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## Snail Induces Mesenchymal Transition and Promotes EGFR TKI Resistance in NSCLC Cells Harboring EFGR Kinase Domain Mutations

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LOYOLA UNIVERSITY CHICAGO

SNAIL INDUCES MESENCHYMAL TRANSITION AND PROMOTES EGFR TKI  
RESISTANCE IN NSCLC CELLS HARBORING EGFR KINASE DOMAIN  
MUTATIONS

A THESIS SUBMITTED TO  
FACULTY OF THE GRADUATE SCHOOL  
IN CANDIDANCY FOR THE DEGREE OF  
MASTER OF SCIENCE

MOLECULAR PHARMACOLOGY AND THERAPEUTICS

BY

RUTU S. GANDHI

CHICAGO, IL

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*To my wonderful parents for  
their love and support*

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## LIST OF ABBREVIATIONS

AB/AM	Antibiotic / Antimycotic
ALK	Anaplastic Large cell Lymphoma Kinase
CT	Computed Tomography
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EML4	Echinoderm Microtubule Associated Protein-Like 4
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular signal Regulated Kinase
FITC	Fluorescein isothiocyanate
GEM	Gemcitabine
GFP	Green Fluorescent Protein
GPCR	G-Protein Coupled Receptor
GSK3- $\beta$	Glycogen Synthase Kinase 3 beta
Hsp90	Heat Shock Protein 90
MAPK	Mitogen Activated Protein Kinase
mTOR	Mammalian Target Of Rapamycin
NSCLC	Non-Small Cell Lung Cancer
PBS	Phosphate Buffered Saline

PI3K	Phosphatidylinositol 3- Kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PTEN	Phosphatase and tensin homolog
RTK	Receptor Tyrosine Kinase
SCLC	Small Cell Lung Cancer
SCP	Small C-terminal domain phosphatase
S6	Ribosomal protein S6
TGF- $\beta$	Transforming Growth Factor beta
TKI	Tyrosine Kinase Inhibitor
VEGF	Vascular Epidermal Growth Factor

## **ABSTRACT**

Lung cancer is the leading cause of cancer related death, accounting for one third of all deaths from cancer worldwide. About 85-90% of lung cancers are non-small cell lung cancer (NSCLC). Treatment approach for NSCLC varies depending on the stage of cancer, overall health of the patient, lung function and symptoms. The common treatment options involve surgery, radiation therapy or chemotherapy involving combination of platinum based compounds. Chemo and radio-therapy offers short-term improvement in disease related symptoms in patients with advanced NSCLC; however the impact of chemo and radio-therapy on quality of patient's life remains a major concern.

Molecular targeted therapies provide a treatment option in addition to conventional cancer treatments. The efficacy of the targeted therapies remains restricted to the cancer cells with oncogene addiction. Oncogene addiction refers to the phenomenon in which cancer cells that contain multiple genetic and epigenetic changes remain addicted to one or a few genes for both maintenance of the malignant phenotype and cell survival. One such oncogene that has been extensively targeted for treatment of NSCLC is mutant epidermal growth factor receptor (EGFR), which is effectively blocked by EGFR tyrosine kinase inhibitors (TKIs). However, the development of resistance to EGFR TKIs continues to be the major limitation in the treatment of NSCLC. Resistance to EGFR TKIs in several

cancers including NSCLC has been associated with the loss of canonical epithelial protein E-cadherin, suggesting involvement of EMT in conferring EGFR TKI resistance. Transcription factors are known to play a central role in inducing EMT.

TGF- $\beta$ , a secreted protein and a potent inducer of EMT is known to directly activate transcription factor SNAIL to exert its effect. Our lab previously found that transcription factor SNAIL was significantly upregulated in *in vitro* model of EMT generated by chronic exposure of EGFR mutant NSCLC cell line to TGF- $\beta$ . Further, TGF- $\beta$  exposed EGFR mutant NSCLC having high SNAIL expression were resistant to EGFR TKIs. Moreover, withdrawal of TGF- $\beta$  exposure resulted in reversal of mesenchymal phenotype and restored sensitivity of EGFR mutant NSCLC to EGFR TKIs. Consequently, we hypothesized that SNAIL induced mesenchymal transition promotes EGFR TKI resistance in EGFR mutant NSCLC. We further hypothesized that acquired EGFR TKI resistance can be reversed by promoting epithelial phenotype. To test our hypothesis, we developed an *in vitro* model of EMT by ectopically overexpressing SNAIL in EGFR mutant NSCLC cells. Our studies showed that ectopic SNAIL overexpression rendered EGFR mutant NSCLC resistant to EGFR TKI.

Further to investigate if EGFR TKI sensitivity of EGFR mutant NSCLC is restored on reversing mesenchymal phenotype, we developed doxycycline inducible SNAIL expressing EGFR mutant NSCLC cell lines. We were able to control epithelial and mesenchymal phenotype by doxycycline inducible SNAIL expression. Thus far, we developed an *in vitro* model of EMT which can be used to investigate

molecular mechanisms affecting EGFR TKI sensitivity in EGFR mutant NSCLC. Further studies to investigate whether or not reversing mesenchymal phenotype restores EGFR TKI sensitivity of EGFR mutant NSCLC need to be done.

In summary, our data suggests that development of drugs targeting transcription factor SNAIL is a promising strategy in overcoming EGFR TKI resistance and enhancing the efficacy of molecular targeted therapy in EGFR mutant NSCLC.

## **CHAPTER ONE**

### **INTRODUCTION**

#### **Pathophysiology of Lung Cancer**

Lung cancer is the leading cause of cancer related death, accounting for one third of all deaths from cancer worldwide (1). The most common symptoms of lung cancer include persistent cough or chest pain. Other symptoms include hemoptysis, malaise, weight loss, dyspnea and hoarseness of voice (2). Presence of lung cancer can be determined from patient's history, physical examination, routine laboratory evaluations, chest x-ray, chest computed tomography (CT) scan with infusion of contrast material or biopsy (2). The vast majority of lung cancers are carcinomas - malignancies arising from epithelial cells (3). Lung cancers are categorized by the size and appearance of the malignant cells under the microscope. The two broad classes are non-small cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC) (3). These 2 types of lung cancer are often treated very differently.

About 85-90% of lung cancers are NSCLC (4). NSCLC is a heterogeneous aggregate of histologies. The most common histologies include squamous cell carcinoma, adenocarcinoma and large cell carcinoma (2). NSCLC arises from the epithelial cells of the lung from the central bronchi to terminal alveoli. The histological type of NSCLC correlates with site of origin reflecting the variation in respiratory tract epithelium of the bronchi to alveoli. Squamous cell carcinoma



usually starts near a central bronchus. Adenocarcinoma and large cell carcinoma usually originate in peripheral lung tissue. These histologies are classified together because approaches to their diagnosis, staging, prognosis and treatment are similar (2). Although NSCLCs are associated with cigarette smoking, adenocarcinomas may be found in patients who have never smoked (2, 4).

### **Treatment of NSCLC**

Treatment approach for NSCLC varies depending on the stage of cancer, overall health of the patient, lung function and symptoms. The common treatment options involve surgery, radiation therapy or chemotherapy involving combination of platinum based compounds (4). Surgery is the most potential curative option for this disease if the tumor is diagnosed in an early stage. Surgical options involve pneumonectomy-surgical removal of entire lung, lobectomy-surgical removal of an entire section (lobe) of a lung and segmentectomy or wedge resection-surgical removal of a part of a lobe (4). Chemotherapy offers short-term improvement in disease related symptoms in patients with advanced NSCLC. Most often a combination of 2 chemotherapeutic agents is used as a treatment regimen for NSCLC. Some of the chemotherapeutic agents used for treatment of NSCLC are cisplatin, carboplatin, paclitaxel, gemcitabine, vinblastine and etoposide (4). However the impact of chemotherapy on the quality of patient's life remains a major concern (5). For people with advanced NSCLC who meet certain criteria, targeted

therapies may be added to the treatment as an adjuvant to chemotherapy or as a first line treatment.

### **Targeted therapies for NSCLC**

Targeted therapies are medications that are designed to treat cancer by interfering with the specific molecular abnormalities or biochemical pathways that drive the abnormal growth and spread of cancer (2). Targeted therapies are designed to predominantly attack abnormal signaling molecules so that they would be more effective and have less side effects as compared to traditional chemotherapeutic agents. Over the past decade, a multitude of targeted agents have been explored for the treatment of advanced NSCLC and clinical trials so far have yield encouraging results.

### **Angiogenesis inhibitors**

Angiogenesis or development of new blood vessels to supplement nutrition and oxygenation to the tissue is an essential process for the growth of tumors. Vascular epidermal growth factor (VEGF) is an important growth factor that control angiogenesis in normal and tumor cells (6). VEGF is frequently overexpressed in NSCLC and associated with tumor progression. Bevacizumab (Avastin), a humanized mouse monoclonal antibody that binds to VEGF has been shown to provide significant survival benefits when used in combination with platinum based chemotherapy as a first line treatment for a subset of advanced NSCLC (3, 7).

However in phase II study of this combination treatment, high rate of fatal pulmonary hemorrhages was found to be associated with bevacizumab treatment which limits the use of this drug (7).

### **Epidermal growth factor receptor inhibitors**

Epidermal growth factor receptor (EGFR) is a cell surface receptor, a member of ErbB family of receptor tyrosine kinases (8). Binding of EGFR to its cognate ligands leads to autophosphorylation of receptor tyrosine kinase and subsequent activation of EGFR signaling pathways. Activation of EGFR signaling pathways has many effects including cell proliferation, differentiation and survival. These effects are mediated by a series of signaling mechanisms such as activation of mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (9, 10). Although present in normal cells, EGFR is frequently overexpressed in a variety of tumor cells and is associated with poor prognosis and decreased survival (8, 11). More than 60% of NSCLC have altered expression of EGFR, as a result EGFR is considered to be an important therapeutic target for treatment of NSCLC (11, 12). Currently available EGFR targeted therapies for NSCLC include small molecule EGFR tyrosine kinase inhibitors (TKIs) - erlotinib (Tarceva), gefitinib (Iressa) and monoclonal antibodies targeting the extracellular domain of EGFR - cetuximab (Erbix) and panitumumab (Vectibix) (3, 11, 13). Although erlotinib has shown to prolong patient survival in large phase III trials, patients who initially respond to EGFR TKIs eventually develop resistance (14). Further, there was no clinical benefit when gefitinib was combined with

chemotherapy in two similar trials INTACT-1 and INTACT-2 (15, 16). Although the pharmacological profile of erlotinib and gefitinib is similar, one reason attributed to the failure of gefitinib in INTACT-1 and INTACT-2 trials is ethnic variability (17). Gefitinib is found to be more effective in female patients of Asian origin and non-smokers indicating that an appropriate patient selection based on genetic and clinical characteristic should be employed for both future clinical trials and clinical use of gefitinib (17). Clinical use of monoclonal antibodies cetuximab and panitumumab for advanced NSCLC is very limited owing to the large variations in responses observed during clinical trials evaluating efficacy of these drugs. Although cetuximab in combination with chemotherapy showed significant efficacy in Phase II trials (18, 19), subsequent phase III trials BMS-099 (cetuximab plus taxane/carboplatin) and FLEX (cetuximab plus cisplatin/vinorelbine) failed to observe a significant improvement in overall survival of patients with advanced NSCLC (20, 21). The controversial results of trials involving cetuximab necessitates the ongoing search to identify a selection marker which might identify a population of NSCLC patients that benefit with cetuximab treatment.

### **Anaplastic large cell lymphoma kinase inhibitors**

Anaplastic large cell lymphoma kinase (ALK) gene encodes a receptor tyrosine kinase that is normally expressed only in certain neuronal cells. ALK gene can be oncogenic either due to formation of a fusion gene as a result of genetic rearrangements, gene amplification or mutations in ALK gene itself. ALK

rearrangements are identified in about 7% of NSCLC patients (22). Majority of rearrangements in NSCLC result due to complex deletion and inversion in chromosome 2p resulting in Echinoderm microtubule associated protein-like 4 (EML4)-ALK fusion gene product. EML4-ALK fusion protein results in constitutive ALK kinase activity contributing to carcinogenesis (22). FDA approved crizotinib (Xalkori) to treat certain advanced NSCLC expressing EML4-ALK fusion gene (23). Early clinical studies showed dramatic response of crizotinib among EML4-ALK positive patients and phase III trials testing the efficacy of crizotinib versus standard chemotherapy in advanced ALK positive lung cancer demonstrated higher rate of progression free survival with crizotinib treatment as compared to chemotherapy (24). However, multiple novel mutations in ALK that confer resistance to crizotinib have been identified, limiting the clinical use of crizotinib (25, 26).

### **RAS/RAF/MEK/ERK pathway inhibitors**

RAS/RAF/MEK/ERK pathway is an important route that regulates cell proliferation and survival. A number of cell surface receptors including EGFR can activate RAS/RAF/MEK/ERK pathway (27). The RAS/RAF/MEK/ERK pathway is a kinase cascade where a number of kinases from RAF to MEK to ERK are sequentially activated in response to an extracellular growth signal. RAS family of proto-oncogene that consists of KRAS, HRAS and NRAS are plasma membrane bound G proteins that regulate a number of signaling pathways involved in cell survival, proliferation and differentiation (28). In about 10-15% of NSCLC RAS

signaling pathway is aberrantly activated due to KRAS mutations and contributes to poor prognosis and tumor metastasis (29). A number of agents targeting RAS oncogenes are being studied of which farnesyl transferase inhibitors (FTIs) such as tipifarnib (Zarnestra) and lonafarnib are under clinical investigations for KRAS mutations harboring NSCLC (30). The RAS/RAF/MEK/ERK signaling pathway can also be activated by perturbations of upstream components of RAS. Aberrant expression or mutational activation of EGFR as observed in NSCLC can lead to hyperactivation of RAS causing upregulated RAS/RAF/MEK/ERK signaling (31, 32). In an effort to inhibit hyperactivated RAS/RAF/MEK/ERK signaling pathway, dual specificity kinase-MEK, a mitogen activated protein kinase kinase, which acts further downstream along the RAS/RAF/MEK/ERK pathway is being explored as a drug target for treatment of NSCLC with advanced malignancy (33). Preclinical studies of MEK inhibitors such as CI-1040, PD-0325901 and AZD6244 show promising antitumor activity (33, 34). However, phase II trials of these compounds failed to meet the primary efficacy end point and due to lack of responses coupled with safety issues, the trials were closed (35, 36).

