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A Role For Lipid Rafts In Egfr Tki Resistance In Breast Cancer

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A ROLE FOR LIPID RAFTS IN EGFR TKI RESISTANCE IN BREAST CANCER

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DEDICATION

The work herein is dedicated to my friends and family. I could not have gotten this far without a lot of help. There is one whom I owe my entire life to. I thank God every day for bringing me this far. Through the trials and tribulations I know He is and always will be there.

I would like to thank my dear friend, Sharon Mitchell. She may not be a scientist, but she sure knows how to keep me sane. Whether it was a laugh or a hug, she has been there constantly. Thank God a one-hour drive would never stop her. Now I can only hope she will move with me!

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ABBREVIATIONS

1. EGF: epidermal growth factor
2. EGFR: epidermal growth factor receptor
3. HER2-4: human EGFR-related 2-4
4. AREG: amphiregulin
5. TGFα: transforming growth factor alpha
6. BTC: betacellulin
7. HB-EGF: heparin binding-epidermal growth factor
8. FRET: fluorescence resonance energy transfer
9. PI3K: phosphoinositide 3 kinase
10. SH2 or SH3: src homology 2 or 3
11. MAPK: mitogen activated protein kinase
12. SOS: son of sevenless
13. STAT: signal transducer and activator of transcription
14. PLCγ: phospholipase C gamma
15. Gab1: Grb2-assciated protien 1
16. VEGF: vascular endothelial growth factor
17. mAbs: monoclonal antibodies
18. TKIs: tyrosine kinase inhibitors
19. FAK: focal adhesion kinase
20. SFK: Src family kinase
21. DRM: detergent resistant microdomain
22. BCR: b-cell receptor

23. MBCD: methyl-beta cyclodextrin
24. Cbp: Csk-binding protein
25. HMG: 3-hydroxy 3-methyl gultaryl
26. ER: estrogen receptor
27. SERMs: selective estrogen receptor modulators
28. PR: progesterone receptor
29. PBS: phosphate buffered saline
30. CI-value: combination index value
31. WCL: whole cell lysate
32. SCLC: small cell lung cancer
33. FAS: fatty acid synthase

Chapter 1

1.1 Epidermal Growth Factor Receptor

A. EGFR discovery and identification of EGFR family of proteins

Discovery/Structure of EGFR

The epidermal growth factor receptor (EGFR) was discovered as a result of the finding that epidermal growth factor (EGF), a potent mitogen, showed specific, rapid, and reversible binding to the cell surface (Hollenberg and Cuatrecasas, 1973; O'Keefe et al., 1974; Carpenter et al., 1978; Das et al., 1978; Linsley et al., 1979). Sequence and structural analysis showed that EGFR contains three primary domains: a 621 amino acid extracellular EGF binding domain, a 23 amino acid transmembrane domain, and a 542 amino acid cytoplasmic domain (Fig. 1.1). The ligand binding domain of EGFR was further subdivided into four sub-domains, domains I, II, III and IV. Domains I and III were shown to be directly involved in ligand binding to the receptor (Lax et al., 1989), while domain II was necessary for receptor dimerization, and domain IV was required for localization to membrane microdomains (Heldin, 1995; Garrett et al., 2002; Ogiso et al., 2002; Yamabhai and Anderson, 2002). The transmembrane domain, along with an N-terminal signal sequence, were responsible for directing EGFR to the plasma membrane where it becomes a type I integral membrane protein (Ullrich and Schlessinger, 1990). The cytoplasmic domain of EGFR has been demonstrated to contain a tyrosine kinase domain and c-terminal regulatory region that is autophosphorylated upon kinase activation (Buhrow et al., 1982; Cohen et al., 1982a; Cohen et al., 1982b; Buhrow et al., 1983). Specifically, EGFR autophosphorylation occurs on tyrosines 992, 1068, 1086, 1148, and 1173 (Downward et al., 1984a).

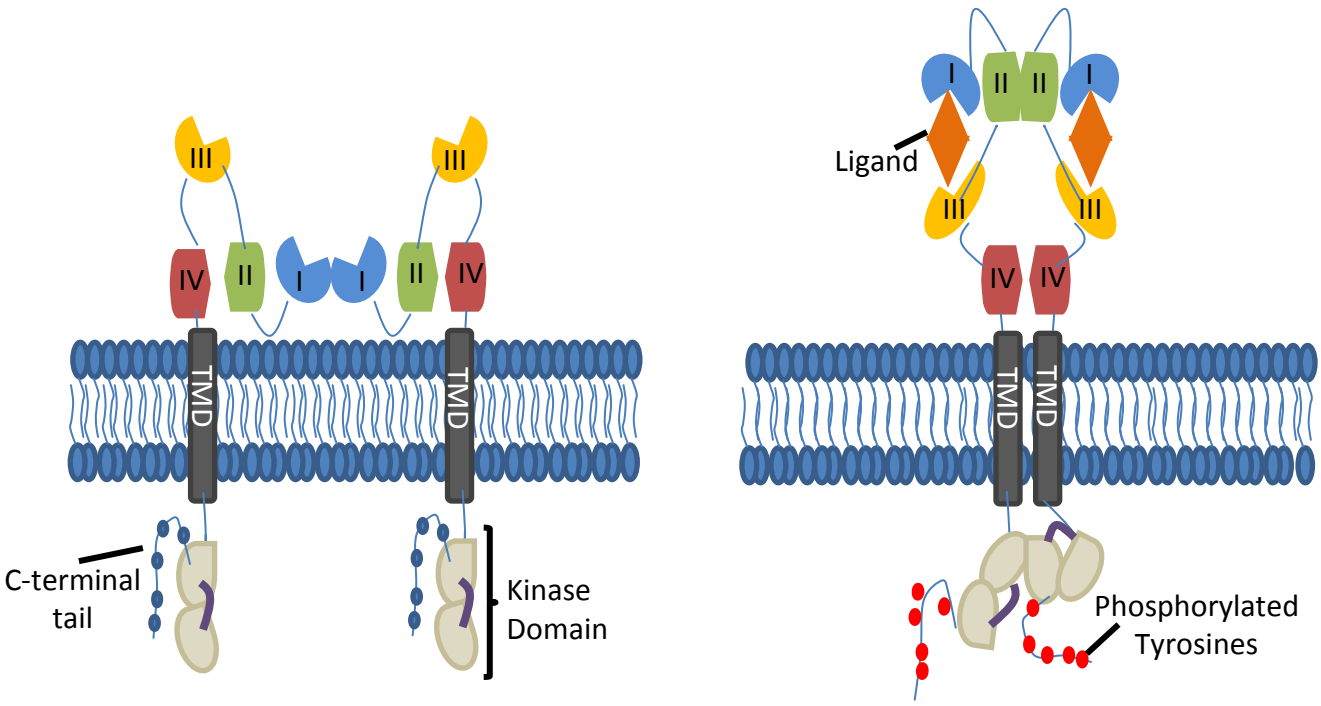


Figure 1.1: EGFR structure, dimerization, and activation. Before activation, EGFR exists as an inactive dimer in the membrane. Upon ligand binding, a conformational change occurs in both the extracellular and intracellular domains leading to autophosphorylation of the receptor complex.

Phosphorylation of these residues results in the recruitment and activation of downstream signaling molecules that participate in cell growth, survival, migration, differentiation, and angiogenesis [reviewed in (Jorissen et al., 2003)].

EGFR tyrosine kinase family members

Further analyses of the structural and functional qualities of EGFR revealed a common protein family. The EGFR tyrosine kinase family consists of four members that include EGFR, HER2, HER3, and HER4.

HER2

Coussens and colleagues discovered a receptor tyrosine kinase with high homology to EGFR, human EGFR-related 2 (HER2), while other laboratories identified the same protein in human mammary carcinoma cells, naming it ErbB2 (Coussens et al., 1985; King et al., 1985). HER2 is the most homologous to EGFR in the family, and is thought to be the major partner for heterodimerization (Tzahar et al., 1996; Graus-Porta et al., 1997). HER2 contains 82% structural homology with EGFR in the kinase domain, 33% in the c-terminus, and 44% in the extracellular domain (Earp et al., 1995). HER2 has no known ligand, thus is thought to be activated following heterodimerization, however, when HER2 is over-expressed, it may form active homodimers (Tzahar et al., 1996).

HER3

HER3 was discovered through cDNA homology screenings of EGFR in 1989 (Kraus et al., 1989). HER3 possesses a 59% structural similarity to EGFR within the kinase domain, 24% in the c-terminus and 36% in the extracellular domain (Earp et al., 1995). This receptor

lacks intracellular kinase activity, and therefore, becomes phosphorylated only when heterodimerized (Guy et al., 1994b; Kim et al., 1998). Neuregulins, rather than the EGFR ligands described later, are the ligands for HER3 (Stove and Bracke, 2004).

HER4

The final EGFR family member, HER4 was also discovered through cDNA homology screening of EGFR (Plowman et al., 1993a). HER4 presents a 79% structural similarity to EGFR in the kinase domain, 28% in the c-terminus, and 48% in the ligand-binding domain (Earp et al., 1995). As with HER3, neuregulins are the primary ligands for HER4 (Stove and Bracke, 2004).

B. Activation of EGFR

i. EGFR Ligands

EGFR has six known ligands that all posses an EGF-like domain that may be sufficient to confer binding specificity (Beerli and Hynes, 1996). Ligands for EGFR include EGF, amphiregulin, transforming growth factor alpha, betacellulin, heparin-binding EGF-like growth factor, and epiregulin (Marquardt et al., 1984; Shoyab et al., 1989; Higashiyama et al., 1991; Shing et al., 1993; Toyoda et al., 1995). These proteins differ in sequence identity (with ~25% homology between each other), as well as glycosylation, presence of heparin-binding domains, and other biochemical properties (Harris et al., 2003). Primarily, these proteins all have cysteine rich EGF modules. The restrictive spacing of these residues and the splicing and functional placement of this module distinguishes these ligands from other EGF module-containing proteins (Groenen et al., 1994; Van Zoelen et al., 2000; van der Woning et al., 2006). Despite diversity in primary sequences, the tertiary structures of EGFR

ligands are all similar. Specifically, orientation of two beta sheet domains, a short omega loop, and a leucine at the fifth position past the sixth cysteine have been shown to be similar or identical in all EGFR binding ligands (Harvey et al., 1991; Matsunami et al., 1991; Hommel et al., 1992; Barbacci et al., 1995; Chau et al., 1996; Jacobsen et al., 1996; Louie et al., 1997; van de Poll et al., 1997; Ballinger et al., 1998).

Ligand binding to the receptor results in an amplification of EGFR activation such that occupancy of 20% of receptors by ligand results in maximal cellular proliferation (Hollenberg and Cuatrecasas, 1973). However, this amplification may be contingent on the differential binding affinities of the ligands for EGFR, as well as their ability to recycle the receptor or target the receptor for degradation due to the pH stability of the ligand-receptor complex (French et al., 1995; Olayioye et al., 2000). Most EGFR ligands act in an autocrine or paracrine fashion (Olayioye et al., 2000). Activation of EGFR ligands requires proteases which release the ligand from the cell surface and remove the regulatory domains (Lee et al., 1985; Massague and Pandiella, 1993; Harris et al., 2003). These ligands are summarized below and in Table 1.1.

EGF

Epidermal growth factor (EGF) was discovered as a peptide growth factor purified from mouse salivary glands by Cohen and colleagues. EGF treatment in newborn mice led to early tooth eruption and eyelid opening (Cohen, 1962). EGF is produced in a precursor form that needs to be proteolytically cleaved to be functional. The active form of EGF is a 53 amino acid peptide that binds specifically to EGFR (Salomon et al., 1995).

Ligand	Size (processed amino acids)	Homology to EGF (%)	EGFR receptor affinity (nM)	Other EGFR family member binding	Overexpression	Knockout	References
EGF	53	_	1.9	-	Hyperproliferation of skin cells	No phenotype	Salomon et al.,1995, Jones et al., 1999
AREG	78-84	38	90	-	Inflammatory skin lesions	Decreased ductal development in the mammary gland	Shoyab et al., 1989, Brown et al., 1998, Adam et al., 1995, Thompson et al., 1996, Neelam et al., 1998, Jones et al., 1999
TGFalpha	50	30-40	9.2	-	Hyperproliferation of liver, metaplasia of pancreas, breast carcinoma	No phenotype	Salomon et al., 1995, Salomon et al., 1990, Massague and Pandiella, 1993, Jones et al., 1999
BTC	80	32	1.4	HER4	Growth retardation and pulmonary/cardiac distress	No phenotype	Sasada et al., 1993, Shing et al., 1993, Jones et al., 1999
HB-EGF	75	43	7.1	HER4	Hyperplasia of skin and heart	Lethal; cardiac abnormalities	Higashiyama et al., 1991, Schneider and Wolf, 2009, Jones et al., 1999
Epiregulin	46	37	2800	HER4	Susceptibility to cancer causing intestinal damage	No phenotype	Toyoda et al., 1995, Shelly et al., 1998, Komurasaki et al., 2002, Jones et al., 1999

Table 1.1: Structure, affinity, and function properties of EGFR ligands.

AREG

Amphiregulin (AREG) was discovered as an EGFR ligand produced by MCF-7 breast adenocarcinoma cells. It was named "amphi" due to an apparent, but yet to be validated, growth inhibitory effect in human neuroblastoma and adenocarcinoma cell lines, and a growth stimulatory effect in human fibroblast, cervical, ovarian, and other breast carcinoma cell lines (Shoyab et al., 1988). AREG is also synthesized as a precursor protein that is cleaved from the membrane, to produce a 78-84 amino acid peptide that shares 38% homology with EGF (Shoyab et al., 1989; Brown et al., 1998a). Like EGF, AREG binds specifically to EGFR. The binding affinity for AREG to EGFR is less than EGF due potentially to a methionine substitution in a conserved region of the ligand (See Table 1.1) (Adam et al., 1995; Thompson et al., 1996; Neelam et al., 1998; Jones et al., 1999).

TGFα

Transforming growth factor alpha (TGFα) was discovered as a low molecular weight polypeptide able to induce transformation of normal rat kidney fibroblasts (de Larco and Todaro, 1978). TGFα is a 50 amino acid peptide when fully processed with 30-40% sequence homology to EGF (Salomon et al., 1995). This ligand binds specifically and with high affinity to EGFR (Table 1.1) (Salomon et al., 1990; Massague and Pandiella, 1993). It is expressed during normal embryogenesis and in a number of adult tissues including regenerating populations of epithelial cells (Kudlow and Bjorge, 1990; Yasui et al., 1992).

Betacellulin

Betacellulin (BTC) was identified from conditioned media of pancreatic beta cell tumor lines, and binds to both EGFR and HER4 with high affinity (Table 1.1) (Sasada et al., 1993;

Jones et al., 1999). BTC is an 80 amino acid peptide that shares 32% homology with EGF (Sasada et al., 1993; Shing et al., 1993). BTC is also expressed in many mesenchymal and epithelial cell lines, particularly in the pancreas, liver, kidney, and small intestine, as well as the heart, lung, colon, testis, and ovary (Sasada et al., 1993; Seno et al., 1996; Dunbar et al., 1999; Dunbar and Goddard, 2000).

HB-EGF

Heparin-binding epidermal growth factor (HB-EGF) was purified originally from conditioned medium of a human lymphoma cell line (Higashiyama et al., 1991). HB-EGF is a 75 amino acid peptide that shares 43% homology with EGF. HB-EGF binds to EGFR with high affinity (Table 1.1), and is also capable of binding HER4 and, potentially, N-arginine dibasic convertase (Higashiyama et al., 1991; Schneider and Wolf, 2009). HB-EGF is highly controlled, both in timing and distribution, as it is induced within the uterine luminal epithelium at the site of blastocyst apposition approximately six hours before uterine implantation (Paria et al., 1999). HB-EGF is the only EGFR ligand whose loss results in lethality due to defects in cardiac and lung function [reviewed in (Schneider and Wolf, 2009)].

Epiregulin

Epiregulin was purified from conditioned media of NIH 3T3/clone T7 cells in a 46 amino acid soluble form (Toyoda et al., 1995). Epiregulin is 37% homologous to EGF (Toyoda et al., 1995). Epiregulin is capable of binding to both EGFR and HER4, however, it may preferentially activate EGFR-containing heterodimers (Shelly et al., 1998). This ligand is expressed primarily in placenta and peripheral blood leukocytes, and in cancers of the

bladder, lung, kidney, pancreas, and colon (Toyoda et al., 1997; Zhu et al., 1999). It is a stronger mitogenic signal as compared to EGF, although epiregulin has a far weaker binding affinity for EGFR (Table 1.1) (Toyoda et al., 1995; Jones et al., 1999; Komurasaki et al., 2002).

Mice with disruption in EGFR ligand expression have indentified functional redundancy between EGFR ligands, as well as specific contributions of individual ligands to EGFR-mediated pathways (Luetteke et al., 1999). Loss of EGF gene expression shows no true phenotype alone, however over expression results in hyperproliferation of epidermal basal layer cells (Schneider and Wolf, 2009). Knockout mouse models have shown that AREG expression is related to ductal development (Luetteke et al., 1999). Overexpression of AREG results in psoriasis-like inflammatory lesions (Schneider and Wolf, 2009). Overexpression of TGFα results in hyperproliferation of the liver, metaplasia of the pancreas, and breast carcinoma, while, in contrast to AREG, knockout models showed no developmental phenotype in the mammary gland (Matsui et al., 1990; Sandgren et al., 1990; Sandgren et al., 1995). As with EGF, no phenotype is reported due to loss of BTC alone, however, overexpression results in growth retardation and an abnormally large heart and lungs which result in pulmonary and cardiac distress (Schneider and Wolf, 2009). Mice lacking the ability to shed HB-EGF develop severe heart abnormalities (similar to HB-EGF knockout) while mice expressing constitutively soluble HB-EGF suffer with hyperplasia of the skin and heart (Yamazaki et al., 2003). Knockout of epiregulin results in no developmental abnormalities, however, epiregulin knockout mice are more susceptible to cancer-predisposing intestinal damage (Lee et al., 2004).

ii. Homodimerization

In 1987, Yarden and Schlessinger described a reversible aggregation of EGFR upon EGF stimulation, suggesting that EGFR may dimerize (Yarden and Schlessinger, 1987). Early evidence of this possible dimerization has been described by Zidovetzki and colleagues, and was hypothesized to have a role in EGFR function (Zidovetzki et al., 1981; Schreiber et al., 1983). Initial studies utilizing covalent cross-linking reagents have suggested that EGFR dimers are formed upon ligand-binding to the receptor (Cochet et al., 1988; Lax et al., 1989; Lax et al., 1991; Tanner and Kyte, 1999). However, more recent technologies such as fluorescence resonance energy transfer (FRET), single-molecule imaging of EGFR, and biophysical studies, have implied the existence of pre-formed dimers of EGFR on cell membranes (Gadella and Jovin, 1995; Sako et al., 2000; Teramura et al., 2006; Clayton et al., 2007). Further studies have indicated that dimerization and activation of the receptor are independent events (Van de Vijver et al., 1991; Yu et al., 2002). Unfortunately, limitations of crystallography techniques used to analyze the membrane bound EGFR have prevented the analyses of an intact EGFR molecule. However, crystallography has demonstrated that asymmetric orientation of the intracellular domains of the receptor pair is required before activation of the dimer can occur (Groenen et al., 1997; Zhang et al., 2006). In the active asymmetric dimer, the c-terminal lobe of one kinase domain and the n-terminal lobe of the second are in contact (Fig. 1.1) (Zhang et al., 2006). The donor kinase then promotes activity of the acceptor kinase (Zhang et al., 2006). In the absence of ligand, the dimer is non-functional due to the extracellular domain having a compact structure where the domain II hairpin loop is buried (Burgess et al., 2003; Ferguson et al., 2003). Upon ligand binding to domains I and III of the extracellular region of the receptor, activation occurs due to a change

in conformation of the receptor dimer complex that brings domain II from each receptor molecule in contact (Heldin, 1995; Garrett et al., 2002; Ogiso et al., 2002). This is thought to bring the intracellular kinase domains in closer proximity to allow autophosphorylation to occur (Fig. 1.1) (Zhang et al., 2006).

iii. Regulation of EGFR via internalization and degradation of the receptor

The earliest notion of EGFR internalization was proposed by Carpenter and Cohen in 1976. Their pulse chase experiments suggested that, after approximately 10 hours, EGF binding to membrane receptors decreased. New synthesis of DNA and RNA were required for binding efficiency to be recovered (Carpenter and Cohen, 1976). This ten hour time point correlates with the discovered half life of EGFR (Stoscheck and Carpenter, 1984). Silver grains with electron microscopy, fluorescence, and affinity labeling were all employed to analyze the loss of EGFR described by Stoscheck and Carpenter. These analyses uncovered concomitant loss of EGF-binding activity or fluorescence, and loss of EGF:EGFR complex or staining at the cell surface with appearance of defined degradation products or increased EGF fluorescence in lysosomal fractions (Das and Fox, 1978; Gorden et al., 1978; Haigler et al., 1978; Schlessinger et al., 1978). Internalization of the ligand-receptor complex is mediated via coated pits and coated vesicles (Schlessinger, 1986; Carpenter, 1987). This degradation is inhibited by lyosomal inhibitors, suggesting a role of lysosomes in the degradation of EGFR. Also, inhibitors of vesicular trafficking impair EGF-induced degradation of EGFR, suggesting that EGFR is trafficked through vesicles into the lysosomes (Stoscheck and Carpenter, 1984).

