug overexpression increases invasive ability of HBEC3 cells. Slug overexpressing cell lines were cultured in 3D lung organotypic culture for 12 days. **A)** H3-NEG **B)** H3-SLUG

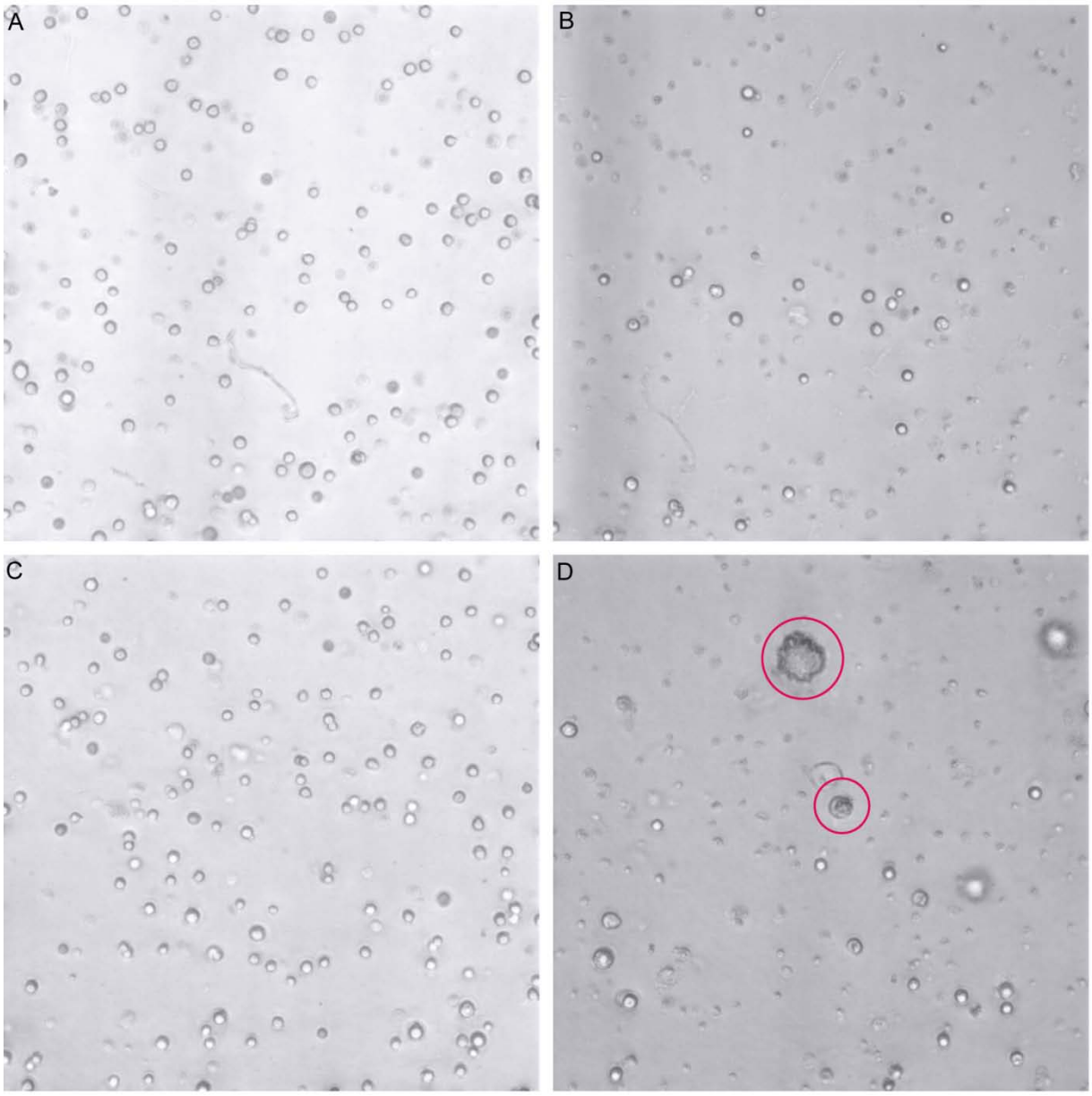

Figure 12. Slug overexpression induces anchorage independent growth in HBEC2 cells. Anchorage independent growth assay of HBEC2 Slug overexpressing cells. Images taken of 30 consecutive planes per well and compressed into a single focused image of all planes. **A)** H2-NEG day1 **B)** H2-NEG day 21 **C)** H2-SLUG day 1 **D)** H2-SLUG day 21.