### **PI3K/AKT pathway inhibitors**

PI3K/AKT signal transduction pathway regulates cell survival and proliferation and is implicated in the development and progress of various tumors (37). Of note, mutations in PIK3CA, the catalytic subunit of PI3K occurs in approximately 5% of NSCLC resulting into constitutive activation of PI3K/AKT signaling pathway (38).

Drugs that inhibit PI3K are being developed for treatment of NSCLC harboring PIK3CA mutations. Phase I studies testing the safety profile of one such compound BEZ235- a competitive dual PI3K/mammalian target of rapamycin (mTOR) inhibitor in combination with everolimus -mTOR inhibitor for advanced solid tumors including NSCLC are ongoing and early trial results showed that the combination is well tolerated by patients (39).

### **Heat shock protein 90 inhibitors**

Heat shock protein 90 (Hsp90) is a member of the heat shock protein/chaperone family, which assists in the folding of newly synthesized proteins in the cell as well as in protein refolding after environmental insults (40). EGFR and several other kinases that contribute to deregulated signaling and proliferation in human cancers rely on the Hsp90 chaperone for their conformational maturation (41). A subset of NSCLC has been reported to be extremely sensitive to treatment with Hsp90 inhibitors *in vitro* as well as *in vivo* (42, 43). Potent disruption of EGFR maturation with Hsp90 inhibitors makes these drugs attractive targets for in depth clinical investigation for use in treatment of NSCLC. A phase II/III trial GALAXY-1 testing the efficacy of Hsp90 inhibitor ganetespib in combination with chemotherapeutic agent docetaxel has shown beneficial effects of ganetespib on overall survival and progression free survival of patients with advanced metastatic lung cancer (44). These encouraging results are further being confirmed in a phase

III trial GALAXY-2 to successfully bring ganetespib to patients with advanced lung cancer (45).

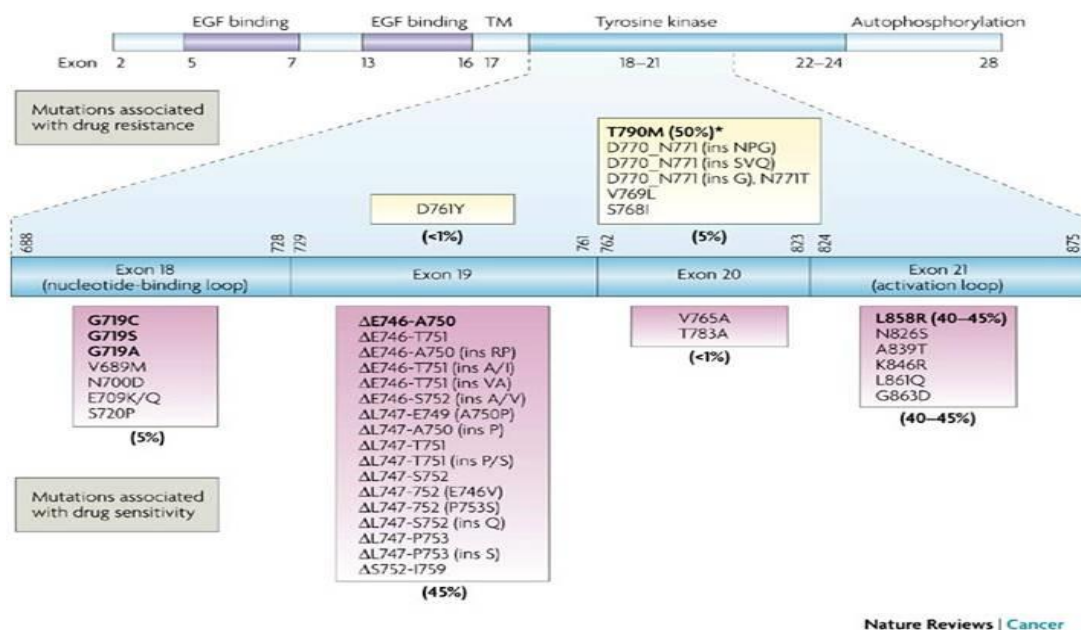
### **EGFR TKIs and EGFR mutations**

The original rationale for development of EGFR targeted therapies was that EGFR is more abundantly expressed in lung carcinoma tissue than in adjacent normal lung (46). However systematic clinical trials revealed variability in clinical response of patients with advanced NSCLC to EGFR TKIs (47). This variability was explained by the discovery of somatic mutations present in the kinase domain of EGFR gene of a subset of NSCLC patients (31, 32). EGFR kinase domain mutations are present in about 10% of cases in North America and Western Europe, 30–50% of cases in individuals of East Asian descent and are frequently associated with adenocarcinomas with bronchioalveolar features that arise in non-smokers and women (48-50).

Entire EGFR kinase domain is encoded by exons 18-24 of which EGFR kinase domain mutations target four exons 18-21 (figure1) (48, 51, 52). EGFR kinase domain mutations are either small in-frame deletions or amino acid substitutions clustered around the ATP-binding pocket of the enzyme (31, 48, 51, 52). The most prevalent mutations are in-frame exon 19 deletions (residues 747-750) accounting for about 45% and L858R substitution in exon 21 accounting for 40-45% of EGFR mutations in NSCLC (Figure1)(53). Nucleotide substitutions in exon 18 and in-frame insertions in exon 20 account for another 5% of EGFR mutations in



NSCLC (53). These mutations often referred to as activating mutations, increase the kinase activity of EGFR leading to hyperactivation of downstream cell proliferation and anti-apoptotic pathways and consequently confer oncogenic effects (53-55). As a result, NSCLCs become dependent on the mutant EGFR for cell survival and proliferation, representing a model of oncogene addiction (53). Additionally, kinase domain mutations render the EGFR extremely sensitive to small molecule EGFR TKIs, which compete with ATP to reversibly bind to the kinase domain of mutant EGFR and inhibit the functioning of mutant EGFR (53). Due to shutting down of mutant EGFR with EGFR TKIs, NSCLCs undergo oncogene crisis and a significant regression in tumor size is observed in patients with EGFR mutant NSCLC (53). As a result, the approval of small molecule EGFR TKIs for treatment of EGFR mutant NSCLC was very well acclaimed although the limitations of their efficacy due to development of drug resistance have become readily apparent (51, 52, 56)



**Figure 1. Gefitinib and erlotinib sensitizing kinase domain mutations of EGFR in NSCLC.** A representation of epidermal growth factor receptor (EGFR) showing the distribution of exons in extracellular domain (EGF binding), transmembrane domain (TM) and intracellular region comprising the tyrosine kinase and autophosphorylation domains. Exons 18–21 in the tyrosine kinase region where the relevant mutations are located are expanded (represented by the cyan bar), and a detailed list of EGFR mutations in these exons that are associated with sensitivity (magenta boxes) or resistance (yellow boxes) to gefitinib or erlotinib is shown. Sharma *et al. Nature Reviews Cancer* 7, 169–181 (March 2007) | doi: 10.1038/nrc2088(53).

### Mechanisms of EGFR TKI resistance

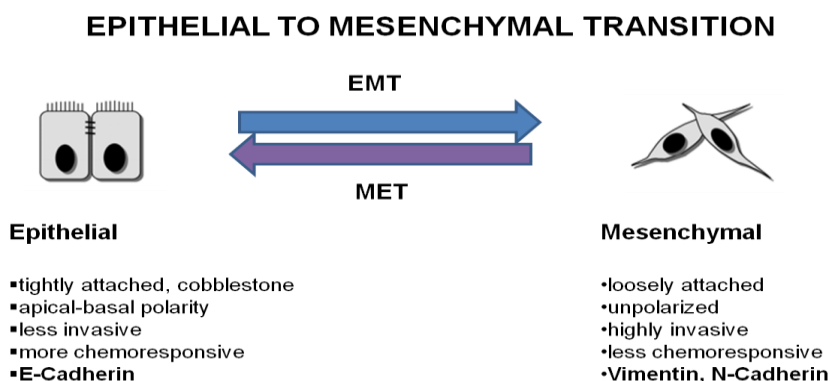
Most NSCLCs with EGFR mutations achieve a marked response to treatment with EGFR TKIs gefitinib or erlotinib. However, despite this initial dramatic response of EGFR mutant tumors to EGFR TKIs, acquired resistance develops within few months of treatment (51, 52, 56). In an effort to understand the mechanisms

underscoring acquisition of drug resistance, Sequist and colleagues undertook a comprehensive genetic and histological analysis of 37 NSCLC patients in 2011 (57). All of the 37 patients had activating EGFR mutations; 54% of the patients had exon 19 deletion mutation and 41% had the exon 21 L858R substitution mutation and had responded clinically to either gefitinib or erlotinib but subsequently developed resistance to EGFR TKIs (57). Tumor tissues were collected both before and after EGFR TKI treatment and were analyzed for the presence of genetic alterations using a clinical genotyping platform, the SNaPshot assay (57). They reported that about 49% patients acquired a second site T790M mutation in exon 20 of EGFR, which has been associated with acquired resistance to gefitinib and erlotinib (48, 52). 5% patients developed MET amplification resulting into overexpression of Met receptor tyrosine kinase, which activates downstream intracellular signaling independent of EGFR, facilitating cancer cell survival. Other mechanisms of acquired drug resistance reported by this study were mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene (5%), EGFR amplification (8%) and transformation to SCLC (14%). However, resistance mechanisms for the remaining 30% of the patients remained elusive. The study reported that approximately half of the remaining 30% of these EGFR TKI resistant NSCLCs present with an epithelial to mesenchymal transition (EMT) phenotype (57).

## **Epithelial to mesenchymal transition**

An epithelial to mesenchymal transition (EMT) is a critical development process where a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, undergoes multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extracellular matrix (ECM) components (58). The completion of an EMT is signaled by the degradation of underlying basement membrane and the formation of a mesenchymal cell that can migrate away from the epithelial layer in which it originated.

The molecular hallmarks of EMT are loss of epithelial traits such as adhesion protein E-cadherin, tight junction protein ZO-1 and upregulation of canonical mesenchymal proteins N-cadherin and Vimentin (59). Multiple signaling changes can induce EMT and all these signaling changes converge at transcription factors. EMT is induced and regulated by transcriptional reprogramming via transcription factors such as SNAIL, SLUG, ZEB-1 and TWIST (59, 60).



**Figure 2. Epithelial to mesenchymal transition.** A process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells along with down regulation of E-cadherin and upregulation of N-cadherin and vimentin proteins. Modified from Claudia Palena *et al.* Experimental Biology and Medicine (Maywood) 2011 236: 537 DOI: 10.1258/ebm.2011.010367 (61).

### Regulation of EMT

EMT process can be controlled by intrinsic oncogenic activation, such as KRAS mutation (62) or Her2 overexpression (63). EMT can also be triggered by external stimuli from microenvironment, which is composed of the extracellular matrix (such as collagen and hyaluronic acid), cancer-associated fibroblasts, immune cells and many secreted soluble factors such as Wnt ligands including Wnt3A, Wnt5A, Wnt5B, Wnt6 or Wnt10A, transforming growth factor- $\beta$  (TGF- $\beta$ ), hedgehog, epidermal growth factor, hepatocyte growth factor and cytokines such as tumor necrosis factor- $\alpha$ , interleukin-6 (Figure 3)(64). These growth factors or inflammatory cytokines can exert their effects in autocrine or paracrine manners. In addition, hypoxic environment can also induce EMT of cancer cells (65). Cell-cell interaction, such as Notch signal is another mechanism to trigger EMT process

(Figure 3)(64, 65). Interactions between the cancer cells and other local inflammatory cells or stromal cells may play an important role in the induction of EMT. Besides, intracellular cross talk between these EMT signaling pathways make the regulation of EMT more complex. Recent findings exemplify how the complex interplay between extra- and intracellular signals can trigger EMT and cancer progression (66). These signaling pathways orchestrate an elaborate gene program and protein network needed for the establishment of mesenchymal phenotypes.

### **EMT in cancer progression**

While EMT is a critical process during development and wound healing, recently properties of EMT have been implicated in human pathology, including fibrosis and cancer metastasis (59, 67). Not surprisingly, many of the same signaling pathways and transcription factors important to physiologic instances of EMT are also activated during pathologic EMT (59). The association of EMT and cancer progression has been revealed in several types of cancer including breast cancer, prostate cancer, pancreatic cancer and hepatoma (68, 69). Detailed analyses of tumor biopsies of NSCLC patients have revealed EMT phenotype in a number of EGFR TKI resistant tumors (57). Circulating tumor cells of a subset of NSCLC patients showed mesenchymal phenotype suggesting a link between EMT and lung cancer progression (70). Additionally, *in vitro* models of drug resistant EGFR mutant NSCLC are reported to have EMT phenotype (71). EMT has been reported to be a key step in progression of tumors towards metastasis and invasion (72). Moreover,

cancer cells undergoing an EMT have demonstrated increased resistance to apoptosis and chemotherapeutic drugs and to acquire traits reminiscent of those expressed by stem cells (73).

### **EMT and drug resistance**

A number of studies have shown that EMT plays a role in conferring resistance to cancer cells against conventional therapeutics (74). EMT has been reported to be a biomarker of de novo EGFR TKI resistance in a subset of head and neck carcinoma and lung carcinoma. (75, 76). Several studies have reported that human NSCLC cells containing wildtype EGFR show a variable sensitivity to EGFR TKI treatment *in vitro* and *in vivo* and the sensitivity of these cells and xenografts to EGFR TKI treatment can be predicted by whether the cells have undergone EMT or not (75, 76). Precisely, NSCLC with wildtype EGFR which were insensitive to EGFR TKI treatment showed evidences of EMT (75, 76). Ectopic expression of epithelial protein E-cadherin in NSCLC cells enhanced gefitinib sensitivity (77) which suggests that EMT contributes to resistance of EGFR TKIs. Although the relationship between EMT and drug resistance has been established, the mechanisms underlying drug resistance and EMT remain elusive. Recently, AXL receptor tyrosine kinase has been reported to be activated and is thought to be the regulator of drug resistance in EMT positive EGFR mutant NSCLC (78-80). Three independent studies investigating the role of AXL in acquired EGFR TKI resistance demonstrate strong correlation between mesenchymal phenotype and AXL activation, suggesting that

AXL is capable of replacing the function of mutant EGFR in EGFR mutant NSCLC and breast cancer cells (78-80). Although these studies demonstrate that increased AXL expression and activation is associated with mesenchymal signature and drug resistance, none of these studies directly address the potential mechanisms underlying AXL upregulation.