While it was previously thought that EGFR is solely degraded, as no recycling of the receptor had been described (King et al., 1980; Krupp et al., 1982; Lyall et al., 1985), it was later determined by Beguinot and colleagues that the receptor has the capacity to recycle back to the membrane. Essentially, internalization of the receptor does not automatically target the receptor for degradation. An ubiquitin ligase, known as Cbl, is responsible for targeting EGFR for lysosomal degradation. Recruitment of Cbl to the receptor in early endosomes promotes receptor ubiquitination and loss of Cbl results in receptors recycling to the plasma membrane (Levkowitz et al., 1998). A specific EGFR tyrosine residue, 1045, is essential for Cbl-mediated EGFR degradation, and mutation of this site leads to extended EGFR signaling (Levkowitz et al., 1999).

iv. EGFR family receptor associations

EGFR family members are capable of forming both homo and heterodimers. EGF, HB-EGF, and BTC are known to stimulate dimerization between EGFR and HER2 (Goldman et al., 1990; Wada et al., 1990; Graus-Porta et al., 1997). HER2:EGFR heterodimers are more stable on the cell surface than other EGFR containing dimers. They are also preferentially recycled rather than sent for degradation. This is due to being less stable in the early endosomal environment, thus Cbl dissociates from the complex, allowing the receptors to recycle to the cell surface (Lenferink et al., 1998). HER2 heterodimerization with EGFR also slows the rate of ligand dissociation from EGFR, prolonging and strengthening the activation of downstream signaling (Karunagaran et al., 1996). Both EGF and neuregulins can stimulate dimerization between EGFR and HER3 (Graus-Porta et al., 1997; Pinkas-Kramarski et al., 1998). HER3 has been shown to effectively associate with the p85α subunit of phosphoinositide-3-kinase (PI3K) due to the presence of consensus motifs present in the

intracellular domain of the receptor (Fedi et al., 1994; Prigent and Gullick, 1994). As such, EGFR heterodimerization with HER3 is thought to be a mechanism by which EGFR mediates PI3K activity (Soltoff et al., 1994). HER2:HER3 heterodimers have also been described, and these form in the presence of neuregulins (Sliwkowski et al., 1994). In 1996, Zhang and colleagues found that co-expression of EGFR and HER4 leads to phosphorylation of EGFR in response to heregulins, and phosphorylation of HER4 in response to EGF, suggesting that functional heterodimers form between these two proteins (Zhang et al., 1996). EGF has also been demonstrated to stimulate dimers of EGFR:HER4 in cell lines (Graus-Porta et al., 1997). Functional dimers of HER4 and HER2 have been shown in artificial systems (Plowman et al., 1993b). In contrast, co-expression of HER3 and HER4 did not result in increased cellular foci in response to ligand stimulation, suggesting that these two proteins either do not functionally interact, or that their interaction does not result in increased cellular survival and proliferation (Zhang et al., 1996). More recent evidence suggests a role in HER3:HER4 dimerization in the sustained activation of PI3K in colorectal carcinoma (Lee et al., 2009). Together, these possible dimerization pairs can alter the properties of stability and functionality of the EGFR family of receptors, and as such, add important diversity to EGFR signaling (Riese et al., 1995; Riese et al., 1996).

C. Interactions and Signaling

The EGFR family is a complex system involved in growth factor cellular signaling (Gullick, 2001). Knockout studies determined that EGFR was important in normal growth and development of epithelial cells. Specifically, knockout of EGFR resulted in embryonic lethality or failure of development of the epithelium of multiple organs including the skin, lungs, and gastrointestinal tract (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill

et al., 1995). Indeed, the consequences of activation of EGFR are a number of second-messenger cascades, which promote cell proliferation, angiogenesis, migration, differentiation, and survival (Fig 1.2) (Jorissen et al., 2003). Phosphorylation of EGFR at the plasma membrane results in high-affinity binding sites for Src homology 2 (SH2) domain containing proteins (Pawson, 1995; Pawson, 1997).

i. EGFR substrates

Shc/Grb2

Shc interacts with EGFR via SH2 domains that bind to the phospho-tyrosine residues on EGFR (Lowenstein et al., 1992; Chardin et al., 1993; Gale et al., 1993; Li et al., 1993). Shc is present in three isoforms, p52/46Shc and p66Shc, the first two of which are involved in EGF-induced mitogen activated protein kinase (MAPK) signaling (Migliaccio et al., 2006). Grb2 binds to receptor-bound Shc (via phosphorylation of residues on Shc/SH2 domain), or can bind directly to EGFR via tyrosines 1068 and 1086 (Batzer et al., 1994; Sasaoka et al., 1994). Grb2 also contains two SH3 domains, which allow for interactions with proline-rich sequences, including those of Son of sevenless (SOS) (Pawson, 1995). The Grb2/Shc/EGFR interaction results in the recruitment of SOS to the plasma membrane. SOS is a guanine nucleotide exchange factor which promotes the conversion of Ras-GDP to the active Ras-GTP. Ras then activates Raf, a serine-threonine protein kinase, which in turn phosphorylates and activates MEK1/2, which then activates ERK1/2 (MAPK) (Marshall, 1994; Pawson, 1995; Marshall, 1996). Shc null cells demonstrated that Shc is not required for Ras activation to occur, however, p52/46Shc amplifies the signal and enhances Ras activity (Lai and Pawson, 2000). Induction of this pathway results in marked increases in cellular

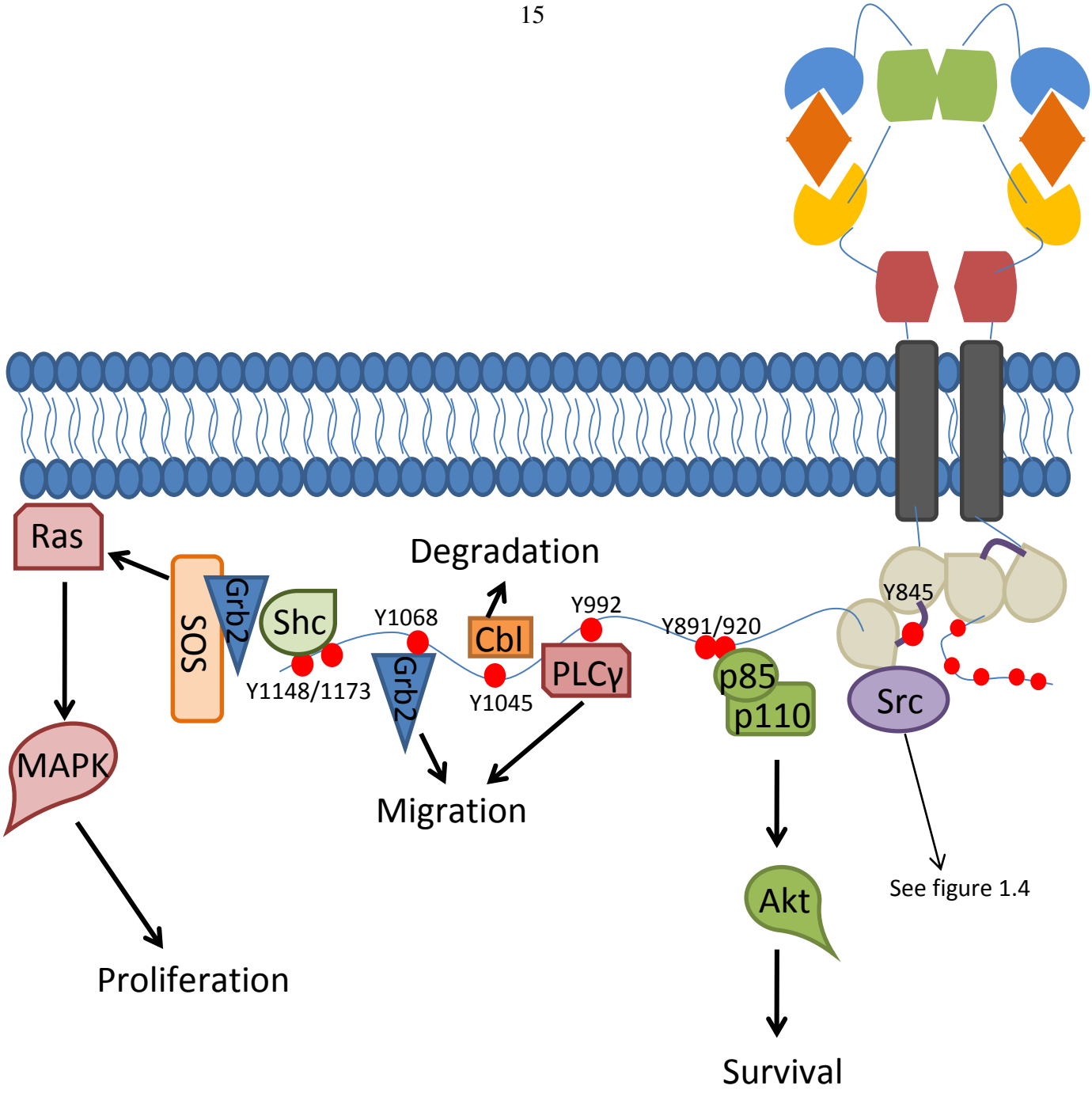


Figure 1.2: EGFR signaling pathways. Phosphorylation of EGFR on tyrosine residues leads to recruitment of proteins involved in downstream signaling pathways. Tyrosine 845 is involved in c-Src signaling. Tyrosines 891 and 920 facilitate PI3K recruitment leading to Akt activation and cellular survival. Tyrosine 992 is where PLCγ interacts leading to migration pathways. Tyrosine 1045 is responsible for c-Cbl binding, which leads to receptor degradation. Tyrosine 1068 recruits Grb2 directly, leading to cellular migration. Finally, tyrosines 1148 and 1173 bind to Shc, which recruits other proteins leading to Ras activation and MAPK signaling which promote proliferation.

proliferation [reviewed in (Zhang and Liu, 2002)]. Shc itself can also be detected complexed with other proteins including MEKK-1, linking to JNK pathway activation, as well as cadherins, which implies a role for this protein in cell-cell adhesions (Xu et al., 1997b; Pomerance et al., 1998). Grb2 also links EGFR to proteins such as Fak and dynamin which implicate a role for Grb2 in migration of cells (Schlaepfer et al., 1999). Grb2 is also associated with differentiation, as Grb2 null mouse embryos contain defects in this process (Cheng et al., 1998).

Signal transducers and activators of transcription (STAT)

Signal transducers and activators of transcription (STAT) proteins are transcription factors that, when activated by tyrosine phosphorylation, translocate to the nucleus [reviewed in (Jorissen et al., 2003)]. The STAT family of proteins consists of seven members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6), of which, STAT1, STAT3, STAT5a, and STAT5b are known to play a role in cancer [reviewed in (Quesnelle et al., 2007)]. Unlike in cytokine receptor activation of STATs, ligand-dependent phosphorylation of STATs by EGFR does not require Jak kinases (David et al., 1996; Leaman et al., 1996; Park et al., 1996). EGFR has been noted to activate STAT1, STAT3, and STAT5 (Olayioye et al., 1999). STATs may also be constitutively associated with EGFR, and their activation is strictly dependent on EGFR tyrosine kinase activity (David et al., 1996; Olayioye et al., 1999; Xia et al., 2002). Activation of STATs leads to increased transcription of proteins involved in mitogenesis, cell survival, and cell differentiation (Cressman et al., 1995; David et al., 1995; Eilers and Decker, 1995; Eilers et al., 1995; Demoulin et al., 1996; Kordula et al., 1996).

p85α

PI3Ks can be divided into subclasses, of which, class Ia is the only one activated by tyrosine kinase receptors (Jorissen et al., 2003). The regulatory subunit of PI3K, p85α, binds to, and represses, the catalytic subunit of PI3K, p110α. This complex is recruited to receptor tyrosine kinases by interaction between an SH2 domain of p85α and phospho-tyrosine residue of the receptor. Binding of the SH2 domain of p85α to a phospho-tyrosine residue results in a conformational change in the p85α: p110α complex that de-represses the activity of p110α. This de-repression allows p110α to catalyze the phosphorylation of the 3' position of phosphatidylinositols [reviewed in (Vanhaesebroeck et al., 2010)]. p85α associates with EGFR either through ErbB3 heterodimerization (as HER3 is a major binding partner for p85α) or through c-Src phosphorylation of EGFR (Kim et al., 1994; Stover et al., 1995; Ram and Ethier, 1996). Activation of PI3K in this manner leads to phosphorylation of Akt, and subsequent activation of cellular processes such as proliferation, survival, adhesion, and migration [reviewed in (Cantley, 2002)]. Akt activation also results in phosphorylation of Bad, a Bcl family member, that when phosphorylated cannot translocate to the mitochondria to inhibit the survival protein Bcl-Xl, such that, apoptosis does not occur (Zha et al., 1996; Datta et al., 1997).

PLCγ

Phospholipase C gamma (PLCγ), is one of a family of enzymes that catalyze the hydrolysis of phosphatidylinositol bisphosphate (PIP2) to inositol trisphosphate and diacyl glycerol [reviewed in (Rhee et al., 1989)]. This family of enzymes has a common dependence on calcium, and substrate specificity for phosphoinositols. PLCγ shares

structural homology with the Src family tyrosine kinases within the SH2 and SH3 domains (Pawson, 1988; Stahl et al., 1988; Suh et al., 1988). Phosphorylated EGFR provides a docking site for PLCγ (Margolis et al., 1989) to induce EGFR-mediated cell motility but not EGF-induced mitogenesis (Chen et al., 1994a).

Gab1

Gab1, or Grb2-associated protein 1, is a docking protein that binds to EGFR, is phosphorylated, and then acts as a docking center for protein complexes (Holgado-Madruga et al., 1996). In particular, Gab1 contains three p85α PI3K binding sites (Holgado-Madruga et al., 1996), and association of p85α with Gab1 and subsequent activation of PI3K has been shown after ligand stimulation (Holgado-Madruga et al., 1997). Rodrigues and colleagues have demonstrated that the PH domain of Gab1 is sufficient for binding of Gab1 to PIP3 and targeting of Gab1 to the plasma membrane. They have also shown that Gab1 directly binds to EGFR and potentiates EGF-induced MAPK, JNK, and PI3K activity (Rodrigues et al., 2000).

Src Family kinases – see Section 1.2

ii. EGFR localization

While EGFR signaling is well known to occur at the plasma membrane, signaling from EGFR also occurs at alternative localizations including the mitochondria, endosomes, and nucleus. For example, EGFR is functionally active at the mitochondria, where phosphorylation of tyrosine 845 is required for association of EGFR with cytochrome c oxidase II. This co-localization and co-association occurs after ligand-stimulation and re-localization of EGFR to the mitochondria through clathrin mediated endocytosis (Boerner et

al., 2004; Demory et al., 2009). EGFR is also capable of signaling while within endosomes. A number of groups have shown that EGFR is autophosphorylated and catalytically active while in endosomes (Cohen and Fava, 1985; Kay et al., 1986; Kuruvilla et al., 2000). Also, signaling molecules (specifically those that regulate Ras activity – including Grb2, SHC, and Cbl) are associated with EGFR localized within endosomes (Di Guglielmo et al., 1994; Fukazawa et al., 1996; Wang and Moran, 1996; Levkowitz et al., 1998; Clague and Urbe, 2001). Following these findings, Wang and colleagues were able to show that EGFR stimulates signal transduction pathways leading to cell survival from within endosomes (Wang et al., 2002; Sadowski et al., 2009). Localization of EGFR to the nucleus has been shown in a number of cell types (Gusterson et al., 1985; Kamio et al., 1990; Lipponen and Eskelinen, 1994; Tervahauta et al., 1994; Zimmermann et al., 1995). Specifically, Lin and colleagues have shown that EGFR rapidly translocates to the nucleus after ligand stimulation, where it acts as a transcription factor in a complex to activate gene transcription of cellular factors including cyclin D1 (Lin et al., 2001), thus promoting cell cycle progression. These diverse localizations increase the complexity of EGFR signaling.

D. EGFR in cancer

Purification and sequencing of EGFR revealed a close sequence similarity of this receptor to the previously characterized v-erb-B viral oncogene. v-erb-B and EGFR are most similar in the transmembrane and cytoplasmic tyrosine kinase domains (Downward et al., 1984b), suggesting that the functional portion of EGFR may be implicated in the cancer phenotype (Downward et al., 1984b). An oncogene is a gene, the product of which has the ability (via mutation, amplification, or overexpression) to promote transformation of normal cells.

EGFR can be labeled an oncogene as EGFR is over-expressed in many cancer types, and this overexpression has been shown to lead to increase tumorigenicity of cells (Velu et al., 1987).

i. EGFR and hallmarks of cancer

The hallmarks of cancer, as described by Hanahan and Weinberg, include evading apoptosis, sustained angiogenesis, un-regulated cellular proliferation, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). EGFR has been shown to regulate each of these hallmarks. Specifically, by increasing PI3K/Akt signaling, EGFR family receptors can modulate both the extrinsic and intrinsic apoptosis pathways. Also, EGFR is known to constitutively activate Stat3 in head and neck cancer (Grandis et al., 1998), which may lead to sustained expression of the anti-apoptotic protein Bcl-Xl effectively allowing these cells to evade apoptosis (Grandis et al., 2000). Angiogenesis, or neovascularization, is necessary to support growing tumors and supply the nutrients and oxygen the tumor needs for growth. Treatment of cells with small molecule kinase inhibitors of EGFR (which block kinase activity of the receptor) results in decreased vascular epithelial growth factor (VEGF) expression, which, in turn, decreases the ability of vascular epithelial cells to migrate towards tumor cells in vitro. EGFR inhibition also leads to the death of tumor-associated vascular epithelial cells in orthotopic pancreatic tumor models (Bruns et al., 2000; Hirata et al., 2002). The ability to proliferate independently of normal growth inhibitory signals is also a trait of cancer cells. EGFR activation can lead to and modulate cellular proliferation and neoplastic growth (Salomon et al., 1995). Interestingly, studies have shown that inhibition of the EGFR family of proteins leads to proliferative block (Mendelsohn and Baselga, 2000) Therefore, it can be suggested that EGFR is necessary for the limitless replicative potential of cancer cells. EGFR has been shown to influence the movement of a variety of cell types in a ligand-

dependent fashion (Barrandon and Green, 1987; Chen et al., 1994b). As mentioned earlier, PLCγ activation is necessary for EGFR mediated cell movement (Chen et al., 1994a). EGFR has also been suggested to directly affect expression and function of integrins, which mediate cytoskeletal changes associated with focal adhesions and motility (Bellas et al., 1991). EGFR can also promote the metastatic phenotype of cancer cells [reviewed in (Khazaie et al., 1993)].

ii. EGFR in human cancers

Ullrich and colleagues showed the first evidence that EGFR itself may be closely related to tumor cell function, as they discovered that EGFR was amplified at the genetic level in A431 epidermoid carcinoma cells (Ullrich et al., 1984). These data, along with the close structural relationship between EGFR and v-erb-B, suggested that EGFR may be important in the cancer phenotype. Mouse models have demonstrated that overexpression of EGFR induces cellular transformation in the mammary gland (Brandt et al., 2000; Marozkina et al., 2008b), ovary (Marozkina et al., 2008a), uterus (Marozkina et al., 2008a), bladder (Cheng et al., 2002), esophagus (Andl et al., 2003), and brain (Holland et al., 1998). Indeed, as mentioned earlier, EGFR itself has been found to be over-expressed in nearly all tumors of epithelial origin (Earp et al., 1995) Below is a concise review of EGFR in these tumor types.

Gliomas

Over-expression of EGFR occurs in 40% of gliomas and correlates with amplification and mutation of the receptor (Libermann et al., 1985; Wong et al., 1987; Helseth et al., 1988; Yung et al., 1990; Ekstrand et al., 1991; Agosti et al., 1992; Chaffanet et al., 1992; Wikstrand et al., 1995). EGFR expression is a negative prognostic marker in glioblastomas, and is

correlated with higher grade of tumor and reduced overall survival (Hurtt et al., 1992; Hiesiger et al., 1993). While overexpression is one way EGFR is activated in gliomas, mutations of EGFR are also present. The most common is a mutation that deletes part of the extracellular domain (vIII) and yields a constitutively active receptor (Wong et al., 1992; Moscatello et al., 1995).

Prostate

EGFR over-expression occurs in approximately 18% of prostate cancers (Schlomm et al., 2007). This protein is amplified in 3-11% of prostate cancers (Edwards et al., 2003; Schlomm et al., 2007). EGFR expression may serve as a prognostic marker for prostate cancers (Gorgoulis et al., 1992; Wong et al., 1992; Irish and Bernstein, 1993). Di Lorenzo and colleagues investigated the relationship of EGFR expression with prostate cancer in human tumor tissue samples. They found that EGFR expression was significantly correlated with higher Gleason scores and PSA levels, as well as disease relapse in patients (Di Lorenzo et al., 2002). EGFR contributes to prostate cancer growth by activating the androgen receptor (AR) through phosphorylation, promoting further cellular growth of both androgen-dependent and androgen-refractory prostate cancer cells without androgen stimulation (Bonaccorsi et al., 2004; Migliaccio et al., 2006; Bonaccorsi et al., 2007; Leotoing et al., 2007). In contrast to normal prostate, where androgens decrease EGFR expression, prostate cancer cell lines have increased EGFR expression induced by androgens leading to increased cellular proliferation (Liu et al., 1993b). Hammarsten and colleagues have demonstrated that treatment of castrated animals with gefitinib (an inhibitor of EGFR) leads to prostate cancer cell growth inhibition (Hammarsten et al., 2007).

