Role of c-Met and EGFR synergism and identification of targets causing resistance to their inhibitors

BY

Jason Thomas Fong
B.S., University of Illinois Urbana-Champaign, 2006

THESIS

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Defense Committee:

Khalifah Sidik, Chair
Aoshuang Chen
Gnanasekar Munirathinam

This thesis is dedicated to my sister, Megan, without whom it would never have been realized and my thoughts would never be here.

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LIST OF ABBREVIATIONS

TKI	Tyrosine Kinase Inhibitors
EGFR	Epidermal Growth Factor Receptor
c-Met	Hepatocyte Growth Factor Receptor
NSCLC	Non-small Cell Lung Cancer
IC50	Half maximal inhibitory concentration
PFS	Progression Free Survival
mTOR	Mammalian Target of Rapamycin
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
S6 Kinase	Ribosomal protein S6 kinase beta-1
β-catenin	Beta-catenin
DNA	Deoxyribonucleic Acid
PDCD6	Programmed cell death 6
AIF	Apoptosis-inducing factor
HMGB2	High-mobility group protein B2
TOM34	Translocase of outer mitochondrial membrane 34
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
EML4-ALK	echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase
SCLC	Small Cell Lung Cancer
AC	Adenocarcinoma
SCC	Squamous Cell Carcinoma
LCC	Large Cell Carcinoma

LIST OF ABBREVIATIONS (continued)

OS	Overall Survival
RTK	Receptor Tyrosine Kinase
HGF	Hepatocyte Growth Factor
EGF	Epidermal Growth Factor
AKT	RAC-alpha serine/threonine-protein kinase
MAPK	Mitogen-activated protein kinase
ATP	Adenosine Triphosphate
mAb	Monoclonal Antibody
p-EGFR	Phosphorylated Epidermal Growth Factor Receptor
p-c-Met	Phosphorylated c-Met
p-mTOR	Phosphorylated Mammalian Target of Rapamycin
p-S6 kinase	Phosphorylated Ribosomal protein S6 kinase beta-1
ERK	Extracellular-Signal-Regulated Kinase
p-ERK	Phosphorylated Extracellular-Signal-Regulated Kinase
p-4E-BP1	Phosphorylated Eukaryotic translation initiation factor 4E-binding protein 1
β-actin	Beta-actin
RPMI	Roswell Park Memorial Institute
FBS	Fetal Bovine Serum
EDTA	Ethylenediaminetetraacetic Acid
SILAC	Stable isotope labeling by amino acids in cell culture
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

LIST OF ABBREVIATIONS (continued)

PBS	Phosphate-Buffered Saline
BSA	Bovine Serum Albumin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBST	Tris-Buffered Saline Tween 20
MS	Mass Spectrometry
PCR	Polymerase Chain Reaction
MCM2	Minichromosome maintenance protein 2
MCM6	Minichromosome maintenance protein 6
TPR	Translocated promoter region
mRNA	Messenger Ribonucleic acid

SUMMARY

While the use of specific Tyrosine Kinase Inhibitors (TKIs) against epidermal growth factor receptor (EGFR) and Hepatocyte Growth Factor Receptor (c-Met) in Non-Small Cell Lung Cancer (NSCLC) are very effective at increasing patient progression free survival (PFS), their efficacy is limited by the subsequent development of resistance and tumor recurrence. Therefore, to establish the mechanism of TKI resistance in NSCLC, two cell lines (H2170 and H358) were made resistant to c-Met (SU11274) and EGFR (erlotinib) inhibitors (4-6-fold and 11-22-fold increase in IC_{50} of SU11274 and erlotinib, respectively). Rather than focusing on secondary mutations, several of which are currently established, we have studied alternative signaling pathways that may be essential in the development of acquired resistance.

We have developed two models of c-Met/EGFR TKI resistant NSCLC cell lines. In our resistant cell lines, one model displays constitutive phosphorylation of EGFR and increased phosphorylation of c-Met, while the other model exhibits decreased phosphorylation of both EGFR and c-Met. Interestingly, in both models we find a 2-4-fold increased activation of mTOR (mammalian target of rapamycin), its substrates 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) and S6 Kinase (Ribosomal protein S6 kinase beta-1) and increased activation of β-catenin (beta catenin), which plays a role in the Wnt signaling pathway.

To confirm the role of mTOR and Wnt pathways in resistance and to explore options to break this resistance, parental and resistant cell lines were treated with an

mTOR inhibitor, everolimus, and a Wnt inhibitor, XAV939, in addition to c-Met/EGFR TKIs SU11274 and erlotinib. When everolimus was added to the c-Met/EGFR TKI combination treatment, resistant cell lines were found to be significantly ($p<.001$) more susceptible in comparison to parental cells. However in response to XAV939, parental cell lines displayed no sensitivity, while resistant lines displayed a significant decrease in viability when used alone ($p<.001$) or when added to the c-Met/EGFR TKI combination treatment ($p<.001$). These results suggest that targeting the mTOR and Wnt pathways may be viable options for NSCLC patients with acquired resistance to EGFR/c-Met inhibitors. Furthermore, results from DNA sequencing of EGFR exons 18-21 (kinase domain and sites of activating EGFR mutations and secondary resistance mutations) in our resistant cells confirm no T790M or D761Y secondary mutations that commonly cause erlotinib-resistance. Hence, our models would establish a mechanism of inhibitor resistance that is separate from secondary resistance mutations.

In addition to mTOR and the Wnt signaling pathways, we also identified several proteins exhibiting modulated expression in resistant cells. These include downregulation of pro-apoptotic proteins such as PDCD6 (Programmed cell death 6) and AIF1 (Apoptosis-inducing factor), downregulation of total β-catenin, which would increase tumorigenicity, and upregulation of translation proteins such as HMGB2 (High-mobility group protein B2) and TOM34 (Translocase of outer mitochondrial membrane 34). While unconfirmed, these proteins may play a role in EGFR/c-Met TKI resistance and warrant further study.

Overall our studies find modulations in the Wnt and mTOR pathways after resistance is acquired to EGFR and c-Met TKIs in NSCLC, and these modulations could be used as targets to overcome c-Met/EGFR TKI resistance in NSCLC patients.

1. INTRODUCTION

1.1 Background

Lung cancer is the most prevalent form of cancer in the world (1) and is the leading cause of cancer-related mortality in the United States and around the world (2). With over 220,000 new cases in the United States each year, it accounts for 14% of all new cancer cases (3). However, with over 157,000 deaths, lung cancer accounts for 28% of all US cancer related deaths (3). Worldwide, lung cancer accounts for 1.4 million new cases and 1.6 million deaths (4). While the causes of lung cancer can be traced to cigarette, pipe and cigar smoking (5), numerous other factors include: secondhand smoke (6), diet and food (7), alcohol (8), lack of physical activity (9), air pollution (6), occupational exposure (10) and mutations in key genes such as *EGFR*, *EML4-ALK* and *KRAS* (11-14).

Out of all lung cancer cases, Non–small cell lung cancer (NSCLC) accounts for 85% while Small cell lung cancer (SCLC) accounts for 15% of lung cancer cases in the United States (2, 15). Historically, the subtypes of lung cancer were distinguished by surgical pathologists because of their vastly different natural histories, histology and treatment options (16). As defined by the World Health Organization, NSCLC can be further subcategorized into three main histological subtypes: adenocarcinoma (AC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC) (17). Both AC and SCC are commonly associated with smoking (18). AC is the most common form of NSCLC and includes a morphologically heterogeneous group of tumors. While AC is associated with smoking, it is also the most common form of cancer seen in “never

smokers" (individuals who have smoked less than 100 cigarettes over their lifetime) (19-21). AC has a high mortality rate since the disease generally has already metastasized before the development of symptoms (17). LCC is the least common of the three main subtypes of NSCLC, its criteria for diagnosis is not well defined (17) and the clinical behavior of some LCC appears to be similar to that of SCLC (17, 22).

While traditionally lung cancer subtypes were classified by their histological features, in the past decade, pathologists and scientists have identified several driver mutations in lung cancer (13). The most common driver mutations include mutations in the kinase domain of the *KRAS* (30%), *EGFR* (5-15%), and *EML4-ALK* (5-15%) genes (13, 14). Currently, clinicians and researchers are now classifying patients and treatment options by their driver mutations in addition to histology and staging (23). Researchers have now developed Tyrosine Kinase Inhibitors (TKIs) that serve to inhibit the activity of the kinases affected by these driver mutations.

1.2 **Statement of problem**

Advances in the development of TKIs have enabled a new generation of highly specific TKIs against EGFR, and c-Met (24-27) to be used for the therapy of lung cancer patients. However, while these TKIs are on the cutting edge of cancer therapy, their individual efficacies are limited (28). Furthermore, virtually all patients who initially respond to treatment or achieve disease stabilization inherently develop secondary resistance resulting in tumor recurrence and progression (29-32). Thus, for patients with acquired secondary resistance, identifying clinical and molecular predictors in addition

to targeting the mechanism of resistance may result in significantly improved clinical outcomes (33).

1.3 Purpose of study

This investigation elucidates target proteins and pathways involved in the development of TKI resistance. It further attempts to determine combinations of TKIs which could be used to target identified proteins and pathways to overcome TKI resistance. Understanding the effects of TKI resistance in NSCLC will help us understand how modulated signaling pathways can confer resistance and discover new drug targets. Results from this study will elucidate the synergistic signaling effects in NSCLC, enable us to gain a better understanding of how enhanced alternative signaling in NSCLC drive resistance to EGFR TKIs and may lead to the development of therapeutic strategies aimed at reducing the effects of these new signaling pathways.

1.4 Significance of the study

Treatment with TKIs, such as erlotinib, results in development of resistance in all patients within 9.4 to 13.3 months (28, 32, 34). Results from this project could provide clinicians with additional combination treatment options for lung cancer patients to increase overall survival (OS). Resistance to TKIs is an important driver of disease progression, and understanding pathways that mediate resistance is vital to increase patient survival with acquired TKI resistance (26). Furthermore, while the use of combined therapy modalities may limit the ability of tumors to develop resistance to specific TKIs, identifying new targets is the best approach for discovering future

treatment options (26). These clinically relevant studies will move lung cancer research forward as they have great potential for improving the treatment of lung cancer patients.

1.5 **Hypothesis**

We hypothesize that modulation of alternative signaling proteins/pathways could be involved in the development of c-Met/EGFR TKI resistance, and inhibition of modulated signaling proteins/pathways could restore c-Met/EGFR TKI sensitivity.

2. REVIEW OF RELATED LITERATURE

2.1 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are essential proteins for normal cellular development and growth, but they are also involved in the pathogenesis of a variety of tumors, including lung cancer (25). c-Met and EGFR are both RTKs that, along with their respective ligands HGF (hepatocyte growth factor) and EGF (epidermal growth factor), play key roles in the tumor growth, metastasis and angiogenesis of NSCLC (25, 35). EGFR and c-Met share several common signaling pathways, including the AKT and MAPK pathways (36), and are both highly expressed in NSCLC tumors where up to 61% overexpress c-Met and 80% overexpress EGFR (37, 38). Activation of c-Met via the TGFα/EGFR axis resulting in EGFR/c-Met cross talk in tumors is suggested by the fact that c-Met co-immunoprecipitates with EGFR in protein extracts from tumor cells, but not normal hepatocytes (39). Additionally, it has also been previously shown that EGFR and c-Met work synergistically to enhance cell proliferation, apoptosis and downstream signaling in NSCLC (26).

2.2 Clinical response of tyrosine kinase inhibitors

Tyrosine kinase inhibitors (TKIs) inhibit their target receptors very specifically and are currently being used regularly in clinics and clinical trials. Erlotinib is a selective and reversible inhibitor of the EGFR tyrosine kinase domain (28) and works by competing for the ATP binding pocket in the EGFR kinase domain (28). Unfortunately, tumor response to EGFR-specific TKIs in NSCLC is only 10% (gefitinib) and 8.9% (erlotinib) in refractory NSCLC patients (25). However, in studies with patient

populations selected for EGFR activating mutations, the response rate was 55-82% to erlotinib or gefitinib and patient PFS ranged from 9.4 to 13.3 months (34). These response rates and PFS are 3-4-fold greater than those in previously observed studies with platinum-based chemotherapy which had response rates at 20% to 30% and PFS at 3-4 months (40).

SU11274 is also an ATP-competitive small molecule inhibitor of the catalytic activity of c-Met, and while not clinically used to treat patients, it is regularly used *in vitro* to induce c-Met inhibition (26, 41-43). Tivantinib (previously ARQ197), a recent non ATP competitive oral c-Met TKI, binds preferentially to the inactive conformation of c-Met and thereby stabilizes this conformation. The inactive c-Met conformation disrupts downstream signaling, and causes anti-proliferative activity. Tivantinib is currently in Phase III clinical trials and has shown increased PFS from 9.7 to 16.1 weeks when given with erlotinib (31).

2.3 **TKI obstacles in lung cancer therapy**

While TKIs against EGFR and c-Met are on the cutting edge of cancer therapy, their individual efficacies are limited (28), leading to resistance to EGFR TKIs resulting in recurrence of NSCLC (44). As seen *in vitro* and *in vivo* NSCLC studies, *MET* amplification, which is accelerated by autocrine production of its ligand HGF (hepatocyte growth factor), accounts for more than 20% of acquired resistance to EGFR TKIs (45, 46). Furthermore, development of secondary ”gatekeeper” mutations, such as T790M or D761Y, which have been demonstrated *in vitro* and *in vivo* to substantially

suppress the inhibitory effects of EGFR TKIs, accounts for 50% of all acquired resistance to EGFR TKIs in patients (47, 48). These mutations, if present prior to treatment with erlotinib or gefitinib, could also result in primary resistance to EGFR TKI therapy (49). The above two mechanisms of resistance, which have been seen both *in vitro* and *in vivo,* are not mutually exclusive and can occur independently and simultaneously. To explore further mechanisms of resistance, it is important to conduct additional *in vitro* studies to determine target proteins responsible for TKI resistance in NSCLC.

2.4 **TKI combinations in NSCLC**

To overcome EGFR resistance, irreversible EGFR TKIs have been developed and are currently being tested (50, 51). While some are proving mildly effective, irreversible EGFR TKIs still only target tumor cells that are dependent on EGFR signaling and have some toxic effects due to concurrent inhibition of wild-type EGFR (50). To prevent and overcome individual TKI resistance, a combination of inhibitors of c-Met and EGFR has been employed in recent clinical trials with some success (30, 31). Current research has focused on a promising TKI combination: erlotinib plus tivantinib (31, 52). Unfortunately, patients undergoing treatment with these TKIs against EGFR and c-Met, while initially responsive, eventually tend to develop resistance to TKI therapy (29). While results from these trials indicate that median PFS was increased marginally from 9.7 to 16.1 weeks, tumors treated with a combination of tivantinib and erlotinib still developed resistance (31). Only certain subsets (KRAS mutants, non-squamous histology and EGFR wild type status) of patients exhibited significantly

increased PFS (31), suggesting that while tivantinib and erlotinib are effective, new TKIs need to be added to this combination to increase efficacy.