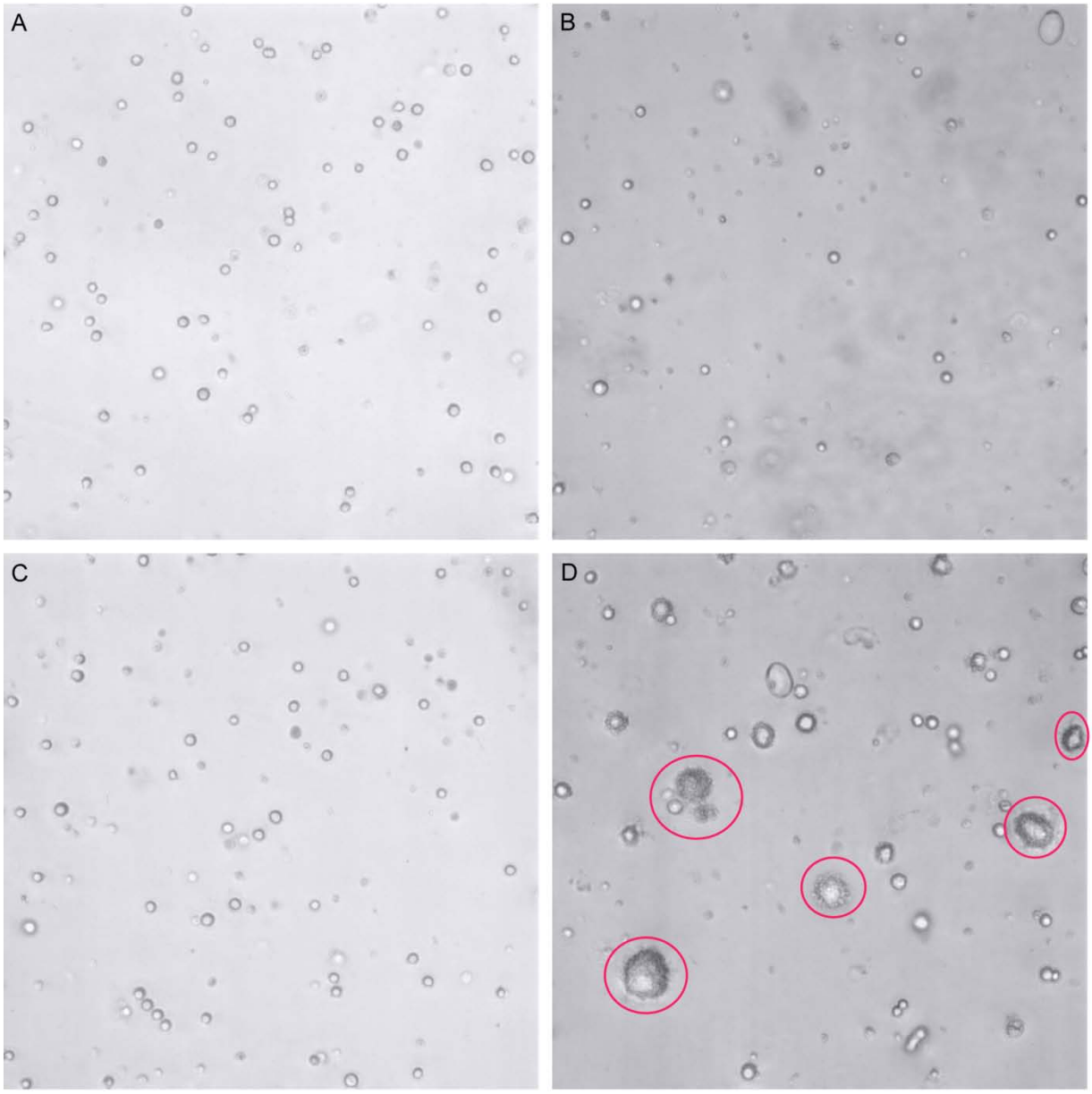

Figure 13. Slug overexpression induces anchorage independent growth in HBEC3 cells. Anchorage independent growth assay of HBEC3 Slug overexpressing cells. Images taken of 30 consecutive planes per well and compressed into a single focused image of all planes. **A)** H3-NEG day1 **B)** H3-NEG day 21 **C)** H3-SLUG day 1 **D)** H3-SLUG day 21.