Pancreatic

EGFR overexpression occurs in ~30-50% of pancreatic carcinomas (Yamanaka et al., 1990; Barton et al., 1991; Korc et al., 1992; Yamanaka et al., 1993). Pancreatic cancer cell lines often contain overexpression of EGFR without gene amplification (Smith et al., 1987; Chen et al., 1990). Treatment of cells with small molecule kinase inhibitors of EGFR results in decreased angiogenesis in orthotopic pancreatic tumor models (Bruns et al., 2000; Hirata et al., 2002).

Lung

EGFR is over-expressed in 40-80% of primary non-small cell lung cancers (NSCLC); however, squamous cell carcinomas generally have higher levels of expression than other types of NSCLC (Berger et al., 1987b; Veale et al., 1987; Dazzi et al., 1989; Di Carlo et al., 1993). This high level of overexpression is due primarily to amplification of the gene encoding for EGFR (Suzuki et al., 2005). EGFR expression has been shown as an important prognostic indicator for NSCLC, as stage III NSCLC was found to have higher levels of EGFR expression compared to stage I and II tumors (Veale et al., 1987). Furthermore, overexpression of EGFR correlates with high levels of metastases, poor differentiation of the tumor, and elevated tumor proliferation rates (Pavelic et al., 1993). As mentioned for gliomas, a mutation that deletes part of the extracellular domain (vIII) yields a constitutively active receptor, and has been found in NSCLC (Garcia de Palazzo et al., 1993). Additional mutations in EGFR also occur in lung cancer. Many of these, including the L858R mutation, result in sensitivity to EGFR inhibitors (Costa and Kobayashi, 2007). This conferred sensitivity to EGFR inhibitors has led to FDA approval of the EGFR TKIs gefitinib and

erlotinib. However, other mutations, such as the T790M mutation, result in acquired resistance to EGFR kinase inhibition (Kobayashi et al., 2005; Pao et al., 2005). Thus, screening of patients for EGFR mutational status in lung cancer may allow further approval of EGFR-based therapies for the sub-populations of lung cancer patients who possess these mutations.

Colon

Approximately 8% of all colorectal carcinomas over-express EGFR. This overexpression is a result of genetic amplification in ~60% of cases (Ooi et al., 2004). While no correlation has been observed between this expression and tumor grade, proliferative index, histological stage, or overall survival (Salomon et al., 1995), Mayer and colleagues have shown that patients whose tumors are over 50% positive for EGFR expression have worse prognosis compared to those with less than 50% of their tumor positive for EGFR (Mayer et al., 1993). Also, EGFR expression is elevated in more highly metastatic primary colon tumors than those with a less aggressive phenotype (Radinsky, 1993). Cetuximab (Erbitux), an EGFR-directed monoclonal antibody, is currently in clinical use in colorectal cancers.

Ovarian

EGFR is over-expressed in 35-70% of primary ovarian carcinomas (Battaglia et al., 1989; Bauknecht et al., 1989a; Bauknecht et al., 1989b; Bauknecht et al., 1990; Johnson et al., 1991; Morishige et al., 1991b; Berns et al., 1992; Henzen-Logmans et al., 1992; Owens et al., 1992; Scambia et al., 1992; Bauknecht et al., 1993). Such overexpression occurs without gene amplification (Zhang et al., 1989; Bauknecht et al., 1990; Berns et al., 1992; Kohler et al., 1992). EGFR expression was found to be significantly associated with high risk of

ovarian carcinoma progression (Scambia et al., 1992). As with gliomas and lung cancers, the vIII constitutively active mutant of EGFR is expressed in ovarian carcinomas (Wong et al., 1992; Moscatello et al., 1995). Proliferation of ovarian cancer cells can be significantly inhibited in vitro utilizing EGFR monoclonal antibodies or anti-TGFα antibodies (Morishige et al., 1991a; Morishige et al., 1991b; Stromberg et al., 1992).

Liver

EGFR over-expression occurs in 30-60% of hepatocellular carcinomas, however little is known about the mechanism of this over-expression (Nonomura et al., 1988; Ito et al., 2001). Nevertheless, over-expression of EGFR in this cancer type correlates with high proliferation, advanced stage, and poor prognosis (Ito et al., 2001). Also, EGFR, as well as ErbB3, and the ligands HB-EGF, TGFα, BTC, and AREG are over-expressed in human hepatocellular carcinoma tissues (Ding et al., 2004; Avila et al., 2006; Breuhahn et al., 2006; Castillo et al., 2006; Berasain et al., 2007). Production of EGFR ligands has been suggested to influence the growth of premalignant liver epithelial cells (Drucker et al., 2006). Activation of EGFR in a ligand-dependent manner in liver cancer cells has been demonstrated to potentiate the aggressive behavior of such cells (Lin et al., 2006).

Bladder

EGFR expression is higher in malignant bladder as compared to normal bladder epithelium, yet EGFR is rarely genetically amplified (Neal et al., 1985; Berger et al., 1987a; Messing et al., 1987; Neal et al., 1989; Messing, 1990; Lonn et al., 1993). EGFR expression in these tumors correlates with invasive potential, poor tumor differentiation, decreased

survival, increased recurrence, and increased rate of progression in bladder cancers (Neal et al., 1985; Neal et al., 1989; Smith et al., 1989; Neal et al., 1990).

Esophagus

Approximately 35% of primary esophageal tumors express EGFR (Iihara et al., 1993) and EGFR is genetically amplified in primary human esophageal carcinomas (Hollstein et al., 1988; Lu et al., 1988). Survival rate is significantly lower in patients with tumors that over-express EGFR as compared to patients with no EGFR overexpression (Iihara et al., 1993). In these tumors, TGFα is also highly expressed, and this expression correlates with short doubling time of esophageal cancer cells (Kim et al., 1991; Thornley and Jones, 1992).

Head & Neck

EGFR is over-expressed in oral squamous cell carcinomas (SCC) (Todd and Wong, 1999), and this over-expression often times occurs without gene amplification [reviewed in (P et al., 2002)]. Ligand-independent activation of EGFR through E-cadherin and subsequent MAPK phosphorylation allows adhesion-mediated survival for this cancer type (Shen and Kramer, 2004). EGFR is known to constitutively activate Stat3 in head and neck cancer (Grandis et al., 1998). Ligand stimulation of EGFR was shown to stimulate anchorage-independent growth in oral SCC cell lines (Lee et al., 1990). Resistance of oral SCCs to chemotherapy and radiotherapy can be pharmacologically reversed through the utilization of inhibitors of EGFR function (Bonner et al., 2002; Raben et al., 2002).

Gastric

Over-expression of EGFR has been noted in approximately 33% of advanced stage gastric carcinomas (compared to 3.8% in early stage or non-malignant samples) (Yasui et al., 1988). Such over-expression is rarely due to genetic amplification in this form of cancer (Kim et al., 2008). EGFR expression has been suggested as a useful prognostic indicator for gastric cancer (Yasui et al., 1988; Lemoine et al., 1991). EGFR expression occurs more frequently in well-differentiated advanced stage adenocarcinomas; however there is no apparent association of EGFR expression with tumor grade or size (Yasui et al., 1988; Lemoine et al., 1991).

Breast – see Section 1.4

E. Inhibiting EGFR activity

i. Monoclonal Antibodies

In 1981, John Mendelsohn and colleagues initially hypothesized that blocking the binding site for EGFR ligands using a monoclonal antibody (mAbs) might be an effective treatment strategy for cancer [reviewed in (Mendelsohn and Baselga, 2000)]. In the years following that hypothesis, mAbs directed toward EGFR have been developed and applied in the clinic, leading to treatment regimens for cancer [reviewed in (Herbst et al., 2001)]. Specifically, mAb 225 (later derived into C225, a humanized murine chimeric version of the mAb) was isolated in 1983. This antibody was later developed into a cancer therapeutic for head and neck cancer. There are currently five EGFR targeting antibodies in development including Cetuximab (Erbitux), Matuzumab, Nimotuzumab (TheraCIM), Panitumumab (Vectivix), and Zalutumumab (Wheeler et al., 2010). These inhibitors have been introduced in clinical trials

both as monotherapies and as combinatorial therapy with chemo- or radio-therapies in colorectal, head and neck, pancreatic, and lung cancers (Mendelsohn, 2004). EGFR-directed mAbs compete with ligand binding and then down-regulate receptor expression, leading to inhibition of cell growth by arresting cells in G1 (Wu et al., 1996; Waksal, 1999). While mAbs can directly stimulate immune response in patients, leading to complement-mediated cytotoxicity or antibody-dependent cell-mediated cytotoxicity (Harris and Mastrangelo, 1989), at least in the case of cetuximab, the primary mechanism of action is related to the disruption of EGFR-mediated signaling (Mendelsohn, 1997). It has been suggested that antibodies against HER2 require endocytic sorting and Cbl to exert their antitumor effects, however, it has yet to be shown if antibodies specific to EGFR act in the same manner (Klapper et al., 2000). To date, two EGFR targeting monoclonal antibodies have been FDA approved for use in cancer patients. Specifically, cetuximab (Erbitux) was approved for use in combination with irinotecan or alone if the patient cannot tolerate irinotecan in metastatic colorectal cancer in 2004, and for head and neck cancer in 2006. Also, panitumumab (Vectibix) was approved for EGFR-expressing advanced colorectal cancer with disease progression on or following fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens in 2006.

ii. Small molecule tyrosine kinase inhibitors (TKIs)

TKIs target the intracellular ATP-binding pocket of the tyrosine kinase moiety of EGFR. In xenograft models and human tumor-derived cell lines, these inhibitors have shown dose dependent tumor growth inhibition alone, or in combination with drugs and/or radiation (Ciardiello, 2000). Clinically, TKIs have demonstrated anti-tumor activity in head and neck cancer (Feng et al., 2007; Reuter et al., 2007) where approximately 10% respond to the

inhibitor, non-small cell lung cancer (Jubelirer et al., 2006), and glioblastomas (Mellinghoff et al., 2005) where again 10-20% of patients respond. Glioblastoma patients that responded were vIII mutants. Those that responded in non-small cell lung cancer have been demonstrated to possess the L858R EGFR mutation (Costa and Kobayashi, 2007). Currently, five EGFR tyrosine kinase inhibitors are in clinical trials including erlotinib (Tarceva), gefitinib (Iressa), vandetanib (Zactima), lapatinib (Tykerb), and pelitinib (Wheeler et al., 2010). Gefitinib (Iressa), an EGFR specific TKI, was approved in 2004 for monotherapy treatment for advanced, chemotherapy-refractory non-small cell lung cancer based on an 11-18% response rate in two phase II clinical trials (Mendelsohn and Baselga, 2000), but approval was later revoked do to failure to produce survival benefit alone or with chemotherapy in three phase III trials (Giaccone et al., 2004; Herbst et al., 2004; Thatcher et al., 2005). Erlotinib (Tarceva) was approved for treatment of non-small cell lung cancer and pancreatic cancer, and recently has been approved for maintenance of locally advanced or metastatic NSCLC that is not progressing after four cycles of platinum based chemotherapy (2010). Lapatinib, a dual EGFR/HER2 inhibitor, has also been recently approved, in combination with letrozole, in post menopausal women with hormone receptor positive metastatic breast cancers that over-express HER2 (2010).

Resistance to EGFR TKIs is a common occurrence in cancer. Generally, resistance can occur by two mechanisms. First, *de novo* or intrinsic resistance may occur. This type of resistance is thought to be due to lack of tumor dependence upon EGFR (Baselga and Arteaga, 2005). Also, EGFR-independent or constitutive activation of effectors downstream of EGFR could contribute to *de novo* resistance. For example, failure to inhibit PI3K/Akt, by mutation or alternative activation, also prevents response to EGFR inhibitors (Bianco 2003,

Cheng 1992, Cheng 1996, Forgacs 1998, Lu 1999, Hoilestelle 2007). EGFR-independent activation of c-Src after interaction with other tyrosine kinases or integrins may lead to activation of signaling pathways downstream of EGFR [reviewed in (Bianco et al., 2007)]. Activated Ras (and thereby persistent MAPK signaling) is associated with intrinsic resistance to both gefitinib and cetuximab in NSCLC (Janmaat et al., 2003), and resistance to gefitinib in breast cancer cells (Normanno et al., 2006). In addition, glioblastomas containing the EGFRvIII mutation are relatively resistant to gefitinib due to persistent Akt phosphorylation after PTEN loss (Kuan et al., 2001). Acquired resistance may also occur after initial response to EGFR inhibitors. This may be due to alternative activation of proangiogenic pathways, as EGFR inhibition can lead to subsequent down-regulation of tumor-induced, VEGF-mediated angiogenesis (Ciardiello et al., 1996; Petit et al., 1997; Perrotte et al., 1999). It has been demonstrated that altered control of these angiogenic pathways can induce resistance to EGFR inhibitors *in vivo* (Viloria-Petit et al., 2001). Also, activation of alternative tyrosine kinases (such as IGF-1R or c-Met), has also been shown as a potent mechanism of acquired resistance to EGFR blockade (Jones et al., 2004). While alterations in EGFR protein such as the L858R mutation or deletion mutants may lead to sensitivity to EGFR inhibitors (Lynch et al., 2004; Paez et al., 2004), secondary mutations, including the T790M mutation, can occur and lead to acquired resistance to EGFR inhibitors (Kobayashi et al., 2005; Pao et al., 2005).

1.2. Src Family Kinases (SFKs)

A. Structure and Activation of SFKs

c-Src, the prototypical member of the Src family kinases, was discovered in 1976 as a mammalian homologue of the transforming agent in avian sarcoma virus, v-Src (Stehelin, 1976; Stehelin et al., 1976). This family of non-receptor tyrosine kinases contains nine members that differ based on tissue specific expression. The first subfamily, Lck, Blk, Lyn, and Hck, are fairly restricted in their expression to hematopoietic cells. The second subfamily, c-Src, Yes, Fyn, Yrk, and Fgr, are ubiquitously expressed (Bolen and Brugge, 1997). These proteins have structural homology consisting of an N- terminal sequence, unique domain, a SH3 domain, a SH2 domain, and a tyrosine kinase domain (Fig 1.3) [reviewed in (Tsygankov and Shore, 2004)]. Within the c-terminal tyrosine kinase domain are two phosphorylation sites that are critical to the function of the protein. First, phosphorylation on tyrosine 527 negatively regulates SFK activity. Phosphorylation of this site occurs via the Csk-family of protein tyrosine kinases (Okada et al., 1991; Bergman et al., 1992; Sabe et al., 1992; Chow et al., 1993; Nada et al., 1993; Superti-Furga et al., 1993; Takeuchi et al., 1993; Thomas et al., 2006). The SH2 domain of SFKs binds to tyrosine 527 after phosphorylation on this site (Roussel et al., 1991; Amrein et al., 1993; Liu et al., 1993a; Weijland et al., 1997), which induces the binding of the SH3 domain to the linker region between the SH2 and tyrosine kinase domains, prohibiting binding of ATP, effectively inactivating the protein (Fig. 1.3) (Sicheri et al., 1997; Williams et al., 1997; Xu et al., 1997a; Gonfloni et al., 1999; Schindler et al., 1999; Xu et al., 1999). Dephosphorylation on tyrosine 527 occurs by one of several protein tyrosine phosphatases including CD45, SHP-1, SHP-2,

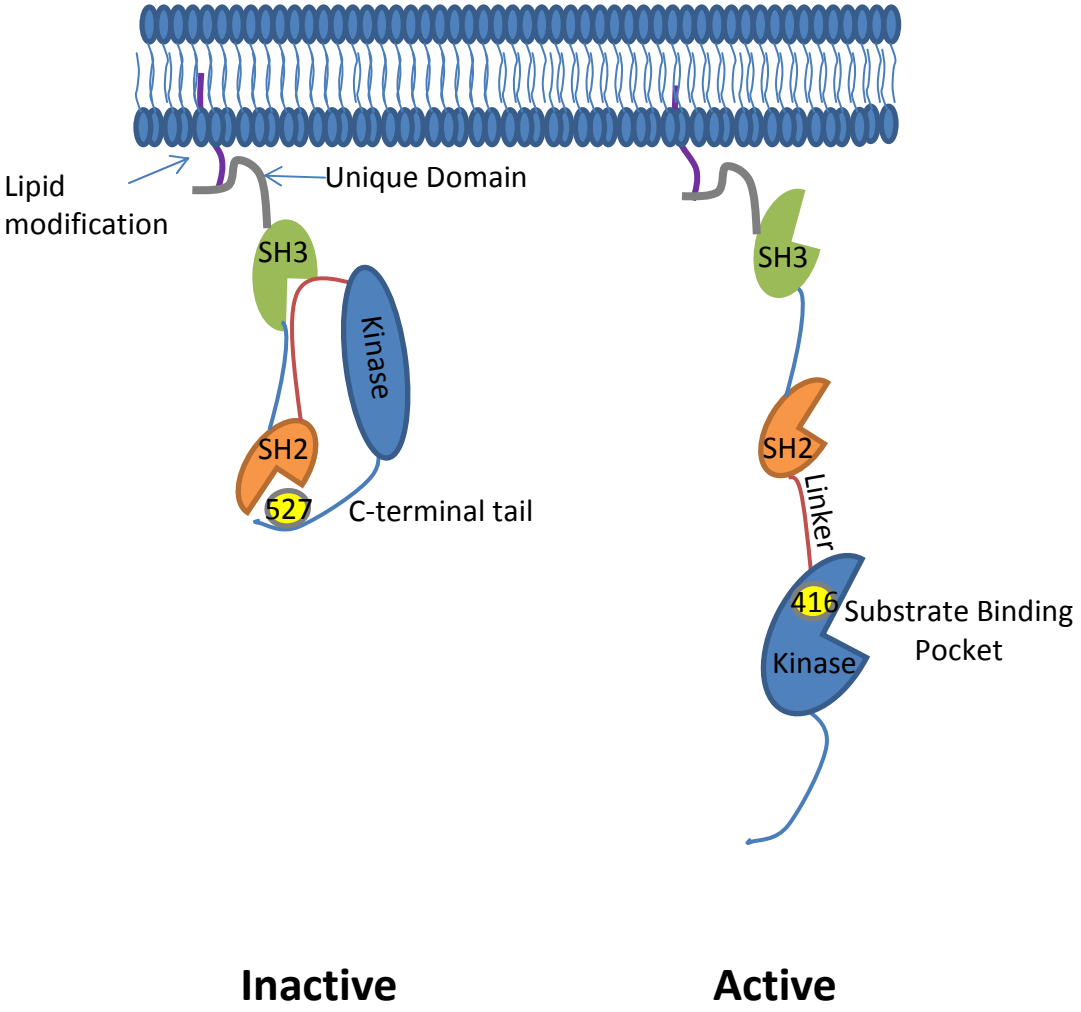


Figure 1.3: c-Src activation. In the inactive conformation, tyrosine 527 on the c-terminal tail of c-Src is phosphorylated, which facilitates binding of the Src SH2 domain to this tail. This binding positions the link region in close proximity to the SH3 domain and prevents the activation of the complex. In the active conformation, tyrosine 416 is phosphorylated leading to opening of the substrate binding pocket and release of the SH2 domain from tyrosine 527, thus repositioning the SH3 and linker domains.

PTP-α, or PTP-λ (Mustelin et al., 1989; Mustelin and Altman, 1990; Mustelin et al., 1992; Shiroo et al., 1992; Zheng et al., 1992; Hurley et al., 1993; Sieh et al., 1993; Biffen et al., 1994; Fang et al., 1994; Peng and Cartwright, 1995; Somani et al., 1997; Harder et al., 1998a; Bjorge et al., 2000). Second, phosphorylation of tyrosine 416, must occur for the protein to be active. Phosphorylation on this site results in displacement from a pocket of hydrophobicity formed within the catalytic domain, resulting in the repositioning of the region and formation of a substrate binding pocket (Fig 1.3) (Yamaguchi and Hendrickson, 1996; Sicheri et al., 1997; Williams et al., 1997; Xu et al., 1997a; Schindler et al., 1999; Xu et al., 1999). Tyrosine 416 is phosphorylated by many proteins, including PDGFR, focal adhesion kinase (FAK), and EGFR (discussed later) (Kypta et al., 1990; Cobb et al., 1994; Schaller et al., 1994; Alonso et al., 1995; Eide et al., 1995; Alexandropoulos and Baltimore, 1996; Moarefi et al., 1997; Chiang and Sefton, 2000). Phosphorylation on tyrosine 416 is sufficient to activate c-Src, even when tyrosine phosphorylation of 527 is present (Sun et al., 1998; Boerner et al., 2004). Thus, dephosphorylation of tyrosine 416 is critical to the inactivation of SFKs. PTP-α and PTP-λ are known to de-phosphorylate tyrosine 416 of c-Src (Zheng et al., 1992; Fang et al., 1994).

SFKs are localized to the plasma membrane, perinuclear regions and endosomal membranes (Silverman et al., 1993; Ley et al., 1994; Resh, 1994). As SFKs contain no transmembrane domain, membrane localization is due, in part, to myristoylation and palmitoylation on N-terminal fatty acid modification sites (Marchildon et al., 1984; Pellman et al., 1985; Peters et al., 1990; Paige et al., 1993; Shenoy-Scaria et al., 1993; Koegl et al., 1994). Localization may also be dependent on the specific protein interactions between

SFKs and cytoskeletal components, cytokines, and growth factor receptors including EGFR (Kaplan et al., 1992; Sandilands et al., 2004; Donepudi and Resh, 2008).

Like the EGFR ligands, SFKs have a high level of functional redundancy. However, loss of c-Src results in the onset of osteoporosis due to defective osteoclast function (Soriano et al., 1991; Boyce et al., 1992). Loss of Lck results in hematopoetic deficiencies (Molina et al., 1992; Molina et al., 1993; Wen et al., 1995) and severe nervous system defects are seen in models where Fyn is knocked out (Grant et al., 1992; Beggs et al., 1994; Miyakawa et al., 1994; Umemori et al., 1994). Yes, Hck, Fgr, and Blk have also been knocked out in mice, however, no distinct phenotypes are seen, suggesting, again, that a high degree of redundancy exists within the family [reviewed in (Lowell and Soriano, 1996)].