Other research has focused on the use of monoclonal antibodies (mAb) such as onartuzumab (one armed anti-c-Met mAb) (53). In a phase II clinical trial, onartuzumab (previously, MetMab, OA-5D5) and erlotinib reduced the risk of death by 3-fold only in a subset of patients positive for c-Met expression by IHC (30, 53). However, onartuzumab in combination with erlotinib in patients with advanced NSCLC did not demonstrate an improvement in PFS or OS in the overall intent to treat population (54). However, in patients with high c-Met expression, a trend towards improvement in both PFS (HR 0.56, $p = 0.0547$) and OS (HR 0.55, $p = 0.1113$) was seen (30). While the use of combined therapy modalities may limit the ability of tumors to develop resistance to specific TKIs (55), understanding the mechanism of resistance is truly the best approach for discovering future treatment targets (56).

Earlier studies indicate that c-Met and EGFR have considerable cross talk (26, 39), which is further substantiated by the fact that HGF can transactivate EGFR and phosphorylation of EGFR can activate c-Met resulting in synergistic tumor growth (57-59). Since crosstalk between c-MET and EGFR receptors increases efficacy for TKI combinations *in vitro* (26), we have decided to determine a combination therapy which could be effective in cells which are resistant to EGFR, c-Met or EGFR/c-Met TKI combination therapy. We believe there may be specific changes in c-Met and EGFR downstream signaling that may enable NSCLC cells to become resistant to EGFR/c-Met

combination therapy. Therefore, to understand how cells develop resistance to c-Met and EGFR TKIs, we have developed H2170 and H358 NSCLC cell lines with acquired resistance to inhibitors of c-Met, EGFR, and a combination of both TKIs. By utilizing these cell lines, we have identified new targets/pathways that confer resistance to c-Met/EGFR TKIs. These targets/pathways could be the basis for future TKI therapy combinations to improve outcome in lung cancer patients.

2.5 New directions for TKIs

Previous studies have shown that an mTOR inhibitor, rapamycin, is able to cooperate with a c-Met inhibitor in NSCLC (60). Additionally, studies have found synergistic effects when using erlotinib and rapamycin or everolimus (oral derivative of rapamycin) combination therapy on cell viability, proliferation and autophagy (61, 62). This combination can also restore gefitinib sensitivity (63). However, these studies only administered EGFR and mTOR inhibitors in combination. In our studies, we have found that mTOR can increase the efficacy of EGFR/c-Met TKI combination therapy. Since other studies have found that using AEE788 (which targets multiple tyrosine kinases such as EGFR, vascular endothelial growth factor receptor, and human epidermal growth factor receptor 2) in combination with mTOR inhibitors are much more effective when compared to a combination of only EGFR and mTOR inhibitors (64-66), we further studied a combination of EGFR/c-Met/mTOR TK inhibitors with Wnt inhibitors and found them to be effective in resistant NSCLC *in vitro*. These studies could be the basis for new clinical trials utilizing c-Met, EGFR, mTOR and Wnt inhibitors that could greatly improve patient prognosis.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents and antibodies

SU11274 (cat. no. S9820) and XAV939 (cat. no. X3004) were obtained from Sigma-Aldrich (St. Louis, MO). Erlotinib (cat. no. E-4007) and everolimus (cat. no. E-4040) were obtained from LC Laboratories (Woburn, MA). Tivantinib (cat. no. CT-ARQ197) was obtained from Chemietek (Indianapolis, IN). All inhibitors were suspended in DMSO and kept in 15µl aliquots at –20°C. HGF (cat. no. 100-39) was obtained from Peprotech (Rocky Hill, NJ), and EGF (cat. no. 8916) was obtained from Cell Signaling Technology (Beverly, MA). All antibodies, as listed in **Table I**, were obtained from the indicated sources and utilized for determination of protein expression by immunoblotting as described in section 3.2.2.4 and diluted as described in the manufacturer's instruction's sheet.

Table I.
ANTIBODIES UTILIZED FOR IMMUNOBLOTTING

Antibody target	Dilution	Source	Provider
p-EGFR (Tyr 1068)	1:1000	Rabbit	Cell Signaling Technology 3777
p-c-Met (Tyr 1003)	1:1000	Rabbit	Cell Signaling Technology 3135
p-mTOR (Ser2448)	1:1000	Rabbit	Cell Signaling Technology, 5536
p-S6 kinase (Tyr421/Ser424)	1:1000	Rabbit	Cell Signaling Technology, 9208
phospho-ERK1/2 (Thr202/Tyr204)	1:1000	Rabbit	Cell Signaling Technology 2532
p-4E-BP1 (Thr37/46)	1:1000	Rabbit	Cell Signaling Technology 2855
Active β-Catenin	1:1000	Rabbit	Millipore 05-665
Total β-Catenin	1:1000	Rabbit	NeoMarker PA6-RB-1491-PABX
β-Actin	1:1000	Mouse	Sigma, 5441
Mouse IgG secondary	1:1000	Horse	Cell Signaling Technology 7076
Rabbit IgG secondary	1:1000	Goat	Cell Signaling Technology 7074

3.1.2 **Cell culture**

Both NSCLC cell lines H358 and H2170 were obtained from the American Type Culture Collection (Rockville, MD, USA, CRL-5807 and CRL-5928, respectively), and were cultured according to their instructions (http://www.ATCC.org) and incubated at 37°C and 7% CO2. Cell lines were grown and maintained in complete Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific, Pittsburg, PA, Cat No: SH3002701) and supplemented with 10%(v/v) Fetal Bovine Serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, S11050), 1% (v/v) Hepes (1mM final concentration) (Invitrogen, 11360), 1% (v/v) Sodium Pyruvate (1mM final concentration) (Invitrogen, 11360) and 1% (v/v) Antibiotic-Antimycotic Solution (100µg/mL streptomycin, 100units/mL penicillin, and 0.25µg amphotericin B/mL) (Invitrogen, 15070-063).

3.1.2.1 **Propagation of lung cancer cells**

Cells were grown in media described above in 6-well plates to approximately 95% confluency, and passed at 1 week intervals as follows: media was aspirated from flasks and replaced with 0.5mL of 0.25% Trypsin/0.1% EDTA (2.5 g/L trypsin and 1.0 g/L EDTA) (Thermo Fisher Scientific, MT-25-053-CI) for 30 seconds. After which, the trypsin solution was aspirated and the cells rinsed again with 1mL 0.25% Trypsin/EDTA for an additional 30 seconds. After which, the trypsin solution was aspirated and the cells rinsed again with 1mL 0.25% Trypsin/EDTA for an additional 30 seconds. After a 5 minute incubation, trypsinized cells were collected into 15mL polypropylene tubes with 2 washes of 5mL medium as described above containing 10% FBS to inactivate trypsin, and centrifuged at 1000 rpm for 3 minutes. After centrifugation, the supernatant was

removed via aspiration, cell pellet was re-suspended in 3mL RPMI media (described previously), and $2x10^5$ plated into replicates of 3 in 6-well plates (1:10 split ratio).

3.1.2.2 **Propagation of lung cancer cells in heavy and light media**

Parental and resistant cells were grown in heavy and light, respectively, stable isotope labeling by amino acids in cell culture (SILAC) media for 6 passages (30 days) using SILAC RPMI (Thermo Scientific) containing 0.1mg/mL "heavy" $^{13}C_6$ L-lysine-2HCl and $^{13}C_6$ $^{15}N_4$ L-Arginine-HCl or "light" L-lysine-HCL and L-Arginine-HCl with Dialyzed FBS. All other conditions are similar to those described in section 3.1.2.1.

3.2 **Methods**

3.2.1 **Cell viability assays using MTT**

To study the effects of the EGFR, c-Met, mTOR and Wnt inhibitors, erlotinib, SU11274, everolimus and XAV939, on cell growth, cell viability was measured by a MTT colorimetric dye reduction assay (Sigma, St. Louis, MO, cat. no. TOX1) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) according to the manufacturer's instructions. Each experiment was done in 96 well plates in six replicate wells for each drug concentration. The IC_{50} value is defined as the concentration needed for a 50% reduction in absorbance is calculated from the MTT assay. To determine the inhibitory effects of EGFR inhibitor erlotinib, c-Met inhibitor SU11274, Wnt Inhibitor XAV939, and mTOR inhibitor everolimus, 5000 cells/well were plated in replicates of six in a 96 well plate as described above and treated after 24 hours at the indicated concentrations of inhibitors in medium with 10% FBS. At 96 hours, cell viability

was measured by adding the MTT reagent, incubating for four hours, after which solubilization solution was added, and absorbance measured spectrophotometrically at a wavelength of 570 nm and background absorbance subtracted at 690 nm from the 570 nm measurement. Percentage of cell viability was determined relative to the control. All drug inhibition studies to study proliferation were performed in media containing 10% FBS. Statistical significance was obtained using an ANOVA test with α at .05 and paired student T tests were used to measure differences between two individual groups.

3.2.2 **Immunoblotting**

3.2.2.1 **Protein extraction and quantification**

For total protein extraction from previously cultured and treated NSCLC cells; cells were washed 2x with ice cold phosphate buffered saline (PBS) and lysed for 60 seconds on ice with 100µL of lysis buffer (phospho-protein extraction buffer (20mM Tris-HCl pH 8, 150mM NaCL, 100mM NaF, 1% NP-40, 10% glycerol (Boston Bioproducts, BP-116P) supplemented with 1mM sodium orthovanadate (Boston Bioproducts, BP-440), and protease inhibitor cocktail from Roche (Roche, Indianapolis, IN, 04-693-124-001). Cells were then scraped off the plate and disrupted by passing 5X through a 26 gauge 3/8 inch needle. Cell lysates were further disrupted using a SONIFIER cell disrupter 350 (Branson Sonic Power, Danbury, CT) using 5 pulses of 10 second each with settings of 15 for percent duty and 3 for output control. Lysate was then centrifuged at 14,000 rpm for 20 min in the cold room, and transferred to new tubes and stored at -80°C.

Estimation of sample protein concentration was performed using the Bradford Method. Briefly, a standard curve was prepared using 1mg/mL Bovine Serum Albumin (BSA) (Sigma, A7906) dissolved in water as shown in **Table II**. Cell lysate samples were prepared in duplicate by diluting 3µL lysate with 97µL water to make total sample volume 100µL. Samples were then mixed with 900µL of prepared Bradford reagent (1 part Bradford, 4 parts water) (Bio-Rad, Hercules, CA, 500-0001) with vortexing. The protein standard curve was generated using a Beckman du 650 spectrophotometer (Beckman Coulter, Brea, CA), and measuring absorbance at 595nm. Protein sample concentration was then obtained by measuring sample absorbance and calculating protein concentration by a formula obtained from the protein standard curve.

Table II.
PROTEIN STANDARD CURVE

Standard	Distilled H_2O	BSA
Blank	100µL	0µL
1µg	99µL	1µL
2µg	98µL	2µL
4µg	96µL	4µL
6µg	94µL	6µL
8µg	92µL	8µL
10µg	90µL	10µL
12µg	88µL	12µL
14µg	86µL	14µL
16µg	84µL	16µL

3.2.2.2 **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis for proteins**

Protein samples were prepared by adding 50µg protein to 4X Laemelli's loading buffer (250mM Tris-HCl ph 6.8, 8% Sodium dodecyl sulfate (SDS), 40% glycerol, 8% β-mercaptoethanol, and 0.02% bromophenol blue (Boston Bioproducts, Boston, MA, BP-110R)). Prepared samples were denatured at 100°C for 8 min, centrifuged for 30 seconds at 14,000 rpm, and loaded into appropriate wells. Prepared protein samples and dual colored protein standards (Bio-Rad, Hercules, CA, 161-0374) were loaded onto a prepared 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoresed for approximately 1.5 hours at 90 volts in a Mini-Protean Tetra Cell (Bio-Rad 165-8005EDU) using the PowerPac Basic Power Supply (Bio-Rad, 164-5050EDU). Running buffer with 25mM Tris (pH 8.3), 0.19M glycine and 0.1% SDS was obtained from Boston Bioproducts (BP-150).

3.2.2.3 **Transfer to nitrocellulose membrane**

After completion of electrophoresis, gels were removed from gel cassettes and proteins were transferred onto a nitrocellulose membrane (Bio-Rad, 162-0112) using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, 170-3940). Samples were transferred in transfer buffer (Boston Bioproducts, BP-190) containing Tris 0.025M (pH 8.4), glycine 0.19M and 20% methanol at 18V for 45 minutes.

3.2.2.4 **Immunodetection**

After transfer, nitrocellulose membranes were rinsed 3x in Tris-buffered Saline (50mM Tris pH 7.4 and .15M NaCl) (BM-301) and 0.05% Tween 20 (TBST), and

blocked for 1 hour using 5% non-fat dry milk (Bio-Rad, 170-6404) in TBST. Membranes were then rinsed 3X in TBST, and then incubated overnight at 4°C with primary antibody, prepared in 2% BSA in TBST at the manufacturer's suggested dilutions. After overnight incubation, primary antibody was removed and membranes were rinsed in TBST 2x for 5 minutes, 2x for 10 minutes, and 2x for 15 minutes. Membranes were then incubated for 1 hr at room temperature with appropriate secondary antibody (diluted 1:1000 in 1% non-fat dry milk in TBST). Membranes were then rinsed 2x for 5 minutes, 2x for 10 minutes, and 2x for 15 minutes in fresh TBST. To visualize proteins, membranes were treated with prepared Pierce® ECL Western Blotting Substrate (Fisher Scientific, Rockford, IL, 32109) for 60 seconds, and exposed to an autoradiography film (Scrip, Inc, Boling Brook, IL, 688-0013) for various time points, after which the film was fixed and developed using the Konica SRX-101A developer (Konica Minolta, Philadelphia, PA).

3.2.2.4.1 **Analysis of immunoblotting results**

To qualitatively determine relative expression of proteins previously immunoblotted, exposed and developed autoradiography films were scanned by a Hewlett-Packard HP Scanjet G3010 scanner and densitometric analysis was done using the Image J software developed by the National Institute of Health. Values presented represent percent of control from a representative experiment.

3.2.3 Specific phosphorylation of c-Met/EGFR and other signaling pathways via HGF/EGF and their inhibition

H358 and H2170 cells that expressed c-Met/EGFR were deprived of growth factors by incubation in serum free medium containing 0.5% BSA for 24 hours with or without inhibitors. After treatment with or without inhibitors in serum free medium, cells were stimulated with or without HGF 40ng/mL for 7.5 minutes or EGF 5ng/mL for 5mins at 37°C. After preparing lysates as described above, the cells were subjected to the standard procedures of immunoblotting as described earlier. Antibodies specifically against phosphorylated c-Met and other proteins were used as described above.