### **EMT transcription factors**

Though the signaling pathways are complex, the hallmark of EMT in cancer is downregulation of E-cadherin, which is also thought to be a repressor of invasion and metastasis (59, 81). E-cadherin, encoded by human CDH1 gene is a cell–cell adhesion glycoprotein gene that participates in homotypic, calcium-dependent interactions to form epithelial adherens junctions and sequester  $\beta$ -catenin (81). Transcription factors promote or inhibit the transcription of a gene by binding to specific motifs on the DNA are implicated in the transcriptional repression of E-cadherin (82). Several transcription factors that are reported to be playing a role in transcriptional repression of E-cadherin, include zinc finger proteins SNAIL, SLUG, ZEB1, ZEB2/SIP1 as well as the basic helix loop-helix factors TWIST and E47 (82). These factors bind to specific sequences in the CDH1 promoter that contain a central core 5'-CACCTG-3' and are denominated E-boxes. By binding to the E-boxes, these transcriptional factors repress the transcription of CDH1 gene (82). These transcription factors can also suppress a subset of genes that encode cadherins, claudins, occludins, plakophilins, MUC1 and cytokeratins to induce EMT



(64, 73). Additionally, these transcription factors are capable of repressing the expression of pro-apoptotic genes such as PTEN, p53, Bid, PUMA and have been associated with resistance to radiotherapy, chemotherapy, endocrine therapy and targeted therapy (64, 73). These transcription factors promote the expression of genes including Sox 2, Nanog, KLF4 and T cell factor-4 that are required for the synthesis of stem cell markers such as aldehyde dehydrogenase 1 (73). Owing to the central role of transcription factors in promoting a mesenchymal, highly invasive and drug resistant phenotype to cancer cells, recently transcription factors are being explored as novel drug targets for treatment of resistant tumors (83).

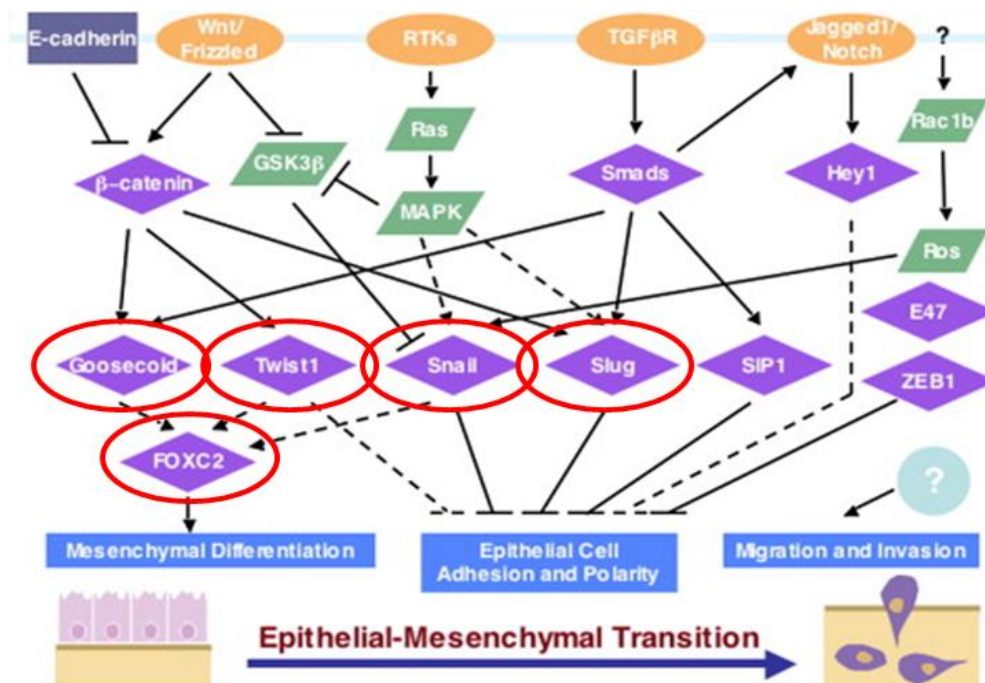
### **Transcription factors as drug targets**

Upregulation of EMT inducing transcription factors has been reported in several carcinomas and has been considered to be a cause of drug resistance. Moreover ectopic overexpression of transcription factors SNAIL and SLUG has been reported to repress E-cadherin expression, induce mesenchymal phenotype and affect sensitivity to chemotherapeutic drugs (60, 84). Role of transcription factors is very well studied with respect to tumor metastasis (85) however, the mechanisms of action of EMT transcription factors with respect to resistance to EGFR TKIs is not well defined at present. In the context of NSCLC, the rationale for investigating if transcription factors induced EMT is a predictor of sensitivity to EGFR TKIs in EGFR mutant NSCLC is that EMT could be used as a biomarker to identify the patients that would not benefit from treatment with EGFR TKIs. Further if EMT is a

mechanism of acquired resistance to EGFR TKIs in EGFR mutant NSCLC, then targeting EMT regulators in order to prevent the induction of EMT or reversing mesenchymal phenotype could be developed as a novel therapy for EGFR TKI resistant EGFR mutant NSCLC. As evident from Figure 3, all the signaling cascades that can induce EMT mediate their actions via transcription factors. The overarching goal of this study is to investigate the role of the transcription factor that acts as a regulator of EMT that can be further developed as a novel drug target to overcome resistance to EGFR TKIs in EGFR mutant NSCLC.

### **Expression of transcription factors in EGFR mutant NSCLC**

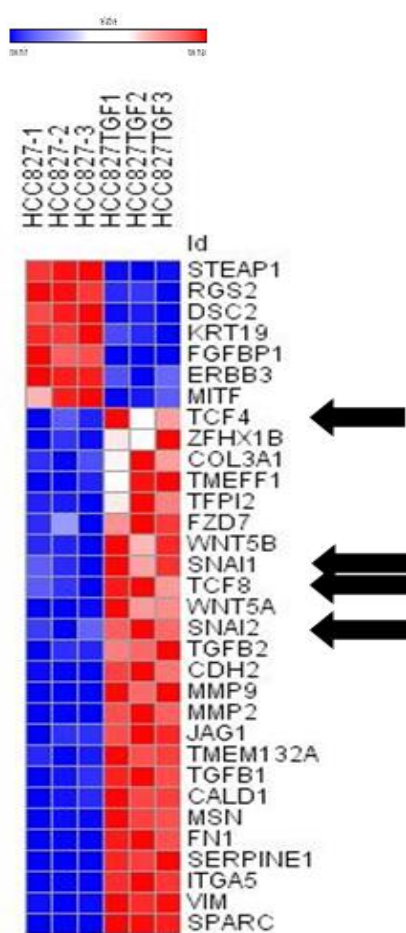
In order to investigate if induction of mesenchymal phenotype is sufficient to confer resistance to EGFR TKIs in EGFR mutant NSCLC, Dr. Shimamura previously developed *in vitro* model of EMT by chronic exposure of EGFR mutant HCC827 NSCLC cell line to TGF- $\beta$  (personal communication), since TGF- $\beta$  is a potent inducer of EMT (86). HCC827 cells are epithelial cells that are exquisitely sensitive to EGFR TKIs due to exon 19 deletion mutation in kinase domain of EGFR. Chronic exposure of HCC827 cells to 10 ng/ml TGF- $\beta$  for 30 days promotes EMT with downregulation of E-cadherin and upregulation of vimentin.



**Figure 3. Multiple signaling cascades converge at EMT-inducing transcription factors.** Several key cellular signal transduction pathways that promote EMT exert their effects via modulation of transcription factors including SNAIL, SLUG, and ZEB1. Intervention of upstream signaling pathways controlling EMT-inducing transcription factors is essential for delineating the strategies to prevent EMT and associated acquired EGFR TKI resistance. Yang *et al.* *Developmental cell*. Volume 14, Issue 6, 10 June 2008, Pages 818-829 (87).

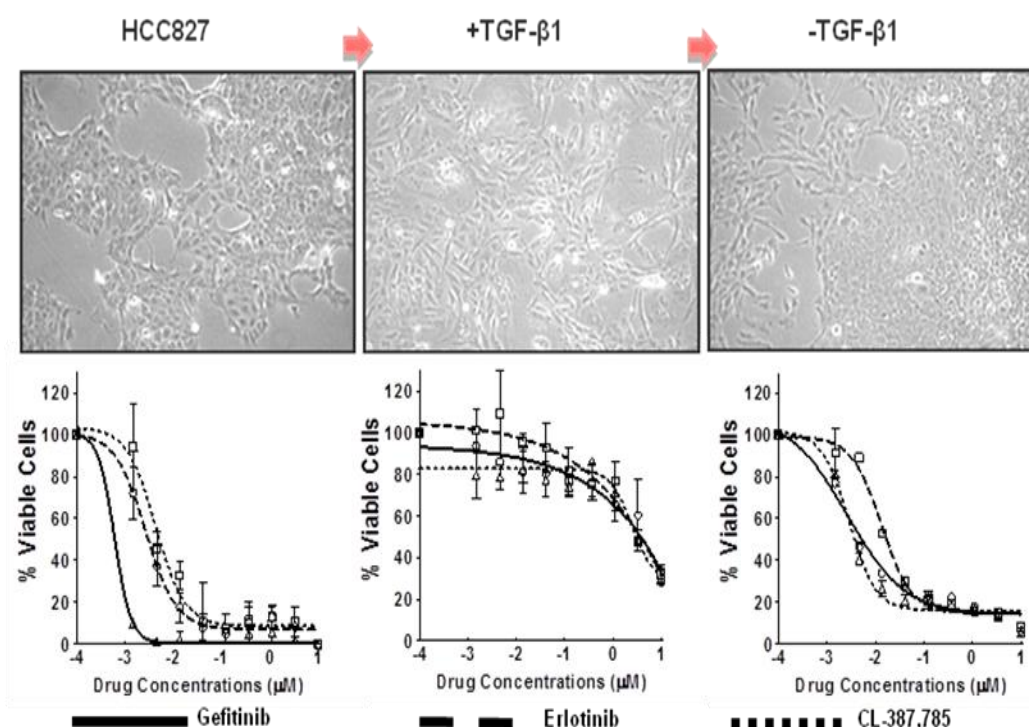
To identify genes uniquely modulated in the mesenchymal HCC827 cells exposed to TGF- $\beta$  but not in parental HCC827 cells, gene expression profiling was performed by isolating total RNA from TGF- $\beta$  treated and untreated cells in triplicates and cRNA was synthesized to hybridize to Human Genome Array U133A2 chips following manufacturer's instructions (Affymetrix, Inc). Differences in signal intensity were normalized using robust multi-array (RMA) method utilized in

Genepattern. 91 EMT-related genes curated from literatures were selected and tested if they were significantly upregulated or downregulated in mesenchymal HCC827 cells using two-sided T-tests and selecting genes that changed more than 3-fold (Figure 4), significant ( $p < 0.05$ , fold change  $> 3$ ) upregulation of EMT associated transcription factors, SNAI1 (SNAIL) and SNAI2 (SLUG) was observed in HCC827 cells exposed to TGF- $\beta$  (Figure 4).



**Figure 4. EMT induced by chronic exposure of EGFR mutant NSCLC cells to TGF- $\beta$  leads to increased expression of EMT inducing transcription factors.** Differential gene expression profiling of TGF- $\beta$  exposed mesenchymal HCC827 cells and parental epithelial HCC827 cells. Genes upregulated or downregulated significantly are shown ( $p < 0.01$  t-test, fold change  $> 2$ ).

Furthermore, HCC827 cells exposed to TGF- $\beta$  and having high levels of SNAIL and SLUG were resistant to EGFR TKIs (Figure 5). Additionally, withdrawal of TGF- $\beta$  from EMT positive HCC827 cells resulted in reversion to epithelial HCC827 cells that are equally sensitive to EGFR TKIs (Figure 5). This experiment suggested that induction of EMT confers resistance to EGFR TKIs in EGFR mutant NSCLC.



**Figure 5. EMT induced by chronic exposure of EGFR mutant NSCLC cells to TGF- $\beta$  confers resistance to EGFR TKI gefitinib and erlotinib.** HCC827 cells chronically exposed to 10 ng/ml TGF- $\beta$  for 30 days were subjected to MTT cell viability assay with gefitinib for 72 hours.

Consequently, we hypothesized that the reversal of mesenchymal phenotype in EGFR mutant NSCLCs with EMT-mediated EGFR TKI acquired resistance to epithelial phenotype should resensitize the resistant cells to EGFR TKIs. To test this hypothesis, we turned our attention to transcription factors that induce EMT. Conditional expression of EMT inducing transcription factors in HCC827 cells might provide a novel platform to induce and reverse EMT and test to EGFR TKI sensitivity and reversibility of EMT mediated EGFR TKI resistance NSCLC.