B. Function of c-Src

For the purposes of this dissertation, we will focus the remainder of this section the prototypical membrane of the Src family, c-Src. c-Src is involved in a number of cellular processes including membrane trafficking, cellular motility and migration, cell cycle progression, apoptosis, and differentiation (Fig. 1.4). First, c-Src has been shown to play a role in the regulation of membrane trafficking. Specifically, c-Src phosphorylates ASAP1, an ADP-ribosylation factor GTPase-activating protein, and association of this protein with Arfs and PIP2 has been shown to be important in actin cyoskeletal remodeling (Brown et al., 1998b; Randazzo et al., 2000). Src also associates with synapsin I, dynamin, synaptophysin, snaptogyrin, and cellugyrin which are all involved in vesicle transport (Barnekow et al., 1990; Onofri et al., 1997; Foster-Barber and Bishop, 1998; Janz and Sudhof, 1998). Second, cellular motility is a process that has also shown a dependence on c-Src. c-Src localizes to

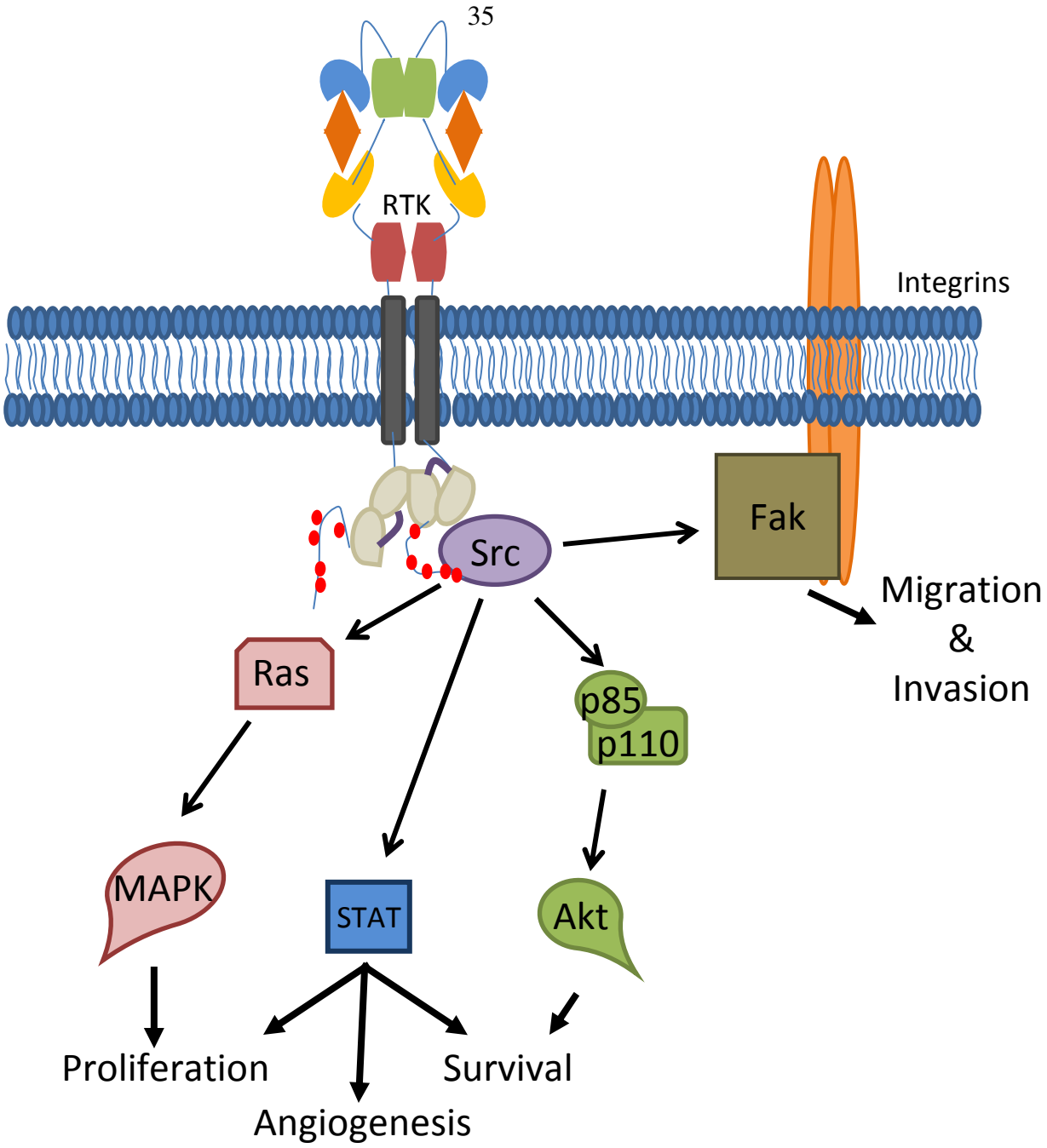


Figure 1.4: c-Src signaling pathways. c-Src is activated through interaction with transmembrane proteins including receptor tyrosine kinases (RTK). Activation of c-Src mediates the Ras/MAPK pathway, leading to proliferation, PI3K/Akt pathway leading to survival, FAK signaling leading to migration and invastion, and STAT pathway leading to proliferation, survival, and angiogenesis.

focal adhesions, where it associates with FAK, a protein tyrosine kinase that is active following the engagement of integrins (Schaller et al., 1993; Cobb et al., 1994; Schaller et al., 1994). This co-association activates Src, which in turn phosphorylates FAK. Subsequently, there is an accumulation of phosphorylated proteins critical to cell motility and migration including p130cas, paxillin, and PI3K (Hildebrand et al., 1993; Schaller et al., 1994; Chen et al., 1995; Polte and Hanks, 1995; Altun-Gultekin and Wagner, 1996; Burnham et al., 1996; Hall et al., 1996; Harte et al., 1996; Vuori et al., 1996; Yokote et al., 1996; Schlaepfer et al., 1997; Schlaepfer and Hunter, 1997). Third, c-Src has been shown to be involved in cell cycle progression. Specifically, c-Src activates Shc and PI3K to modulate DNA synthesis and c-Src phosphorylates SAM68 to regulate mitosis (Augustine et al., 1991; Yamanashi et al., 1992; Pleiman et al., 1993; Taylor and Shalloway, 1993; Fumagalli et al., 1994; Ptasznik et al., 1995; Broome and Hunter, 1996; Luttrell et al., 1996; Pillay et al., 1996; Taylor and Shalloway, 1996). Through these interactions, c-Src may also regulate apoptosis. Specifically, constitutive activation of c-Src leads to rescue of apoptosis induced by cytokine removal, irradiation, chemotherapeutics, or disruption of extracellular matrix interactions (Anderson et al., 1990; Frisch and Francis, 1994; Basu and Cline, 1995; Canman et al., 1995). Lastly, differentiation has also been described to involve c-Src. Constitutive activation of c-Src is sufficient to block differentiation in myoblasts, retinoblasts, and chondroblasts, allowing a self renewal phenotype to persist (Muto et al., 1977; Yoshimura et al., 1981; Alema and Tato, 1987).

C. The interaction between c-Src and EGFR

c-Src interacts with a number of receptor tyrosine kinases including PDGFR, FGFR, CSF-1R, NGF-R, HGF-R, IGF-R, HER2, and EGFR (Luttrell et al., 1988; Kozma and

Weber, 1990; Kypta et al., 1990; Kremer et al., 1991; Courtneidge et al., 1993; Faletto et al., 1993; Zhan et al., 1994; Muthuswamy and Muller, 1995). Interaction between EGFR and c-Src enhances many EGFR-dependent cellular functions including DNA synthesis, protein tyrosine phosphorylation, transformation of mouse fibroblasts and human mammary epithelial cell lines, and tumor formation in nude mice (Luttrell et al., 1988; Wilson et al., 1989; Wilson and Parsons, 1990; Chang et al., 1995; Maa et al., 1995; Boerner et al., 2005; Dimri et al., 2007). Activation of EGFR increases c-Src catalytic activity (Sato et al., 1995a; Sato et al., 1995b; Weernink and Rijksen, 1995). c-Src, in turn, phosphorylates novel sites on EGFR, including tyrosine 1101, 891, 920, and 845 (Maa et al., 1995; Tice et al., 1999). c-Src-dependent phosphorylation on these sites promotes EGFR signaling. Specifically, phosphorylation of tyrosine 845 has been shown to be critical to EGFR-induced mitogenesis (Biscardi et al., 1999a; Tice et al., 1999), while phosphorylation of tyrosines 891 and 920 mediates the binding of PI3K to EGFR (Stover et al., 1995). c-Src may also be important in EGFR crosstalk. c-Src expression mediates crosstalk between estrogen receptor and EGFR (Castoria et al., 1999), as well as between EGFR and the HGF receptor, c-Met (Mueller et al., 2008). Thus, c-Src is an important regulator of EGFR signaling.

D. c-Src in cancer

As c-Src was discovered due to its homology to viral v-Src, a potent oncogene, it was widely believed that c-Src would be involved in the cancer phenotype (Fung et al., 1983). Overexpression of c-Src alone is insufficient to transform mouse fibroblasts in culture, and cannot sustain tumor growth in vivo (Shalloway et al., 1984; Luttrell et al., 1988; Maa et al., 1995). However, other findings have suggested a role for c-Src in tumorigenesis. c-Src is necessary for induction of mammary tumors produced by the polyoma middle T oncogene

(Guy et al., 1994a). Also, expression levels of c-Src are elevated in lung, skin, colon, breast, cervical, parotid, esophageal, gastric, and brain cancers [reviewed in (Biscardi et al., 2000; Irby and Yeatman, 2000)]. While mutations of c-Src are rare in cancer, a mutant form of c-Src that is truncated before the inhibitory phosphorylation site was identified in 1999 by Irby and colleagues in highly metastatic colon carcinoma (Irby et al., 1999).

E. c-Src tyrosine kinase inhibition

Two classes of c-Src inhibitors have been identified and tested in clinical trials: those that alter the interactions of c-Src with other proteins (SH3 and SH2 inhibitors) and those that inhibit the intrinsic tyrosine kinase activity of Src itself [reviewed in (Sawyer et al., 2001)]. The SH3 and SH2 inhibitors are small molecules that mimic the specific structure of SFK SH3 and SH2 domains thereby blocking association of substrates with c-Src. The first active SH3 inhibitor of c-Src was reported in 1999 (Cussac et al., 1999), however, SH3 domains found in various proteins contain common protein sequences, thus specificity might be an issue. The same is true for c-Src SH2 inhibitors, of which, only one has been shown effective *in vivo* [reviewed in (Sawyer et al., 2001)]. Small molecule c-Src tyrosine kinase inhibitors, such as PP1, PP2, SU6656, and dasatinib have also been developed [reviewed in (Sawyer et al., 2001)]. While PP1 and PP2 were found to inhibit Src family tyrosine kinase inhibitors effectively, they also showed efficacy for the PDGF receptor (Hanke et al., 1996). To overcome this, Blake and colleagues at SUGEN Inc., developed SU6656, which more selectively inhibited Src family tyrosine kinases (Blake et al., 2000). These inhibitors have shown to be useful tools in the study of the effects of c-Src kinase in cell models. Most clinically relevant, and important to this dissertation, is the small molecule c-Src tyrosine kinase inhibitor dasatinib. Dasatinib, or BMS-354825, was originally described as an

inhibitor of the oncogenic tyrosine kinase Bcr-Abl (Lombardo et al., 2004). Lombardo and colleagues suggested that dasatinib also could act as a potent inhibitor of SFKs. The IC_{50s} for dasatinib inhibition of SFKs were in the picomolar range, whereas the IC_{50} for Bcr-Abl was closer to 1 nanomolar. Preclinically, dasatinib was found to have antitumor activity in mouse xenograft models of leukemia (Lombardo et al., 2004) which led to the development of clinical trials and the approval of dasatinib for the treatment of Bcr-Abl expressing acute lymphoblastic leukemia in 2006 (Brave et al., 2008). Due to the elevated c-Src expression levels in many solid tumor types and preclinical evidence for an effect of c-Src TKIs on solid tumor growth, dasatinib has gone forward into clinical trials alone, and in combination with other therapeutics, in breast, skin, pancreatic, brain, colorectal, head and neck, ovarian, gastrointestinal, and prostate cancers (Laird et al., 2003; Kim et al., 2010).

1.3. Lipid Rafts

A. Discovery of lipid rafts

Early on, scientists held a two dimensional view of the lipid bilayer. This view was that the plasma membrane was a "fluid mosaic" (Singer, 1972) containing "Icebergs in the sea" (i.e. proteins in a fluid surrounding of lipid). This idea was further perpetuated by the finding that the membrane contained areas that were loosely packed and demonstrated rapid lateral diffusion (Lee, 1977). However, in the early 1950s, flask-shaped invaginations in the plasma membrane were discovered by electron microscopy (Palade, 1953). These invaginations were termed “little caves” or caveolae (Yamada, 1955) and were found to contain the protein caveolin (Glenney, 1992; Rothberg et al., 1992; Scherer et al., 1995; Tang et al., 1996). The identification of caveolin-1 led to the development of biochemical techniques that resulted in

the isolation of low density detergent resistant microdomains (DRMs) (Brown and Rose, 1992; Lisanti et al., 1995). These DRMS are rich in flotillin protein (Smart et al., 1999) as well as glycosphingolipids, gangliosides (including GM-1), cholesterol, and other proteins; however, some DRMs lack caveolin and invagination structure (Mirre et al., 1996; Wu et al., 1997). Simons and Ilkonen coined the term "lipid raft" for the DRMs that do not contain caveolin protein (Simons and Ikonen, 1997). These rafts range in size from 10 to 200 nm in diameter (Varma and Mayor, 1998; Sharma, 2004; Pike, 2006). Because of their high lipid content, lipid rafts float to low densities during gradient centrifugation (Brown and Rose, 1992). However, localization within buoyant fractions after isolation does not constitute co-association or co-localization specifically with plasma membrane lipid rafts as most isolation techniques also result in the isolation of caveolae, as well as lipid rafts from organelles [reviewed in (Simons and Ikonen, 1997)]. As recently as 2006, a consensus definition of what a lipid raft is was developed at the Keystone Symposium of Lipid Rafts and Cell Function, stating that "Lipid rafts are small (10-200nm) heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions" (Fig 1.5) (Pike, 2006). Lipid rafts have been isolated from many cell types including epithelial cells, fibroblasts, hematopoietic cells, endothelial cells, adipocytes, muscle cells, neurons, and even yeast [reviewed in (Brown and London, 1997)].

B. The controversy of lipid rafts

It was suggested originally by Simons and colleagues that the presence of glycosphingolipid rich lipid rafts may be an artificial finding. Their hypothesis was that rather than the lipids interacting to form rafts in intact cells, the lipids may interact during the

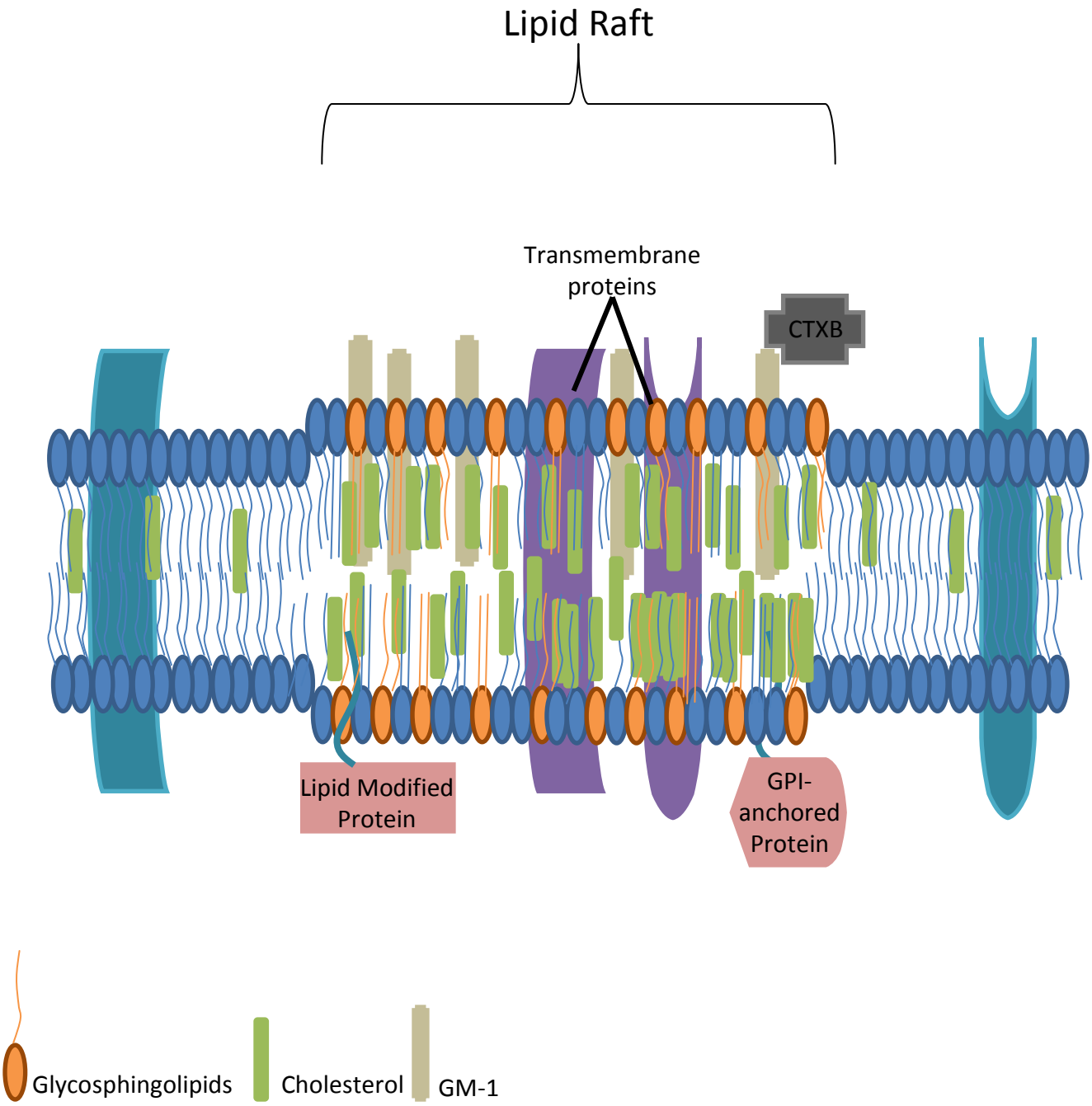


Figure 1.5: The structure of lipid raft membrane microdomains. Lipid rafts contain elevated levels of cholesterol, glycosphingolipids, and gangliosides (including GM-1). Transmembrane proteins, as well as various lipid modified proteins (including GPI-anchored proteins) have the ability to associate with lipid rafts. A commonly used method of detection for lipid rafts takes advantage of the ability of cholera toxin subunit B (CTXB) to bind GM-1.

extraction process (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990). Further indication that lipid rafts may not be present as structures within the plasma membrane is the finding that detergents themselves can cause redistribution of proteins on the cell surface. For example, Triton X-100 extraction results in GPI-anchored proteins enriched in biochemically isolated DRMs, while other proteins, such as the transferrin receptor, are readily solubilized (Hooper and Turner, 1988; Hooper and Bashir, 1991; Sargiacomo et al., 1993; Mayor and Maxfield, 1995). However, Mayor and Maxfield performed immunofluorescent staining and electron microscopy of GPI-anchored proteins in the absence and presence of detergent. They found that treatment of cells with Triton X-100 was sufficient to induce clustering of GPI-anchored proteins, suggesting that membrane domains enriched in GPI-anchored proteins are an artificial product of detergent treatment (Mayor and Maxfield, 1995). However, in 1998, Schroeder and colleagues found that DRMs were not formed spontaneously during Triton X-100 treatment, as exogenously applied radiolabelled sphingomyelin (a main component of DRMs) is not incorporated into membranes previously identified as detergent soluble (Schroeder et al., 1998). In summary, detergent extraction did not spontaneously produce DRMs where there were none previously. A number of groups utilized single particle tracking of GM-1, chemical cross-linking of GM-1 to other proteins, FRET between GM-1 and these proteins, and immunofluorescent microscopy, to show that lipid rafts exist in living cells (Sheets et al., 1997; Friedrichson and Kurzchalia, 1998; Harder et al., 1998b; Simson et al., 1998; Varma and Mayor, 1998). More recently, non-detergent methods have addressed concerns over detergent "artifacts." These methods isolate lipid rafts from the cell by mechanical lysis in detergent free buffers

followed by ultracentrifugation. These findings, along with the studies using microscopy techniques, support the presence of lipid rafts (Macdonald and Pike, 2005).

C. Rafts in cellular functions

Rafts are thought to first assemble within the golgi, where sphingolipids are synthesized (van Meer, 1989). From there, these domains travel through vesicles to the plasma membrane and other cellular organelles. At the membrane, lipid rafts participate in many cellular functions, including endocytosis and cellular signaling.