3.2.4 Immunofluorescence

10,000 cells were plated on chamber slides, allowed to adhere for 24 hours and then kept in serum free medium with 0.5% BSA overnight. Cells were then treated with ± EGF for 15 min, fixed with 1:1 acetone:methanol and visualized with p-EGFR (Y1068) primary antibody and anti-rabbit DyLight 488 secondary antibody (Thermo Fisher Scientific) on a Zeiss Axio Observer Z1 fluorescent microscope. Total cell fluorescence was measured using ImageJ to measure fluorescence intensity over 8 microscopic fields per condition. Values were averaged and corrected for background. Statistical significance was obtained using an ANOVA test with α at .05 and paired student T tests were used to measure differences between groups.

3.2.5 **Mass spectrometry**

3.2.5.1 **Lysate collection**

To prepare samples for mass spectrometry (MS) analysis, parental and resistant cells grown in Light and Heavy SILAC media, respectively, were plated on 60mm petri dishes, allowed to adhere for 24 hrs, kept in serum free medium (0.5% BSA) overnight, treated with/without erlotinib/SU11274 and with/without EGF/HGF and lysed as described in section 3.2.2.1. 50µg of each sample (parental/light and resistant/heavy) were mixed equally before electrophoresis (4-15% SDS-PAGE). Gels were stained with a GelCode Blue Stain (Thermo Fisher, 24590) to visualize protein bands.

3.2.5.2 **In-Gel tryptic digestion**

Bands were excised, destained, reduced, alkylated and digested (In-Gel Tryptic Digest Kit, Thermo Fisher, 89871). Briefly, gels bands were excised into 2 × 2 mm pieces. 400µl of destaining solution (Thermo Fisher, 89871) was added to the gel pieces. Samples were incubated at 37°C for 30 minutes with shaking. Destaining solution was removed and discarded. This was repeated until gel slices were completely destained. 100µl of reducing buffer (Thermo Fisher, 89871) was added to the tube to completely cover gel slices and incubated at 60°C for 10 minutes. Samples were allowed to cool, and reducing buffer was then removed and discarded from tube. 100µl of alkylation buffer (Thermo Fisher, 89871) was added to the tube and sample was incubated in the dark at room temperature for 1 hour. Alkylation buffer was removed and discarded from tube. The sample was washed by adding 400 µl destaining buffer to

the tube and incubated at 37°C for 15 minutes with shaking. Destaining buffer was removed and discarded from tube. This was repeated once. Gel pieces were shrunk by adding 200µl of acetonitrile (Thermo Fisher, 89871), incubating for 15 minutes at room temperature, removing acetonitrile and allowing gel pieces to air-dry for 15 minutes. Gel pieces were swollen by adding 100 µl of activated trypsin solution to the tube, incubated at room temperature for 15 minutes. Samples were then digested by adding 250µl digestion buffer to the tube, and incubated at 30°C overnight with shaking. The next day, the digestion mixture was removed and placed in a new tube. To further extract peptides, 100µl 5% formic acid solution was added to gel pieces and incubated for 5 minutes. After which the formic acid solution was removed and added to the digestion mixture. 100µl acetonitrile was added to gel pieces and incubated at 37°C for 15 minutes with shaking. Acetonitrile solution was removed and also added to the digestion mixture. The peptides in the digestion mixture were lyophilized to near dryness and reconstituted with 10µl of 0.1% formic acid.

3.2.5.3 **Mass spectrometry run and analysis**

Reconstituted parental and resistant peptides were run through a Thermo Scientific LTQ Orbitrap XL ETD high resolution mass spectrometer. Differences in target proteins were identified and compared between heavy (resistant) and light (parental) labeled peptides. Identified proteins had a minimum of 2 unique peptides and a False Discovery Rate < 0.05% to confirm verification of the protein. Mass spectra of heavy peptides containing $^{13}C_6$ $^{15}N_2$ L-lysine and $^{13}C_6$ $^{15}N_4$ L-Arginine was shifted to the right of the light peptide spectra by a mass to charge ratio (m/z) of 4 or 5. Due to the shift in

m/z, the mass spectrometer can compare the expression levels of the heavy (resistant) and light (parental) labeled peptides. Identified proteins were plotted on semi-log graphs and sorted by average SILAC ratios to determine most up- and down-regulated proteins (**Figure 1**).

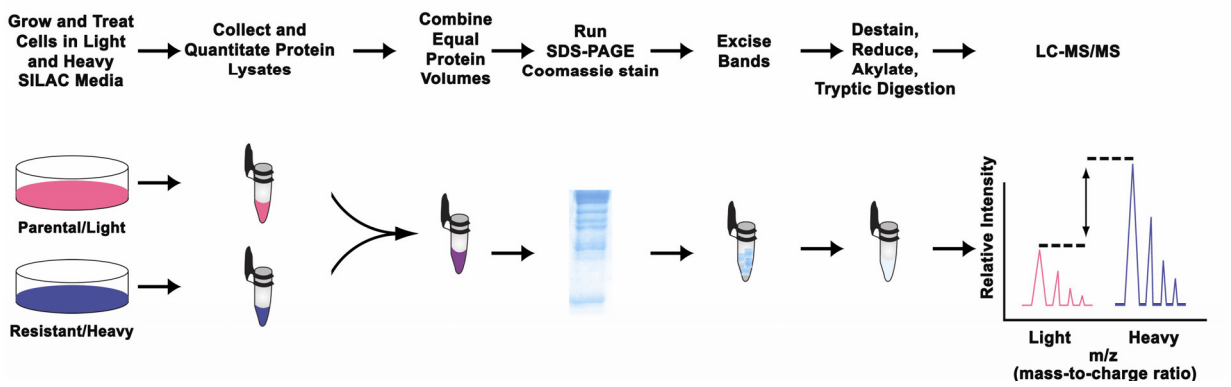

Figure 1: Schematic workflow for sample preparation for mass spectrometry samples. H358 Parental and Resistant cells were grown for 6 passages (30 days) using SILAC RPMI. 50µg of each sample (parental/light and resistant/heavy) were equally mixed before 4-15% SDS-PAGE. Gels were stained with GelCode Blue Stain. Bands were excised, destained, reduced, alkylated and digested.

3.2.6 DNA sequencing

5 million parental and resistant H2170 and H358 cells were plated on 150 mm diameter petri dishes. Cells were allowed to adhere and grow in media as described above. DNA was extracted using the Qiagen DNeasy® Blood & Tissue kit (Qiagen, Valencia, CA, 69504) following their protocol. Prior to Polymerase Chain Reaction (PCR), primers were obtained from Integrated DNA Technologies (Coralville, Iowa) and re-suspended in molecular grade water (Invitrogen, 10977-015) to obtain a

concentration of 10μM. PCR was then performed to amplify EGFR exons 18-21 using primers described by Paez et al (67) (listed in **Table III**), using the AmpliTaq Gold® PCR Master Mix (Applied Biosystems, Foster City, CA, 4327058). The master mix was then aliquoted into reaction tubes, after which the genomic 100ng DNA template was added as suggested by manufacturer. PCR was run on an Eppendorf Mastercycler Gradient (Hamburge, Germany, 384) according to the following PCR cycle protocol:

1. 5 minute incubation at 95°C
2. 30 cycles of:
   15 seconds at 95°C
   15 seconds at 50°C
   60 seconds at 72°C
3. 7 minute incubation at 72°C
4. Hold at 4°C

Table III.
PRIMER SEQUENCES USED FOR PCR

Title	Sequence
EGFR 18 Forward	5′- TCC AAA TGA GCT GGC AAG TG -3′
EGFR 18 Reverse	5′- TCC CAA ACA CTC AGT GAA ACA AA -3′
EGFR 19 Forward	5′- GTG CAT CGC TGG TAA CAT CC -3′
EGFR 19 Reverse	5′- TGT GGA GAT GAG CAG GGT CT -3′
EGFR 20 Forward	5′- ATC GCA TTC ATG CGT CTT CA -3′
EGFR 20 Reverse	5′- ATC CCC ATG GCA AAC TCT TG -3′
EGFR 21 Forward	5′- GCT CAG AGC CTG GCA TGA A -3′
EGFR 21 Reverse	5′- CAT CCT CCC CTG CAT GTG T -3′

The PCR products were purified using the GeneJET^{TM} PCR Purification Kit (Thermo Fisher, K0701) and sent to the University of Illinois DNA Services Facility for sequencing.

4. RESULTS

4.1 Preliminary studies

4.1.1 Determining inhibition of parental cell lines

To determine the IC_{50} (half maximal inhibitory concentration) of SU11274 and erlotinib in parental H2170 and H358 cells, cells were plated in replicates of 6 at 1000 cells/well in 96 well plates. Cells were allowed to adhere to the surface for 24 hours in complete medium, after which media was aspirated and replaced with complete RPMI medium at increasing concentrations of SU11274 or erlotinib. After 96 hours of treatment, the IC_{50} of each cell line was determined by MTT assay using the Sigma MTT assay kit as outlined in section 3.2.1. Briefly, the MTT reagent was added and allowed to incubate for 3 hours. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals which are insoluble in aqueous solutions. The formazan crystals were solubilized in MTT solubilization reagent (acidified isopropanol) and plates read at 570nm using a Biotek plate reader. IC_{50}s determined are displayed in **Table IV.**

4.1.2 Erlotinib- and SU11274-sensitive H358 and H2170 cells were made resistant to either or both of the drugs

To identify the appropriate concentrations of SU11274, erlotinib, and a combination of both TKIs for the development of resistant cell lines, each cell line was treated with progressively increasing concentrations of SU11274 (2.5-12μM) (26), erlotinib (0.5-12μM) (68), or both SU11274 (1.25-10μM) and erlotinib (0.25-10μM) for 96 hrs. The IC_{50} for each TKI or EGFR/c-Met TKI combination was calculated for each

resistant cell line (**Table IV**). After treating cells with increasing concentrations of SU11274 (26), erlotinib (68) or both, five individual resistant clones from single cells were isolated, expanded and checked for stable resistance after each serial passage (69). Resistant cells were grown in the absence of SU11274, erlotinib or a combination for 6 passages and found to be still resistant. Isolated resistant clones grew in concentrations 4-6-fold higher of SU11274, 11-22-fold higher of erlotinib, and in combination 6-fold higher of SU11274 and 16-32-fold higher erlotinib.

Table IV.

IC_{50} OF TYROSINE KINASE INHIBITORS AND COMBINATIONS

Cell lines	SU11274	Erlotinib	Combination
H2170 Parental	2.5μM	0.5μM	1.25μM SU11274/0.25μM Erlotinib
H2170 Resistant	12μM	11μM	8.0μM SU11274/8.0μM Erlotinib
H358 Parental	2.5μM	1μM	1.25μM SU11274/0.5μM Erlotinib
H358 Resistant	11μM	11μM	8.0μM SU11274/8.0μM Erlotinib

4.2 Experimental results

4.2.1 SU11274 resistant cells are also cross resistant to the oral c-Met inhibitor, tivantinib

To test the inhibitory effects of tivantinib, an effective oral c-Met TKI that is currently in clinical trials (31, 70), on our parental and SU11274-resistant cells, cells were plated in 96 well plates in replicates of six. Cells were then treated with increasing concentrations of tivantinib (.01-.2μM) for 24 hours, after which an MTT viability assay was performed (70). We found that while tivantinib inhibited parental cells significantly,

its effect on SU11274-resistant cells was minimal (**Figure 2**). A 3-fold decrease ($p<0.01$) in inhibition was seen in H2170-resistant cells compared to parental cells at 0.01μM and 0.1μM tivantinib. At 0.1μM tivantinib, parental cells were inhibited by 32.8% ± 3.6% in comparison to untreated parental cells, while resistant cells were only inhibited by 10.7% ± 3.7% in comparison to untreated resistant cells ($p<0.01$). Similar results were seen in SU11274-resistant H358 cells treated with 0.1μM tivantinib, where a 10-fold decrease in inhibition was seen in resistant cells compared to parental cells (1.2% ± 5.6% and 11.8% ± 3.4%, respectively, $p<.01$). In H2170 cells incubated with 0.2μM of tivantinib we observed a 2-fold decrease in inhibition when comparing parental and resistant cells (46.9% ± 2.2% and 24.2% ± 2.2%, respectively $p<.001$). H358 parental and resistant cells followed a similar trend at 0.2μM tivantinib exhibiting a 3-fold decrease (25.9% ± 1.4% and 7.0% ± 4.5%, $p<.01$). Munshi et al have also shown sub-μM sensitivity to tivantinib in other NSCLC cell lines (26). These data suggest that our findings could also be applied to patients with resistance to tivantinib which is currently in clinical trials (31).

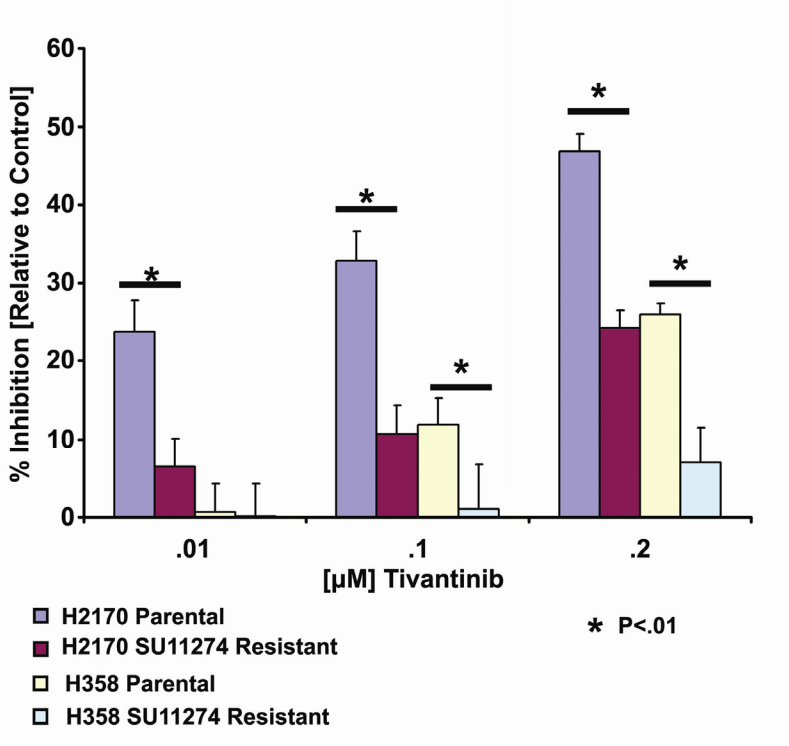

Figure 2: SU11274 resistant cells are cross resistant to tivantinib. 3000 cells were plated and treated with tivantinib (.01-.2µM) for 24 hrs after which an MTT viability assay was performed. SU11274 resistant H2170 and H358 cells showed a 2-10-fold decrease in sensitivity to the anti-proliferative effect of tivantinib compared with parental cells. This data indicates that our cell lines are cross resistant to tivantinib. Differences between groups were found to be statistically significant by student T test analysis.