As evident from Figure 4, EMT positive and EGFR TKI resistant HCC827 cells generated by chronic exposure to TGF- $\beta$  showed high levels of EMT inducing transcription factors SNAIL and SLUG. The role of transcription factor SLUG in NSCLC has been previously investigated and SLUG has been reported to be a metastasis promoting transcription factor which acts by upregulating matrix metalloproteinase-2 and increasing angiogenesis (85, 88). However the role of SLUG in regulating sensitivity to EGFR TKIs is still unclear. Transcription factor SNAIL is more potent than SLUG in repressing E-cadherin due in part to the higher affinity of SNAIL to the promoter region of E-cadherin (82). Additionally, previous findings from our laboratory and literatures suggest that transcription factor SNAIL acts directly downstream of TGF- $\beta$  in mediating EMT by suppressing E-cadherin expression (Figure 4)(89, 90). Hence, to control epithelial and mesenchymal phenotypes in EGFR mutant NSCLC cells, we sought to stably and conditionally express SNAIL in EGFR mutant NSCLC cell line. Such an *in vitro* model of SNAIL overexpression will enable us to systematically evaluate the potential of transcription

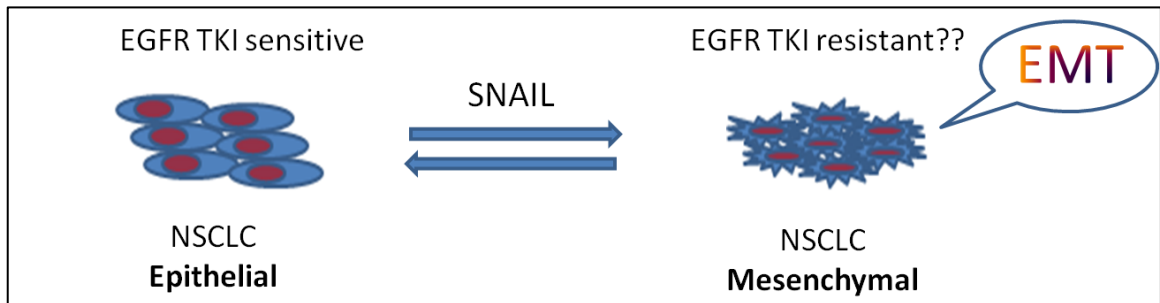
factor SNAIL as a novel drug target for treatment of EGFR TKI resistant EGFR mutant NSCLC.

## CHAPTER TWO

### PROJECT OVERVIEW

#### Hypothesis

We hypothesize that overexpression of EMT transcription factor SNAIL induces mesenchymal phenotype and is sufficient to confer EGFR TKI resistance in EGFR mutant NSCLC. We further hypothesize that reversing the mesenchymal phenotype or promoting epithelial phenotype will resensitize EGFR mutant NSCLC to EGFR TKIs.



Testing this hypothesis helps us answer the most clinically relevant question, if reversing EMT should be developed as a therapeutic target to overcome resistance to EGFR TKIs. The hypothesis will be tested by studies proposed in following two specific aims.



## **Specific Aims**

**1: Determine if transcription factor SNAIL induces mesenchymal phenotype and confers EGFR TKI resistance in EGFR mutant NSCLC.**

1a. Ectopically overexpress SNAIL in EGFR mutant NSCLC cells to promote mesenchymal phenotype.

1b. Determine if SNAIL overexpression is sufficient to confer resistance to EGFR TKIs.

**2: Determine if mesenchymal phenotype induced by transcription factor SNAIL is reversible and is sufficient to reverse SNAIL induced EGFR TKI resistance.**

2a. Establish and characterize EGFR mutant NSCLC cells with inducible expression of SNAIL.

2b. Determine the reversibility of mesenchymal phenotype by conditional expression of SNAIL.

2c. Determine if reversing mesenchymal phenotype by conditional expression of SNAIL is sufficient to restore sensitivity of EGFR mutant NSCLC to EGFR TKIs.

## **CHAPTER THREE**

### **MATERIALS AND METHODS**

#### **Cell lines**

HCC827 NSCLC cell lines were obtained from American Type Culture Collection (ATCC). Stable GFP and SNAIL overexpressing HCC827 cells were cultured in RPMI 1640 supplemented with 5 % Fetal Bovine Serum (FBS) and 1 % antibiotic-antimycotic (AB/AM)(10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone). HCC827 cells with inducible expression of GFP and SNAIL were cultured in RPMI 1640 supplemented with 5 % tetracycline free FBS and 1 % AB/AM. 293LTV cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1 % ABAM. 293FT cells were maintained in complete medium containing 0.1 mM MEM non-essential amino acids, 4 mM L-glutamine, 1 mM sodium pyruvate, 500 µg/mL Geneticin (G418), 10 % FBS and 1 % AB/AM.

#### **Generation of HCC827 cells stably overexpressing C-terminal V5-tagged SNAIL**

HCC827 cells with stable V5 tagged SNAIL expression were generated using PCR cloning system with Gateway® Technology with pDONR221 & OmniMax 2 competent cells, Catalog number 123535-029 (Invitrogen, Carlsbad, CA). attB

*SNAIL* (gateway specific substrate) was generated by a Polymerase Chain Reaction (PCR). *SNAIL* sequence cloned into pBabe *SNAIL* retroviral plasmid (Addgene, Cambridge, MA) was amplified by PCR using AccuPrime® Taq DNA polymerase high fidelity enzyme (Invitrogen, Carlsbad, CA) and primers containing a 25-base pair attB sequence followed by *SNAIL* forward or reverse primer sequence (Table 1). Further, using the gateway recombination cloning technology with Clonase II enzyme (Invitrogen, Carlsbad, CA), we subcloned attB *SNAIL* generated by PCR into entry vector pENTR304 (Addgene, Cambridge, MA) to generate entry clone pENTR304 *SNAIL*. In a consequent recombination reaction, pENTR304 *SNAIL* was subcloned into destination vector pEXP304 (Addgene, Cambridge, MA) which has a C-terminal V5 tag to generate expression clone pEXP304 *SNAIL*-V5. Entry clone for GFP - pENTR304 GFP (Addgene, Cambridge, MA) was subcloned into destination vector pEXP304 (Addgene, Cambridge, MA) to generate expression clone pEXP304 *GFP*-V5. Subclones pENTR304 *SNAIL*, pENTR304 *GFP*, pEXP304 *SNAIL*-V5, pEXP304 *GFP*-V5 were verified by restriction digest and sequencing using Genewiz DNA sequencing services (South Plainfield, NJ). Lentivirus coding for *SNAIL* and *GFP* expression vectors were generated by transfection of 293LTV cells using TransIT® transfection reagent (MIRUS, Madison, WI). HCC827 cells were infected with these lentiviruses in the presence of polybrene (10 µg/ml). 24 hours after infection, medium was replaced and cells were maintained in RPMI 1640 media supplemented with 5% Fetal Bovine Serum (FBS), 1 % (AB/AM) and blasticidin 20

µg/ml. Single cell colonies were isolated, expanded and screened for stable ectopic expression of SNAIL and GFP.

**Table 1. SNAIL Primers**

Gene	Primer Sequence	Product size (bp)
SNAIL	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGG AGATAGAACCATGCCGCGCTCTTTCCTCGTCAGG  Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTAGCGGG GACATCCTGAGCAGCC	800

### **Generation of HCC827 cell line with inducible SNAIL overexpression**

Doxycycline inducible HCC827 SNAIL and GFP cell lines were generated by using ViraPower HiPerform T-REx Gateway® Expression System, Catalog number A11141 (Invitrogen, Carlsbad, CA). plenti 3.3/TR (tet repressor) vector was transfected into 293FT cells to generate lentivirus coding for plenti 3.3tetR. HCC827 cells were infected with lentivirus codings for plenti3.3/TR to generate HCC827tetR cell line with constitutive expression of tet repressor. Single cell colonies were isolated and screened for highest expression of tet repressor. *SNAIL* and *GFP* entry clones pENTR *SNAIL* and pENTR *GFP* respectively were subcloned into pLenti6.3/TO/V5-DEST plasmid vector using gateway recombination cloning

technology to generate expression vectors with components of tetracycline regulated (TET ON) system. Lentivirus coding for pLenti6.3/TO/V5-*SNAIL* and pLenti6.3/TO/V5-*GFP* were generated by transfection of 293FT cells using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA). HCC827tetR (highest tet repressor expressing HCC827 clone) cells were infected with pLenti *SNAIL-V5* and pLenti *GFP-V5* lentiviruses in the presence of polybrene (10 µg/ml). 24 hours post infection, medium was replaced and cells were maintained in RPMI 1640 supplemented with 5% Tetracycline free FBS, 1 % ABAM, G418 1000 µg/ml and blasticidin 20 µg/ml. Pooled stable cell lines were screened for the ability of doxycycline to induce relevant transgene expression.

### **Drug treatments**

Gefitinib and XL-184 were purchased from ChemieTek (Indianapolis, IN). 17DMAG and AUY922 were purchased from LC laboratories (Woburn, MA). Stock solutions of drugs were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and maintained at -20 °C. Drugs were diluted to 1 mM using DMSO for a working solution and used at concentrations ranging from 0.001 to 10 µM.

### **Western blot analysis**

Whole cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml leupeptin (Cell Signaling Technology, Danvers, MA) supplemented with halt protease and phosphatase inhibitor cocktail containing AEBSF-HCl, aprotinin, bestatin, E-64, EDTA, leupeptin, pepstatin A (Pierce, Rockford, IL). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) and equivalent amounts (40 µg) of cell lysates were subjected to SDS-PAGE on 4 % to 20 % gradient gels. V5 tag antibody was purchased from Invitrogen (Carlsbad, CA). Snail, E-cadherin, Vimentin, Claudin, Akt/pAkt, Erk/pErk, pEGFR, MET/pMET, HER3/pHER3 antibodies were purchased from Cell signaling technology (Danvers, MA). EGFR antibody was purchased from Santacruz Biotechnology (Dallas, TX) and tetR antibody was purchased from Boca Scientific Inc (Boca Raton, FL).

### **Cell proliferation assay**

Cells were seeded at a density of 3,000 cells per well in 96-well plates and cultured in the presence of drugs alone or in combination for 72 hours. After 72 hours, cells were incubated at 37 °C in the presence of Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies Inc., Rockville, Maryland) reagent for 3-4 hours. CCK-8 reagent is a highly water soluble tetrazolium salt which is reduced to a yellow

colored formazan dye by the activity of dehydrogenases in the cells. The amount of yellow formazan dye is directly proportional to the number of living cells which is estimated by measuring the optical density of cells at 450 nm. After incubation with CCK-8 reagent, optical density was measured at 450 nm by reading the plates using microplate reader (BioTek, Winooski, VT). Data were graphically displayed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA). The curves were fitted using a nonlinear regression model with a sigmoidal dose response.

#### **Crystal violet formaldehyde assay**

To account for the slower growth rate of mesenchymal HCC827 SNAIL-V5 CL7 as compared to HCC827 GFP cells (determined by growth curve analysis), HCC827 SNAIL-V5 CL7 were seeded at a density twice that of HCC827 GFP cells. HCC827 GFP ( $0.3 \times 10^6$  cells/well) and HCC827 SNAIL-V5 CL7 ( $0.6 \times 10^6$  cells/well) were seeded in duplicates in 6 well plates. Cells were cultured either with DMSO or 1  $\mu$ M EGFR TKI gefitinib for 72 hours. After 72 hours, cells were washed with 1 ml PBS(+/-) to remove dead cells and the cells attached to the plates were stained with 2 ml crystal violet formaldehyde solution (0.05 %w/v crystal violet, 1% formaldehyde, 1X PBS, 1% methanol). Crystal violet formaldehyde solution stains proteins of cells. The intensity of violet staining is directly proportional to the number of living cells attached to the plate.

### **Flow cytometric analysis of apoptosis**

0.3X10<sup>6</sup> cells/well HCC827 and HCC827 SNAIL-V5 CL7 were seeded in duplicates in a 6 well plate and allowed to adhere to the plate for 24 hours. Cells were cultured in the presence of either DMSO or gefitinib (1 µM) for 48 hours. After 48 hours cells were washed twice with cold cell staining buffer (Biolegend, San Diego, CA) and resuspended in annexin V binding buffer (Biolegend, San Diego, CA) at a concentration of 0.1X10<sup>7</sup> cells/ml. 100 µl of cell suspension was transferred to a 5 ml tube and stained with 10 µl FITC annexin V and 10 µl propidium iodide (PI) (Biolegend, San Diego, CA) for 30 minutes after which percentage apoptosis was determined by FACS analysis using BD canto II machine (BD Biosciences, San Jose, CA). Results were analyzed using Flowjo software (Tree Star Inc., Ashland, OR).

### **Estimation of apoptosis by Poly ADP ribose polymerase cleavage**

0.3X10<sup>6</sup> cells were seeded in duplicates in 6-well plates and allowed to adhere to the plate for 24 hours. Cells were then treated with either DMSO or gefitinib (1 µM) for 48 hours. After 48 hours whole cell lysates were prepared using lysis buffer containing SDS. Lysates were analyzed for percentage Poly ADP ribose polymerase (PARP) cleavage by 3-plex apoptosis assay using Luminex analyzer (Millipore, Danvers, MA). Luminex analyzer color-codes tiny beads, called microspheres, into 500 distinct sets. Each bead set can be coated with a reagent specifically designed to quantify cleaved PARP (cleavage sites: aspartic acid 214 and glycine 215) in cell



lysate, allowing the capture and detection of cleaved PARP from the sample. The results are analyzed by xPONENT® v3.1 Software of the xMAP technology operating system (Millipore, Danvers, MA).  $\beta$ -actin was used to normalize the levels of PARP between different cell lines.

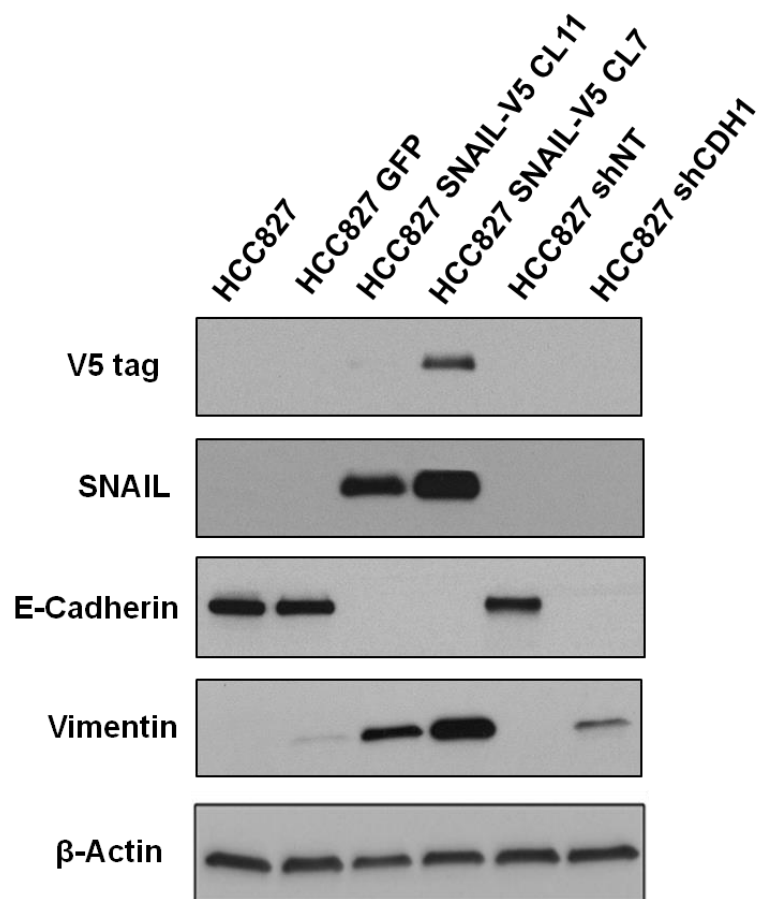
### **Phospho-receptor tyrosine kinase proteome array**

$1 \times 10^6$  cells were seeded in duplicates in 6-well plates and allowed to adhere to the plate for 24 hours. Cells were then treated with either DMSO or gefitinib ( $1 \mu\text{M}$ ) for 24 hours. After 24 hours cells whole cell lysates were prepared by solubilizing the cells at  $1 \times 10^7$  cells/mL in receptor tyrosine kinase (RTK) array specific lysis buffer (R&D Systems, Minneapolis, MN).  $100 \mu\text{g}$  of DMSO or gefitinib ( $1 \mu\text{M}$ ) treated whole cell lysate were applied to each array and the array was developed following RTK array protocol (R&D systems Catalog # ARY001B).