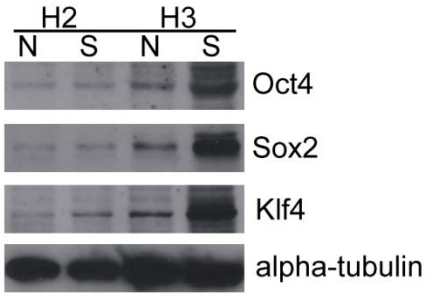

Figure 14. Slug overexpression induces genes associated with maintaining a stem cell phenotype. Western blot of HBEC2 and HBEC3 Slug overexpressing cells.

CHAPTER 2: The Effects of Hepatocyte Growth Factor in Non Small Cell Lung Cancer

Introduction

Hepatocyte Growth Factor

Hepatocyte Growth Factor (HGF) and its receptor c-Met are only found in vertebrates and are necessary for organ branching morphogenesis and limb and central nervous system development. HGF was first discovered as a “scatter factor” based on its ability to scatter epithelial cells grown in culture, as well as act as a morphogen and a liver growth factor [110]. HGF is secreted by fibroblasts and other mesenchymal cells which are located in close proximity to Met expressing epithelial cells. In normal adult tissues, HGF levels are increased following liver, kidney, or heart injury. In cancer it is known to induce EMT as well as angiogenesis, proliferation, and anti-apoptosis [111]. In the lung tumor microenvironment, HGF is commonly secreted by stromal cells or infiltrating neutrophils, bound to in the extracellular matrix, or produced by tumor cells to induce an autocrine signal [111,112]. Upon ligand binding, c-Met is capable of activating a number of signaling cascades which includes but is not limited to Rac/Rho, PI3K/Akt, and MAPK/Erk. Besides autocrine signaling, constitutive activation of c-Met can be achieved by gene amplification, allowing for constitutive dimerization and autophosphorylation of c-Met. Increased HGF and Met amplification are implicated in EGFR-TKI resistance, as both HGF/Met signaling and Met amplification are both able to activated PI3K/Akt signaling that allows bypass of activated EGFR pathway [61,113]. Aberrant HGF/c-Met signaling is common to many epithelial cancers including lung, colon, and breast cancers, and is an independent prognostic indicator of poor survival [21,114,115]. HGF is found to be elevated at all stages of NSCLC, and is associated with increased recurrence and metastasis regardless of tumor stage [116].

Sim1

Single Minded 1 (Sim1) is the mammalian homologue of the *Drosophila* dSim, which controls central nervous system development and tubular structures such as airway passages in flies. Mammalian and fly Sim proteins, which are highly conserved, are part the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors which, in mammals, includes Sim1, Single Minded 2 (Sim2), Arylhydrocarbon Receptor (AhR), Hypoxia Inducing Factor 1α (HIF-1α), and the Arylhydrocarbon Receptor Nuclear Translocater (Arnt). This family of transcription factors heterodimerize with Arnt in order to regulate gene expression. Members of the bHLH/PAS family of transcription factors can regulate activity of other family members through binding competition for Arnt [117,118].

Sim1, which is expressed in mammalian lung, brain, kidney, and skeletal muscle tissues, has been shown to activate genes associated with hypoxia under normoxic conditions [118]. Under hypoxic conditions, cells rapidly increase HIF-1α expression by preventing its degradation through the ubiquitin–proteosome pathway. Stabilization of HIF-1α allows for it to dimerize with Arnt (also known as HIF-1β) and bind to the Hypoxia Response Element (HRE) in promoters. HRE binding and activation by HIF-1α activates genes associated with angiogenesis, cell proliferation, survival, and glucose metabolism [119]. Sim1, which is known to bind the Central Midline Enhancer (CME) sequence, can also bind to HRE sequences due to their identical core sequences [120]. Sim1 has been shown to induce the erythropoietin gene, *EPO*, under normoxic conditions by binding to the HRE in its promoter. Erythropoietin is an angiogenic hormone that regulates the hematopoiesis of erythroid cells (red blood cells) [121]. The ability of Sim1 to regulate erythropoietin indicates that Sim1 has the potential to induce angiogenesis independent of hypoxic signaling; an ability that may be subverted by cancer if Sim1 expression becomes dysregulated.