4.2.2 **Effect of EGF and erlotinib on EGFR phosphorylation and signaling proteins in two resistant NSCLC models**

We then studied the mechanism of resistance to erlotinib in two NSCLC models. Erlotinib-resistant H2170 cells (clone H2170-E1) exhibit constitutively autophosphorylated p-EGFR (Y1068) (19-fold increase) in the absence of its ligand EGF (**Figure 3A**), while erlotinib-resistant H358 cell lines (clone H358 E4) exhibited a 6-fold decrease in p-EGFR (Y1068) (**Figure 3B**). Results from **Figure 3A** were validated

with immunofluorescence, demonstrating that EGF has a minimal effect on EGFR phosphorylation in erlotinib resistant H2170 cells suggesting autophosphorylation of EGFR may be due to a mutation in EGFR (**Figure 3C, D**). When total fluorescence units were measured, we found a 3.8- and 1.7-fold increase in corrected total cell fluorescence units while comparing parental to resistant cells in the absence and presence of EGF ($p<.01$). Interestingly, the presence and absence of EGF had no significant difference in fluorescence on H2170 erlotinib resistant cells ($p=.4$). In erlotinib-resistant H358-E4 and H2170-E1 cells, there was a 2-4-fold increase in p-mTOR (S2448), after erlotinib treatment (**Figure 3A, B**). Interestingly, a 2-fold upregulation of p-S6 kinase (T389) was also observed in H2170-E1 and H358-E4 cells in the presence of erlotinib (**Figure 3A, B**) and 2-fold upregulation was seen in the absence of erlotinib in H2170 cells. Furthermore, p-4E-BP1 (T37/46), was also upregulated 2-fold, in H2170-E1 erlotinib-resistant cells (**Figure 3A**). Additionally, a 2-5-fold upregulation of downstream signaling protein p-ERK (T202/Y204) was seen in both resistant cell lines. Our results indicate that the mTOR pathway and other receptors could upregulate p-S6 kinase and p-4E-BP1 thereby mediating resistance through two separate mechanisms in H2170 and H358 NSCLC models.

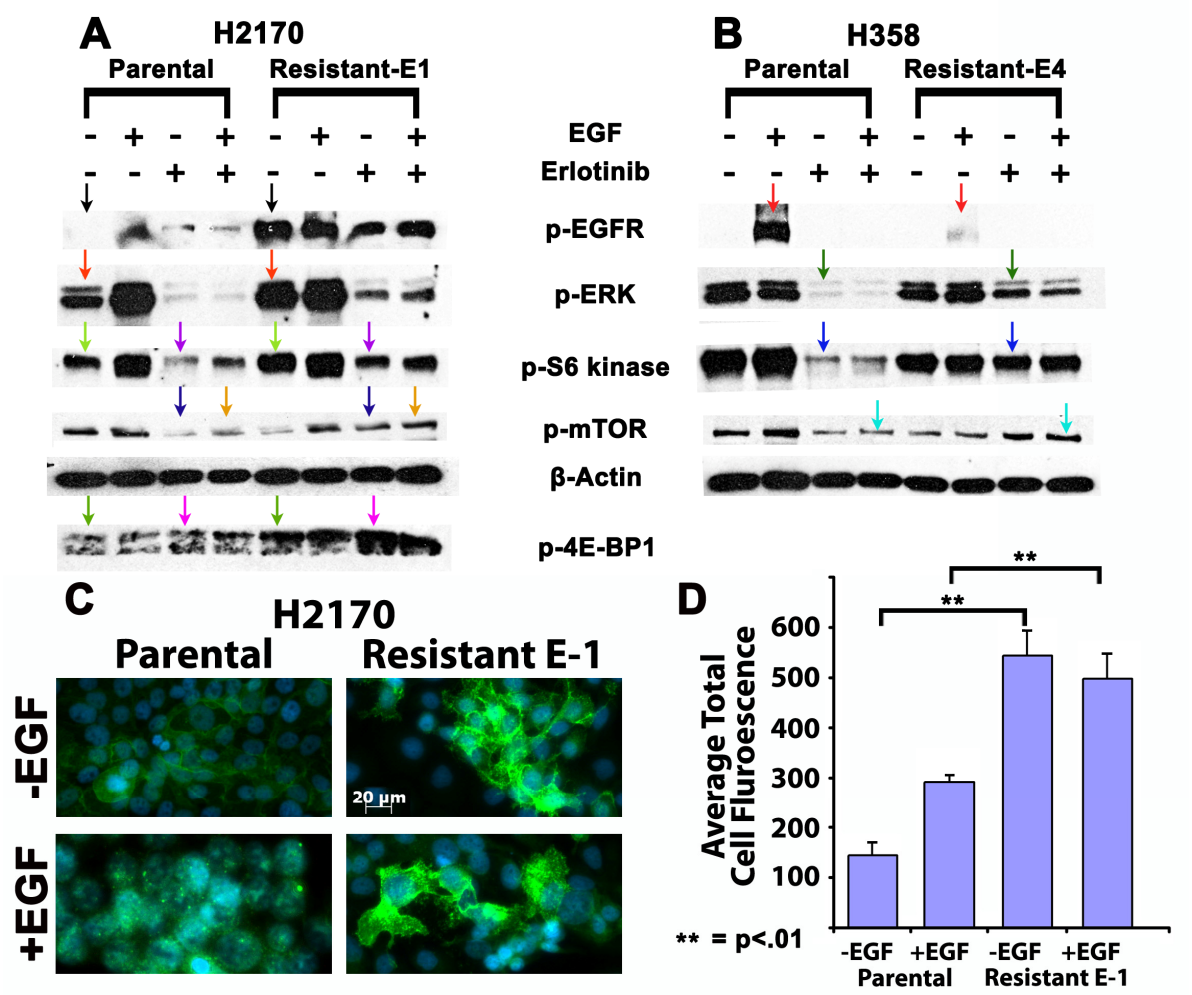

Figure 3: Upregulation of the mTOR pathway may cause erlotinib resistance. **A.** and **B.** EGFR is autophosphorylated in H2170-E1 and downregulated in H358-E4 resistant cell lines. p-mTOR (S2448) and its downstream signaling protein p-S6 kinase (T389) and p-4E-BP1 (T37/46) are upregulated in both resistant cell lines. H2170 and H358 parental and resistant cell lines were kept in serum free medium (0.5% BSA) overnight and then treated with or without 7.0μM of erlotinib for 24 hours after which cells were stimulated with 10ng/mL of EGF for 2.5 min. Autophosphorylation of EGFR on Y1068 was seen in the absence of EGF in H2170-E1 cells which was not seen in H358-E4 cells. Upregulation of p-mTOR and its downstream proteins p-S6 kinase (T389) and p-4E-BP1 (T37/46) were seen in resistant lines ± erlotinib. H2170-E1 cells show increased EGFR phosphorylation ± EGF indicating that H2170 resistant cells may have autophosphorylation of EGFR. **C.** To confirm autophosphorylation of EGFR, cells were plated on chamber slides, allowed to adhere for 24 hrs and then kept in serum free medium overnight. Cells were then treated with ± EGF for 15 min, fixed with acetone:methanol and visualized with p-EGFR (Y1068) primary antibody and anti-rabbit DyLight secondary antibody on a Zeiss Axio Observer Z1 fluorescent microscope. **D.** Graph showing relative average total cell fluorescence units per 8 microscopic fields. There was a 3.8-fold increase in fluorescence when comparing parental to resistant cells in the absence of EGF.

4.2.3 **Effect of HGF and SU11274 on c-Met phosphorylation and signaling proteins in two NSCLC models**

To investigate models of resistance to SU11274 in NSCLC, we analyzed the expression levels of proteins in the presence and absence of the ligand HGF and c-Met SU11274. H2170 (clone H2170-S1) and H358 (clone H358-S2) SU11274 resistant cells exhibited a 4- and 1.5-fold downregulation of p-c-Met (Y1003) respectively (**Figure 4A, B**) as analyzed by immunoblotting analysis. Downregulation is independent of SU11274 since it was seen even after 6 passages of drug withdrawal. We also found inhibition of p-c-Met (Y1003) in the presence of SU11274. This indicates that H2170-S1 and H358-S2 do not employ p-c-Met as a means of resistance, suggesting a separate mechanism of resistance. Interestingly, we also found a 20-fold upregulation of p-S6 kinase, a protein downstream of mTOR that is involved in cancer cell survival (71), in untreated H2170-S1 cells and a 2-fold upregulation was seen in cells treated with HGF and SU11274 (**Figure 4A**). A 2-fold upregulation in p-4E-BP1 (T37/46), a protein downstream of mTOR that promotes tumorigenicity (71), was seen in both H2170-S1 and H358-S2 resistant cells (**Figure 4A, B**). These results indicate that the mTOR pathway, a key regulator of the growth of cancer cells (71), may be involved in mediating resistance. To further study alternative cell signaling in H2170-S1 cells, we examined p-ERK (T202/Y204) and active β-catenin, which is involved in the Wnt signaling pathway (72), in response to HGF. We found that p-ERK remained stable for at least 120 min in H2170-S1 cells compared to only 30 min of stability in the parental cell lines (**Figure 4C, D**). Interestingly, we also found that in un-stimulated cells, basal levels of active β-catenin were 2-fold higher and remained high (3.6-fold) for 120 min

after HGF treatment in H2170-S1 cells compared to those in parental cells at 60 min incubation (**Figure 4C**). Due to the involvement of S6 kinase, p-4E-BP1, p-ERK and β-catenin, these results suggest cross talk between the c-Met, mTOR and Wnt pathways.

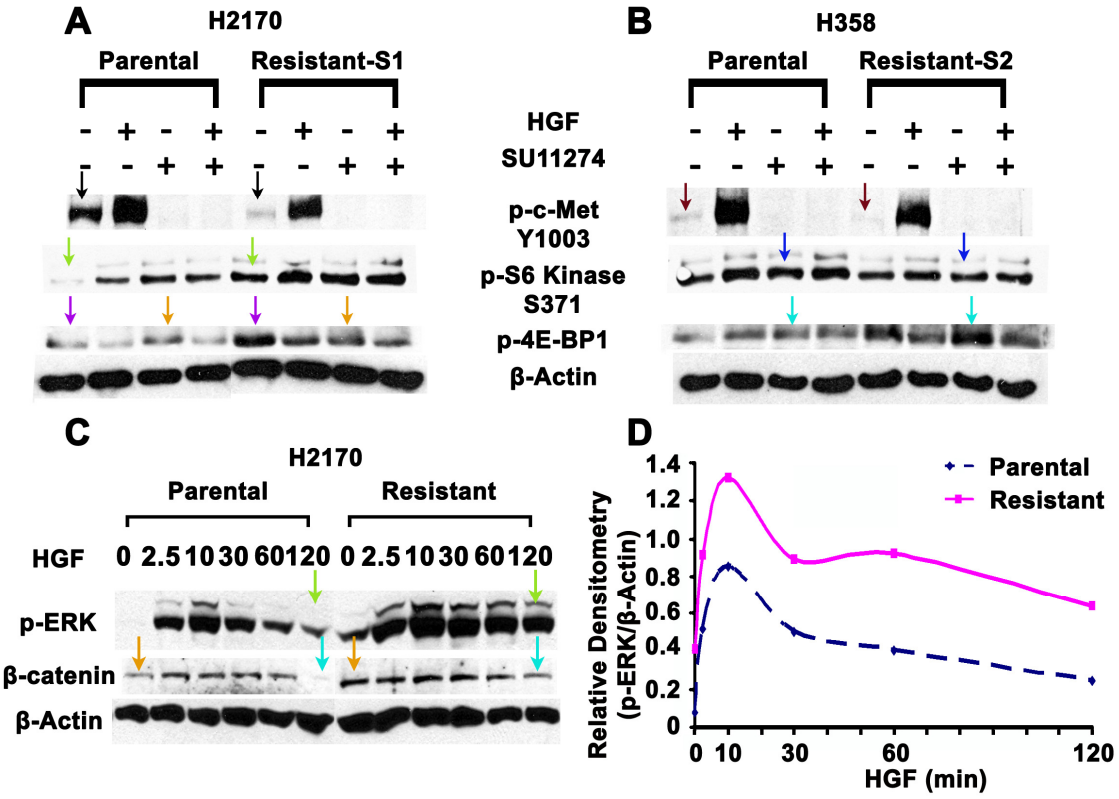

Figure 4: Upregulation of the mTOR pathway may cause SU11274 resistance. **A** and **B** p-4E-BP1 is upregulated in H358-S2 and H2170-S1 SU11274 resistant cells. Cells were kept in serum free medium (0.5% BSA) overnight and then treated with or without 8.0μM SU11274 for 24 hours. Cells were then stimulated with 40ng/mL of HGF for 2.5 min after which immunoblot analysis was performed. Downregulation of p-c-Met (Y1003) was seen in both cell lines. Upregulation of p-S6 kinase (S371) was observed in H2170-S1 cells. Upregulation of p-4E-BP1 (T37/46) was observed in both cells lines ± SU11274. **C.** In H2170-S1 cells, HGF induced prolonged p-ERK signaling compared to parental cells. Cells were kept in serum free medium for 48 hours and then stimulated with 40ng/mL of HGF. Immunoblotting indicated that in H2170-S1, HGF activated p-ERK (T202/Y204) which remained high for 120 min compared the parental lines where p-ERK expression was downregulated at 60 min. Basal levels of active β-catenin were also 2-fold higher and remained high (3.6-fold) for 120 min after HGF treatment in H2170-S1 cells compared to those in parental cells at 60 min incubation. **D.** Relative densitometry of p-ERK/β-actin showing results described above.