## CHAPTER FOUR

### RESULTS

**Ectopic SNAIL overexpression in EGFR mutant NSCLC:** Transcription factor SNAIL is known to induce mesenchymal phenotype in cultured cells (82, 91). To systematically analyze the phenotypic changes associated with SNAIL expression we decided to generate an *in vitro* model of ectopic SNAIL overexpression in EGFR mutant NSCLC. We ectopically overexpressed SNAIL in EGFR mutant HCC827 NSCLC cells and identified 2 subclones HCC827 SNAIL-V5 CL 7 and HCC827 SNAIL-V5 CL 11 with highest ectopic expression of SNAIL (Figure 6). We next investigated if ectopic SNAIL overexpression is sufficient to induce an EMT in HCC827 SNAIL-V5 CL 7 and HCC827 SNAIL-V5 CL 11 cells. We observed that canonical epithelial marker E-cadherin is significantly downregulated whereas canonical mesenchymal marker vimentin is upregulated in HCC827 SNAIL-V5 CL11 and HCC827 SNAIL-V5 CL7 as compared to HCC827 and HCC827 GFP cells (Figure 6).

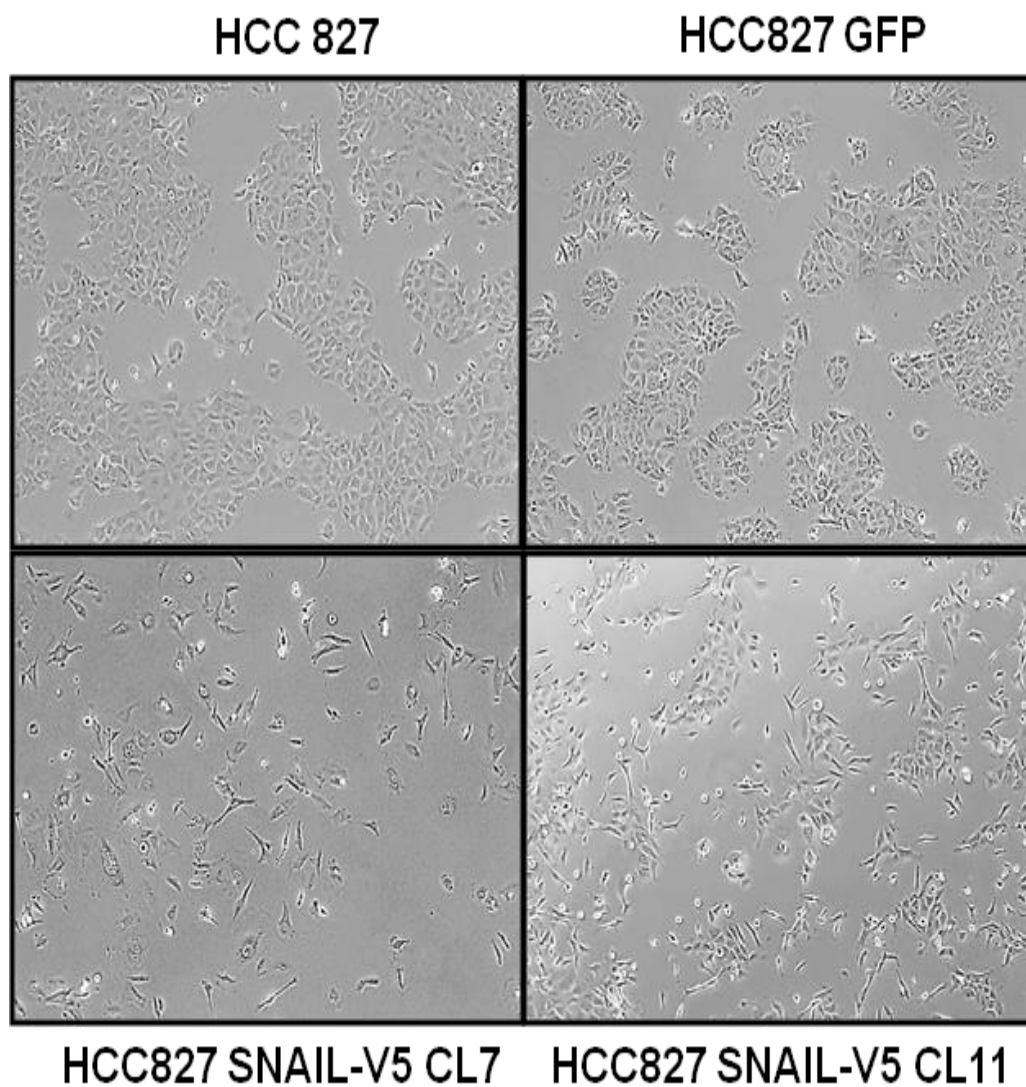


**Figure 6. Ectopic SNAIL overexpression in HCC827 cells promotes mesenchymal phenotype.** Western blot analysis of HCC827, HCC827 GFP, HCC827 SNAIL-V5 clone (CL)-11 and HCC827 SNAIL-V5 clone (CL)-7. Whole cell lysates were resolved on SDS-PAGE and Western blot was performed using indicated antibodies.  $\beta$ -Actin serves as loading control. HCC827 shCDH1 are cells with E-cadherin knockdown respectively. HCC827shNT serves as negative control for E-cadherin knockdown & serve as positive control for EMT markers.

**Morphology of EGFR mutant NSCLC with ectopic SNAIL overexpression:** Cells that transition into a mesenchymal state undergo morphological changes (59), hence we sought to investigate changes in cell shape and growth pattern in ectopic SNAIL overexpressing HCC827 cells. We observed that parental HCC827 and HCC827 GFP cells displayed the classic cobblestone epithelial morphology and tight cell-cell junctions of epithelial cells while HCC827 SNAIL-V5 CL7 and HCC827 SNAIL-V5 CL11 HCC827 exhibited a fibroblastic morphology and assumed a scattered growth pattern (Figure 7).

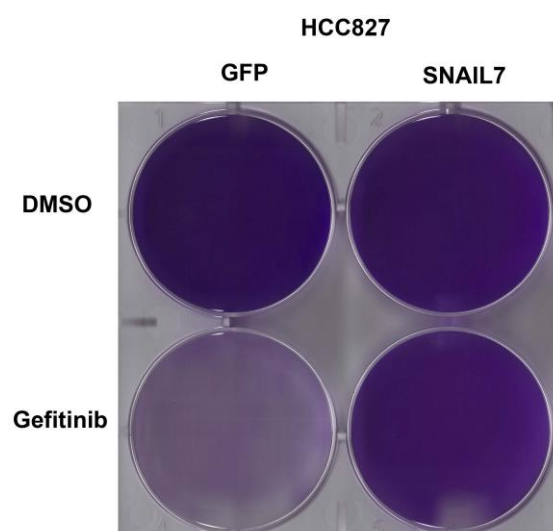
**Ectopic SNAIL overexpression alters sensitivity of EGFR mutant NSCLC to EGFR TKI:** Ectopic expression of EMT transcription factors has been reported to alter sensitivity of cancer cells to EGFR TKIs (84, 92), hence we investigated if ectopic SNAIL expression affects EGFR TKI sensitivity of EGFR mutant NSCLC. When investigated by crystal violet formaldehyde assay (Figure 8) we observed that either gefitinib or DMSO (vehicle) treatment in HCC827 SNAIL overexpressing cells did not compromise cell viability whereas gefitinib treatment in HCC827 GFP cells resulted in significant cell loss evidenced by absence of stained cells and significant percent control reduction in crystal violet staining (Figure 8). We estimated percentage apoptosis induced by gefitinib by flow cytometric analysis and observed that gefitinib treatment induced 2 fold more cell death in HCC827 cells (17.04 % apoptosis) when compared with DMSO treated HCC827 cells (9.91 % apoptosis) while there was no change in percentage apoptosis in HCC827 SNAIL

overexpressing cells treated with (6.17 % apoptosis) or without (6.8 % apoptosis) gefitinib.

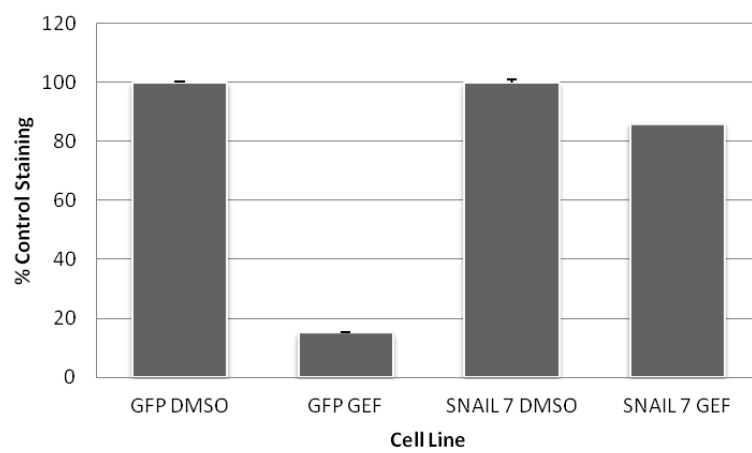


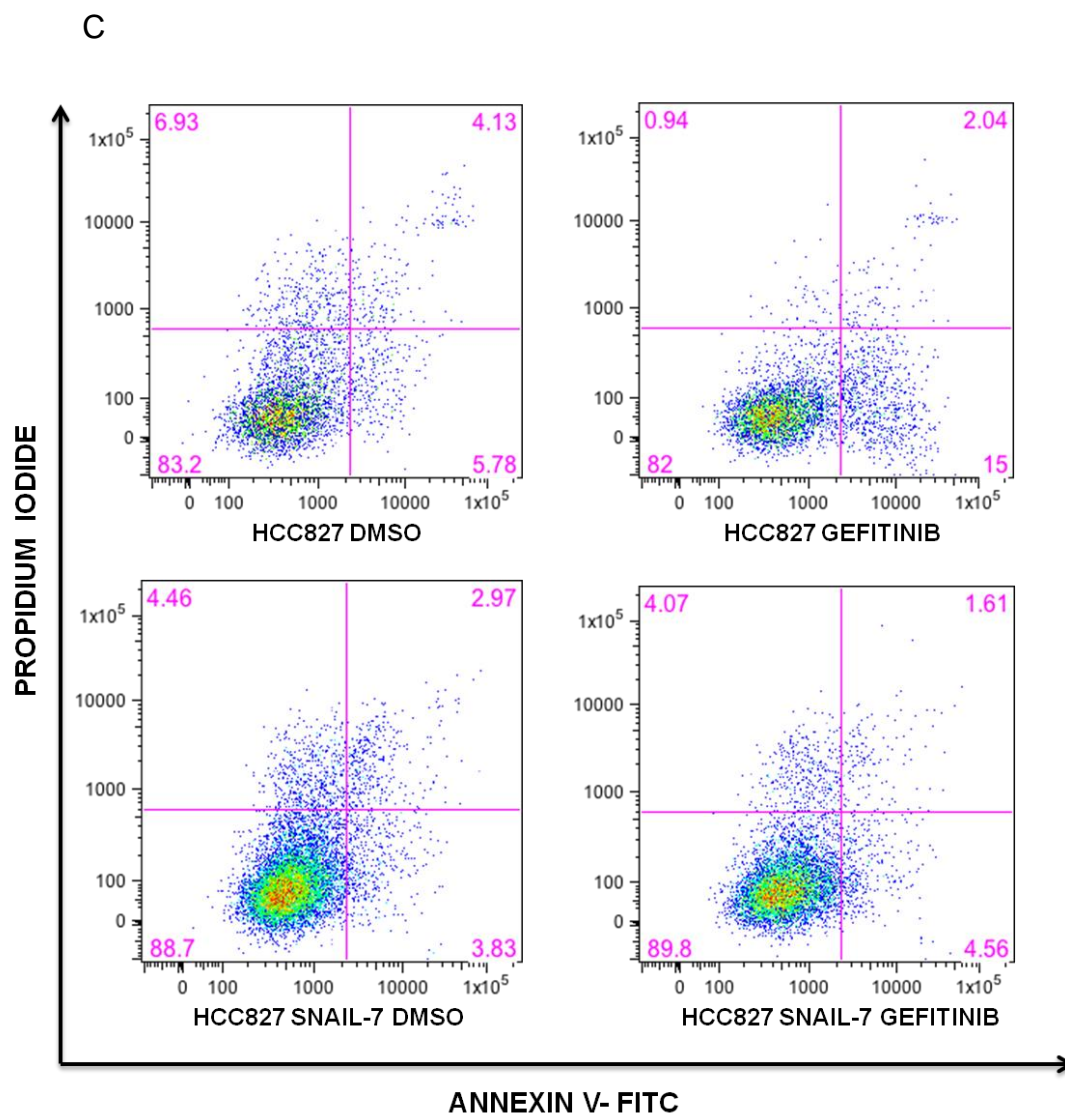
**Figure 7. Morphological changes in HCC827 cells with ectopic SNAIL overexpression.** Figure shows bright field microscopic images (4X magnification) which reveal flattened, fibroblastic morphology & scattered growth pattern of HCC827 SNAIL-V5 CL7 & HCC827 SNAIL-V5 CL11.