The endocytotic pathway has been proposed to involve lipid rafts. Both clathrin dependent and independent mechanisms of endocytosis have been shown to involve lipid rafts (Puri et al., 1999) as evidenced by the fact that lipid rafts are themselves are found in early endosomes that are recycled back to the cell surface or Golgi apparatus (Puri et al., 1999; Mukherjee and Maxfield, 2000). Lipid rafts are also involved in the cellular internalization of toxins. For example, cholera toxin, the infectious agent of *vibrio cholerae*, requires lipid rafts for entry into human cells (Orlandi and Fishman, 1998). Specifically, GM-1, a lipid raft specific ganglioside, is the target binding partner for cholera toxin subunit B (Fig 1.5), and the cellular function of cholera toxin is solely triggered when GM-1 is present in lipid rafts (Wolf et al., 1998).

Lipid rafts contain high concentrations of signaling molecules (Chang et al., 1994; Lisanti et al., 1994; Hope and Pike, 1996; Wu et al., 1997). Different classes of signaling proteins localize within lipid rafts including GPI-anchored proteins, transmembrane proteins, receptor tyrosine kinases, G-protein coupled receptors, and Src family kinases (Skibbens et al., 1989; Sargiacomo et al., 1993; Danielsen and van Deurs, 1995). These rafts may facilitate

signaling through localizing components of a signaling pathway together or localizing distinct signaling pathways within the same raft to facilitate crosstalk. Lipid rafts also negatively regulate signaling by sequestering molecules and preventing their association with molecules required to activate the proteins [reviewed in (Zajchowski and Robbins, 2002)]. The B cell receptor (BCR) is an excellent example of the capability of lipid rafts to regulate the signaling abilities of proteins. Specifically, in immature B cells, BCR does not translocate to lipid rafts after activation, and signaling pathways activated downstream lead to apoptosis rather than activation of the cell (Sproul et al., 2000). However, in mature B-cells, BCR rapidly translocates into lipid rafts, where it interacts with the Src family kinase member Lyn to promote signaling (Cheng et al., 1999; Petrie et al., 2000). Interestingly, in mature B cells infected with Epstein-Barr virus, this movement into lipid rafts is impaired, preventing BCR signaling activation (Dykstra et al., 2001).

D. The effect of lipid rafts on EGFR and c-Src signaling

Of the many proteins that are capable of localizing to lipid rafts, EGFR and c-Src are the most relevant to this dissertation. EGFR has been demonstrated to localize within lipid rafts in a variety of human cancer cell lines (Ringerike et al., 2002; Sun et al., 2002; Abulrob et al., 2004; Macdonald and Pike, 2005; Oh et al., 2007; Schley et al., 2007), monkey kidney cells (Peres et al., 2003), vascular smooth muscle cells (Zuo et al., 2004), and Chinese hamster ovary cells (Macdonald and Pike, 2005). The localization of EGFR to lipid rafts was difficult to detect initially, as the biochemical techniques used to isolate lipid rafts initially involved detergent extraction. EGFR is lost from lipid raft fractions when Triton X-100 is the detergent used for biochemical fractionation (Pike and Casey, 1996; Gustavsson et al., 1999). When other, less stringent, detergents are utilized in lipid raft isolations, EGFR

remains associated with lipid rafts (Roepstorff et al., 2002). The advent of detergent-free methodologies of biochemical raft isolation, as well as fluorescent and EM based techniques, have since confirmed the localization of EGFR to lipid rafts (Couet et al., 1997b; Waugh et al., 1999). Most recently, Hofman and colleagues utilized fluorescence resonance energy transfer (FRET) microscopy to show co-localization of EGFR with lipid raft specific gangliosides (Hofman et al., 2008). Mineo and colleagues have demonstrated that approximately 60% of EGFR is contained within low density caveolae and non-caveolae raft fractions (lipid rafts) in human fibroblasts (Mineo et al., 1999). Unlike localization to the nucleus (Kalderon et al., 1984; Lanford and Butel, 1984), mitochondria (Omura, 1998), endoplasmic reticulum (Munro and Pelham, 1987), peroxisome (Gould et al., 1989), or caveolae (Couet et al., 1997a), there is no conserved signaling motif present in all lipid raft localized proteins [reviewed in (Brown, 2006)]. However, Yamabhai and Anderson utilized deletion and chimera constructs to map the region of EGFR that is responsible for localization to lipid rafts. Their results have suggested that the second cysteine-rich region of EGFR is responsible for localization of this protein to lipid rafts, however, the mechanisms by which this region promote such localization have yet to be determined (Yamabhai and Anderson, 2002).

There is evidence that lipid rafts play both negative and positive roles in EGFR signaling. First, lipid rafts are inhibitory to EGFR signaling functions in that lipid raft localization of EGFR inhibits EGF binding (Pike and Casey, 2002; Ringerike et al., 2002; Roepstorff et al., 2002), decreases receptor autophosphorylation (Pike and Casey, 2002; Ringerike et al., 2002; Westover et al., 2003), and reduces the activation of PLCγ, Gab1 and Ras (Chen and Resh, 2002). Subsequently, activation of MAPK and p38 MAPK is abrogated (Liu et al., 1996;

Anderson, 1998; Furuchi and Anderson, 1998; Schley et al., 2007). In contrast, increased EGFR signaling as a result of lipid raft localization of EGFR has also been demonstrated. Specifically, EGFR localization to lipid rafts recruits Shc and Grb2 after EGF binding (Biedi et al., 2003; Ridyard and Robbins, 2003; Yang et al., 2004), activates PI3Kinase activity (Pike and Casey, 1996; Pike and Miller, 1998), and mediates Akt signaling (Oh et al., 2007). For example, in Vero cells (non-transformed monkey kidney cells), using cholesterol depletion via methyl-beta cyclodextrin (MBCD), Peres and colleagues found that lipid rafts provide a platform to bring together EGFR, PI3K, and PIP2, creating an environment favorable for PI3K activation, and thereby leading to Akt activation (Peres et al., 2003). Thus, while lipid rafts inhibit EGFR signaling to the MAPK pathway, they also facilitate EGFR signaling via the PI3K/Akt pathway.

As mentioned earlier, Src family kinases contain lipid modifications on the N-terminus. In the case of Fyn and Yes, this is a double modification with both myristate and palmitate, whereas c-Src is singly modified with myristate (Paige et al., 1993; Koegl et al., 1994; Shenoy-Scaria et al., 1994; Robbins et al., 1995; Yurchak and Sefton, 1995). Src family kinases are capable of localizing to lipid rafts (Liu et al., 1997; Furuchi and Anderson, 1998; Davy et al., 2000). While other family members are likely to associate preferentially to lipid rafts through their double lipid modification, c-Src contains basic residues in the unique domain that, in combination with the single lipid modification on c-Src, promotes interaction with lipid rafts (Sigal et al., 1994). In particular, c-Src has been shown to localize to lipid rafts in neuronal (Mukherjee et al., 2003; Kasai et al., 2005), hematopoietic (Stoddart et al., 2002), and madin-darby canin kidney cells (Shenoy-Scaria et al., 1994), as well as skeletal myoblasts (Smythe et al., 2003), murine fibroblasts (Robbins et al., 1995), and cervical and

lung cancer cell lines (Shenoy-Scaria et al., 1994; Arcaro et al., 2007). Lipid rafts also play a dual role in Src family kinase signaling. While lipid rafts are important in mediation of the c-Src dependent activation of PI3K/Akt signaling in human SCLC cell lines (Arcaro et al., 2007) and in the co-localization FAK to promote early contact signaling in cells (Baillat et al., 2008), lipid rafts also provide a platform for Csk binding protien (Cbp) to bring Csk, an endogenous inhibitor of c-Src, in close proximity to its substrate to down regulate SFK activity (Torgersen et al., 2001).

E. Lipid rafts in cancer

In the early 1900s, long before the discovery of lipid rafts, deposition of cholesterol was detected in various tumor types [reviewed in (Patra, 2008)]. It has been postulated that increased membrane cholesterol, and thereby lipid rafts, contributes to oncogenic pathways of cell signaling [reviewed in (Patra, 2008)]. Neuroblastoma and melanoma cell lines have specifically shown the presence of lipid rafts in cancer cells (Brown and London, 1997)). Levels of lipid rafts have also been shown to be elevated in prostate and breast cancer cell lines as compared to normal cell lines(Hazarika et al., 2004; Li et al., 2006).

There is evidence to suggest that lipid rafts may be essential in anti-cancer therapeutics. Lipid rafts cluster apoptosis-inducing death receptors for anti-cancer therapeutics (Sun, 2005). Specifically, lipid rafts mediate the response of colon adenocarcinoma cells to the chemotherapeutic cisplatin, as treatment of cells with the cholesterol-sequestering reagent nystatin prevents Fas clustering in these cells which is necessary for the induction of cisplatin-induced apoptosis (Lacour et al., 2004). Also, edelfosine, an anti-leukemic drug, induces apoptosis quickly in leukemia cells where activation of Fas death receptor and

ligand-independent recruitment of Fas into lipid rafts occurs (Gajate et al., 2000; Gajate and Mollinedo, 2001; Gajate et al., 2004; Gajate and Mollinedo, 2007). Other anti-tumor drugs, including resveratrol (Delmas et al., 2003), aplidin (Gajate and Mollinedo, 2005), perifosine (Gajate and Mollinedo, 2007), and anandamide (DeMorrow et al., 2007), work via a similar mechanism. Lipid rafts also facilitate the entry of chemotherapeutics into cancer cells. For example, the anti-cancer drug class of alkylphospholipids have toxic effects against many tumor types (Unger et al., 1989; Munder and Westphal, 1990; Mollinedo et al., 1997; Ruiter et al., 1999) and, in 2007, van der Luit and colleagues demonstrated that all alkyphospholipids utilize lipid rafts for entry into cancer cells, specifically lymphoma cells, where they induce apoptosis (van der Luit et al., 2007).

While lipid rafts may be useful in targeting cancer cells with anti-cancer therapeutics, they also play a role in oncogenesis. First, proliferative signaling and migration are increased due to the presence of lipid rafts. In cervical cells, the known oncogenic virus HPV-16 E5 is found at increased levels within lipid rafts, leading to enhanced activation of oncogenic signaling and proliferation (Suprynowicz et al., 2008). In breast cancer, knockdown of lipid raft specific Src family kinases impairs cell adhesion and cell cycle progression (Hitosugi et al., 2007). Also, in migrating cells, establishment of polarity between the front and rear of the cell is of vital importance for cellular motility. Manes and colleagues have demonstrated that lipid rafts help to establish this polarity through re- localization of proteins including chemokine receptors, (Manes et al., 1999). Lipid rafts also regulate survival in cancer cells. Li and colleagues found that breast and prostate cancers are more sensitive to apoptotic stimuli after cholesterol depletion, due, in part, to decreased lipid raft content and a decrease

in Akt activity following that depletion of lipid rafts (Li et al., 2006). Thus, lipid rafts mediate an escape from apoptosis in cancer cells.

F. Depletion of lipid rafts

Both lipid rafts and caveolae require cholesterol for structure (Rothberg et al., 1990), thus pharmacological depletion of cholesterol has become an important tool in the study of these domains. Methods commonly used to alter cholesterol levels within cells include lipid-free lipoprotein-mediated cellular lipid efflux (which also removes phospholipids from the membrane) [reviewed in (Oram and Yokoyama, 1996)], cholesterol extraction (Ohtani et al., 1989; Kilsdonk et al., 1995; Neufeld et al., 1996), and inhibition of cholesterol biosynthesis (Kandutsch and Chen, 1974; Endo et al., 1976a; Alberts et al., 1980; Berkhout et al., 1996; Brown et al., 1998a). Of these methodologies, cholesterol extraction and inhibition of cholesterol biosynthesis are important to this dissertation.

i. Cyclodextrins

The main methodology to extract cholesterol from cellular membranes is the use of cyclodextrins. The first reported isolation of a cyclodextrin substance was in 1891 when Villivers isolated a product from *Bacillus amylobacter* [reviewed in (Szejtli, 1998)]. There are three well known cyclodextrins, alpha, beta, and gamma, as well as several less characterized larger cyclodextrins, which are cyclic oligosaccharides that differ in the number of glucopyranose units (alpha with six, beta with seven, and gamma with eight) [reviewed in(Szejtli, 1998)]. Cyclodextrins have a barrel like structure with hydrophillic sites on the outside, and a hydrophobic core. Water molecules present in the core of cyclodextrins create an energetically unfavorable environment, and thus a reactive complex.

Molecules that are less polar than water, such as cholesterol, will be readily substituted within the core of the cyclodextrin molecule [reviewed in (Szejtli, 1998)]. Cyclodextrins can be useful to aid in drug delivery through complex formation of a drug with the molecule, or in research as discussed below. While toxicities of cyclodextrins were originally reported, further studies have not found *in vivo* toxicity in most models, however, beta cyclodextrin is relatively unused for *in vivo* applications due to its high affinity toward membrane lipid components which could result in hemolysis [reviewed in (Szejtli, 1998)]. However, this high affinity for membrane lipids can be exploited for research use *in vitro*. For example, the methyl derivative of beta-cyclodextrin (MBCD) effectively removes cholesterol from the plasma membrane (Kilsdonk et al., 1995; Klein et al., 1995; Yancey et al., 1996; Furuchi and Anderson, 1998; Hao et al., 2001; Parpal et al., 2001; Kanzaki and Pessin, 2002) thereby reducing the main structural component of lipid rafts and caveolae.

ii. Statins

The inhibition of cholesterol biosynthesis with statin-type drugs is another effective means of reducing levels of lipid rafts in cells. Statins were originally discovered for their function of reducing activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, which, in turn, abrogated cholesterol biosynthesis. Therefore, Endo and colleagues spent two years and searched over 6,000 microbes for one that would inhibit this enzyme. *Penicillium citrinum* produces an inhibitor to lipid synthesis that was a new compound, mevastatin (Endo et al., 1976b), which inhibited HMG CoA reductase at nanomolar concentrations (Endo et al., 1976b). In 1980, mevastatin was utilized in a Japanese clinical study in heterozygous patients with familial hypercholesterolemia. In this study, the HMG CoA reductase inhibitor showed the largest reduction in plasma cholesterol of these patients ever seen (Yamamoto et

al., 1980). In 1976, Merck & Co. isolated a molecule similar to mevastatin from *Aspergillus terreus*. Lovastatin was more effective than mevastatin (Tobert et al., 1982), and was FDA approved in 1987 (Stossel, 2008). However, the landmark discovery that cholesterol reduction via statins was associated with inhibition of cardiovascular events was not established until 1994 (Stossel, 2008). This, is turn, lead to the development of other statin-type drugs, many of which are approved and in clinical use. Indeed, statins are one of the most widely prescribed drugs in America (Collisson et al., 2003) and are well tolerated in patients (Law et al., 2003).

Statins inhibit cholesterol biosynthesis by competitive inhibition of the committed step of isoprenoid and sterol synthesis (Fig 1.6) (Endo et al., 1976a; Corsini et al., 1995). Specifically, statins inhibit HMG-CoA reductase by binding to the active site, sterically hindering the binding of substrate to the enzyme (Istvan and Deisenhofer, 2001). There are two groups of statins, including type I (lovastatin, pravastatin, simvastatin) which are all similar in structure to the originally isolated mevastatin, and type II (fluvastatin, cerivastatin, atorvastatin, and rosuvastatin) which are fully synthetic inhibitors (Istvan and Deisenhofer, 2001). These drugs can be ranked according to their hydrophobicity. The most lipohilic statin is cerivastatin, followed by lovastatin, simvastatin, fluvastatin, atorvastatin, rosuvastatin, and finally pravastatin (McTaggart et al., 2001). Only highly lipophilic statins are capable of permeating the cell membrane directly to affect cellular signaling (Katz, 2005). In addition to inhibiting the production of cholesterol, statins also promote the growth of new blood vessels (Kureishi et al., 2000), stimulate bone formation (Mundy et al., 1999), decrease oxidative modification of LDL, and have anti-inflammatory effects (Davignon and Laaksonen, 1999). While there is evidence that statins may have clinical benefit in other

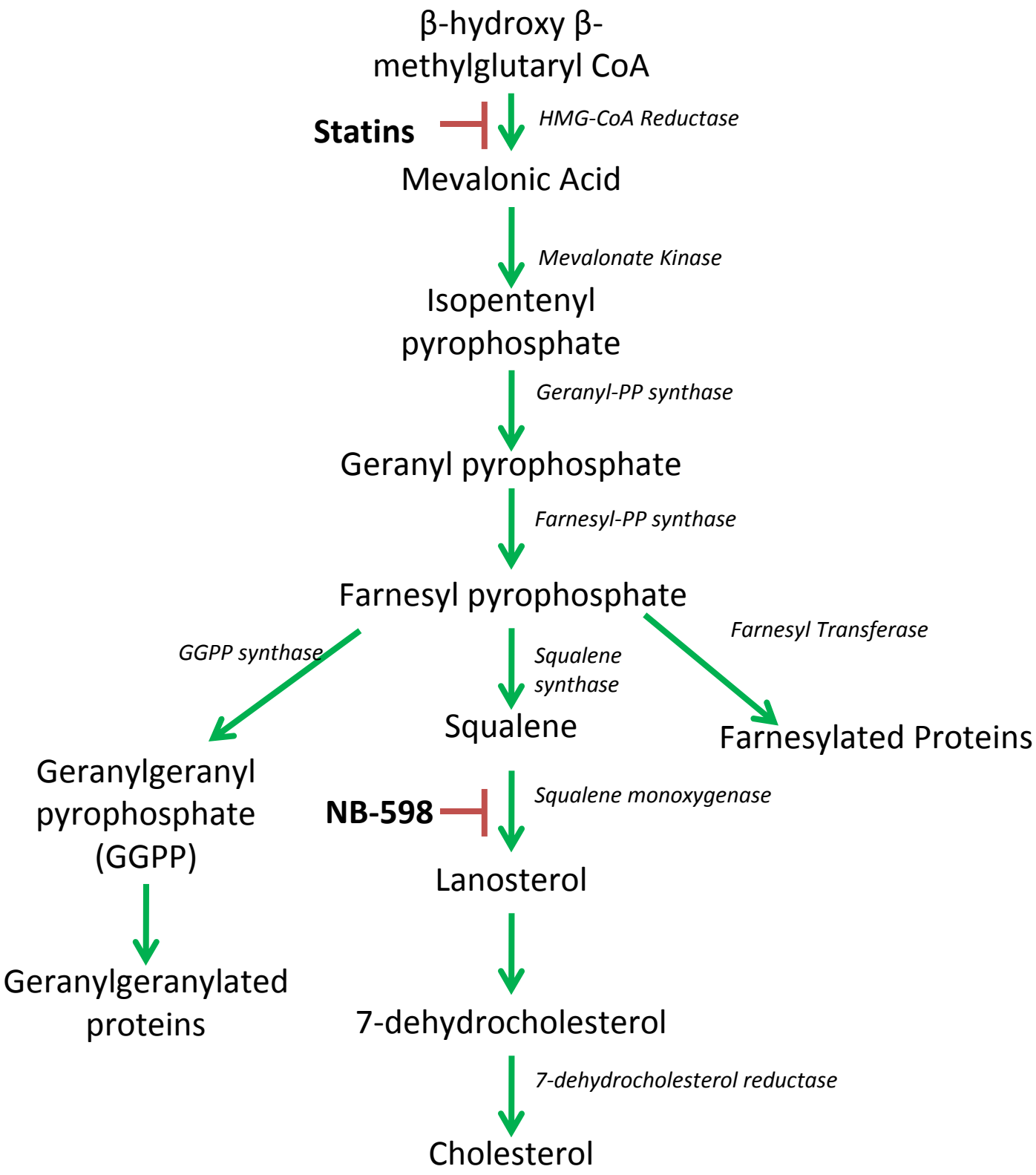


Figure 1.6: The cholesterol biosynthetic pathway and its inhibitors.

diseases including Alzheimer's, multiple sclerosis, bacteremia, and HIV (Vaughan, 2003; Almog et al., 2004; del Real et al., 2004; Vollmer et al., 2004), the information pertinent to this dissertation is the benefit of statins in cancer. Interestingly, cancer cells, as compared to normal cells, generally express elevated levels of HMG-CoA reductase and low-density lipid receptor (LDL), making them more sensitive than normal cells to depletion of HMG-CoA reductase activity (He et al., 1997; Liao, 2002).

As a single agent, statins have shown anti-cancer effects *in vitro*. Specifically, in preclinical models, statins have anti-cancer effects as single agents in breast (Campbell et al., 2006; Kotamraju et al., 2007), melanoma (Depasquale and Wheatley, 2006; Glynn et al., 2008), lung (Glynn et al., 2008), lymphoma (Cafforio et al., 2005), myeloma (van de Donk et al., 2003), brain (Jones et al., 1994; Girgert et al., 1999; Macaulay et al., 1999), prostate (Sivaprasad et al., 2006), renal (Woodard et al., 2008) and pediatric leukemia, rhabomyosarcoma and medulloblastoma (Dimitroulakos et al., 2001). However, the epidemiological data on the use of statins as a singular agent in cancer prevention are mixed (Olsen et al., 1999; Blais et al., 2000; Coogan et al., 2002; Kaye et al., 2002; Beck et al., 2003; Cauley et al., 2003; Boudreau et al., 2004; Graaf et al., 2004; Katz, 2005). These results are confounded by the lack of information on the type of statin used or by the reporting of the use of pravastatin, a hydrophilic statin that has no known anti-cancer activity *in vitro* (Campbell et al., 2006). When the hydrophobicity of the statin drug is taken into account, there is a 47% reduction in the relative risk of colorectal cancer (Poynter et al., 2005) and an 18% reduction in breast cancer incidence (Cauley et al., 2006). Also, hydrophobic statins may act to prevent cancer recurrence. For example, post-diagnosis statin

use is associated with decreased risk of both breast and prostate cancer recurrence (Kwan et al., 2008; Gutt et al., 2010; Hamilton et al., 2010).