4.2.4 **The growth of H2170 and H358 resistant cells are inhibited by everolimus and XAV939**

In order to test whether H358 and H2170 cells resistant to SU11274 and erlotinib are sensitive to an mTOR inhibitor (everolimus), H358/H2170 NSCLC cell lines were treated with 1μM of everolimus. In H358 cells, treatment with 1μM of everolimus inhibited the growth of parental cells by 40% and that of SU11274/erlotinib-resistant cells by only 20%. Interestingly, the same concentration of everolimus inhibited the growth of parental cells completely and that of SU11274/erlotinib resistant cells by 65% when used in combination with either SU11274 (8μM) or erlotinib (8μM) (**Figure 5**). 95% inhibition of cell growth was seen in resistant H358 cells when everolimus was used with both SU11274 and erlotinib (**Figure 5A**). Similar results were also seen in H2170 cells (99% inhibition of growth) (**Figure 5B**).These results indicate that the mTOR pathway has an essential role in c-Met/EGFR TKI resistance and could be combined with EGFR and c-Met TKIs as a drug target for complete inhibition of cell growth. We then further tested the efficacy of Wnt inhibition in our resistant cells. H2170 parental and erlotinib/SU11274 resistant NSCLC cell lines were treated with increasing concentrations of XAV939 (Wnt inhibitor) and an MTT viability assay performed. Interestingly, parental cells showed little to no response to XAV939. However, resistant cells were inhibited in a dose-responsive manner (**Figure 5B**), suggesting that the Wnt signaling specifically affected resistant cells. Furthermore, when XAV939 was further combined with erlotinib/SU11274, an 85% decrease in viability in resistant cells was observed, which suggests that XAV939 could be combined with current EGFR/c-Met TKIs. Statistical significance for both data sets were determined by a two-way ANOVA

with replication and α at .05 ($p<.01$), after which individual paired student T tests were performed to determine significance between untreated diluent and treatment groups. In summary, targeting of Wnt and mTOR may be a possible solution to overcome EGFR and c-Met TKI resistance in NSCLC.

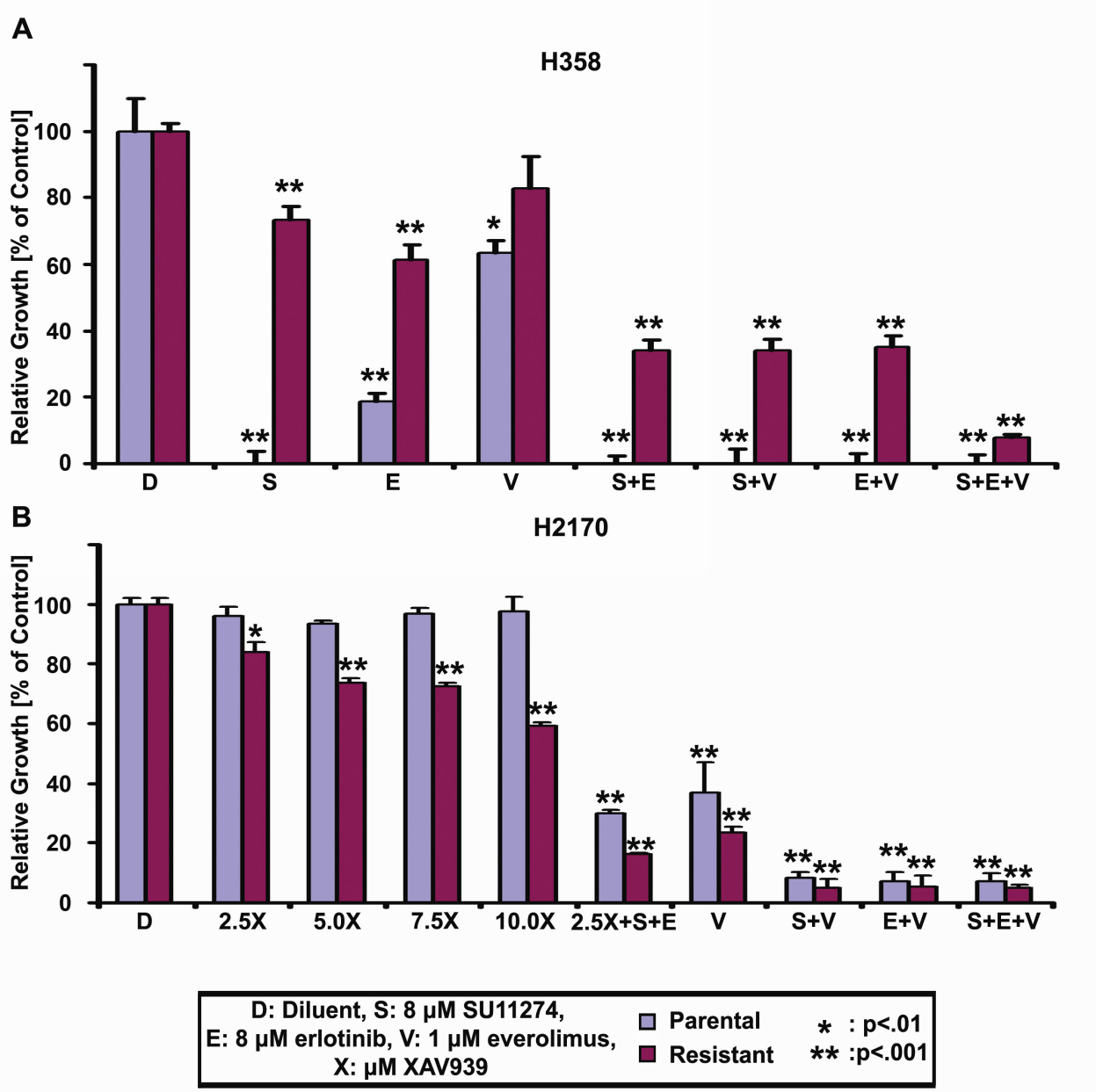

Figure 5: Resistant cells are susceptible to mTOR and Wnt inhibition. Growth of SU11274/erlotinib resistant H358/H2170 NSCLC cell lines is inhibited significantly by everolimus only in the presence of SU11274 and erlotinib. Cells were treated as described for 96 hours after which an MTT viability assay was performed. **A.** In H358 cells, inhibition of growth by 95% occurred when everolimus was used with both SU11274 and erlotinib. Inhibition is comparable to that seen in the parental cells. **B** Parental H2170 cells show little to no inhibition when given increasing concentrations of XAV939. Conversely, resistant cells treated with XAV939 are inhibited in a dose responsive manner. There was a 1.8-fold increase in combination with XAV939, SU11274 and erlotinib and a 1.5-fold increase in combination with everolimus, SU11274 and erlotinib ($p<.01$).

4.2.5 **Mass spectrometry results**

4.2.5.1 **Protein identification**

To further elucidate differences between our parental and resistant cells, we measured protein expression levels using the Thermo Scientific LTQ Orbitrap XL ETD high resolution mass spectrometer. Mass spectra of peptides containing heavy labeled $^{13}C_6$ $^{15}N_2$ L-lysine and $^{13}C_6$ $^{15}N_4$ L-Arginine are shifted to the right of the light peptide spectra by a mass to charge ratio (m/z) of 4 or 5. The shift in m/z, can be measured by the mass spectrometer which can then compare the different expression levels of the heavy (resistant) and light (parental) labeled peptides. However, before the output of differential protein expression could be analyzed, all proteins were plotted on a semi-log graph and sorted by average SILAC ratios to determine the most up-and downregulated proteins (**Figure 6**).

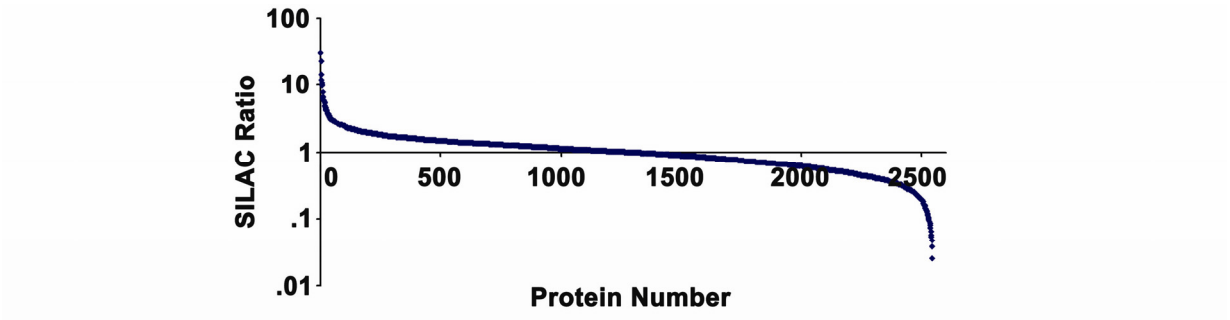

Figure 6: Identified proteins were plotted on a semi-log graph and sorted by average SILAC ratios (protein expression ratio). Identified proteins had a minimum of 2 unique peptides and a False Discovery Rate < 0.05% to confirm verification of the protein.

4.2.5.2 **Proteins involved in DNA replication, translation, cell death and tumorigenicity are upregulated in resistant cells**

Of the over 2500 unique proteins identified via mass spectrometry, the majority were either unchanged in their protein expression levels or had little bearing to NSCLC therapy. Therefore, to measure differences, we focused on proteins that were either up- or down-regulated more than 1.5-fold. This narrowed the list to approximately 200 proteins. As seen in **Table V**, we then further reduced the list to eight proteins that have significant potential to influence cell survival and growth. Among these eight proteins the downregulation of one protein, total β-Catenin, has been validated by western blotting (**Figure 7**). Validation of the other proteins will be conducted in future studies.

Table V
FUNCTIONS OF MODULATED PROTEINS IN RESISTANT CELLS

Protein	Function	Fold Change
Total β-Catenin	The majority is present as component of an E-cadherin/catenin adhesion complex composed of at least E-cadherin/CDH1 and β -catenin/CTNNB1, and possibly α-catenin/CTNNA1; the complex is located to adherens junctions where it is necessary to stabilize the junction. Loss of total β-catenin would imply decreased junctions, and lead to a more invasive and metastatic tumor. (73).	0.50
MCM2/ MCM6	Essential proteins involved in DNA replication initiation and elongation in eukaryotic cells (74). Increased MCM2 expression is also an independent predictor of poor prognosis in NSCLC patients (75).	1.65
TPR	Activates oncogenic kinases and the mitotic spindle checkpoint and increases tumorigenicity of lung cancer (76).	1.57
Syntenin	Adapter protein that may be required for the targeting of TGFA to the cell surface in the secretory pathway. Syntenin has also been shown to interact with Frizzled, a family of Wnt receptors, and other Wnt co-receptors (77).	1.85
HMGB2	DNA binding protein that associates with chromatin and have the ability to bend DNA. It preferentially binds single-stranded DNA. Overexpression of HMGB2 is associated with tumor aggressiveness, growth, poor prognosis of hepatocellular carcinoma and increased sensitivity to cisplatin (78).	2.32
TOM34	Imports cytosolically synthesized pre-proteins into mitochondria that help to keep newly synthesized precursors in an unfolded import compatible state. Tom34 is upregulated in colon cancer and knockdown of Tom34 with siRNA drastically inhibited cell growth. Tom34-/- knockout mice experience no loss of function, indicating its potential as a target (79).	2.51
PDCD6	Calcium-binding protein required for T-cell receptor-, Fas-, and glucocorticoid-induced cell death. May mediate Ca2+-regulated signals along the death pathway. Interaction with DAPK1 can accelerate apoptotic cell death by increasing caspase-3 activity (80).	0.36
AIF	Dual role in controlling cellular life and death; during apoptosis, it is translocated from the mitochondria to the nucleus to function as a pro-apoptotic factor in a caspase-independent pathway. Plays a critical role in caspase-independent, pyknotic cell death in hydrogen peroxide-exposed cells (81).	0.56

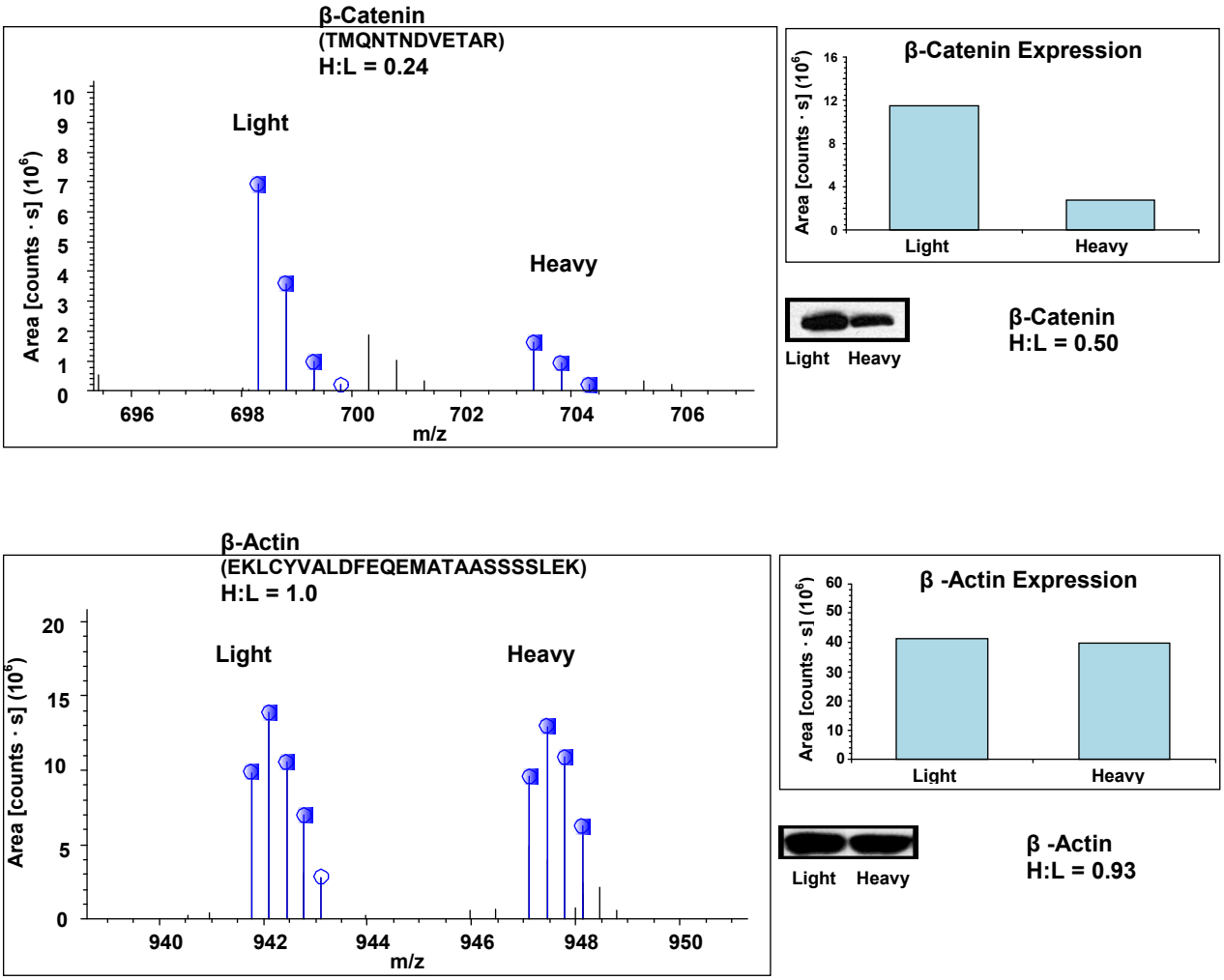

Figure 7: Validated proteins. Representative MS spectra of heavy and light peptides for total β-catenin and β-actin (control). Mass spectra of heavy peptides containing $^{13}C_6$ $^{15}N_2$ L-lysine and $^{13}C_6$ $^{15}N_4$ L-Arginine are shifted to the right of the light peptide spectra by a mass to charge ratio (m/z) of 4 or 5, respectively for +2 ionized peptides. Peptide identification and SILAC quantitation was performed using Thermo Scientific Proteome Discoverer v1.3 software. Downregulation of total β-catenin is confirmed by immunoblotting, while β-actin exhibits similar expression for both MS analysis and immunoblotting. Immunoblots of protein samples show comparable protein levels to those observed by MS analysis.