A



B



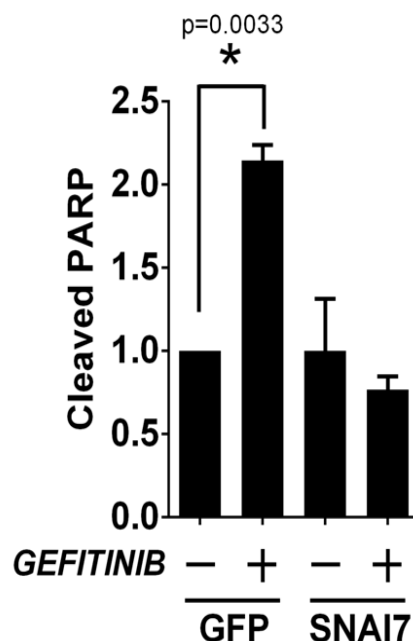


**Figure 8. Gefitinib treatment does not compromise viability of cells ectopically expressing SNAIL-V5.** (A) HCC827 GFP cells ( $0.3 \times 10^6$  cells/well) and HCC82SNAIL-V5 CL7 ( $0.6 \times 10^6$  cells/well) were seeded in 6 well plate and allowed to adhere to the plate for 24 hours, after which cells were treated with DMSO or 1  $\mu$ M gefitinib for 72 hours. After 72 hours cells were washed with PBS (+) to remove dead cells and then stained with crystal violet formaldehyde solution. Intensity of violet color produced after staining with crystal violet is proportional to number of viable cells. Gefitinib or DMSO treatment in HCC827 SNAIL-V5 CL7 cells did not compromise cell viability whereas gefitinib treatment in HCC827 GFP cells compromised cell viability as evidenced by absence of violet staining. (B)

Quantification of crystal violet staining indicates that gefitinib treatment results in 85 % control reduction in crystal violet staining of HCC827 GFP cells whereas only 15 % control decrease in staining of HCC827 SNAIL 7. Results shown here are representative of 2 independent experiments analyzed in duplicates. Error bars indicate standard deviation. (C) Flow cytometric analysis using annexin V-FITC and propidium iodide staining indicates that 48 hours of 1  $\mu$ M gefitinib treatment does not induce apoptosis in SNAIL overexpressing HCC827 cells.

**Ectopic SNAIL overexpressing EGFR mutant NSCLC cells are resistant to EGFR TKI induced apoptosis:** EGFR TKIs are known to induce marked apoptosis in EGFR mutant NSCLC (55). Cancer cells with EMT phenotype are resistant to apoptosis induced by chemotherapeutic agents including cisplatin, paclitaxel (93, 94). We investigated if mesenchymal phenotype associated with ectopic SNAIL expression inhibits EGFR TKI induced apoptosis in EGFR mutant NSCLC. We determined the levels of PARP cleavage induced by gefitinib treatment in HCC827 cells with SNAIL overexpression and observed that gefitinib induced apoptosis is attenuated on ectopic SNAIL overexpression in HCC827 cells (Figure 9).



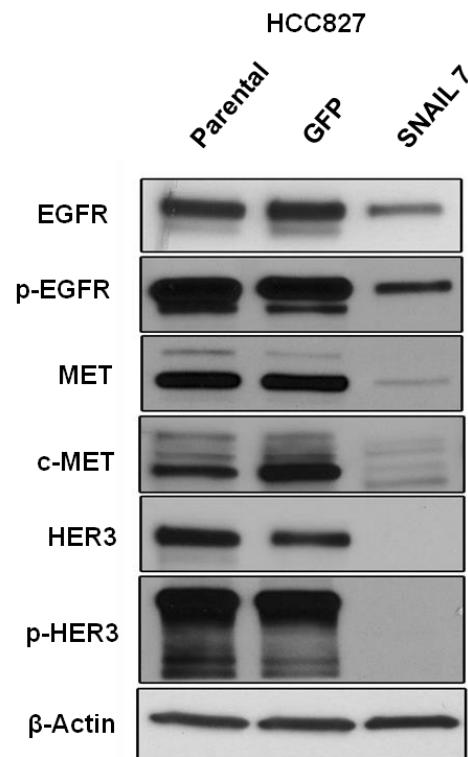


**Figure 9. Gefitinib treatment does not induce apoptosis in SNAIL overexpressing EGFR mutant NSCLC.**  $0.3 \times 10^6$  cells/well were seeded in duplicates in 6 well plate, allowed to adhere to plate for 24 hours and treated with DMSO or gefitinib ( $1 \mu\text{M}$ ) for 48 hours. After 48 hours, whole cell lysate were prepared using lysis buffer containing SDS and lysates were analyzed for percentage PARP cleavage using 3-plex luminex assay.  $\beta$ -Actin was analyzed to normalize PARP levels between different cell lines. Error bars indicate standard deviation.

#### **Attenuated EGFR, HER3 and MET receptor signaling in SNAIL overexpressing**

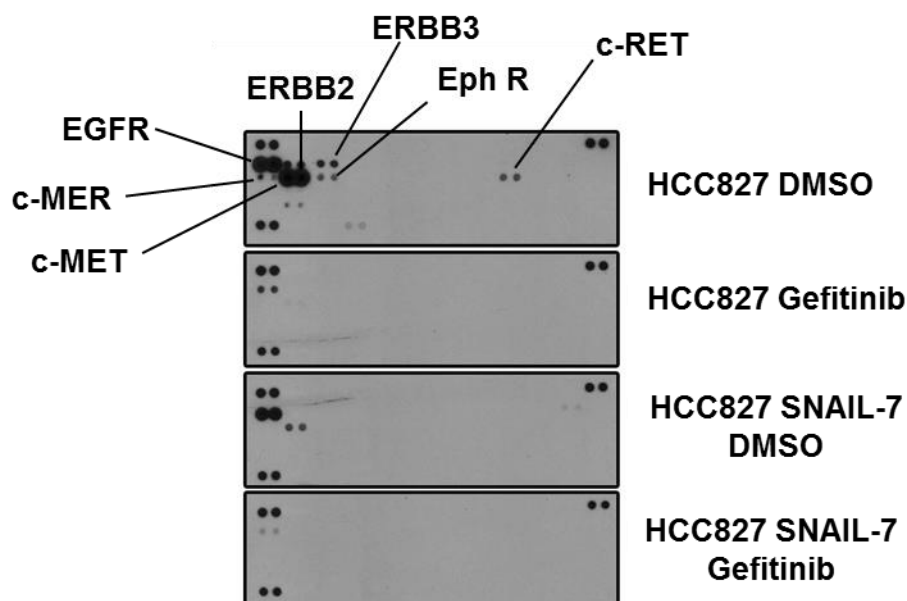
**EGFR mutant NSCLC:** In response to mesenchymal transition, cancer cells shift cellular equilibrium to rely on alternate growth factors and signaling receptors (95, 96). Increased activation of MET and HER3 receptors has been reported in several lung cancer specimen that developed resistance to EGFR TKIs (97, 98). We investigated if ectopic SNAIL overexpression altered the expression and activation

of MET or HER3 receptors in EGFR mutant NSCLC. We observed that EGFR, HER3 and MET receptors are downregulated and their activation is decreased in HCC827 SNAIL-V5 CL7 as compared to HCC827 GFP cells (Figure10).



**Figure 10. Ectopic SNAIL over-expression promotes downregulation of EGFR, MET & HER3 expression.** Western blot analysis indicated a decrease in both expression and activation of EGFR, MET & HER3 receptors on ectopic SNAIL overexpression in HCC827 cells.  $\beta$ -Actin was analyzed as a loading control.

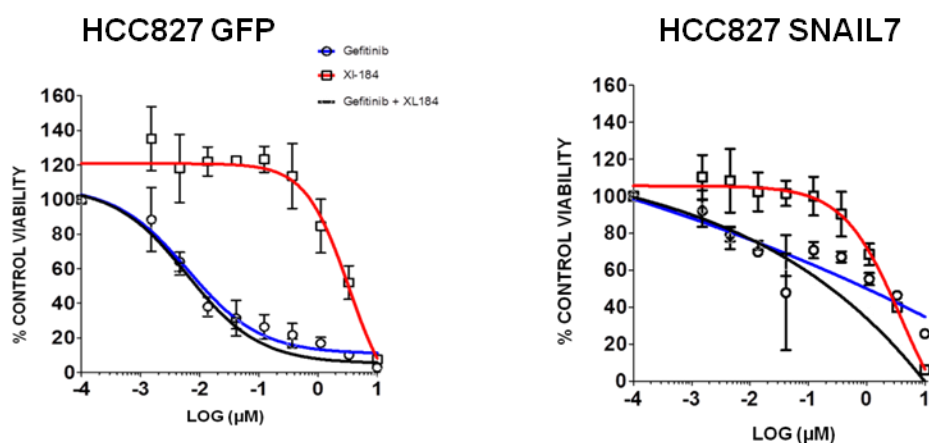
**None of the known 42 receptor tyrosine kinases seem to be the driver of drug resistance in SNAIL overexpressing EGFR mutant NSCLC:** Aberrant activation of several receptor tyrosine kinases has been reported to be sufficient to confer EGFR TKI resistance and drive the survival of carcinoma cells (99, 100). To examine if EGFR TKI resistant SNAIL overexpressing EGFR mutant NSCLC have increased activation of either known or potential novel driver of drug resistance which is capable of replacing function of mutant EGFR (96), we performed a RTK array analysis of HCC827 GFP and HCC827 SNAIL7 cells with or without treatment with gefitinib. The analysis did not identify the activation of HER3, MET, IGF-1R or any other well-known driver of drug resistance in EGFR mutant NSCLC (Figure 11). We observed increased activation of AXL in HCC827 SNAIL7 cells treated with DMSO, however the phosphorylation of AXL is lost upon treatment of HCC827 SNAIL7 cells with gefitinib (1  $\mu$ M) (Figure 11).



**Figure 11. RTK array failed to identify a RTK that could be potentially activating cell survival and proliferation signals in EGFR TKI treated SNAIL overexpressing HCC827 cells.** HCC827 cells expressing GFP or SNAIL7 were treated with either DMSO or gefitinib (1  $\mu$ M) for 24 hours after which whole cell lysates were subjected to RTK profiling. Each RTK is assayed in duplicates. The dots represent activated receptor tyrosine kinase.

**Ectopic SNAIL expressing EGFR mutant NSCLC cells are insensitive to AXL inhibitor XL-184:** Activation of AXL receptor tyrosine kinase has been reported to be a mechanism of drug resistance in a subset of EMT positive EGFR mutant NSCLC (78-80). Furthermore, our RTK analysis showed activation of AXL receptor in SNAIL overexpressing HCC827 cells although the activation of AXL was inhibited on treatment with EGFR TKI gefitinib (Figure 11). To investigate if the survival of

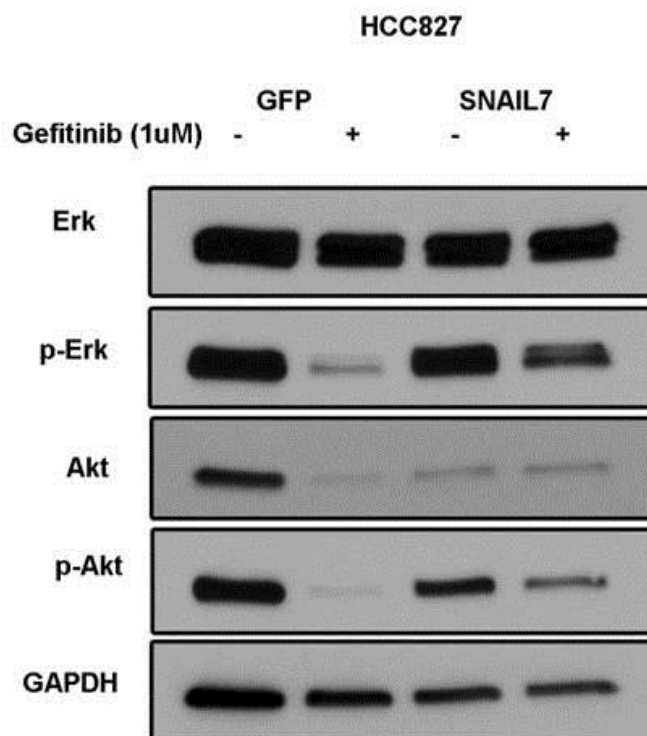
mesenchymal EGFR mutant NSCLC is dependent on AXL activation, we treated HCC827 SNAIL overexpressing cells with AXL inhibitor XL-184 alone or in combination with gefitinib and determined percent control viability of the cells after 72 hours of drug treatment. XL-184 is a compound that was originally developed as a VEGFR2 inhibitor but is also reported to inhibit AXL receptor with the  $IC_{50}$  being 7 nM for Axl. XL-184 alone or in combination with gefitinib did not compromise viability of HCC827 SNAIL7 cells (Figure 12).



**Figure 12. AXL inhibitor does not block the proliferation of HCC827 SNAIL7.** Exponentially growing HCC827 GFP and SNAIL7 cells were treated with indicated concentrations of gefitinib, XL-184 and a combination of both the drugs. At 72 hours, CCK-8 assay was performed and the viability of each sample was normalized to that of DMSO treated cells.

**Pharmacological inhibition of EGFR does not deplete PI3K/AKT and RAS/RAF/MEK/ERK signals in SNAIL overexpressing EGFR mutant NSCLC:** PI3K/AKT and RAS/RAF/MEK/ERK signaling pathways have been shown to

cooperate to promote cell survival and proliferation on NSCLC (101). A number of receptor tyrosine kinases such as EGFR, HER2, HER3, IGF-1R, PDGFR activate cell survival and proliferation signals (96). Surprisingly, none of the known potential drivers of cell survival and proliferation were activated in HCC827 SNAIL7 cells (Figures 10 and 11), yet SNAIL overexpressing HCC827 cells were resistant to treatment with EGFR TKI (Figures 8, 9 and 12) hence we decided to investigate whether or not cell survival and proliferation signals are still active in HCC827 SNAIL7 cells treated with gefitinib. We observed that even after 48 hours of treatment with gefitinib (1  $\mu$ M), PI3K/AKT and RAS/RAF/MEK/ERK pathways are active in HCC827 SNAIL7 cells while these pathways are inhibited on gefitinib (1  $\mu$ M) treatment of HCC827 GFP cells (Figure 13).