In combination therapy, there is more hope for statins as anti-cancer drugs. In preclinical *in vivo* studies, lovastatin enhances the response to chemotherapy in a mouse model of melanoma (Feleszko et al., 1998; Feleszko et al., 2002). Also, clinical studies have suggested that statin drugs may increase sensitivity to chemotherapy or radiotherapy at therapeutic doses in prostate, rectal, and hepatocellular carcinomas (Kawata et al., 2001; Katz et al., 2005). Statins also improve the efficacy of small molecule tyrosine kinase inhibitors in glioblastoma cells (Cemeus et al., 2008). In addition, Mantha and colleagues have shown that the effects of gefitinib, an EGFR TKI, can be potentiated by large doses of statins in head and neck squamous cell carcinoma, non-small cell lung carcinoma, and cervical carcinomas (Mantha et al., 2005). However, the doses used in this study were far above selective concentrations for these inhibitors, and, as such, may be inhibiting a myriad of other proteins.

Statins are a widely used research tool for the reduction of lipid rafts. As mentioned earlier, lipid rafts and caveolae depend on cholesterol for structure. Thus, the inhibition of cholesterol biosynthesis by statins can reduce the amount of membrane cholesterol, thereby reducing lipid rafts (Endo et al., 1976a; Alberts et al., 1980). However, statins are not necessarily specific for cholesterol. As seen in figure 1.6, statins may also inhibit isoprenoid synthesis. Thus, while statins may have a primary effect on cholesterol, they may also inhibit protein prenylation leading to other cellular effects. Generally speaking cholesterol content is unaffected when statin treatment decreases protein prenylation (Ghittoni et al., 2005; Paintlia et al., 2008), and effects deemed to be related to protein prenylation are typically

seen after short duration (under 24 hour) statin treatment, whereas effects requiring longer treatment times are generally indicated as cholesterol based effects (deCathelineau and Bokoch, 2009). Therefore, the use of drugs, such as NB-598, that inhibit downstream effectors in the cholesterol biosynthesis pathway (Fig. 1.6), should be utilized to tease out the effects of statins that are based on depletion of prenylation versus cholesterol (Horie et al., 1990).

1.4. Breast Cancer

Breast cancer is currently one of the leading causes of cancer death in American women [reviewed in (Maughan et al., 2010)]. Approximately one in eight women will be afflicted with breast cancer in her lifetime [reviewed in (Maughan et al., 2010)]. As such, an understanding of the molecular mechanisms of breast cancer, and research on how to effectively treat these cancers, is of vital importance. In 2000 and 2001, Perou and Sorlie and colleagues established distinctive molecular sub-types of breast cancers (Perou et al., 2000; Sorlie et al., 2001). These genetic classifications were found to correlate well with clinical implications of these diseases (Sorlie et al., 2001).

A. Molecular sub-types of breast cancer

The first sub-type, normal-like, contained tumor samples as well as normal breast specimens that are typified by expression of genes that are "normally" expressed in basal epithelial cells and adipose cells (Perou et al., 2000).

The second sub-type, luminal breast cancers, can be further stratified into two groups, luminal A and luminal B (Sorlie et al., 2001). Luminal A breast cancers are approximately

45% positive for the estrogen receptor (ER) alpha and express high levels of luminal cell keratins 8/18 (Perou et al., 2000), but have low or no expression of HER2. Luminal A breast cancers have the best prognosis (Sorlie et al., 2001). Luminal B breast cancers express many of the same components as luminal A (including ER), and have expression of HER2 (Sorlie et al., 2001). As such, luminal B-type breast cancers have a less favorable outcome than luminal A-type breast cancers (Sorlie et al., 2001). While chemotherapeutics and surgery with radiation are standards of care in the breast cancer world, targeted therapeutics have become more commonly utilized [reviewed in (Maughan et al., 2010)]. Indeed, after approval of selective estrogen receptor modulators (SERMs), which are utilized for treatment of ER positive luminal breast cancers, the mortality rates of US women with breast cancer have declined significantly (Altekruse SF, 1975-2007) suggesting that targeted therapeutics may be more beneficial than previous therapies. Specifically, tamoxifen, an estrogen receptor antagonist, was the first SERM to be approved for use clinically. Currently, tamoxifen is used either alone or in sequence with an aromatase inhibitor for the treatment of ER positive breast cancers [reviewed in (Maughan et al., 2010)]. Aromatase inhibitors have also shown clinical efficacy in luminal breast cancers, and three have been approved for use alone or in sequence with tamoxifen including anastrozole, exemestane, and letrozole (Baum et al., 2003; Coombes et al., 2004; Thurlimann et al., 2005).

The third sub-type, HER2 positive breast cancer, is characterized by expression of the EGFR family member HER2, and GRB7, with low or no estrogen receptor expression (Perou et al., 2000). These cancers have a worse prognosis than luminal breast cancers, however, a better prognosis than basal-type breast cancers (described below) (Sorlie et al., 2001). Trastuzumab (Trastuzumab), a monoclonal antibody inhibitor of the HER2 receptor, has

been clinically approved for the treatment of HER2+ breast cancer. Trastuzumab targets HER2 by binding to domain IV of the extracellular domain of the receptor and activates immune effecter cells to the ErbB2-overexpressing tumor (Sliwkowski et al., 1999; Clynes et al., 2000; Cho et al., 2003) The use of trastuzumab has significantly improved the disease-free and overall survival rates for women with HER2+ breast cancer (Romond et al., 2005; Smith et al., 2007). Recently, a dual HER2/EGFR inhibitor, lapatinib, was approved for the treatment of HER2+ breast cancer (Ryan et al., 2008).

The final breast cancer sub-type is basal-type. These tumors represent approximately 15-20% of all breast cancers and are defined by elevated expression of cytokeratin 5 and 17, laminin, and fatty acid-binding protein 7, and lack of estrogen receptor expression (Perou et al., 2000; Millikan et al., 2008). Although basal-type breast cancers do have a high chemotherapy response rate (45%), they remain associated with the worst patient prognosis (Sorlie et al., 2001; van de Rijn and Rubin, 2002; Rouzier et al., 2005; Fulford et al., 2006; Laakso et al., 2006). Basal-type breast cancers tend to be triple-negative, in that they do not express ER, progesterone receptor (PR), or HER2 [reviewed in (Seal and Chia, 2010)]. As such, no targeted therapeutic has been approved for the treatment of this type of breast cancer. Thus, the current standard of care for basal-type breast cancers is surgery with radiation and/or chemotherapy [reviewed in (Chen and Russo, 2009)]. Taking into account the decreased mortality rates after approval of targeted therapeutics for luminal and HER2 positive breast cancers, it would be advantageous to develop targeted therapeutics for basal-type breast cancers, which, as mentioned before, are the worst breast cancers in terms of prognosis.

B. EGFR in breast cancer

HER2 is probably the most widely known EGFR family member to be implicated in breast cancer. Initially, HER2 was found to be closely related to the transforming oncogenes *neu* and c-erb-B2 (Shih et al., 1981; Schechter et al., 1984; Coussens et al., 1985; Schechter et al., 1985). Much like these oncogenes, over-expression of HER2 in transgenic mouse models induces transformation in the mammary gland (Pierce et al., 1991; Guy et al., 1994b; Eccles, 2001). It was later discovered that the HER2/*neu* oncogene was amplified in breast cancer cell lines and tissue samples (King et al., 1985; Slamon et al., 1987). Specifically, amplification of HER2 is seen in 20-30% of all breast cancers and over-expression of HER2 correlates with poor clinical outcome (Slamon et al., 1987; Ross et al., 1998; Menard et al., 2001). More recently, Perou and colleagues discovered that HER2-positive breast cancers share a similar genetic profile (Perou et al., 2000). Due, in part, to the high level of HER2 expression, inhibitors to HER2, such as trastuzumab and lapatinib, have been developed. These inhibitors increase patient survival by increasing the time to progression of HER2 positive breast cancers (Slamon and Pegram, 2001).

EGFR is expressed in 14-91% of all breast cancers (Sainsbury et al., 1987; Klijn et al., 1992; Ferrero et al., 2001; Tsutsui et al., 2002). This wide range is most likely due to the myriad of techniques utilized to analyze breast cancer specimens for EGFR, including autoradiography, immunohistochemistry, immunoezymatic assay, and gene transcript analyses. There are conflicting data on the quality of EGFR expression as a prognostic indicator in breast cancer (Rampaul et al., 2005). These conflicts are due to studies being restricted based on small cohort size, short follow-up times, variable adjuvant therapies, and differing detection methodologies. For example, in a study performed by Fox and colleagues

370 patients were examined over a period of eighteen months. Utilizing a ligand-binding assay to detect EGFR, no correlation was reported between EGFR expression and size, stage, or grade of tumor. However, multivariate analysis of their data revealed EGFR as a prognostic indicator for relapse-free survival (Fox and Das, 1979). A more recent study by Tsuitsu and colleagues utilized immunohistochemistry to detect EGFR in 1029 cases of breast cancer over forty-six months and found that EGFR was an independent prognostic factor (Tsutsui et al., 2002). This study, as well as several other large studies, has shown that EGFR over-expression is a negative prognostic variable in breast cancer (Bolla et al., 1990; Toi et al., 1991; Jardines et al., 1993). More specifically, EGFR is over-expressed in 60% of basal-type breast cancers (Livasy et al., 2006; Siziopikou and Cobleigh, 2007).

EGFR contributes to the normal development of the mammary gland and deregulation of EGFR is a contributing factor for breast cancer (Hanahan and Weinberg, 2000; Harari et al., 2007). Unlike brain and lung cancers (as described earlier), deregulation of EGFR through activating mutations is rare in breast cancer (Rae et al., 2004; Weber et al., 2005). Genetic amplification of EGFR occurs in approximately 6% of breast cancers (Kersting et al., 2004; Bhargava et al., 2005; Milanezi et al., 2008), however; over-expression at the protein level seems to be the primary mechanism by which EGFR becomes deregulated. Such over-expression is controlled through transcriptional up-regulation and/or protein stabilization (Fox and Harris, 1997).

Other mechanisms that result in deregulated EGFR signaling include increased ligand expression and receptor crosstalk (Arteaga, 2002; Goswami et al., 2005; Milanezi et al., 2008). Elevated ligand expression is one mechanism by which EGFR is deregulated in breast cancer leading to the transformed phenotype. Specifically, over-expression of TGFα, an

EGFR ligand, in transgenic mouse models leads to a hyperplastic phenotype, as well as tumor formation in the mammary gland (Matsui et al., 1990; Sandgren et al., 1990; Sandgren et al., 1995). TGFα promotes the growth of breast cancer cell lines, and is expressed at higher levels in estrogen receptor negative breast cancers (Perroteau et al., 1986; Bates et al., 1988; Salomon et al., 1990). Cross-breeding between TGFα and HER2 over-expressing mice results in synergistic tumorigenesis due to increased EGFR transactivation of HER2 (Muller et al., 1996). This particular EGFR ligand is expressed in 40-70% of primary and metastatic breast tumors (Bates et al., 1988; Dublin et al., 1993; Murray et al., 1993; Ciardiello et al., 1996). AREG is also expressed in breast cancer cell lines, although there is evidence that ER-positive breast cancers express increased levels of AREG as compared to ER-negative cell lines (Plowman et al., 1990). EGF is also upregulated in breast cancer cell lines where it induces PI3K activity resulting in proliferation (Harris, 1989; Atalay et al., 2003). Receptor crosstalk is also capable of inducing EGFR deregulation. G-protein coupled receptors activate EGFR-dependent signaling. Specifically, Andreev and colleagues have utilized genetic knockout models to show that c-Src and Pyk2 (another protein tyrosine kinase) mediate activation of EGFR by G-protein coupled receptors leading to activation of MAPK signaling (Andreev et al., 2001). c-Met-dependent activation of EGFR has also been noted in breast cancer (Mueller et al., 2008; Mueller et al., 2010). In particular, c-Met is responsible for EGFR-kinase independent phosphorylation of EGFR in breast cancer cells. Co-treatment of these cells with EGFR and c-Met inhibitors resulted in abrogation of EGFR phosphorylation and inhibition of breast cancer cell growth (Mueller et al., 2008).

EGFR expression is twice as frequent in ER/PR negative breast cancer than positive (Klijn et al., 1992), and, in fact, cellular proliferation in estrogen receptor negative breast

cancers is dependent on EGFR signaling (Biswas et al., 2001). When EGFR and estrogen receptor are co-expressed, it is thought that the signaling pathways of these two molecules may be intertwined. For example, EGFR activation results in phosphorylation and activation of nuclear estrogen receptor. Also, plasma-membrane localized estrogen receptor is able to hijack the EGFR signaling cascade for signal transduction (Levin, 2003). EGFR may also be involved as a mechanism of resistance to endocrine therapy (Yarden et al., 1996; Yarden et al., 2001). Specifically, estrogen-receptor positive breast cancer cell lines with acquired resistance to tamoxifen, an estrogen receptor antagonist, have increased levels of EGFR and HER2 expression as compared to the parental cell line (McClelland et al., 2001; Knowlden et al., 2003). Also, treatment naive breast cancers that have elevated EGFR and/or HER2 expression are more likely to be resistant to estrogen directed therapeutics as compared to low expressing cells (Gee et al., 2001). As EGFR and HER2 are increased on tamoxifen resistant breast cancers, lapatinib, a dual EGFR/HER2 inhibitor (described earlier), has been used in preclinical studies in an effort to overcome such resistance (Chu et al., 2005).

As described previously, both monoclonal antibodies and small molecule tyrosine kinase inhibitors directed against EGFR have been designed and tested in clinical trials. In breast cancer, however, the results have been less than hopeful. While lapatinib, a small molecule was approved in 2007 in HER2 positive breast cancer in patients that have failed trastuzumab therapy (Ciardiello et al., 1996; Geyer et al., 2006), lapatinib has failed to show efficacy in HER2 negative breast cancers, including those of the basal sub-type (Finn et al., 2007; Di Leo et al., 2008). The monoclonal antibody cetuximab has shown promise *in vitro*, however, clinical trials combining cetuximab with chemotherapeutics have shown negative results (Modi et al., 2006; Shiu et al., 2008; Burness et al., 2010). Other small molecule EGFR TKIs

have been tested in clinical trial, however, most ER-negative breast cancers fail to respond to EGFR-specific tyrosine kinase inhibitors [reviewed in (Atalay et al., 2003)].

C. c-Src in breast cancer

c-Src expression and activity are elevated in breast tumor tissue and cell lines as compared to matched non-tumor tissue (Ottenhoff-Kalff et al., 1992; Verbeek et al., 1996; Belsches-Jablonski et al., 2001; Reissig et al., 2001). Furthermore, inhibition of Src kinase activity results in decreased proliferation, adhesion, and invasion of breast cancer *cells in vitro* (Finn et al., 2007; Green et al., 2009). c-Src expression correlates with breast cancer recurrence, and with poor clinical outcome (Aligayer et al., 2002; Wilson et al., 2006). c-Src effectively promotes the mitogenic effects of estrogen (Shupnik, 2004), and as such, has been implicated in the tumorigenic phenotype of estrogen receptor positive breast cancers (Herynk et al., 2006). c-Src kinase activity is elevated in *in vitro* models of tamoxifen resistant breast cancers, independent of its gene or protein level (Hiscox et al., 2006). In these tamoxifen resistant cells, inhibition of c-Src kinase activity with a small molecule tyrosine kinase inhibitor is sufficient to abrogate invasion and migration (Hiscox et al., 2006), suggesting a role for c-Src in resistance to estrogen targeted therapies. c-Src has also been implicated in the metastatic process in breast cancer. Kinase activity of c-Src is implicated in the highly metastatic phenotype of HER2-type breast cancer [reviewed in (Kim et al., 2010)]. Also, there is an established association between c-Src and metastases to the bone and other sites (Myoui et al., 2003; Rucci et al., 2006; Jallal et al., 2007).

The interaction between EGFR and c-Src is apparent in breast cancer. As mentioned earlier, co-overexpression of EGFR and c-Src occurs in a subset of human breast cancer cell

lines and tumor tissues (Maa et al., 1995). The interaction between EGFR and c-Src induces oncogenesis through increased EGFR signaling (Luttrell et al., 1988; Wilson et al., 1989; Maa et al., 1995; Biscardi et al., 1998; Olayioye et al., 1999). Inhibition of c-Src kinase activity reduces EGFR-mediated proliferation of breast cancer cells (Wilson et al., 1989; Roche et al., 1995). c-Src also plays a role in transactivation of EGFR in breast cancer. For example, activation of EGFR by urokinase-type plasminogen activator (uPA) is c-Src-dependent, and leads to breast cancer invasion (Guerrero et al., 2004).

Due to the apparent role of c-Src in breast cancer growth, drug resistance, and metastasis, tyrosine kinase inhibitors of c-Src are currently in clinical trial. Dasatinib, one such c-Src tyrosine kinase inhibitor, is undergoing trials for basal-type breast cancers, as it is efficacious in preclinical models (Finn et al., 2007; Huang et al., 2007). Also, as c-Src expression alone is insufficient to cause transformation (Shalloway et al., 1984), c-Src inhibitors are also being explored in combination therapies with HER family inhibitors, VEGF inhibitors, PDGFR inhibitors, and chemotherapeutics (Boudny and Nakano, 2003; Griffiths et al., 2004; Hatake et al., 2007).

D. Lipid rafts and breast cancer

Lipid rafts play a role in the growth and survival of breast cancer cells. Findings from multiple studies have indicated a role for lipid rafts in the growth of estrogen receptor positive breast cancer (Auricchio et al., 1996; Chambliss et al., 2000; Kelly and Levin, 2001; Marquez et al., 2001; Razandi et al., 2002; Li et al., 2003; Song et al., 2004; Marquez et al., 2006). Also, disruption of lipid rafts by cholesterol depletion results in increased sensitivity to apoptotic stimuli due to a decrease in Akt signaling in breast cancer cells, as well as

epidermoid carcinoma and prostate cancer cells (Li et al., 2006). Lipid rafts also play a role in migration and metastasis in breast cancer. Manes and colleagues have shown a role for lipid rafts in migration of breast adenocarcinoma cell lines. Specifically, the establishment of front-rear polarity after growth factor stimulation, which is required for chemotaxis of cancer cells, is dependent on lipid rafts (Manes et al., 1999). Furthermore, EGF-induced chemotaxis (Liu et al., 2007), the formation of invadopodia, and extracellular matrix degradation by breast cancer cell lines are lipid raft dependent (Yamaguchi et al., 2009). Together, these results suggest a role for lipid rafts in metastasis of breast cancer.

Epidemiological data regarding the use of statins (reviewed earlier in this chapter) as singular agents in breast cancer prevention or treatment are mixed (Beck et al., 2003; Cauley et al., 2003; Kwan et al., 2008). However, in regards to breast cancer recurrence, the data on post-diagnosis statin use are positive. Specifically, a 2007 study by Kwan and colleagues found that starting lipophilic statin treatment after diagnosis resulted in a significant decrease in the risk of breast cancer recurrence (Kwan et al., 2008).

Preclinical data for the use of statins as part of combinatorial therapies in breast cancer has also gained support (Katz, 2005). For example, HER2, a member of the EGFR family of receptor tyrosine kinases, is expressed in approximately 30% of breast cancer, and as such, HER2 has been an attractive therapeutic target in breast cancer (Slamon et al., 1987; Pierce et al., 1991). However, the response rate for trastuzumab, a clinically approved HER2 inhibitor, is only 34%, indicating *de novo* resistance to the therapeutic (Cardoso et al., 2002; Vogel et al., 2002). HER2 has been demonstrated to localize to lipid rafts (Harder et al., 1998b; Nagy et al., 1998; Nagy et al., 2002), and modulation of lipid rafts through fatty acid synthase inhibition has been shown to act synergistically with trastuzumab in breast cancer

cells (Menzo et al., 1993). These data suggest that statins may be an effective therapeutic agent in breast cancer.

1.5 Significance

Breast cancer is currently one of the leading causes of cancer death amongst American women. As described, breast cancer is subdivided into molecular sub-types by the genetic signature of the tumor. Of those, basal-type breast cancers have the fewest treatment options. Unlike luminal and HER2+ breast cancers, there are no currently approved targeted therapeutics for basal-type breast cancers, therefore, cytotoxic chemotherapy regimens, along with surgery and radiation, remains the standard of care. After the approvals of tamoxifen and trastuzumab, the mortality rates of American women with breast cancer declined, suggesting that approval of targeted therapeutics may be the key to lowering the rate of death from breast cancer. Identification of a targeted therapy for basal-type breast cancers is therefore of the utmost importance. Nearly 60% of basal-type breast cancers express the EGFR. As such, EGFR may be an attractive therapeutic target in this cancer sub-type. However, these breast cancers fail to respond to EGFR tyrosine kinase inhibition. Thus, the work herein seeks to discover mechanisms of resistance to EGFR tyrosine kinase inhibition. Levels of cholesterol rich lipid rafts are up-regulated in breast cancer cells as compared to normal mammary epithelial cells. Depletion of cholesterol in breast cancer cells results in increased sensitivity to apoptotic stimuli, suggesting that, due to this elevation breast cancer cells are dependent on signaling within lipid rafts. In support of this idea, retrospective studies of women with breast cancer found that women who took statins to lower cholesterol saw a decrease in the recurrence of their cancer compared to women who did not take statins. c-Src, a non-receptor tyrosine kinase, also localizes to these lipid rafts to promote signaling

pathways. c-Src and EGFR interact in breast cancer, and their interaction has been implicated in transformation and tumorigenesis. c-Src is capable of phosphorylating EGFR on novel sites, which lead to increased association of signaling molecules with the receptor. Thus, we hypothesized that the interaction of these two proteins within lipid rafts may mediate resistance to EGFR tyrosine kinase inhibition in breast cancer cells.