4.2.6 **DNA Sequencing**

Our studies indicate that H2170 erlotinib resistant cells may have an activating EGFR mutation which needs to be identified in future studies. Interestingly results of DNA Sanger Sequencing of PCR products from H2170 and H358 parental and resistant cells indicate that parental and resistant cells do not have secondary resistance point mutations T790M or D761Y which are acquired after erlotinib/gefitinib treatment (48).

5. DISCUSSION

5.1 mTOR signaling may contribute to c-Met/EGFR TKI resistance

Molecularly targeted TKIs have become integral to the therapy used by clinicians to combat NSCLC. However, acquired resistance to TKIs has severely limited the extent to which this therapy can be employed effectively. Contrary to previous studies which have focused on EGFR mutations (47, 82), we have concentrated on alternative signaling pathways. We studied two NSCLC model cell lines which show either upregulation (H2170) or downregulation (H358) of p-EGFR and downregulation of p-c-Met. However, both cell lines show modulation of both the mTOR and Wnt pathways in EGFR/c-Met TKI resistant cells which implies that both cell lines, despite different mechanisms of resistance, are susceptible to mTOR and Wnt inhibition. Our study is the first to date to show that *both* the mTOR and Wnt signaling pathways may contribute to acquired EGFR/c-Met TKI resistance.

As seen in **Figures 3** and **4**, p-mTOR, p-S6 kinase, and p-4E-BP1 are upregulated in resistant cell lines. To validate the role of mTOR in resistance and to attempt to subsequently break this resistance, parental and resistant cell lines were treated with the mTOR inhibitor everolimus in addition to c-Met and EGFR TKIs. Inhibition of mTOR alone did not significantly inhibit the growth of H358 and H2170 resistant lines, but when used in combination with EGFR/c-Met TKIs, resistance to EGFR/c-Met TKIs was overcome (**Figure 6A and B**). Our results further confirm previous studies showing a relationship between an mTOR linked pathway in EGFR TKI resistance (64, 65) and another study that found synergistic effects with an

EGFR/mTOR TKI combination (gefitinib and genistein, respectively) in T790M positive NSCLC cells (83). However, our results additionally demonstrate a clear link between non phosphorylated EGFR (**Figure 3B**) (T790M negative), c-Met inhibitor resistance and the mTOR pathway in NSCLC (**Figure 4A, B and 5A**). To our knowledge, this is the first study indicating that targeting the mTOR pathway could be a potential therapeutic target that is irrespective of EGFR secondary mutations in NSCLC patients.

5.2 Wnt signaling may contribute to c-Met/EGFR TKI resistance

Additionally, in **Figure 3C**, we show upregulation of active β-catenin (modulated by the Wnt pathway) in resistant cells. Similar to the results with mTOR inhibition, inhibiting the Wnt pathway using XAV939 resulted in a significant reduction in viability of EGFR/c-Met TKI resistant cells (**Figure 4B**) which could also make targeting of the Wnt pathway a plausible option for EGFR/c-Met TKI resistant NSCLC. In previous studies, when the Wnt signaling pathway is hyperactive, it has been shown to be involved in NSCLC development, in modulation of mTOR expression and in tumorigenicity (84, 85). While no link between Wnt and c-Met is established in NSCLC, studies have shown cross talk between HGF/c-Met and Wnt/β-catenin pathways that is essential for HGF-induced tumor invasion in breast cancer (86). However, the mechanism of how the Wnt pathway contributes to EGFR/c-Met TKI resistance is currently unknown. We speculate that the Wnt pathway, known to activate mTOR (84), may be working synergistically with the mTOR pathway by using proteins common to both, such as GSK3, to increase tumorigenicity (**Figure 5**). Additionally, upregulation of p-ERK (as seen in **Figure 2 and 3**) may phosphorylate GATA-6 which may in turn

stimulate transcription of Wnt7b, a known canonical Wnt pathway activator (87-89). p-ERK is perhaps activated by a different mechanism in H2170 and H358 cells. In H2170 resistant cells, the constitutive phosphorylation of p-EGFR could stimulate p-ERK. However, because both p-EGFR and p-c-Met are downregulated in H358 resistant cells (**Figure 2 and 3**) we suspect that in H358 both the Wnt pathway and p-ERK are activated by a positive feedback loop in which Wnt is activated, which phosphorylates p-ERK, which phosphorylates GATA-6, which stimulates more Wnt7b transcription (90) (**Figure 5**). These mechanisms are substantiated by our results demonstrating that inhibition of either the mTOR or Wnt signaling pathways in NSCLC overcomes EGFR/c-Met TKI resistance, thus restoring the ability of EGFR/c-Met TKIs to inhibit tumor growth and survival.

Interestingly, sensitivity to Wnt inhibition appears to be seen mainly in resistant lines. As shown in **Figure 4B**, XAV939 had no statistically significant effects on parental cell viability, while XAV939 had significant effects on resistant cells at the same concentrations implicating Wnt as an essential pathway in EGFR/c-Met TKI resistance. Conversely, everolimus showed efficacy on parental lines individually and was more effective on resistant lines when used in combination with erlotinb and SU11274 (**Figure 4A and B**). The role of the mTOR pathway in resistance mechanisms is seen by a 2-4-fold increase in p-mTOR in resistant H2170 and H358 cells over that of parental lines in response to treatment with erlotinib (**Figure 2A and B**). Thus, the mTOR pathway, in resistant lines, appears to be strongly activated when challenged with EGFR/c-Met TKIs. This further implies the need to target resistant NSCLC with

multiple inhibitors in order to prevent resistance to a single inhibitor treatment which could be caused by activation of alternative signaling mechanisms in resistant cells.

Furthermore, while there is no known link between c-Met TKI resistance and mTOR in NSCLC, previous studies have shown that c-Met inhibitors could cooperate with an mTOR inhibitor (60). Regardless of the mechanism, in both H358 and H2170 resistant cell lines we observed no phosphorylation of c-Met when treated with SU11274. This suggests that SU11274, while effective in inhibiting the phosphorylation of c-Met, has little effect on inhibiting downstream signaling necessary for cell growth and survival. As resistant cell lines have been shown to proliferate in the presence of SU11274, this would suggest that targeting alternative pathways would be necessary to inhibit cell growth. Since our cells are cross resistant to tivantinib (**Figure 1**), our results may apply to patients with acquired resistance to erlotinib and tivantinib combinations.

5.3 **Alternative signaling may play a vital role in the presence/absence of secondary RTK mutations in c-Met/EGFR TKI resistance**

Previous studies have shown that mutations in EGFR (T790M) are the primary cause of resistance (47), and therefore the simple remedy would be to target these mutations. Thus, many researchers have focused on designing irreversible TKIs against EGFR that would still be effective against T790M mutations and thereby prevent the selection of secondary resistance clones (50). Unfortunately, to date, no EGFR TKI or mAb has been approved that can effectively counteract the T790M mutation (50). In contrast, this study focused on alternative signaling pathways that appear to be

upregulated and sustained in EGFR/c-Met resistant lines. By targeting alternative signaling pathways that are downstream or parallel to EGFR, we have shown decreased cell viability of TKI resistant cells. When translated into clinical practice, this novel therapy has the potential to greatly increase patient PFS. Targeting additional pathways besides EGFR in NSCLC patients has already been shown to be effective in clinical trials with erlotinib and tivantinib combinations and erlotinib and MetMab combinations (30, 31). Therefore, the additional targeting of the mTOR and Wnt pathways may further improve drug efficacy. Furthermore, in the event that the anti-T790M mutation can be targeted by TKIs, additional pathways such as the mTOR and Wnt signaling pathways may still cause additional tumorigenicity (**Figure 2B**). When mTOR and Wnt signaling are upregulated, even after downregulation of p-EGFR, cells may continue to proliferate as seen in our H358 cell model (**Figure 5A**).

5.4 Mass spectrometry results

As seen in **Table V**, we list changes in expression of eight different proteins that may have roles in EGFR/c-Met TKI resistance. Decreased total β-catenin (not to be confused with active β-catenin) would lead to fewer cell-to-cell junctions, and lead to a more invasive and metastatic tumor (73). While it is interesting that pro-apoptotic proteins such as PDCD6 and AIF (80, 81) are downregulated in resistant cells, suggesting that resistant cells are better able to survive, it would be clinically challenging to target these proteins in patients. Interestingly, HGF, via its downstream effector, focal adhesion kinase, downregulates AIF and thereby induces cisplatin resistance in NSCLC cells (91). Furthermore, knockout of focal adhesion kinase

increased AIF expression and restored sensitivity (91). Therefore, *indirect* targeting of AIF by inhibiting focal adhesion kinase may restore AIF expression and sensitivity in our cells. However, proteins such as MCM2/6, TPR, HMGB2, and TOM34, which are upregulated in resistant cells and play important roles in replication, tumorigenesis, growth and translation (74-76, 78, 79), could be directly targeted to overcome EGFR/c-Met TKI resistance. Syntenin may be the most interesting since syntenin may be involved in the recycling of the Wnt membrane receptor, Frizzled (77), this provides further evidence of Wnt signaling being involved in c-Met/EGFR TKI resistance.

5.5 **Future studies**

Our studies indicate that it is necessary to prevent secondary acquired resistance and, ideally, a combination of inhibitors could initially be given to untreated patients to prevent secondary resistance (64). Development of new therapeutics that target multiple tyrosine kinases could supplement or might be another approach in addition to the presently used highly specific TKIs (64, 65). While our studies provide evidence that the mTOR and Wnt signaling pathways contribute to acquired EGFR/c-Met TKI resistance, further work is needed to translate these findings into clinical applications. Experiments comparing parental and resistant cells' susceptibility to mTOR and Wnt inhibition *in vivo* would be needed to validate our findings before they could be translated into patient therapy.

Further studies would be necessary to clarify the exact mechanism by which both the mTOR and Wnt pathways are stimulated after TKI therapy. We show

upregulation of p-ERK which might stimulate GATA-6, (**Figure 8**) which in turn promotes transcription of Wnt7b mRNA which could account for the Wnt activation.

Interestingly, we also found that parental cells grow faster than resistant cells under no drug treatment which would imply that resistant cells may be more biologically similar to cancer stem cells. Cancer stem cells tend to grow slower and be more drug resistant than normal cancer cells (92). This is interesting since erlotinib resistant NCI-H1650 cells have been shown to demonstrate cancer stem cell like traits (93). Furthermore, increased expression of Wnt signaling (which is seen in our resistant lines) has been shown to increase the proliferation of cancer stem cells and promote resistance to apoptosis (94). Additionally, inactivation of AIF in normal stem cells renders them resistant to cell death even in the absence of serum (95). Since we observe downregulation of AIF in our resistant cells, which act similar to cancer stem cells, the resistant cells may also be much better able to survive in serum-free conditions. These results suggest cancer stem cell like traits in our resistant lines. However, additional studies would be necessary to confirm their cancer stem cell like traits and validate any cancer stem cell biomarkers.

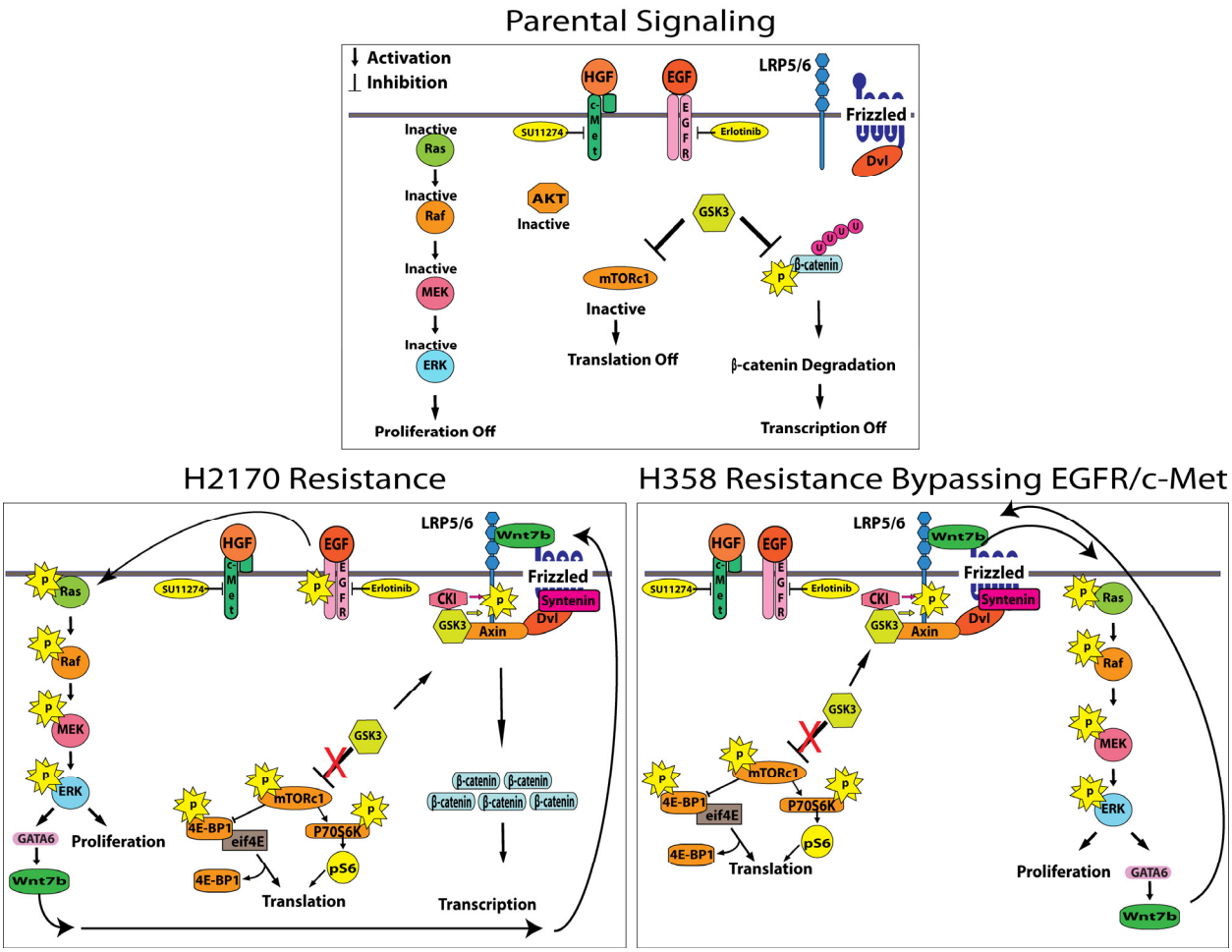

Figure 8: Potential pathway of parental, H2170 resistant and H358 resistant signaling pathways after treatment with ligand and inhibitors. Parental cells are inhibited and show no alternative signaling pathways. H2170 cells exhibit upregulated c-Met and EGFR and signal downstream to mTOR, Wnt and MAP kinase pathways. H358 cells do not utilize EGFR or c-Met and instead Wnt creates a feedback loop that constantly stimulates mTOR and the MAP kinase pathways.