Runx3

Runx3 is another transcription factor that has been dysregulated in cancer. It is a member of the Runt family of transcription factors, which also includes Runx1 and Runx2, and is located at chromosome 1p36.11-1p36.13. Frequent deletions of the short arm of chromosome 1p, mapped between 1p36.1 and 1p36.2, where Runx3 is located, have been observed in lung cancer. Re-expression of this region of chromosome 1 has been shown to reduce carcinogenesis [122]. This reduction in carcinogenesis may be in part related to Runx3 expression. Although no functional studies of Runx3 have been performed in lung cancer specimens, it is known that loss of Runx3 in gastric cancer prevents apoptosis of gastric epithelial cells [123]. Loss of Runx3 in NSCLC tumor lines may allow for greater survival and cell proliferation.

MicroRNA

MicroRNAs (miRNA) are small non-coding RNA sequences that bind to 3' UTR sequences of mRNA and either block protein translation or lead to mRNA degradation. Primary miRNAs (pri-miRNA) are expressed as polycistronic RNAs that are then cleaved by the RNase III enzymes, Drosha and Dicer, to become mature miRNAs between 18-24nt long [124]. Their short 2-7nt seed sequences are capable of binding with partial complementation. This permissive binding ability allows for a single miRNA to effect large numbers of diverse targets across many pathways and cellular functions [125,126]. miRNA sequences have been implicated in cancer progression, acting as both tumor suppressors and oncogenes by inhibiting mRNA targets related to cell proliferation, apoptosis, and cell differentiation [127].

Mir-155 is an oncogenic miRNA that is located within the BIC gene. BIC is not highly conserved among species, except for the 138bp region encoding mir-155. BIC is regulated by Myc, which is a transcription factor often upregulated in cancer and allows for uncontrolled

growth [127,128]. Although miRNA expression tends to be tissue specific, mir-155 has been shown to be increased in Burkett and Hodgkin's lymphomas, breast cancer, and lung cancer [106,127,129,130,131,132]. Mir-155 along with another miRNA, mir-146b, have been shown to predict overall survival in lung cancer patients [130].

Along with mir-155, the mir-17-92 cluster of miRNAs have been found to be overexpressed in a similar set of malignancies; B-cell lymphomas, breast, lung, and hepatocellular carcinoma . This is not surprising as both mir-155 and the mir-17-92 cluster are regulated by Myc [127,133]. Myc directly regulates the primary mir-17-92 transcript, a polycistronic pri-miRNA that encodes mir-17-5, mir-17-3p, mir-18a, mir-19a, mir-20a, mir-19b-1, and mir-92-1, which are overexpressed in different combinations in different tumor types [134]. The mir-17-92 cluster as a whole has been shown to induce proliferation, survival, and angiogenesis in cancer [135]. Specifically in lung cancer, mir-20a and mir-17-5p have been shown to promote apoptosis resistance when overexpressed in NSCLC cells [129].

Materials and Methods

Cell Culture

HBEC3, HBEC4, HBEC7 and HBEC3 mutant Kras and Vector cell lines were a kind gift from Dr. John D. Minna. A427, A549, H292, H358, H1299, H1650, and H2122 cell lines were purchased from ATCC. HBEC cells were cultured in KSFM supplemented with 0.2ng/ml rEGF and 30µg/ml Bovine Pituitary Extract (Life Technologies) at 37ºC and 5% CO_2. HBEC cells are trypsinized in 0.25%Trypsin-EDTA and neutralized using Trypsin Neutralization Solution (Lonza). NSCLC cell lines were maintained in RPMI 1640 (Cellgro) supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Life Technologies). All cell lines were tested for mycoplasma contamination using MycoAlert Assay (Lonza). All cell lines were genotyped using the Cell ID System (Promega) at the UCLA Genotyping Core. Genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) and amplified using AmpliTaq Gold DNA Polymerase (Roche).