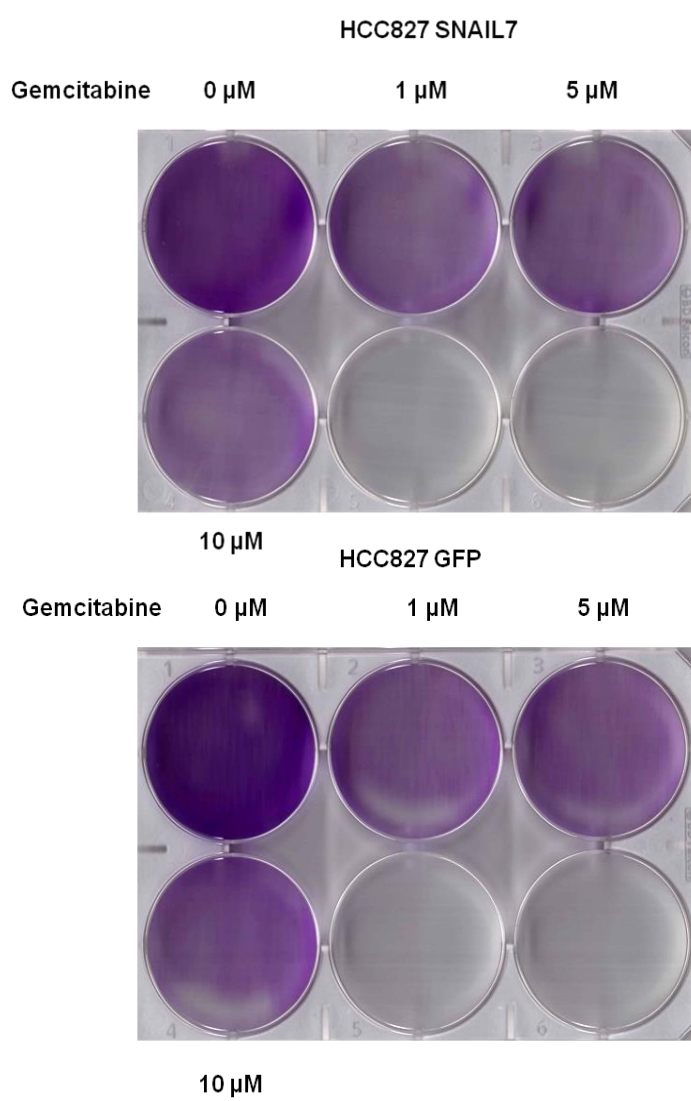


**Figure 13. SNAIL overexpressing HCC827 cells retains cell survival and proliferation signals upon EGFR inhibitor treatment.** Exponentially growing HCC827 GFP and SNAIL7 cells were cultured in the presence of DMSO or gefitinib (1  $\mu$ M) for 48 hours after which whole cell lysates were subjected to Western blotting using indicated antibodies. Sustained activation of Erk and Akt signals was observed in HCC827 SNAIL7 cells even after 48 hours of gefitinib treatment while gefitinib treatment inhibited Erk and Akt phosphorylation in HCC827 GFP cells. GAPDH was assessed as loading control.

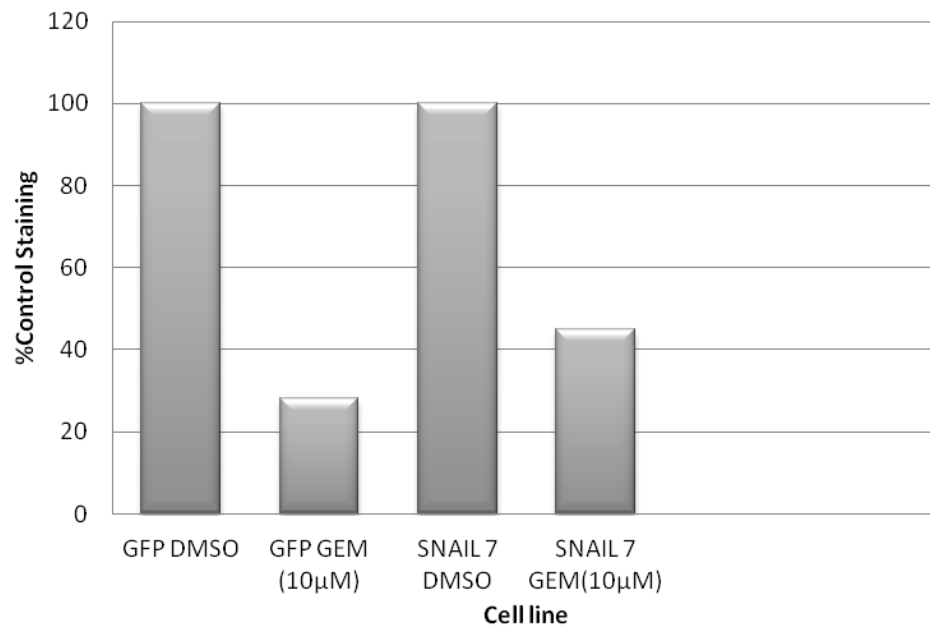
**SNAIL overexpressing EGFR mutant NSCLC cells are sensitive to chemotherapeutic agents:** EMT induced by transcription factors SNAIL and SLUG has been associated with chemo-resistance in ovarian cancer due to altered expression of genes involved in cell cycle regulation and drug transport (84). We investigated if ectopic SNAIL overexpression in EGFR mutant NSCLC renders the cells resistant to chemotherapeutic agents in general or if it is an EGFR TKI specific resistance mechanism. HCC827 GFP and SNAIL7 cells were treated for 72 hours with 4 different concentrations of a chemotherapeutic agent gemcitabine which is a nucleoside analog known to arrest tumor growth and induce apoptosis in a number of cancers (102). We observed that both HCC827 SNAIL7 and HCC827 GFP cells were equally sensitive to 72 hours of treatment with gemcitabine, indicating that ectopic SNAIL overexpression does not affect sensitivity of EGFR mutant NSCLC to chemotherapeutic agents (Figure 14).



A

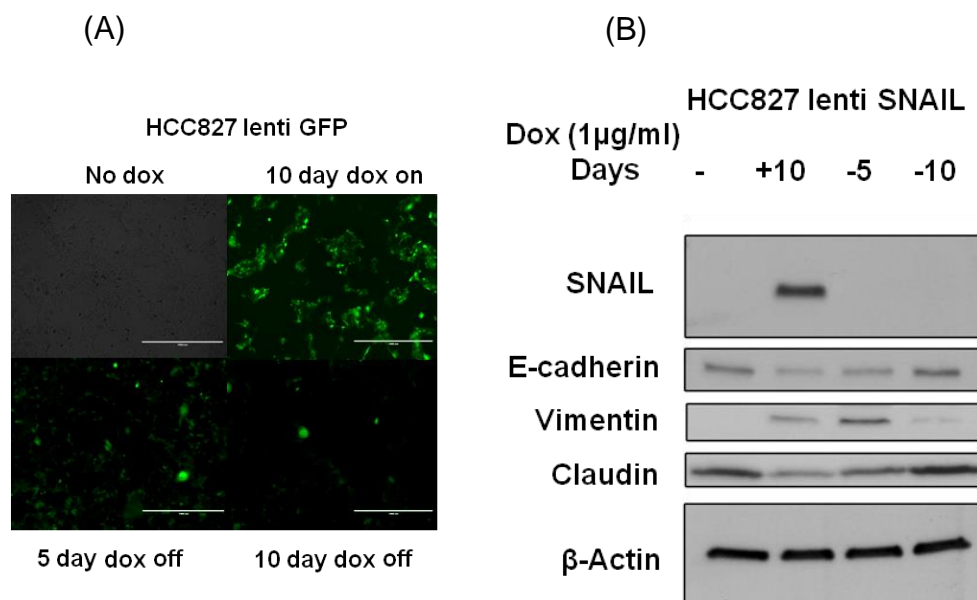


B



**Figure 14. Gemcitabine induces apoptosis in SNAIL overexpressing EGFR mutant NSCLC.** (A) Exponentially growing HCC827 GFP cells and HCC827 SNAIL-V5 CL7 cells were seeded at  $0.3 \times 10^6$  cells/well in 6 well plate and allowed to adhere to the plate for 24 hours, after which cells were treated with indicated dose of gemcitabine for 72 hours. Effect of gemcitabine (GEM) treatment on cell viability was assessed by staining with crystal violet formaldehyde solution. (B) Quantification of crystal violet staining post gemcitabine treatment indicates significant reduction in percent control staining of HCC827 GFP as well as SNAIL7 cells. This suggests that gemcitabine treatment compromised cell viability of both HCC827 GFP and SNAIL7 cells. Results shown here are representative of two independent experiments analyzed in duplicates. Error bars indicate standard deviation.

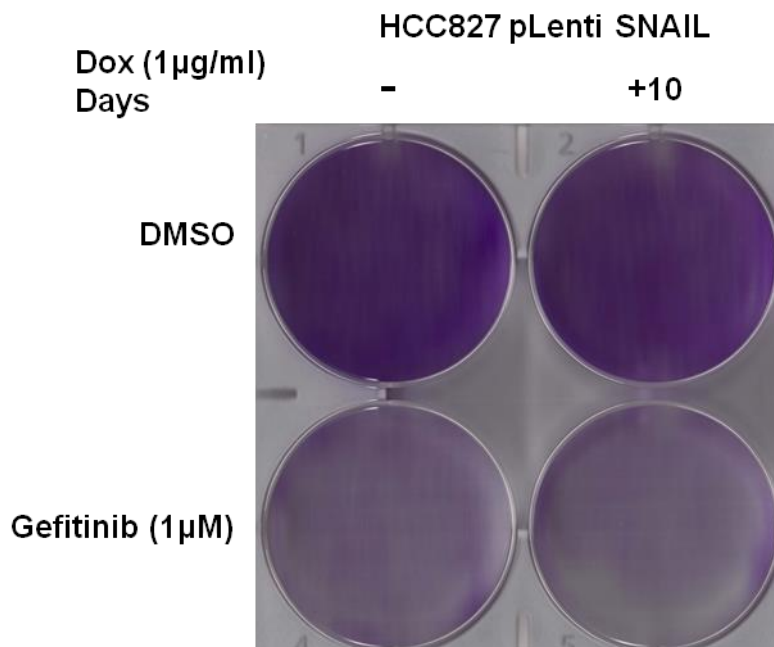
**Inducible SNAIL expression controls the expression of EMT markers and reversal of EMT in EGFR mutant NSCLC:** EMT in theory is a reversible process (59), so we wanted to determine if EMT induced by ectopic SNAIL overexpression in EGFR mutant NSCLC is reversible. We developed doxycycline inducible GFP and doxycycline inducible SNAIL expressing HCC827 cell lines. We observed GFP expression after days of doxycycline treatment of HCC827 cells expressing inducible GFP vector (Figure 15A). Further expression of GFP was repressed after 10 days of doxycycline removal from culture media of cells, indicating that gene expression is under doxycycline control (Figure 15A). After 10 days of doxycycline induced SNAIL expression in HCC827 cells expressing inducible SNAIL vector, epithelial markers E-cadherin, claudin were downregulated and mesenchymal marker vimentin was upregulated (Figure 15B). After 10 days of removal of doxycycline from the cell culture media, the expression of SNAIL was inhibited, expression of epithelial markers E-cadherin, claudin was restored and mesenchymal marker vimentin was downregulated (Figure 15B).



**Figure 15. Conditional expression of GFP and SNAIL in EGFR mutant NSCLC.**

(A) A representative image of HCC827 cells expressing inducible GFP vector. Expression levels of GFP in HCC827 lenti GFP cells changed as expected, on treatment of cells with or without doxycycline. (B) Western blot analysis of EMT markers in HCC827 cells with inducible SNAIL expression. HCC827 cells expressing inducible SNAIL vector were treated with or without doxycycline (1  $\mu$ g/ml) for the indicated time after which whole cell lysates were immunoblotted with antibodies against SNAIL, E-cadherin, vimentin and claudin.  $\beta$ -Actin serves as loading control.

**Inducible SNAIL expressing EGFR mutant NSCLC are sensitive to EGFR TKI after 10 days of doxycycline induced SNAIL expression:** Mesenchymal EGFR mutant NSCLCs with stable SNAIL expression are resistant to EGFR TKI (Figures 8 and 9), therefore we investigated if inducible SNAIL expression alters EGFR TKI sensitivity of EGFR mutant NSCLC. When investigated by crystal violet viability assay, we observed that gefitinib treatment resulted in significant cell loss in inducible SNAIL expressing HCC827 cells treated with and without doxycycline for 10 days as evidenced by absence of stained cells (Figure 16).



**Figure 16. 10 days of doxycycline induced SNAIL expression is not sufficient to confer EGFR TKI resistance in EGFR mutant NSCLC.** Inducible SNAIL expressing HCC827 cells, treated with or without doxycycline (1 µg/ml) for 10 days were seeded at a density of  $0.3 \times 10^6$  cells/well in duplicates in 6 well plate and allowed to adhere to the plate for 24 hours, after which cells were treated with DMSO or 1 µM gefitinib for 72 hours. After 72 hours, cells were washed with PBS (+/+ ) to remove dead cells and then stained with crystal violet formaldehyde solution. Intensity of violet color produced after staining with crystal violet is proportional to number of viable cells. As evidenced by absence of crystal violet staining, gefitinib treatment compromised viability of inducible SNAIL expressing HCC827 cells treated with and without doxycycline for 10 days.

## **CHAPTER FIVE**

### **DISCUSSION**

Development of molecular targeted therapies provides a treatment option in addition to conventional cancer treatments. Molecular targeted drugs interfere with and block specific molecular pathways involved in cancer growth. However, the efficacy of the targeted therapies remains restricted to the cancer cells with oncogene addiction (103). Oncogene addiction refers to the phenomenon in which cancer cells that contain multiple genetic and epigenetic changes remain addicted to one or a few genes for both maintenance of the malignant phenotype and cell survival (53, 103). One such oncogene that has been extensively targeted for the treatment of NSCLC is mutant EGFR, which is effectively blocked by EGFR TKIs (11). However, the development of resistance to EGFR TKIs continues to be the major limitation in the treatment of EGFR mutant NSCLC (57).