5.6 Conclusion

In summary, our studies have demonstrated that alternative Wnt and mTOR signaling pathways are mechanisms by which NSCLC may become resistant to erlotinib and c-Met TKIs (**Figure 8**). Wnt and mTOR may add to EGFR and c-Met signaling as in the case of H2170 cells, causing enhanced survival or, replace EGFR and c-Met signaling as in the case of H358 cells, allowing these cells to survive and proliferate. However, the targeting of Wnt and mTOR in both cases may enable us to overcome EGFR and c-Met TKI resistance in NSCLC. To our knowledge, this is the first study showing a relationship between the mTOR and Wnt signaling pathways and acquired EGFR/c-Met TKI resistance, and suggests a novel treatment modality to overcome this acquired resistance seen in NSCLC patients.

CITED LITERATURE

1. T. J. Key, A. Schatzkin, W. C. Willett, N. E. Allen, E. A. Spencer and R. C. Travis: Diet, nutrition and the prevention of cancer. Public Health Nutr. 1A:7:187-200, 2004.

2. J. R. Molina, P. Yang, S. D. Cassivi, S. E. Schild and A. A. Adjei: Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc. 5:83:584-594, 2008.

3. A. Jemal, R. Siegel, J. Xu and E. Ward: Cancer statistics, 2010. CA Cancer J Clin. 5:60:277-300, 2010.

4. A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward and D. Forman: Global cancer statistics. CA Cancer J Clin. 2:61:69-90, 2011.

5. A. G. Shaper, S. G. Wannamethee and M. Walker: Pipe and cigar smoking and major cardiovascular events, cancer incidence and all-cause mortality in middle-aged British men. Int J Epidemiol. 5:32:802-808, 2003.

6. P. Boffetta: Human cancer from environmental pollutants: the epidemiological evidence. Mutat Res. 2:608:157-162, 2006.

7. N. I. Krinsky and E. J. Johnson: Carotenoid actions and their relation to health and disease. Mol Aspects Med. 6:26:459-516, 2005.

8. J. L. Freudenheim, J. Ritz, S. A. Smith-Warner, D. Albanes, E. V. Bandera, P. A. van den Brandt, G. Colditz, D. Feskanich, R. A. Goldbohm, L. Harnack, A. B. Miller, E. Rimm, T. E. Rohan, T. A. Sellers, J. Virtamo, W. C. Willett and D. J. Hunter: Alcohol consumption and risk of lung cancer: a pooled analysis of cohort studies. Am J Clin Nutr. 3:82:657-667, 2005.

9. I. M. Lee: Physical activity and cancer prevention--data from epidemiologic studies. Med Sci Sports Exerc. 11:35:1823-1827, 2003.

10. P. Boffetta: Epidemiology of environmental and occupational cancer. Oncogene. 38:23:6392-6403, 2004.

11. S. J. Hwang, L. S. Cheng, G. Lozano, C. I. Amos, X. Gu and L. C. Strong: Lung cancer risk in germline p53 mutation carriers: association between an inherited cancer predisposition, cigarette smoking, and cancer risk. Hum Genet. 3:113:238-243, 2003.

12. X. Li and K. Hemminki: Inherited predisposition to early onset lung cancer according to histological type. Int J Cancer. 3:112:451-457, 2004.

13. W. Pao and N. Girard: New driver mutations in non-small-cell lung cancer. Lancet Oncol. 2:12:175-180, 2011.

14. P. J. Roberts, T. E. Stinchcombe, C. J. Der and M. A. Socinski: Personalized medicine in non-small-cell lung cancer: is KRAS a useful marker in selecting patients for epidermal growth factor receptor-targeted therapy? J Clin Oncol. 31:28:4769-4777, 2010.

15. T. Sher, G. K. Dy and A. A. Adjei: Small cell lung cancer. Mayo Clin Proc. 3:83:355-367, 2008.

16. D. C. Paech, A. R. Weston, N. Pavlakis, A. Gill, N. Rajan, H. Barraclough, B. Fitzgerald and M. Van Kooten: A systematic review of the interobserver variability for histology in the differentiation between squamous and nonsquamous non-small cell lung cancer. J Thorac Oncol. 1:6:55-63, 2011.

17. F. R. Hirsch, A. Spreafico, S. Novello, M. D. Wood, L. Simms and M. Papotti: The prognostic and predictive role of histology in advanced non-small cell lung cancer: a literature review. J Thorac Oncol. 12:3:1468-1481, 2008.

18. S. D. Stellman, J. E. Muscat, S. Thompson, D. Hoffmann and E. L. Wynder: Risk of squamous cell carcinoma and adenocarcinoma of the lung in relation to lifetime filter cigarette smoking. Cancer. 3:80:382-388, 1997.

19. C. A. Moran: Pulmonary adenocarcinoma: the expanding spectrum of histologic variants. Arch Pathol Lab Med. 7:130:958-962, 2006.

20. S. Couraud, G. Zalcman, B. Milleron, F. Morin and P. J. Souquet: Lung cancer in never smokers - A review. Eur J Cancer. 2012.

21. A. Charloux, E. Quoix, N. Wolkove, D. Small, G. Pauli and H. Kreisman: The increasing incidence of lung adenocarcinoma: reality or artefact? A review of the epidemiology of lung adenocarcinoma. Int J Epidemiol. 1:26:14-23, 1997.

22. W. D. Travis, R. I. Linnoila, M. G. Tsokos, C. L. Hitchcock, G. B. Cutler, Jr., L. Nieman, G. Chrousos, H. Pass and J. Doppman: Neuroendocrine tumors of the lung with proposed criteria for large-cell neuroendocrine carcinoma. An ultrastructural, immunohistochemical, and flow cytometric study of 35 cases. Am J Surg Pathol. 6:15:529-553, 1991.

23. I. Petersen: The morphological and molecular diagnosis of lung cancer. Dtsch Arztebl Int. 31-32:108:525-531, 2011.

24. M. Sattler and R. Salgia: The MET axis as a therapeutic target. Update Cancer Ther. 3:3:109-118, 2009.

25. E. Pisick, S. Jagadeesh and R. Salgia: Receptor tyrosine kinases and inhibitors in lung cancer. ScientificWorldJournal. 4:589-604, 2004.

26. N. Puri and R. Salgia: Synergism of EGFR and c-Met pathways, cross-talk and inhibition, in non-small cell lung cancer. J Carcinog. 7:9, 2008.

27. F. Janku, I. Garrido-Laguna, L. B. Petruzelka, D. J. Stewart and R. Kurzrock: Novel therapeutic targets in non-small cell lung cancer. J Thorac Oncol. 9:6:1601-1612, 2011.

28. Y. Xu, H. Liu, J. Chen and Q. Zhou: Acquired resistance of lung adenocarcinoma to EGFR-tyrosine kinase inhibitors gefitinib and erlotinib. Cancer Biol Ther. 8:9:572-582, 2010.

29. J. G. I. Laux, R. Just, K. Brady, J. Li, B. Schwartz, R. Savage, E. Garmey, L. Rosen J.: Phase I dose escalation trial (ARQ 197–111) evaluating combination of selective c-Met inhibitor ARQ 197 and erlotinib. Journal of Clinical Oncology. 15S:27:2009.

30. D. R. Spigel, T. J. Ervin, R. Ramlau, D. B. Daniel, J. H. Goldschmidt, G. R. Blumenschein, M. J. Krzakowski, G. Robinet, C. Clement-Duchene, F. Barlesi, R. Govindan, T. Patel, S. V. Orlov, M. S. Wertheim, J. Zha, A. Pandita, W. Yu, R. L. Yauch, P. H. Patel and A. C. Peterson: Final efficacy results from OAM4558g, a randomized phase II study evaluating MetMAb or placebo in combination with erlotinib in advanced NSCLC. Clin Oncol. 29: abstr 7505:2011.

31. L. V. Sequist, J. von Pawel, E. G. Garmey, W. L. Akerley, W. Brugger, D. Ferrari, Y. Chen, D. B. Costa, D. E. Gerber, S. Orlov, R. Ramlau, S. Arthur, I. Gorbachevsky, B. Schwartz and J. H. Schiller: Randomized Phase II Study of Erlotinib Plus Tivantinib Versus Erlotinib Plus Placebo in Previously Treated Non-Small-Cell Lung Cancer. J Clin Oncol. 24:29:3307-3315, 2011.

32. M. Batus, M. J. Fidler and P. D. Bonomi: Primary and secondary therapeutic strategies for EGF receptor pathway inhibition in non-small-cell lung cancer. Expert Rev Anticancer Ther. 10:10:1589-1599, 2010.

33. C. A. Carter and G. Giaccone: Treatment of nonsmall cell lung cancer: overcoming the resistance to epidermal growth factor receptor inhibitors. Curr Opin Oncol. 2:24:123-129, 2012.

34. J. A. Engelman and P. A. Janne: Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. Clin Cancer Res. 10:14:2895-2899, 2008.

35. M. C. Stella and P. M. Comoglio: HGF: a multifunctional growth factor controlling cell scattering. Int J Biochem Cell Biol. 12:31:1357-1362, 1999.

36. J. A. Engelman, K. Zejnullahu, T. Mitsudomi, Y. Song, C. Hyland, J. O. Park, N. Lindeman, C. M. Gale, X. Zhao, J. Christensen, T. Kosaka, A. J. Holmes, A. M. Rogers, F. Cappuzzo, T. Mok, C. Lee, B. E. Johnson, L. C. Cantley and P. A. Janne: MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 5827:316:1039-1043, 2007.

37. P. C. Ma, R. Jagadeeswaran, S. Jagadeesh, M. S. Tretiakova, V. Nallasura, E. A. Fox, M. Hansen, E. Schaefer, K. Naoki, A. Lader, W. Richards, D. Sugarbaker, A. N. Husain, J. G. Christensen and R. Salgia: Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res. 4:65:1479-1488, 2005.

38. T. Kanematsu, S. Yano, H. Uehara, Y. Bando and S. Sone: Phosphorylation, but not overexpression, of epidermal growth factor receptor is associated with poor prognosis of non-small cell lung cancer patients. Oncol Res. 5:13:289-298, 2003.

39. M. Jo, D. B. Stolz, J. E. Esplen, K. Dorko, G. K. Michalopoulos and S. C. Strom: Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. J Biol Chem. 12:275:8806-8811, 2000.

40. J. H. Schiller, D. Harrington, C. P. Belani, C. Langer, A. Sandler, J. Krook, J. Zhu and D. H. Johnson: Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med. 2:346:92-98, 2002.

41. N. Puri, S. Ahmed, V. Janamanchi, M. Tretiakova, O. Zumba, T. Krausz, R. Jagadeeswaran and R. Salgia: c-Met is a potentially new therapeutic target for treatment of human melanoma. Clin Cancer Res. 7:13:2246-2253, 2007.

42. M. Sattler, Y. B. Pride, P. Ma, J. L. Gramlich, S. C. Chu, L. A. Quinnan, S. Shirazian, C. Liang, K. Podar, J. G. Christensen and R. Salgia: A novel small molecule met inhibitor induces apoptosis in cells transformed by the oncogenic TPR-MET tyrosine kinase. Cancer Res. 17:63:5462-5469, 2003.

43. E. Vergani, V. Vallacchi, S. Frigerio, P. Deho, P. Mondellini, P. Perego, G. Cassinelli, C. Lanzi, M. A. Testi, L. Rivoltini, I. Bongarzone and M. Rodolfo: Identification of MET and SRC activation in melanoma cell lines showing primary resistance to PLX4032. Neoplasia. 12:13:1132-1142, 2011.

44. J. R. Sierra, V. Cepero and S. Giordano: Molecular mechanisms of acquired resistance to tyrosine kinase targeted therapy. Mol Cancer. 9:75, 2010.

45. J. Bean, C. Brennan, J. Y. Shih, G. Riely, A. Viale, L. Wang, D. Chitale, N. Motoi, J. Szoke, S. Broderick, M. Balak, W. C. Chang, C. J. Yu, A. Gazdar, H. Pass, V. Rusch, W. Gerald, S. F. Huang, P. C. Yang, V. Miller, M. Ladanyi, C. H. Yang and W. Pao: MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci U S A. 52:104:20932-20937, 2007.

46. A. B. Turke, K. Zejnullahu, Y. L. Wu, Y. Song, D. Dias-Santagata, E. Lifshits, L. Toschi, A. Rogers, T. Mok, L. Sequist, N. I. Lindeman, C. Murphy, S. Akhavanfard, B. Y. Yeap, Y. Xiao, M. Capelletti, A. J. Iafrate, C. Lee, J. G. Christensen, J. A. Engelman and P. A. Janne: Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell. 1:17:77-88, 2010.

47. A. F. Gazdar: Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors. Oncogene. 28 Suppl 1:S24-31, 2009.

48. S. V. Sharma, D. W. Bell, J. Settleman and D. A. Haber: Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer. 3:7:169-181, 2007.

49. R. Dienstmann, S. De Dosso, E. Felip and J. Tabernero: Drug development to overcome resistance to EGFR inhibitors in lung and colorectal cancer. Mol Oncol. 2011.

50. W. Zhou, D. Ercan, L. Chen, C. H. Yun, D. Li, M. Capelletti, A. B. Cortot, L. Chirieac, R. E. Iacob, R. Padera, J. R. Engen, K. K. Wong, M. J. Eck, N. S. Gray and P. A. Janne: Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. Nature. 7276:462:1070-1074, 2009.