Generation of HBEC mutant Kras Cells

HBEC4 and HBEC7 cell lines were plated in 6 well plates and pSRZ-KrasV12 and pSRZ empty vectors, which were a kind gift from Dr. John D. Minna, were delivered to the UCLA Vector Core where viral production and transduction were performed. Transduced cells were selected in Puromycin for 14 days and analyzed by RFLP to determine expression of mutant KrasV12.

Gene and MicroRNA Array Profiling

Gene array and microRNA array profiling were performed at the UCLA Clinical Microarray Core. Gene array was performed using Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Gene array chip images were analyzed using dChip DNA Chip analysis program [136]. MicroRNA array profiling was performed using Exiqon miRCURY LNA microRNA Array.

MicroRNA chips were aligned by hand and scanned by UCLA Clinical Microarray Core. Data was filtered to eliminate signal below noise levels based on the mean fluorescence of negative controls. Mean fluorescent intensities of quadruplicate spots were determined. Normalization factors were calculated by dividing median intensity by mean intensity of each sample. Signal intensity for each miRNA sample was normalized using Normalization Factor and analyzed with dChip.

RNA Isolation and miRNA qPCR

RNA was isolated using the miRNeasy kit (Qiagen) and QIAshredder columns (Qiagen). Cells were plated in T25 or T75 flasks and treated with 40ng/ml HGF for 24 hours. RNA was isolated using microRNeasy RNA isolation kit (Qiagen) according to manufacturer's protocol. Reverse Transcription and qPCR miRNA specific primers were included in Taqman MicroRNA Assay (Applied Biosystems). Reverse Transcription reactions were carried out using Taqman MicroRNA Reverse Transcriptase kit (Applied Biosystems). qPCRs were performed using Taqman Universal Master Mix II, no UNG (Applied Biosystems) on iCycler PCR machine (BioRad) and delta delta Ct analysis was performed with iQ5 software (BioRad).

Western Blotting

Whole cell protein lysates were collected using Radio-Immunoprecipitation Assay (RIPA) buffer supplemented with 1% Triton-X 100, Complete Protease Inhibitor Cocktail (Roche), Sodium Fluoride, activated Sodium Orthovanadate, and phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined using BCA assay (Pierce). Immunoblotting was carried out on 8-10% SDS-PAGE gels and transferred to PVDF membrane (Millipore). Blots were blocked in either 5% milk or 5% Bovine Serum Albumin in Tris Buffered Saline containing 0.05% Tween-20 (Sigma) for 1 hour. Following antibody incubations, blots were developed using either Western Lightning Plus ECL (Perkin Elmer) or SuperSignal Chemiluminescent (Pierce) followed by

exposure to film. Antibodies were purchased as follows: Sim1 (Sigma), Runx3 (Active Motif), Alpha-tubulin (Cell Signaling).

Results

Gene Array

To determine genes regulated by HGF/c-Met signaling, seven NSCLC and HBEC3 mutant Kras and vector cells were analyzed by gene array and miRNA profiling. Kras mutant HBEC cells were included because Kras is commonly mutated and constitutively activated in NSCLC. Both HGF signaling and Kras are present in tumors and both are associated with drug resistance, specifically EGFR-TKI drug resistance [8,113]. Cell lines with constitutive Met phosphorylation were excluded from array analysis. HBEC3 mutant Kras and Vector and NSCLC cell lines were treated with 40ng/ml HGF for 24 hours. Total RNA including small RNAs were isolated and subjected to gene array and microRNA array profiling at the UCLA Clinical Microarray Core. Several genes were changed between HGF and untreated controls, but the direction and specific genes that changed were not consistent across cell lines. Two genes, Sim1 and Runx3, were selected for validation by western blot analysis due to mild fold changes in the same direction across three or more cell lines (Table 1).

Expression of Sim1 and Runx3 after HGF treatment

Sim1 and Runx3 were analyzed by western blot. Sim1 does not change in NSCLC cells (Fig. 1A). Mild increases in Sim1 were only seen in H1299, while Sim1 remains unchanged in the remaining NSCLC cell lines. In HBEC3 mutant Kras cells, Sim1 decreased following HGF treatment relative to untreated mutant Kras cells. Sim1 expression did not change in HBEC3 mutant Vector cells; although there was an overall increase in Sim1 expression between untreated Vector and Kras HBEC3 cells. Unfortunately, this pattern was not consistent with other HBEC mutant Kras cell lines (Fig. 1B). Runx3 protein was not expressed in any of the NSCLC cell lines assayed (Fig. 1C). Antibody was validated with Raji cell lysates, a positive control suggested by the antibody manufacturer.