Chapter 2

2. Materials and Methods

2.1 Reagents - Gefitinib was provided by AstraZeneca (Wilmington, DE). All other reagents were purchased from Sigma or VWR unless otherwise noted.

2.2 Cell lines - The SUM series of cell lines were obtained from Dr. Stephen Ethier (Wayne State University/Karmanos Cancer Institute, Detroit, MI). The remaining cell lines were purchased from ATCC (Manassas, VA). The growth conditions for each cell line are as follows. SUM 52, SUM 149, SUM 159, SUM 185, SUM 225, and SUM 229 cells are grown in 5%IH media (Ham's F-12 media, supplemented with 5% FBS, 1μg/ml hydrocortisone, and 5μg/ml insulin). SUM 1315 cells are grown in 5%IE media (Ham's F-12 media, supplemented with 5% FBS, 10ng/ml EGF, and 5μg/ml insulin). SUM 44 and SUM 190 cells are grown in SFIH media (Ham's F-12 media, supplemented with 1μg/ml hydrocortisone, 5μg/ml insulin, 5mM ethanolamine, 10mM HEPES, 5μg/ml transferrin, 10nM triiodo-thyronine, 50μM sodium selenite, and 5% BSA). SUM 102 and MCF10A cells are grown in SFIHE media (Ham's F-12 media, supplemented with 1μg/ml hydrocortisone, 5μg/ml insulin, 10ng/ml EGF, 5mM ethanolamine, 10mM HEPES, 5μg/ml transferrin, 10nM triiodo-thyronine, 50μM sodium selenite, and 5% BSA). MCF7, SKBr3, T47D, MDA-MB-231 and MDA-MB-468 cells are grown in DMEM+10%FBS media (DMEM media, supplemented with 10% FBS). BT-20 cells are grown in Eagles+NEAA media (Eagle's MEM with 2mM L-glutamine and Earle's BSS adjusted to contain 1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids, 1mM sodium pyruvate, and 10% FBS). BT-549 cells are grown in RPMI+L-GLUT(2mM) media (RPMI-1640, supplemented to contain

1.5g/L sodium bicarbonate, 4.5g/L glucose, 10mM HEPES, 1mM sodium pyruvate, 0.023 IU/ml insulin, and 10% FBS). HCC 1937 and HCC 1954 cells are grown in RPMI+L-GLUT media (RPMI-1640 media with 2mM L-glutamine adjusted to contain 1.5g/L sodium bicarbonate, 4.5g/L glucose, 10mM HEPES, 1mM sodium pyruvate, and 10% FBS). The SUM and HCC cells are cultured in 10% CO_2 and the remaining cells are cultured in 5% CO_2. All media are supplemented with 2.5 µg/ml amphotericin B and 25 µg/ml genatimycin. Information regarding the isolation and estrogen or HER2 receptor status of these cell lines can be found in Table 2.1.

2. 3 Immunoblotting – Breast cancer cell lines were plated at a density of $1X10^6$ cells per 100-mm dish and grown for 48 h. Cells were treated with indicated reagents (1.0 µM gefitinib for 30 min in serum free media, and/or 1 µM lovastatin 72 h, and/or 1.0 µM dasatinib 2 h in serum free media). Media was aspirated, and then cells were washed in 1X PBS containing 1 µM sodium orthovanadate. One milliliter of 1X PBS containing sodium orthovanadate was again added, and cells were scrapped and placed into a conical. Cells were pelleted by centrifugation, and then lysed in CHAPs lysis buffer [10 mM CHAPs, 50 mM Tris (pH 8.0), 150 mM NaCl, and 2 mM EDTA with 10 µM sodium orthovanadate and 1x protease inhibitor cocktail]. Lysates were then centrifuged for 10 min at a speed of 18,000Xg at 4 degrees Celsius. Bradford protein assay was then performed. For immunoblotting, 10 to 100 µg of protein lysate were separated by SDS-PAGE (200 V for 30 min) and transferred to Immobilon P. Membranes were blocked in either 5% nonfat dry milk for 1 h at 25°C or overnight at 4°C (phospho-MAPK), or 5% BSA overnight at 4°C (phospho-SrcY416). Membranes were probed with EGFR (Cell Signaling Technology, Danvers, MA, 1:1000), Akt (Cell Signaling Technology, Danvers, MA, 1:1000), MAPK

Cell Line	Isolation	ER Status	HER2 Status	References
HMEC	Primary			
MCF10A	Immortalized normal mammary cell line			(Soule et al., 1990)
SUM 44	Pleural effusion	+	+	(Forozan et al., 1999)
SUM 52	Pleural effusion	-	+	(Turner et al., 1993; Forozan et al., 1999)
SUM 102	Intraductal carcinoma	-	-	(Forozan et al., 1999; Bertucci et al., 2005; Anders and Carey, 2008)
SUM 149	Invasive ductal carcinoma (inflammatory)	-	-	(Forozan et al., 1999; Bertucci et al., 2005)
SUM 159	Anaplastic carcinoma	-	-	(Forozan et al., 1999)
SUM 185	Pleural effusion	-	+	(Forozan et al., 1999; Zhou et al., 2004)
SUM 190	Invasive ductal Carcinoma (inflammatory)	+	+	(Forozan et al., 1999)
SUM 225	Chest wall recurrence of ductal carcinoma in situ	-	+	(Forozan et al., 1999)
SUM 229	Pleural effusion	-	-	(Forozan et al., 1999)
SUM 1315	Skin metastasis of infiltrating ductal carcinoma	-	+	(Forozan et al., 1999)
MCF7	Pleural effusion	+	-	(Soule et al., 1973; Levenson and Jordan, 1997; Ross and Fletcher, 1998)
T47D	Pleural effusion	+	-	(Keydar et al., 1979; Judge and Chatterton, 1983)
BT 20	Carcinoma	-	-	(Keyomarsi and Pardee, 1993)
BT 474	Invasive ductal carcinoma	+	+	(Lasfargues et al., 1978)
BT 549	Invasive ductal carcinoma	-	-	(Lasfargues et al., 1978)
SKBR3	Pleural effusion	-	+	(Trempe, 1976)
HCC 1937	Primary ductal carcinoma	-	-	(Gazdar et al., 1998; Tomlinson et al., 1998)
HCC 1954	Invasive ductal carcinoma	-	+	(Gazdar et al., 1998)
MDA-MB 231	Pleural effusion	-	-	(Cruciger et al., 1976)
MDA-MB 468	Pleural effusion	-	-	(Cailleau et al., 1978)

Table 2.1: Isolation properties and estrogen receptor/HER2 receptor status of cell lines used.

(Cell Signaling Technology Danvers, MA, 1:500), phospho-Akt (Ser473; Cell Signaling Technology, Danvers, MA, 1:2000), phospho-ERK1/2 (MAPK) (Invitrogen, Carlsbad, CA, 1:500), phospho-SrcY416 (Cell Signaling Technology, Danvers, MA, 1:1000), c-Src (Cell Signaling Technology, Danvers, MA, 1:500), transferrin receptor (Invitrogen, Carlsbad, CA, 1:2000), or flotillin (BD Biosciences, San Jose, CA, 1:1000) antibodies. All antibodies were incubated overnight at 4°C, except for phospho-MAPK and phospho-SrcY416 (2 h at room temperature). Membranes were washed with TBS + 0.1% Tween 20 three times for 10 min, followed by incubation with corresponding secondary antibody and another series of three washes. Incubation with enhanced chemiluminescence (GE Healthcare Buckinghamshire, UK) was followed by exposure to film. Experiments were repeated at least three times and quantified using densitometry (NIH Image).

2.4 In vitro kinase assays – Under normal growth conditions, 1 million cells were grown for 48 h. Cells were washed in 2X in PBS and lysed in solubilization buffer (50 mM HEPES, pH 7.5, 10% glycerol, 0.5% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 50 µg/ml aprotinin, and 400 nM vanadate). Lysates were cleared by centrifugation, quantified, and 0.5 mg of protein was immunoprecipitated using EGFR antibodies (mab108, M. Weber, University of Virginia, Charlottesville, VA). Antibody bound proteins were collected using 40 µl protein A beads (Upstate Biotechnology, Lake Placid, NY) and washed three times in HTG buffer (20 mM HEPES, pH 7.5, 0.1% Triton X-100, and 10% glycerol). For the kinase assay, 40 µl HTG buffer, 4 µl $MnCl_2$ (of 100 mM stock), and 10 µCi ^{32}P-γATP were incubated with the immunoprecipitates for 10 min at 30ºC. The beads were pelleted and the supernatant removed and discarded. The beads were washed twice with solubilization buffer and once with PBS. 40 µl of sample buffer was added to the pellets, the samples were boiled,

and proteins were separated using 7.5% SDS-PAGE. The gels were dried and exposed to film. Each experiment was repeated at least three times.

2.5 ShRNA downregulation of EGFR - To downregulate EGFR expression we utilized 21 EGFR-directed shRNA lentiviral constructs from OpenBiosystems (TRCN0000039633, TRCN0000039634, TRCN0000039635, TRCN0000039636, TRCN0000039637, TRCN0000010329, TRCN0000121067, TRCN0000121068, TRCN0000121069, TRCN0000121070, TRCN0000121071, TRCN0000121202, TRCN0000121203, TRCN0000121204, TRCN0000121205, TRCN0000121206, TRCN0000121327, TRCN0000121328, TRCN0000121329, TRCN0000121330, TRCN0000121331). Three constructs were chosen based on their specific reduction in EGFR expression in our cell models. Specifically, EGFR shRNA #1 = TRCN0000121071 (CCGGCCGTGGCTTGCATTGATAGAACTCGAGTTCTATCAATGCAAGCCACGGTT TTTG), EGFR shRNA #2 = TRCN0000121329 (CCGGCAGCATGTCAAGATCACAGATCTCGAGATCTGTGATCTTGACATGCTGTT TTTG), and EGFR shRNA #3 = TRCN0000121204 (CCGGCCTCCAGAGGATGTTCAATAACTCGAGTTATTGAACATCCTCTGGAGGTT TTTG) showed pertinent effects in our model system. The lentiviruses were packaged using a third generation lentiviral packaging system developed by Didier Trono and colleagues (Lausanne, Switzerland) and purchased from Addgene (Dull et al., 1998). Specifically, Addgene plasmids pMLDg/pRRE (12251), pRSV-Rev (12253), and pMD2.G (12259) were transfected into HEK293T cells with the lentiviral vectors containing the shRNAs using FUGENE6 (Roche). Cellular supernatant was collected on days 2 and 3 after transfection, pooled, and filtered. The lentivirus was titered using HEK293T cells in a 24-well plate,

incubated with increasing volumes (10 to 100 µl) of virus with 8 µg/ml polybrene and selected for via the puromycin selection on the lentiviral vector (1 µg/ml puromycin). Colonies were visualized and used to compare viral preps and between viruses for consistent titers used in experiments. To determine the efficacy of EGFR downregulation in breast cancer cells, equal multiplicity of infection (MOI) of EGFR shRNA virus (or a non-silencing control) was added to the indicated cells in the presence of 8 µg/mL polybrene. Four days later, cell lysates were prepared, separated by SDS-PAGE, and immunoblotted using EGFR antibodies as described above. EGFR was considered knocked down if the densitometric values of at least three experiments demonstrated at least a 50% reduction of EGFR protein expression.

To determine if EGFR downregulation affects cell proliferation in breast cancer cells, the indicated cells were incubated with equal MOI of virus and allowed to proliferate for three days. 1 µg/ml of puromycin was then added to media to select for cells that contain the lentivirus and cells were allowed to proliferate for an additional eight days. The number of cells was quantified using a Beckman Coulter Counter. Briefly, media was aspirated and plates were washed with 1 ml 1X PBS. Cells were then incubated with 500 µL Hepes:MgCl2 solution (0.01 M Hepes, 0.015 M $MgCl_2$ for five minutes, followed by 75 µL ZAP (Ethyl hexadecyldimethylammonium bromide) for 10 min. This solution was then added to 9.5 mL of isoton solution and counted using a coulter counter. Each experiment was repeated at least three times with the following control conditions: no puromycin added to the cells, no viral infection with puromycin selection, and non-silencing control with puromycin selection. The percent of cell growth was determined by using the non-silencing control with puromycin selection as 100% cell growth.

2.6 Immunostaining - Anti-EGFR (mab108) was labeled with Alexa-fluor-488 (Invitrogen, Carlsbad, CA) and Anti-Src (2-17, S. Parsons, University of Virginia, Charlottesville, VA) was labeled with Alexa-flour-594 (Invitrogen, Carlsbad, CA) utilizing Alexa-fluor labeling kits. Cells were plated onto coverslips at a density of $1.5X10^5$ cells per 35mm dish and grown for 48 h. For lipid raft staining, media was aspirated, cells were washed with 1X PBS once, and then incubated with Alexa-fluor-594 labeled cholera toxin subunit B (Invitrogen, Carlsbad, CA) at 1 µg/ml for 10 min on ice prior to fixation (Roepstorff et al., 2002; Liu et al., 2007). Cells were then washed three times in 1X PBS (washed), fixed with formalin for 20 min at room temperature, washed, permeabilized with 0.1 % Triton-x 100 (if applicable) for 2 min on ice, washed, blocked in 20% goat serum for 1 h at room temperature, then incubated with EGFR (Alexa-fluor labeled) or c-Src (2-17 unlabeled for lipid raft staining, 594 labeled for EGFR co-staining) antibody for 1 h (followed by corresponding Alexa-fluor 594 secondary antibody if necessary), washed, quickly washed once in deionized water, and then mounted onto slides with Prolong Gold containing DAPI (Invitrogen, Carlsbad, CA). Imaging was performed via confocal microscopy using a Zeiss Axioplan2 apotome microscope fitted with a 63X 1.25 oil immersion lens at the Microscopy and Imaging Resources Laboratory (Wayne State University, Detroit, MI).

2.7 Biochemical Raft Isolation - Biochemical lipid raft isolation was adapted from established protocols (Macdonald and Pike, 2005). Briefly, cells were plated at a density of $0.5X10^6$ cells in six-100 mm plates for 72 h. Media was aspirated, and then cells were scraped in base buffer [20 mM Tris, pH 7.8, 250 mM Sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 µM sodium orthovanadate], centrifuged at 250Xg at four degrees to pellet cells and lysed in base buffer containing 1X protease inhibitor cocktail (EMD Biosciences, Gibbstown, NJ)

by passing through a 22 gauge X 1.5" needle 40 times. Lysates were centrifuged for 10 min at four degrees and 1000Xg, and the first and second post-nuclear supernatants were combined and frozen at -20°C. This freezing step was required for reproducibility of results, as without it the isolation was never repeatable. Samples were thawed and combined with equal volume of 50% Opti-Prep (Greiner Bio One, Monroe, NC) and 0-20% Opti-Prep gradient was applied. Gradients were centrifuged for 90 min at 52,000Xg and then fractionated into 16 - 0.56 mL fractions from the top of the tube. Fractions were separated via SDS-PAGE, transferred to Immobolin-P (Millipore, Billerica, MA), and immunoblotted utilizing antibodies described above. Fractions were dot blotted with Cholera Toxin Subunit B-HRP (Invitrogen, Carlsbad, CA) to determine GM-1 expression. Incubation with enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) was followed by exposure to film. Experiments were repeated at least three times and quantified using densitometry (NIH Image).

2.8 Immunoprecipitation – Five hundred micrograms of whole cell lysates (as collected for immunoblotting) or two hundred microliters of each lipid raft fractionation (1-7) were pooled and immunoprecipitated with 10 μg of EGFR antibody (mab108) or 10 μg c-Src antibody (2-17) for 1 h at 4°C. Forty microliters of a 50% slurry of protein A agarose beads were added for 30 min. Samples were then pelleted and washed three times in CHAPs lysis buffer, then 25 μL 2X Lamelli buffer was added, and samples were boiled for 5 min. Samples were subjected to SDS-PAGE followed by immunoblotting for EGFR and c-Src. Whole cell lysates were utilized as a migration control. Immunoprecipitation with 10 μg mouse IgG antibody served as a negative control.

2.9 Cholesterol Assay – SUM159 breast cancer cells were plated at a density of $0.5X10^4$ cells per well of a 6-well plate then treated with 1 mM methyl-beta cyclodextrin (MBCD 1 h), 1 μM gefitinib (72 h), 1 μM lovastatin (72 h), 1 μM atorvastatin (LC Laboratories, Woburn, MA; 72 h), or 0.5 μM NB-598 (72 h). Cells were then lysed in CHAPS lysis buffer [10 mM CHAPs, 50 mM Tris (pH 8.0), 150 mM NaCl, and 2 mM EDTA with 10 μM sodium orthovanadate and 1x protease inhibitor cocktail] and Bradford protein assay (Bio-Rad, Hercules, CA) was performed. Cholesterol was measured utilizing the Amplex Red cholesterol assay kit (Invitrogen, Carlsbad, CA). Briefly, 5 μl of sample was diluted into 45 μl 1X reaction buffer and 50 μl Amplex Red buffer [2 U/mL horse radish peroxidase, 2 U/mL cholesterol oxidase and 0.2 U/mL cholesterol esterase] was added in a 96-well plate. Reactions were incubated at 37°C for 30 min to allow for production of hydrogen peroxide due to the reaction of cholesterol esterase and oxidase with cholesterol in the samples. The Amplex Red buffer changes fluorescent color upon exposure to hydrogen peroxide, thus excitation was performed at 540/525nm and emission measured at 620/640nm utilizing filters of a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT) to measure the amount of hydrogen peroxide produced in the samples. Emission readings were averaged and compared to a cholesterol standard curve, then normalized for protein content.

2.10 Growth Assays – Cells were plated at a density of $3.5X10^4$cells per well in 6-well plates on day 0. Every other day starting on day 1, cells were treated with 1 μM gefitinib, 1 μM lovastatin (EMD Biosciences, Gibbstown, NJ), or increasing doses of dasatinib alone or in combination. On days 1, 4 and 8, cells were counted with a Coulter counter (Beckman Coulter, Brea, CA). Briefly, media was aspirated and plates were washed with 1 ml 1X PBS. Cells were then incubated with 500 μL Hepes:$MgCl_2$ solution (0.01 M Hepes, 0.015 M

$MgCl_2$ for five minutes, followed by 75 µL ZAP (Ethyl hexadecyldimethylammonium bromide) for 10 min. This solution was then added to 9.5 mL of isoton solution and counted using a coulter counter. Graphs represent the mean of three individual experiments performed in triplicate.

2.11 MTS Assays – Breast cancer cells were plated at a density of $1\text{-}2X10^3$ in 96-well plates, incubated overnight, and then treated with 0.001-100 µM lovastatin, NB-598, dasatinib, and/or gefitinib for 72 h. Twenty microliters of CellTiter 96 Aqueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI) were added to each well and allowed to incubate at 37°C. Absorbance at 490nm was detected at 2 h using a OpsysMR microplate reader (Dynex, Chantilly, VA). Absorbance units were normalized to the mean of a single dose to compare between experiments. Dose response curves were generated using non-linear sigmoidal dose response curve analyses in GraphPad Prism 4. IC_{50} values were calculated and plotted on isobolograms. The IC_{50} of the primary drug alone was plotted on the y-axis and the IC_{50} of the secondary drug was plotted on the x-axis. The line of additivity was drawn between them. IC_{50} values of the primary drug were calculated at various doses of the secondary drug and plotted on the graph. Points in the graph represent a mean of three independent experiments performed in triplicate. Data points below the line of additivity were considered synergistic.

2.12 Statistics – Student's t-test was performed utilizing the statistical software in GraphPad Prism 4. P-values of <0.05 were considered statistically significant. To perform synergy analyses, the IC_{50} gefitinib was calculated for each dose of lovastatin. The combination index (CI-value) was calculated as follows: (IC_{50} gefitinib at X dose lovastatin)/(IC_{50} gefitinib alone) + (dose of lovastatin)/(IC_{50} lovastatin alone).

Chapter 3

3.1 Introduction

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase whose function has been implicated in many biological processes. When activated, EGFR stimulates signaling pathways involved in cell growth, survival, and migration. While EGFR contains activating mutations in glioblastomas and lung cancer, overexpression is the primary mechanism by which EGFR contributes to breast cancer growth and progression (Wong et al., 1992; Paez et al., 2004). EGFR overexpression occurs in approximately 30% of all breast cancers and correlates with poor clinical prognosis (Sainsbury et al., 1987; Bolla et al., 1990; Toi et al., 1991). Several small molecule tyrosine kinase inhibitors (TKIs) targeting EGFR have been tested in clinical trials with some success in lung and colon cancers. While EGFR TKIs have shown some clinical efficacy in hormone receptor-positive breast cancer (Polychronis et al., 2005; Guix et al., 2008; Cristofanilli et al., 2010), EGFR TKIs lack efficacy in hormone receptor-negative breast cancer (Blagosklonny and Darzynkiewicz, 2003).

The sub-cellular localization of EGFR determines the signaling pathways stimulated by EGFR activation. In fact, EGFR promotes differential signaling depending on receptor localization to endosomes, at the mitochondria, within the nucleus, or on the plasma membrane. Specifically, EGFR localization to endosomes results in ligand-dependent activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways (Sadowski et al., 2009), while mitochondrial localization of EGFR has been implicated in modification of cytochrome c oxidase subunit II activity (Boerner et

al., 2004). Also, EGFR localizes to the nucleus where it may act as a transcription factor (Lin et al., 2001). Perhaps the most well known localization of EGFR is to the plasma membrane, where it modulates both MAPK and Akt signaling pathways [reviewed in (Bianco et al., 2007)].