Resistance to EGFR TKIs in several cancers including NSCLC has been associated with a loss of canonical epithelial protein E-cadherin, suggesting involvement of EMT in conferring EGFR TKI resistance (75, 76). Transcription factors SNAIL, SLUG, ZEB1 and TWIST that are potent repressors of E-cadherin are being extensively studied as potential therapeutic targets to overcome EMT associated drug resistance in a number of cancers (83, 92).

SNAIL, a zinc finger protein functions as transcriptional repressor of E-cadherin by binding to E-box elements of E-cadherin promoter region (82). TGF- $\beta$ , a secreted protein is known to directly activate transcription factor SNAIL by the action of Smad2, Smad3 and Smad4 to induce an EMT (89). TGF- $\beta$  through activation of transcription factor SNAIL represses E-cadherin expression and induces a mesenchymal phenotype (89). In line with this reports, our preliminary experiments indicated that chronic TGF- $\beta$  exposure results in more than 2 fold increase in SNAIL expression in EGFR mutant NSCLC (Figure 4). Further, TGF- $\beta$  exposed EGFR mutant NSCLC cells with high SNAIL expression revealed an EMT gene signature and were resistant to EGFR TKIs (Figures 4 and 5). These results suggest a direct link between TGF- $\beta$  mediated SNAIL activation and EGFR TKI resistance.

The role of transcription factor SNAIL with respect to EGFR TKI resistance in EGFR mutant NSCLC is less explored. To establish a link between expression of SNAIL and EGFR TKI sensitivity, we developed an *in vitro* model of stable ectopic SNAIL expression in HCC827 cells (EGFR TKI sensitive EGFR mutant NSCLC cell line). Investigation of ectopic SNAIL overexpressing subclones revealed that ectopic SNAIL expression was sufficient to alter the expression of canonical EMT markers and induce a mesenchymal phenotype in EGFR mutant NSCLC (Figures 6 and 7). This observation was in line with previous reports that ectopic SNAIL expression induces EMT in several carcinomas (104). One important characteristic of mesenchymal cells is resistance to apoptosis (59, 73). Moreover, ectopic expression of EMT transcription factors is known to alter drug sensitivity of cancer cells (84, 92).



We observed that ectopic SNAIL expression in HCC827 was sufficient to inhibit gefitinib (1  $\mu$ M) induced apoptosis (Figures 8 and 9) suggesting that EMT induced by SNAIL alters the sensitivity of EGFR mutant NSCLC to EGFR TKIs.

Owing to the fact that EMT is a reversible process, we hypothesized that EMT induced by SNAIL in EGFR mutant NSCLC is reversible. Our goal was to test if reversing the mesenchymal phenotype induced by ectopic SNAIL expression would resensitize EGFR mutant NSCLC to EGFR TKIs. To investigate the reversibility of SNAIL induced EMT, we developed an *in vitro* model of HCC827 cells with doxycycline inducible (Dox On) SNAIL expression. We developed HCC827 cells with doxycycline inducible GFP expression as a control for inducible gene expression system and ensured that gene expression is under doxycycline control (Figure 15A). Expression of SNAIL induced by treatment with doxycycline (1  $\mu$ g/ml) for 10 days, caused EMT like changes in the phenotype of cells. Canonical epithelial markers E-cadherin and claudin were downregulated and canonical mesenchymal marker vimentin was upregulated after 10 days of doxycycline induced SNAIL expression (Figure 15B).

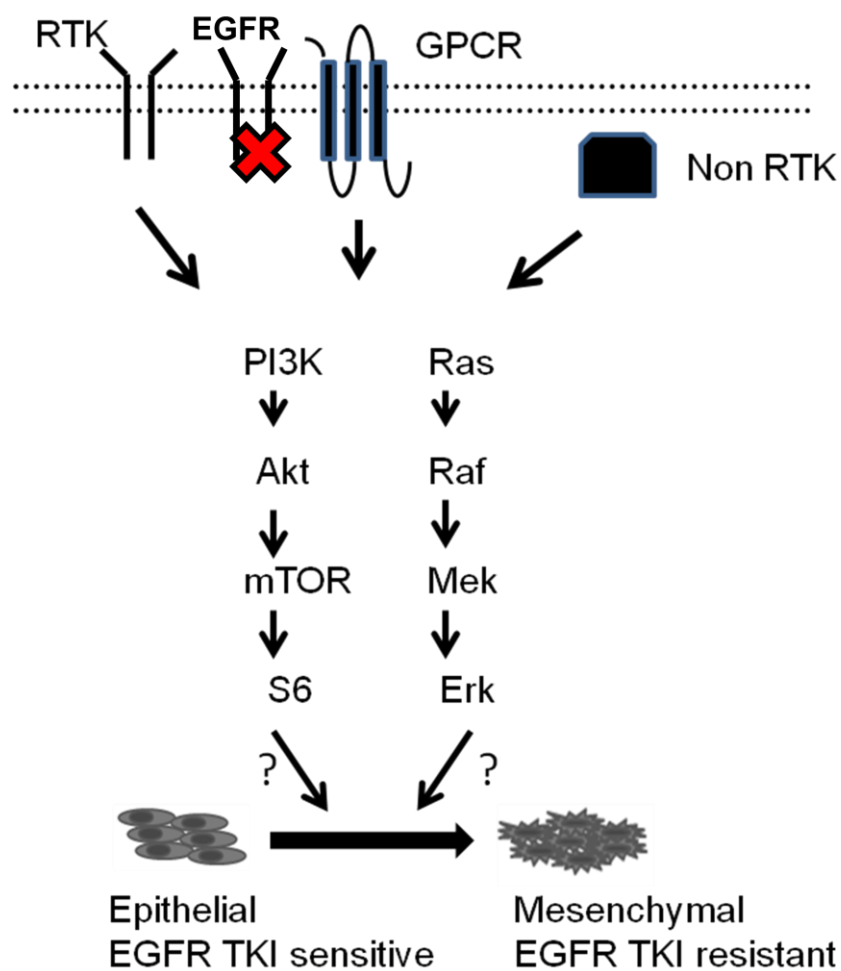
Our observations with stable SNAIL expression in HCC827 cells suggested that mesenchymal phenotype induced by SNAIL expression confers EGFR TKI resistance in EGFR mutant NSCLC (Figures 8 and 9). However after 10 days of doxycycline induced SNAIL expression, HCC827 cells remained sensitive following 72 hours exposure to 1  $\mu$ M gefitinib (Figure 16). One possible explanation for this would be that level of SNAIL expression achieved after 10 days of doxycycline

treatment is sufficient to engage the cells into a mesenchymal state but insufficient to induce a complete EMT to render the cells resistant to EGFR TKIs. There is substantial evidence that SNAIL plays a prominent role in triggering EMT while activation of other EMT regulators including SLUG, ZEB1 or TWIST is essential in maintaining the mesenchymal phenotype (105). It is tempting to speculate that such a subsequent activation of other EMT regulators might be achieved over a period of culturing of HCC827 cells with stable ectopic SNAIL expression but this event is not recapitulated by the short term inducible SNAIL expression in HCC827 cells. Long-term doxycycline exposure of HCC827 cells expressing inducible SNAIL vector might enable complete induction of EMT and render the cells resistant to EGFR TKIs.

Ectopic SNAIL overexpression has been shown to confer resistance to a number of chemotherapeutic agents including paclitaxel, camptothecin, doxorubicin and 5-Fluorouracil in various carcinomas (84, 106, 107). Based on these reports, we expected that ectopic SNAIL overexpressing EMT positive HCC827 cells would be resistant to chemotherapeutic agents. Surprisingly, SNAIL overexpressing HCC827 cells were sensitive to chemotherapeutic agent gemcitabine (Figure 14) suggesting that SNAIL induced drug resistance is specific to EGFR TKIs.

Although a link between EMT induced by transcription factor SNAIL and drug resistance has been established, the mechanisms by which EMT confers insensitivity to EGFR TKIs are poorly understood. One mechanism explaining the survival of EGFR dependent NSCLC even in the absence of EGFR activation is

acquisition of alternate routes to activate PI3K/Akt and Ras/Raf/Mek/Erk pathways (95). EMT can promote the survival and proliferation of cancer cells by activation of alternate receptor tyrosine kinases including HER3, MET, PDGFR, IGF1-R that are capable of activating PI3K/Akt/ and Ras/Raf/Mek/Erk pathways (95, 96). Recently, upregulation of AXL receptor tyrosine kinase has been strongly linked to increased activation of PI3K/Akt/ and Ras/Raf/Mek/Erk pathways in EMT positive EGFR TKI resistant models of NSCLC (78, 79). However, ectopic SNAIL overexpressing HCC827 cells did not show increased activation of any of the known promoter of cellular survival and proliferation (Figures 10 and 11). Further, treatment of SNAIL overexpressing HCC827 cells with AXL inhibitor XL-184 (1  $\mu$ M) for 72 hours did not compromise the viability of cells, suggesting that AXL is less likely to be regulating cell survival and proliferation of SNAIL overexpressing HCC827 cells (Figure 12). Additionally, 48 hours exposure of SNAIL overexpressing HCC827 cells to 1  $\mu$ M gefitinib did not suppress phosphorylations of Akt and Erk (Figure 13 suggesting that novel receptor or non-receptor tyrosine kinase or G protein coupled receptor pathways are established in the HCC827 SNAIL overexpressing cells to offset the loss of mutant EGFR induced activation of PI3K/Akt and Ras/Raf/Mek/Erk pathways, thereby acting as promoters of EGFR inhibitor resistance (Figure 17). Future studies designed to investigate this novel driver of cell survival and proliferation in SNAIL induced EMT model of EGFR mutant NSCLC could provide a potential drug target to treat resistant NSCLC.



**Figure 17. A schematic of proposed mechanism of EGFR TKI resistance in EGFR mutant NSCLC.** A novel receptor tyrosine kinase (RTK), G protein coupled receptor (GPCR) or non-RTK might be activating PI3K/Akt and Ras/Raf/Mek/Erk pathway to promote cell survival and proliferation even in the absence of EGFR activation.

Literatures suggest that TGF- $\beta$  is a potent inducer of EMT that requires activation of transcription factor SNAIL to exert its effects (89, 108). EGFR mutant NSCLC cells induced to mesenchymal state by chronic exposure to TGF- $\beta$  express high level of SNAIL and are insensitive to EGFR TKIs, suggesting that the biological

activities induced by the expression of SNAIL might affect drug sensitivity (Figures 4 and 5). Interestingly, SNAIL knockdown in cisplatin resistant pancreatic cancers and gemcitabine resistant ovarian cancers has been shown to restore sensitivity of the cancer cells to cisplatin and gemcitabine (93, 109). These evidences suggest that the inhibitors of SNAIL activity might prevent SNAIL-dependent mesenchymal transition to restore or gain cellular sensitivity to EGFR TKIs.

Due to the fact that transcription factors mediate their actions mostly through protein-protein interactions rather than enzymatic activities, they have been traditionally regarded as undruggable targets. Recently rigorous efforts are being undertaken to understand the mechanisms of action of transcription factors that would enable rational designing of drugs targeting transcription factors (110, 111). A group of researchers have developed a novel SNAIL inhibitor GN-25 that was shown to suppress SNAIL and reverse SNAIL-induced mesenchymal phenotype to epithelial phenotype through genetic reprogramming of EMT genes (110). Another group of researchers have reported targeted inactivation of SNAIL family transcription factors by use of novel Co(III) E-box conjugate (111). Co(III) E-box conjugate is a Schiff base compound that selectively inhibits transcription factors that harbor zinc finger domain and bind to E-boxes. Such a dual specificity of Co(III) E-box conjugate might allow for targeted SNAIL disruption with very few off target effects. Additionally, phosphorylation of SNAIL on serine residues 104 and 107 by glycogen synthase kinase- $\beta$  (GSK3 $\beta$ ) has been reported to unmask the nuclear export sequence facilitating nuclear exit and subsequent ubiquitination of SNAIL

(82). This action is counteracted by a small C-terminal domain phosphatase (SCP) that stabilizes SNAIL in the nucleus (82). Agents that might potentially phosphorylate SNAIL at serine residues 104 and 107 and target SNAIL towards ubiquitination or agents that might inhibit the activation of SCP could be developed as a way to prevent and inhibit SNAIL activation. Concomitant administration of EGFR TKIs and SNAIL inhibitors might serve as a preventive treatment for the induction of mesenchymal phenotype with EGFR TKI resistance by retaining EGFR TKI sensitivity of epithelial EGFR mutant NSCLC. Furthermore, molecular chaperone Hsp90 has been reported to bind to and stabilize phosphorylated SNAIL in response to DNA damage (112). Consequently, destabilization of SNAIL by Hsp90 inhibitors might reverse SNAIL-dependent mesenchymal phenotype back into epithelial phenotype, which could be sensitive to EGFR TKIs. Taken together, our data suggests that development of drugs targeting transcription factor SNAIL is a promising strategy in overcoming EGFR TKI resistance and enhancing the efficacy of molecular targeted therapy in EGFR mutant NSCLC.

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## **VITA**

Rutu Gandhi was born in Mumbai, India on March 24, 1989 to Rupa and Shailesh Gandhi. She received Bachelor of Pharmaceutical Sciences degree from Gujarat University (Ahmedabad, India) in May 2011.

While studying pharmaceutical science, Rutu got interested in studying cancer therapeutics and decided to move to United States to explore the field of cancer drug research. In August of 2011, Rutu joined the department of Molecular Pharmacology & Experimental Therapeutics at Loyola University Medical Center (Maywood, IL). Shortly thereafter, she joined the laboratory of Dr. Takeshi Shimamura where she studied epithelial to mesenchymal transition as a mechanism of resistance to epidermal growth factor receptor tyrosine kinase inhibitors in EGFR mutant non-small cell lung cancer.

After finishing her M.S., Rutu plans to work as a researcher in pharmaceutical industry to continue her pursuit of exploring the field of cancer drug research.