MicroRNA Array

To determine if HGF regulated any miRNA sequences, miRNA array profiling was performed. Mild fold changes were seen in a handful of microRNAs, including mir-155 and mir-20a, which have both been proposed function in an oncogenic capacity in different cancer types, including lung cancer (Table 2). The remaining two miRNAs, mir-874 and mir-7-2*, have no known function and are not associated with any cancer types. Mir-155 and mir-20a were chosen for qPCR validation because both miRNAs are known to be increased in lung cancer they both had mild fold changes in two or more cell lines.

Expression of mir-155 and mir-20a after HGF treatment

No detectable mir-155 could be found in A427 or H358 cell lines. Mild increases in miR-155 occurred in A549 and H1650 cell lines, but not above 1.5 fold, which is not considered significant (Fig. 2A). HBEC2, HBEC4, and HBEC11 parental cell lines were also tested for mir-155 expression following HGF treatment. No significant change in mir-155 expression was found in these cell lines either (Fig. 2B).

Next NSCLC cell lines were tested for mir-20a. No significant change was found in the majority of the cell lines tested; H292, H358, H1299, and H2122 (Fig. 2C). A549 had a clear induction of mir-20a, greater than 2 fold, but it was the only cell line with any significant change.

Discussion

Increased expression of HGF and c-Met amplification are important mediators of tumorigenesis and drug resistance. Here, several NSCLC cell lines were exposed to HGF and their gene and miRNA signatures were analyzed. The NSCLC cell lines tested were of different cancer subtypes and mutational backgrounds, which may have clouded the array studies. Individual cell lines had different mutations, and although some mutations were common to multiple cell lines, such as Kras activating mutations present in A549 and H358, versus p53 deletions present in H1299 and H1650, no two NSCLC lines had the same mutational background. This heterogeneity, which is a hallmark of tumor cells, may affect each cell line's response to HGF and ultimately the gene and miRNA array data. This variation in cellular background may have led to the convolution of array data. Gene array analysis indicated several genes changed in response to HGF, but none consistently among all, or even a small group, of the cell lines tested. Here cell lines were selected on the basis of molecular response to HGF; markers of EMT were analyzed prior to gene array analysis. Although each cell line expressed c-Met and responded to HGF by induction of mesenchymal markers such as Vimentin or N-cadherin, or repression of epithelial markers such as E-cadherin and γ–catenin, none of the cell lines induced the exact same set of EMT markers (data not shown). Although this study did not yield any novel targets of HGF mediated tumorigenesis, HGF is known to affect carcinogenesis through its promotion of proliferation, invasion, motility, angiogenesis, and survival. Further study of HGF in NSCLC using gene array analysis or RNA sequencing techniques (RNAseq) should include larger numbers of NSCLC cell lines in order to increase the statistical power of such a study.

Figures and Tables

	H1650	H2122	A549	H292	H358	H3M Vector	H3M KRAS
Gene	***Fold Change***						
RUNX3	-1.06	27.24	-9.21	1.84	1.61	-1.69	-3.72
SIM1	-12.53	-7.19	-3.27	4.14	-1.07	-2.47	2.03

Table 1. Gene array profiling of NSCLC cell lines treated with HGF. Gene array profiling of NSCLC cell lines treated with 40ng/ml HGF for 24 hours. Differential expression of total mRNA from gene array analyzed using dChip program shown as fold change compared to untreated controls.