51. Z. Tang, R. Du, S. Jiang, C. Wu, D. S. Barkauskas, J. Richey, J. Molter, M. Lam, C. Flask, S. Gerson, A. Dowlati, L. Liu, Z. Lee, B. Halmos, Y. Wang, J. A. Kern and P. C. Ma: Dual MET-EGFR combinatorial inhibition against T790M-EGFR-mediated erlotinib-resistant lung cancer. Br J Cancer. 6:99:911-922, 2008.

52. A. A. Adjei, B. Schwartz and E. Garmey: Early clinical development of ARQ 197, a selective, non-ATP-competitive inhibitor targeting MET tyrosine kinase for the treatment of advanced cancers. Oncologist. 6:16:788-799, 2011.

53. M. Surati, P. Patel, A. Peterson and R. Salgia: Role of MetMAb (OA-5D5) in c-MET active lung malignancies. Expert Opin Biol Ther. 12:11:1655-1662, 2011.

54. M. Sattler, M. M. Reddy, R. Hasina, T. Gangadhar and R. Salgia: The role of the c-Met pathway in lung cancer and the potential for targeted therapy. Ther Adv Med Oncol. 4:3:171-184, 2011.

55. A. B. Turke, K. Zejnullahu, Y. L. Wu, Y. Song, D. Dias-Santagata, E. Lifshits, L. Toschi, A. Rogers, T. Mok, L. Sequist, N. I. Lindeman, C. Murphy, S. Akhavanfard, B. Y. Yeap, Y. Xiao, M. Capelletti, A. J. Iafrate, C. Lee, J. G. Christensen, J. A. Engelman and P. A. Janne: Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell. 1:17:77-88,

56. R. Bianco, V. Damiano, T. Gelardi, G. Daniele, F. Ciardiello and G. Tortora: Rational combination of targeted therapies as a strategy to overcome the mechanisms of resistance to inhibitors of EGFR signaling. Curr Pharm Des. 33:13:3358-3367, 2007.

57. K. P. Xu and F. S. Yu: Cross talk between c-Met and epidermal growth factor receptor during retinal pigment epithelial wound healing. Invest Ophthalmol Vis Sci. 5:48:2242-2248, 2007.

58. T. E. Reznik, Y. Sang, Y. Ma, R. Abounader, E. M. Rosen, S. Xia and J. Laterra: Transcription-dependent epidermal growth factor receptor activation by hepatocyte growth factor. Mol Cancer Res. 1:6:139-150, 2008.

59. J. K. Spix, E. Y. Chay, E. R. Block and J. K. Klarlund: Hepatocyte growth factor induces epithelial cell motility through transactivation of the epidermal growth factor receptor. Exp Cell Res. 15:313:3319-3325, 2007.

60. P. C. Ma, E. Schaefer, J. G. Christensen and R. Salgia: A selective small molecule c-MET Inhibitor, PHA665752, cooperates with rapamycin. Clin Cancer Res. 6:11:2312-2319, 2005.

61. K. Schmid, Z. Bago-Horvath, W. Berger, A. Haitel, D. Cejka, J. Werzowa, M. Filipits, B. Herberger, H. Hayden and W. Sieghart: Dual inhibition of EGFR and mTOR pathways in small cell lung cancer. Br J Cancer. 5:103:622-628, 2010.

62. E. Buck, A. Eyzaguirre, E. Brown, F. Petti, S. McCormack, J. D. Haley, K. K. Iwata, N. W. Gibson and G. Griffin: Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors. Mol Cancer Ther. 11:5:2676-2684, 2006.

63. S. La Monica, M. Galetti, R. R. Alfieri, A. Cavazzoni, A. Ardizzoni, M. Tiseo, M. Capelletti, M. Goldoni, S. Tagliaferri, A. Mutti, C. Fumarola, M. Bonelli, D. Generali and P. G. Petronini: Everolimus restores gefitinib sensitivity in resistant non-small cell lung cancer cell lines. Biochem Pharmacol. 5:78:460-468, 2009.

64. I. Nakachi, K. Naoki, K. Soejima, I. Kawada, H. Watanabe, H. Yasuda, S. Nakayama, S. Yoda, R. Satomi, S. Ikemura, H. Terai, T. Sato and A. Ishizaka: The combination of multiple receptor tyrosine kinase inhibitor and mammalian target of rapamycin inhibitor overcomes erlotinib resistance in lung cancer cell lines through c-Met inhibition. Mol Cancer Res. 8:8:1142-1151, 2010.

65. S. Wedel, L. Hudak, J. M. Seibel, E. Juengel, I. Tsaur, A. Haferkamp and R. A. Blaheta: Combined targeting of the VEGFr/EGFr and the mammalian target of rapamycin (mTOR) signaling pathway delays cell cycle progression and alters adhesion behavior of prostate carcinoma cells. Cancer Lett. 1:301:17-28, 2011.

66. C. R. Zito, L. B. Jilaveanu, V. Anagnostou, D. Rimm, G. Bepler, S. M. Maira, W. Hackl, R. Camp, H. M. Kluger and H. H. Chao: Multi-level targeting of the phosphatidylinositol-3-kinase pathway in non-small cell lung cancer cells. PLoS One. 2:7:e31331, 2012.

67. J. G. Paez, P. A. Janne, J. C. Lee, S. Tracy, H. Greulich, S. Gabriel, P. Herman, F. J. Kaye, N. Lindeman, T. J. Boggon, K. Naoki, H. Sasaki, Y. Fujii, M. J. Eck, W. R. Sellers, B. E. Johnson and M. Meyerson: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 5676:304:1497-1500, 2004.

68. S. A. Milligan, P. Burke, D. T. Coleman, R. L. Bigelow, J. J. Steffan, J. L. Carroll, B. J. Williams and J. A. Cardelli: The green tea polyphenol EGCG potentiates the antiproliferative activity of c-Met and epidermal growth factor receptor inhibitors in non-small cell lung cancer cells. Clin Cancer Res. 15:15:4885-4894, 2009.

69. H. M. Coley: Development of drug-resistant models. Methods Mol Med. 88:267-273, 2004.

70. N. Munshi, S. Jeay, Y. Li, C. R. Chen, D. S. France, M. A. Ashwell, J. Hill, M. M. Moussa, D. S. Leggett and C. J. Li: ARQ 197, a novel and selective inhibitor of the human c-Met receptor tyrosine kinase with antitumor activity. Mol Cancer Ther. 6:9:1544-1553, 2010.

71. D. A. Guertin and D. M. Sabatini: Defining the role of mTOR in cancer. Cancer Cell. 1:12:9-22, 2007.

72. B. T. MacDonald, K. Tamai and X. He: Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell. 1:17:9-26, 2009.

73. G. Li, H. Schaider, K. Satyamoorthy, Y. Hanakawa, K. Hashimoto and M. Herlyn: Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. Oncogene. 56:20:8125-8135, 2001.

74. E. Dehan, A. Ben-Dor, W. Liao, D. Lipson, H. Frimer, S. Rienstein, D. Simansky, M. Krupsky, P. Yaron, E. Friedman, G. Rechavi, M. Perlman, A. Aviram-Goldring, S. Izraeli, M. Bittner, Z. Yakhini and N. Kaminski: Chromosomal aberrations and gene expression profiles in non-small cell lung cancer. Lung Cancer. 2:56:175-184, 2007.

75. N. Ramnath, F. J. Hernandez, D. F. Tan, J. A. Huberman, N. Natarajan, A. F. Beck, A. Hyland, I. T. Todorov, J. S. Brooks and G. Bepler: MCM2 is an independent predictor of survival in patients with non-small-cell lung cancer. J Clin Oncol. 22:19:4259-4266, 2001.

76. P. Peschard and M. Park: From Tpr-Met to Met, tumorigenesis and tubes. Oncogene. 9:26:1276-1285, 2007.

77. D. Wawrzak, A. Luyten, K. Lambaerts and P. Zimmermann: Frizzled-PDZ scaffold interactions in the control of Wnt signaling. Adv Enzyme Regul. 1:49:98-106, 2009.

78. J. H. Kwon, J. Kim, J. Y. Park, S. M. Hong, C. W. Park, S. J. Hong, S. Y. Park, Y. J. Choi, I. G. Do, J. W. Joh, D. S. Kim and K. Y. Choi: Overexpression of high-mobility group box 2 is associated with tumor aggressiveness and prognosis of hepatocellular carcinoma. Clin Cancer Res. 22:16:5511-5521, 2010.

79. K. Terada, S. Ueno, K. Yomogida, T. Imai, H. Kiyonari, N. Takeda, M. Yano, S. Abe, S. Aizawa and M. Mori: Expression of Tom34 splicing isoforms in mouse testis and knockout of Tom34 in mice. J Biochem. 5:133:625-631, 2003.

80. J. H. Lee, S. B. Rho and T. Chun: Programmed cell death 6 (PDCD6) protein interacts with death-associated protein kinase 1 (DAPk1): additive effect on apoptosis via caspase-3 dependent pathway. Biotechnol Lett. 14:27:1011-1015, 2005.

81. J. T. Kim, K. D. Kim, E. Y. Song, H. G. Lee, J. W. Kim, S. K. Chae, E. Kim, M. S. Lee, Y. Yang and J. S. Lim: Apoptosis-inducing factor (AIF) inhibits protein synthesis by interacting with the eukaryotic translation initiation factor 3 subunit p44 (eIF3g). FEBS Lett. 27:580:6375-6383, 2006.

82. M. N. Balak, Y. Gong, G. J. Riely, R. Somwar, A. R. Li, M. F. Zakowski, A. Chiang, G. Yang, O. Ouerfelli, M. G. Kris, M. Ladanyi, V. A. Miller and W. Pao: Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. Clin Cancer Res. 21:12:6494-6501, 2006.

83. H. Zhu, H. Cheng, Y. Ren, Z. G. Liu, Y. F. Zhang and B. De Luo: Synergistic inhibitory effects by the combination of gefitinib and genistein on NSCLC with acquired drug-resistance in vitro and in vivo. Mol Biol Rep. 2011.

84. A. Y. Choo, P. P. Roux and J. Blenis: Mind the GAP: Wnt steps onto the mTORC1 train. Cell. 5:126:834-836, 2006.

85. K. Inoki, H. Ouyang, T. Zhu, C. Lindvall, Y. Wang, X. Zhang, Q. Yang, C. Bennett, Y. Harada, K. Stankunas, C. Y. Wang, X. He, O. A. MacDougald, M.

You, B. O. Williams and K. L. Guan: TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell. 5:126:955-968, 2006.

86. F. I. Huang, Y. L. Chen, C. N. Chang, R. H. Yuan and Y. M. Jeng: Hepatocyte growth factor activates Wnt pathway by transcriptional activation of LEF1 to facilitate tumor invasion. Carcinogenesis. 2012.

87. Y. Adachi, Y. Shibai, J. Mitsushita, W. H. Shang, K. Hirose and T. Kamata: Oncogenic Ras upregulates NADPH oxidase 1 gene expression through MEK-ERK-dependent phosphorylation of GATA-6. Oncogene. 36:27:4921-4932, 2008.

88. Z. G. Li, J. Yang, E. S. Vazquez, D. Rose, F. Vakar-Lopez, P. Mathew, A. Lopez, C. J. Logothetis, S. H. Lin and N. M. Navone: Low-density lipoprotein receptor-related protein 5 (LRP5) mediates the prostate cancer-induced formation of new bone. Oncogene. 5:27:596-603, 2008.

89. J. Weidenfeld, W. Shu, L. Zhang, S. E. Millar and E. E. Morrisey: The WNT7b promoter is regulated by TTF-1, GATA6, and Foxa2 in lung epithelium. J Biol Chem. 23:277:21061-21070, 2002.

90. M. S. Yun, S. E. Kim, S. H. Jeon, J. S. Lee and K. Y. Choi: Both ERK and Wnt/beta-catenin pathways are involved in Wnt3a-induced proliferation. J Cell Sci. Pt 2:118:313-322, 2005.

91. J. T. Chen, C. Y. Huang, Y. Y. Chiang, W. H. Chen, S. H. Chiou, C. Y. Chen and K. C. Chow: HGF increases cisplatin resistance via down-regulation of AIF in lung cancer cells. Am J Respir Cell Mol Biol. 5:38:559-565, 2008.

92. R. Gangemi, L. Paleari, A. M. Orengo, A. Cesario, L. Chessa, S. Ferrini and P. Russo: Cancer stem cells: a new paradigm for understanding tumor growth and progression and drug resistance. Curr Med Chem. 14:16:1688-1703, 2009.

93. G. Ghosh, X. Lian, S. J. Kron and S. P. Palecek: Properties of resistant cells generated from lung cancer cell lines treated with EGFR inhibitors. BMC Cancer. 12:95, 2012.

94. L. Feller, B. Kramer and J. Lemmer: Pathobiology of cancer metastasis: a short account. Cancer Cell Int. 1:12:24, 2012.

95. N. Joza, S. A. Susin, E. Daugas, W. L. Stanford, S. K. Cho, C. Y. Li, T. Sasaki, A. J. Elia, H. Y. Cheng, L. Ravagnan, K. F. Ferri, N. Zamzami, A. Wakeham, R. Hakem, H. Yoshida, Y. Y. Kong, T. W. Mak, J. C. Zuniga-Pflucker, G. Kroemer and J. M. Penninger: Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature. 6828:410:549-554, 2001.

VITA

NAME:	Jason T. Fong
EDUCATION:	B.S., Molecular and Cellular Biology, University of Illinois, Urbana-Champaign, Illinois, 2006
	M.S., Medical Biotechnology, University of Illinois at Chicago, Chicago, Illinois, 2012
PROFESSIONAL EXPERIENCE:	Research specialist, University of Illinois College of Medicine at Rockford, Department of Biomedical Sciences, Rockford, Illinois, 2009-2012.
HONORS:	Recipient of the "UIC Graduate Student Council Travel Award" sponsored by UIC to attend Annual meeting of the American Association of Cancer Research, 2011, Orlando, FL, USA.
	Recipient of the "MBT (masters in medical biotechnology) Graduate Student Research Poster Award" sponsored by Department of Biomedical Science on Research Day at University of Illinois College of Medicine at Rockford, 2011, Rockford, IL, USA.
	Recipient of the "Best Basic Science student Poster Award" sponsored by Department of Biomedical Science on Research Day at University of Illinois College of Medicine at Rockford, 2012, Rockford, IL, USA.
	Recipient of the "Travel Award" sponsored by Department of Biomedical Science to attend 2012 AACR meeting, Chicago, IL, USA.
	Academic Excellence Award, University of Illinois, Chicago, College of Medicine, Rockford, Illinois 2012
	Recipient of the University of Illinois Rockford Health Sciences Innovation in Education, Research & Community Projects Grant, University of Illinois, Chicago College of Medicine, Rockford, Illinois, 2012
PROFESSIONAL MEMBERSHIPS:	American Association for Laboratory Animal Science American Association for Cancer Research American Society of Clinical Oncology