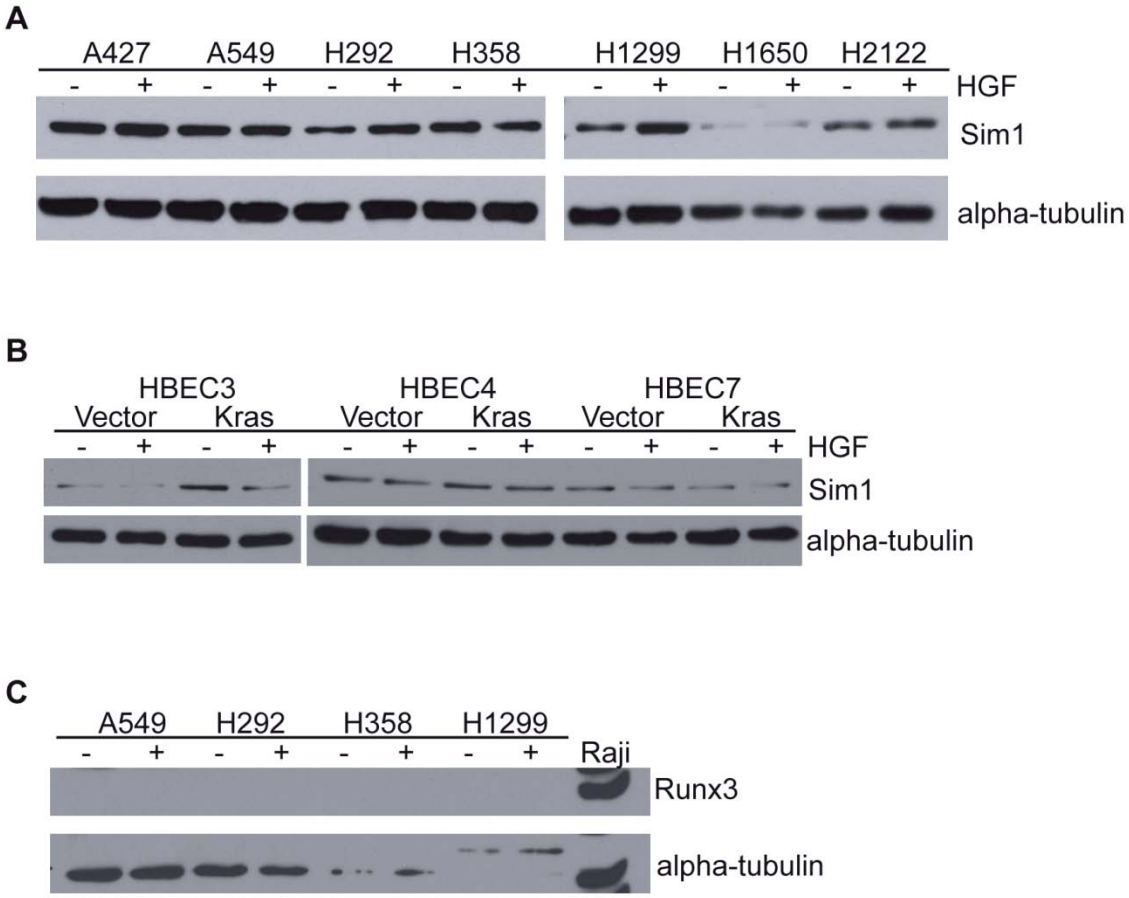

Figure 1. Sim1 does not consistently change among HGF treated NSCLC and HBEC cell lines. NSCLC and HBEC cell lines were treated with 40ng/ml HGF for 24 hours and analyzed by western blot. **A)** NSCLC cell lines blotting for Sim1 expression **B)** HBEC cell lines expressing mutant KrasV12 and matching empty vector controls blotting for Sim1 expression **C)** NSCLC cells blotting for Runx3 expression

	A427	A549	H292	H358	H1299	H1650	H2122	H3M Vector	H3M KRAS
Probe	***Fold Change***								
miR-155	1.15	2.06	2	1.45	2.23	-1.04	1.11	-1.19	-1
miR-20a	1.14	-2.07	1.07	1.22	-1.87	1.03	1.2	-1.01	1.17
miR-874	-1.12	1.11	-1.26	2.31	1.8	-1.04	-1.03	1.09	2.01
miR-7-2*	1.11	2.06	1.81	1.38	2.58	1.1	1.27	-1.15	1.28

Table 2. MicroRNA Array profiling of NSCLC cell lines treated with HGF. MicroRNA array profiling of NSCLC cell lines treated with 40ng/ml HGF for 24 hours. Differential expression of miRNA array analyzed using dChip program shown as fold change compared to untreated controls.

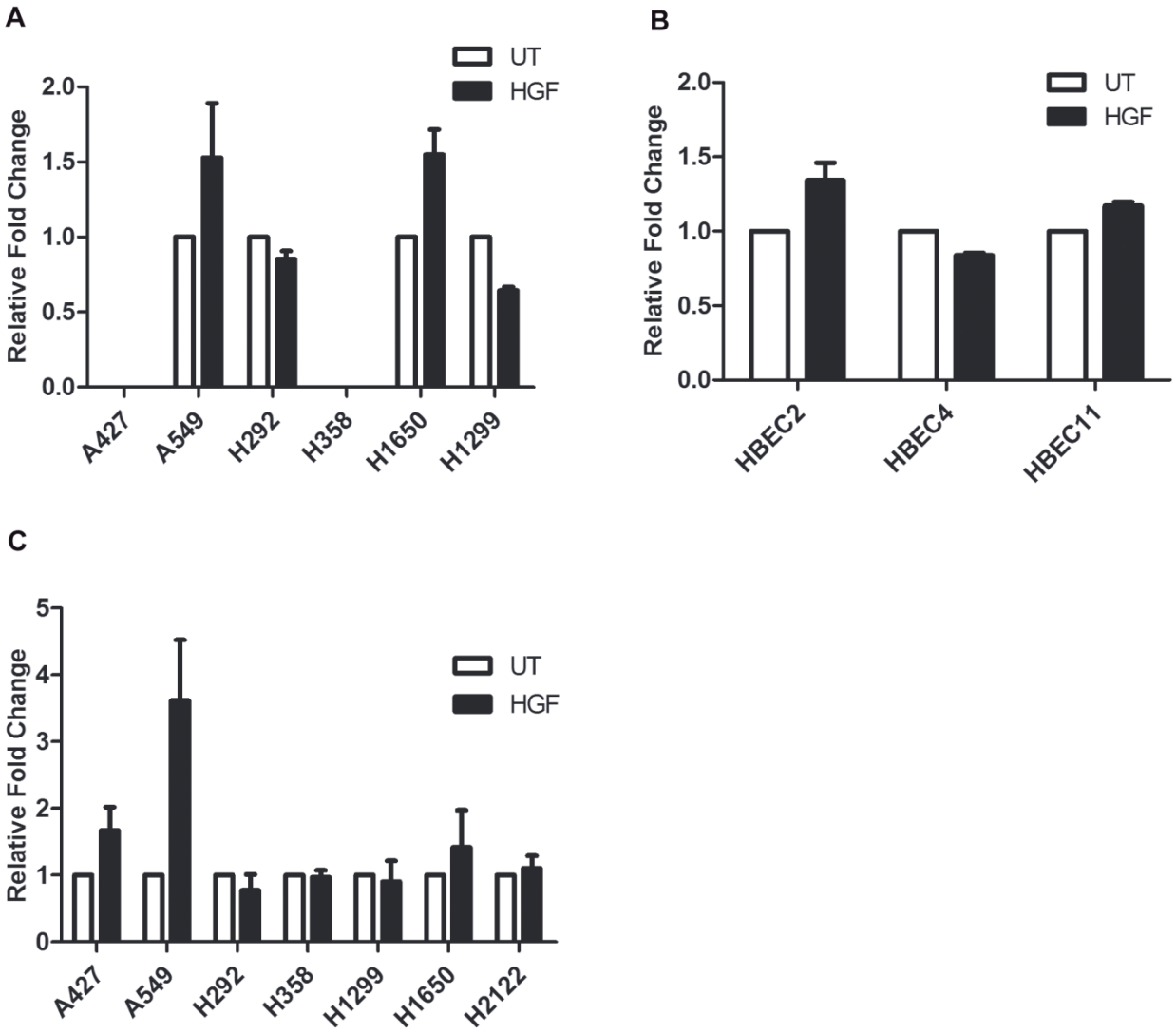

Figure 2. Mir-155 and mir-20a expression levels do not change following HGF treatment. RNA samples from cells treated for 24 hours with 40ng/ml HGF and analyzed using miRNA specific qPCR. **A)** miR-155 expression in NSCLC cells, **B)** miR-155 expression in HBEC cells, **C)** miR-20a expression in NSCLC cells.

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