The plasma membrane contains discrete heterogeneous microdomains (Maa et al., 1995). These microdomains are less fluid than the surrounding bulk plasma membrane, and are enriched in cholesterol, sphingolipids, and gangliosides. They have been termed lipid rafts, and act as platforms for cellular signaling (Simons and Ikonen, 1997). Levels of lipid rafts are increased in melanomas, prostate, and breast cancer cell lines as compared to normal cell lines, which suggests that these structures play a functional role during tumorigenesis (Hazarika et al., 2004; Li et al., 2006). EGFR is one of many proteins shown to exist within lipid rafts, but the effect of EGFR localization to lipid rafts is not well understood. While it has been noted that lipid raft localization of EGFR inhibits ligand binding and subsequent signaling downstream (Chen and Resh, 2002; Roepstorff et al., 2002), other studies have shown that lipid rafts promote EGFR signaling (Zhuang et al., 2002; Peres et al., 2003).

In this chapter, we have found that lipid raft localization of EGFR plays a role in the response of breast cancer cell lines to EGFR TKI-induced growth inhibition. Specifically, EGFR localization to lipid rafts correlated with EGFR TKI resistance. In addition, reduction of cholesterol from lipid rafts sensitized resistant breast cancer cells to the EGFR TKI gefitinib. Significantly, the effects of cholesterol biosynthesis inhibitors and gefitinib were synergistic. Interestingly, while gefitinib abrogated both Akt and MAPK phosphorylation in EGFR TKI sensitive cells, Akt remained phosphorylated in EGFR TKI resistant cell lines. Lovastatin was sufficient to diminish this phosphorylation. Thus, our data suggest that lipid

rafts provide a platform for EGFR-kinase independent activation of Akt in EGFR TKI resistant cell lines.

3.2 Results

A. Resistance of EGFR expressing breast cancer cell lines to EGFR TKIs

The lack of clinical response of breast cancers to EGFR TKIs prevents the use of an excellent targeted agent for the treatment of this disease. To study mechanisms of resistance to EGFR TKIs in breast cancer, we characterized a panel of twenty breast cancer cell lines for EGFR protein expression (Fig. 3.1A). Thirteen of the cell lines analyzed expressed EGFR protein. Interestingly, in twelve of the thirteen EGFR expressing cell lines, EGFR was kinase active under normal growth conditions (Fig. 3.1B). To determine the response of these twelve cell lines to the EGFR TKI gefitinib, we treated the cells with increasing doses of gefitinib, an EGFR TKI, and measured cellular proliferation over time (Table 3.1, Fig. 3.2). These experiments identified seven EGFR TKI resistant cell lines: SUM159, SUM229, BT20, BT549, HCC1937, MDA-MB231, and MDA-MB468 (Fig 3.2B). Breast cancer cells resistant to gefitinib-induced growth inhibition were also shown to be resistant to other EGFR selective TKIs, including the irreversible inhibitor CI-1033 (data not shown). Others have found similar patterns of sensitivity and resistance to EGFR inhibitors in breast cancer cell lines (Helfrich et al., 2006).

In order to determine if gefitinib effectively inhibits EGFR kinase activity in these breast cancer cells *in vitro* kinase assays were performed. We have previously published that 0.1 μM gefitinib completely abrogates EGFR kinase activity as measured by ^{32}P incorporation into EGFR via autophosphorylation (Mueller et al., 2008). Interestingly, we found that in

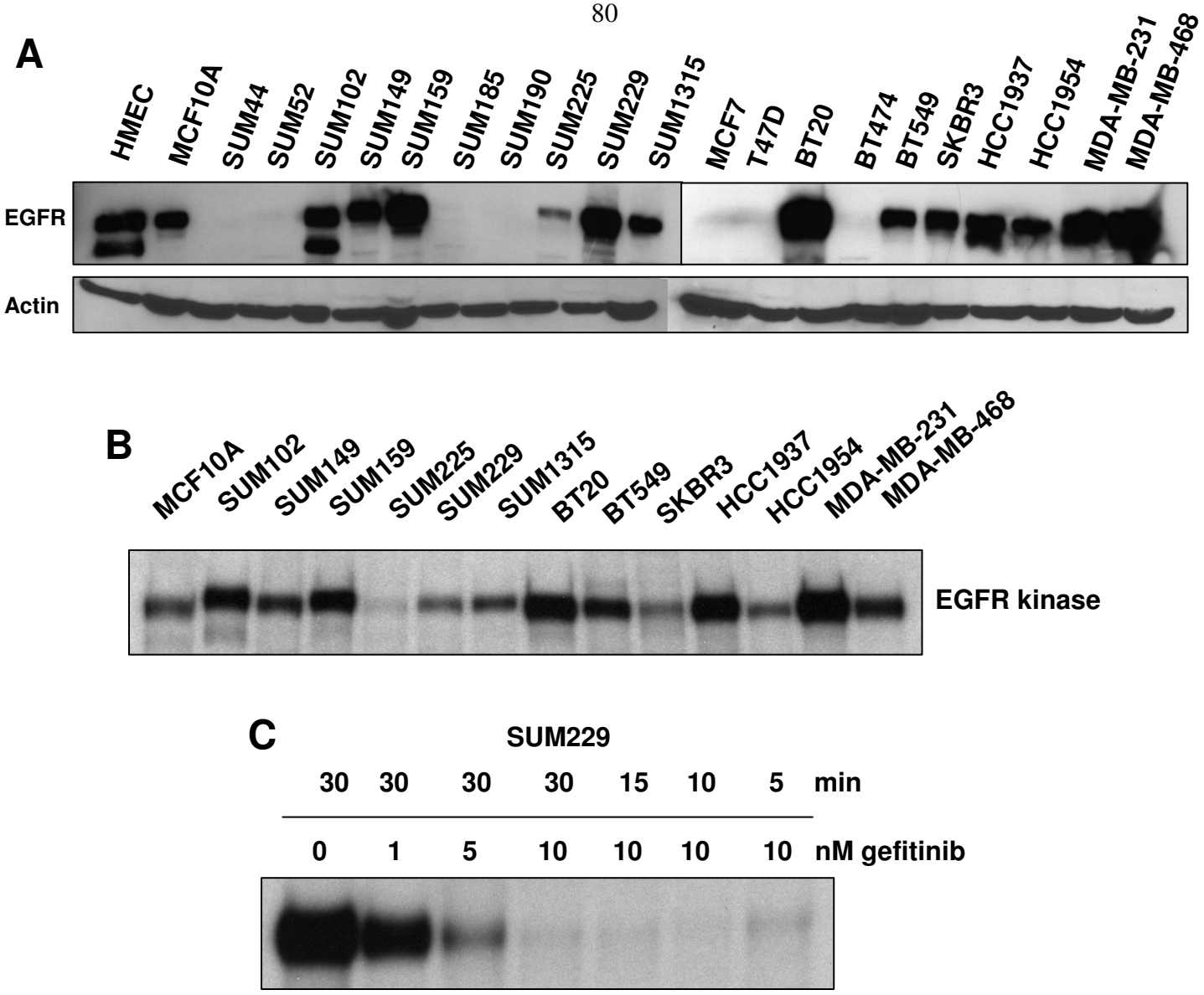
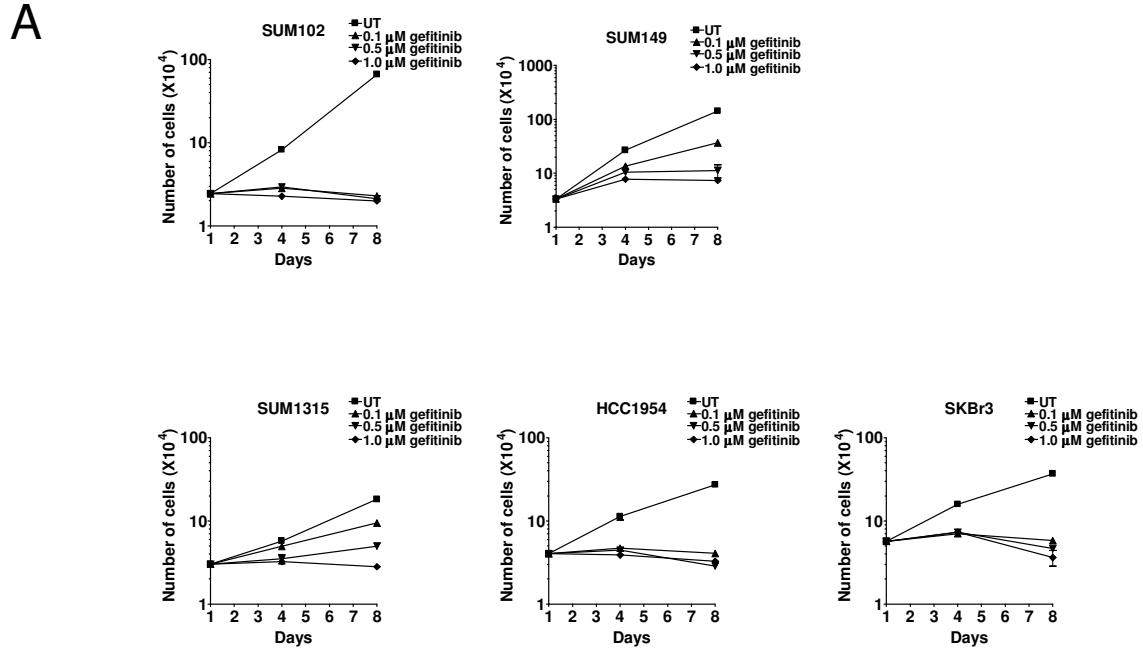


Figure 3.1 EGFR is expressed and kinase active in breast cancer. Breast cancer cell lines were grown under normal growth conditions. (A) Cells were lysed and 100 µg of lysate was separated by SDS-PAGE, transferred to PVDF, and immunoblotted with EGFR or β-actin antibodies. Immunoblots were repeated at least three times. (B) Cells were lysed in kinase-buffer and immunoprecipitated with EGFR antibodies. Kinase assays were performed with ^{32}P-γATP incorporated into EGFR as the substrate for EGFR kinase activity. (C) Kinase assays were performed as described after indicated treatment times and doses of gefitinib in SUM 229 cells. {These experiments were performed by Julie Boerner, PhD}



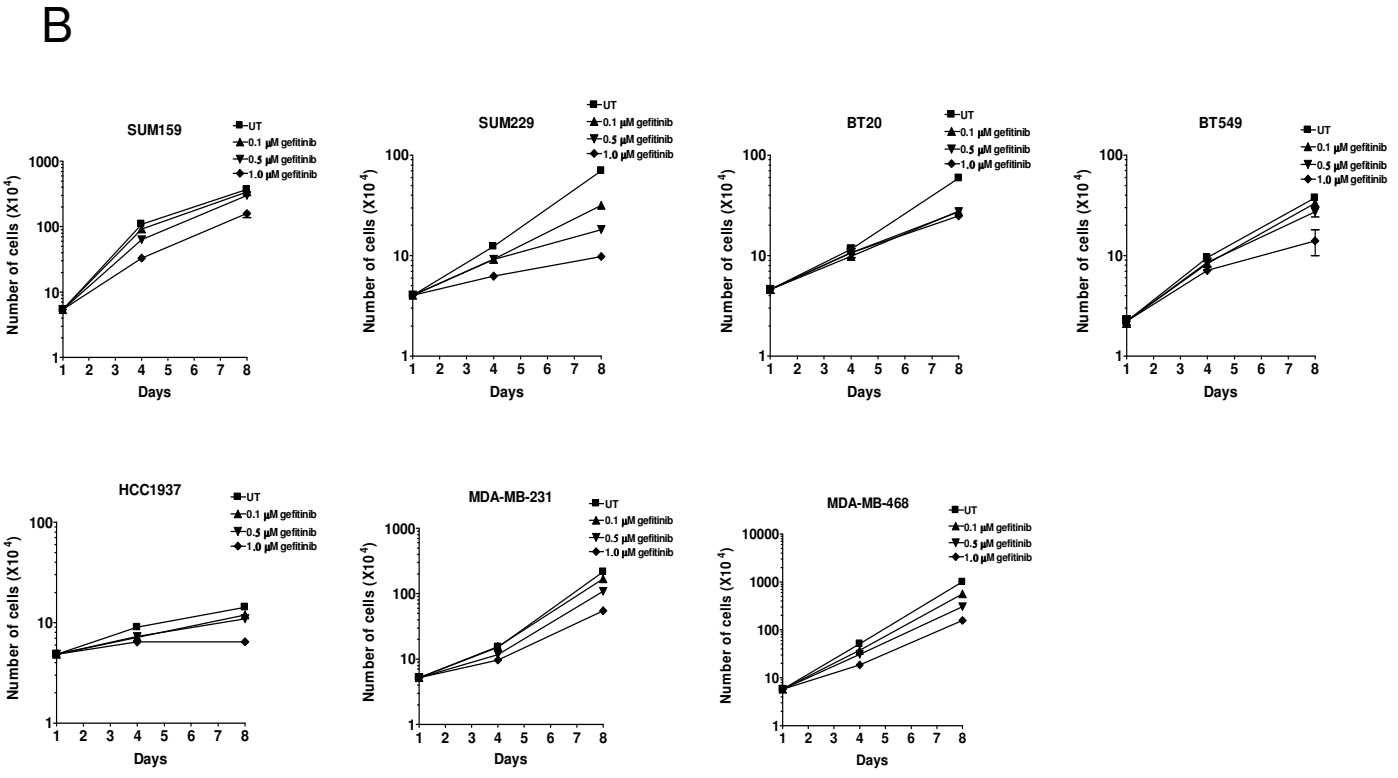


Figure 3.2 Seven of thirteen EGFR expressing breast cancer cell lines are resistant to EGFR TKI induced growth inhibition. Cellular proliferation assays were performed in the presence of increasing doses of gefitinib for eight days. Cell counts were taken on days 1, 4, and 8. Error bars represent the standard error of the mean. Proliferation assays were performed in triplicate and repeated at least three times. (A) Sensitive cells (B) Resistant cells. {These experiments were performed by Julie Boerner, PhD}

Cell Line	Gefitinib Sensitivity
SUM 102	Sensitive
SUM 149	Sensitive
SUM 159	**Resistant**
SUM 229	**Resistant**
SUM 1315	Sensitive
BT20	**Resistant**
BT549	**Resistant**
SKBR3	Sensitive
HCC1937	**Resistant**
HCC 1954	Sensitive
MDA-MB 231	**Resistant**
MDA-MB 468	**Resistant**
H3255	Sensitive
H1650	**Resistant**

Table 3.1: Breast cancer cell lines differ in their sensitivity to EGFR TKIs. Proliferation of breast cancer cell lines in the presence of increasing doses of gefitinib was determined as described for Figure 3.2. Sensitive cell lines are those where less than 1 μM inhibited proliferation, while resistant (**bold**) cells lines are those that continued proliferation in the presence of 1 μM gefitinib.

five of the seven EGFR TKI resistant breast cancer cells, tyrosine phosphorylation was maintained in the absence of EGFR kinase activity which we have evidence to support occurs via transphosphorylation by other activated tyrosine kinases (Mueller et al., 2008). Here, we added to these findings by determining the minimal dose and time of gefitinib required to completely inhibit EGFR kinase activity (Fig. 3.1C). We found that as little as 10 nM gefitinib for five minutes was sufficient to deplete EGFR kinase activity in these cells. Therefore, EGFR kinase activity was successfully inhibited by the doses of gefitinib utilized in these studies in both EGFR TKI sensitive and resistant cell lines.

Although EGFR kinase activity is not required for the growth of EGFR TKI resistant cell lines, the previously described maintenance of EGFR phosphorylation in the absence of kinase activity (Mueller et al., 2008) suggests that the protein itself may still be required for proliferation. Thus, to directly determine if proliferation of EGFR TKI resistant cells requires EGFR protein expression, we used EGFR-targeting shRNA lentiviral infection to down-regulate EGFR protein expression. Twenty-one EGFR shRNA constructs were screened for efficiency of knocking down EGFR expression, as measured by immunoblotting. Three EGFR shRNA constructs consistently decreased EGFR protein expression (Fig. 3.3A). Construct three gave the best knockdown, as there was at least a 50% reduction in EGFR protein of all cell lines tested when compared to the non-silencing shRNA control. In order to determine if knockdown of EGFR was sustained over the period utilized to conduct growth assays, SUM159 and SUM229 cells were infected with EGFR shRNA, and grown with puromycin selection for two weeks. As seen in Figure 3.3B, EGFR protein expression remained reduced at two weeks in both cell lines, demonstrating that EGFR #3 shRNA sufficiently knocks down EGFR expression over the time period necessary for

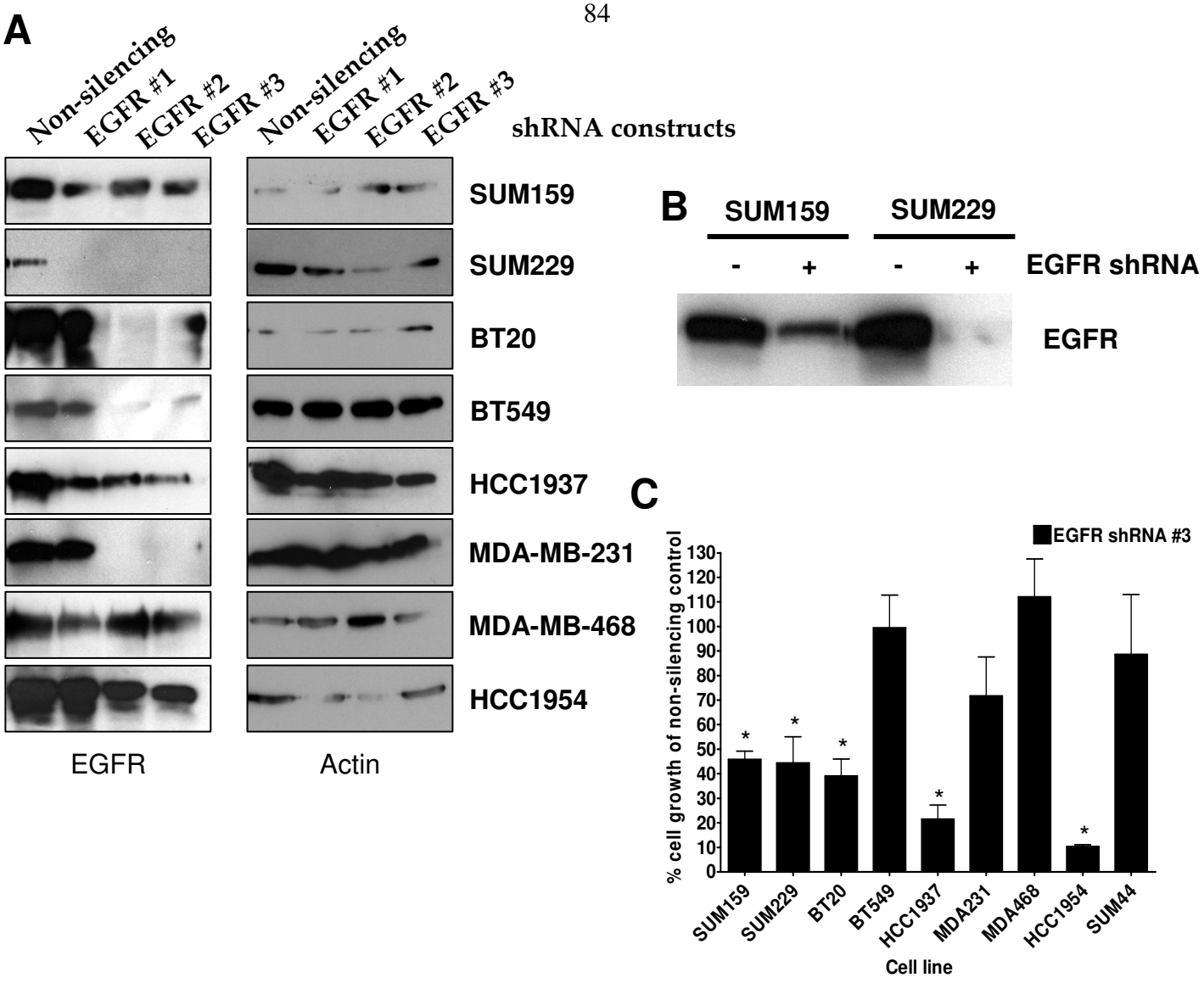


Figure 3.3: EGFR protein expression is required for growth in four of seven EGFR TKI resistant breast cancer cell lines. (A) Cells were incubated with equal MOI of virus for four days, lysed, separated by SDS-PAGE, and immunoblotted for EGFR and β-actin. (B) SUM 159 and SUM 229 cells were grown under selection pressure for two weeks post-infection, then lysed, separated by SDS-PAGE, and immunoblotted for EGFR (C) Cells were plated at 1,000 cells/well of a 6-well plate and inoculated with EGFR shRNA #3 lentivirus or non-silencing control lentivirus. Three days later, the media was changed to puromycin containing media for eight days. Cells were then counted using a Beckman Coulter Counter. Experiments were performed at least three times with the error bars representing the standard error of the mean. Statistical analyses were performed utilizing Student's t-test, * = $p>0.05$. {These experiments were performed by Julie Boerner, PhD}

growth assays to be performed. Additionally, SUM44 cells, which do not express EGFR (Fig. 3.1A) were utilized as a negative control, and HCC1954 cells which are sensitive to EGFR TKIs (Table 3.1, Figure 3.2A) were utilized as a positive control. Notably, BT549, MDA-MB231, and MDA-MB468 cells continued to grow after a decrease in EGFR protein expression (Fig. 3.3C). This non-dependence on EGFR protein expression in these three cells lines may be a result of genetic alterations in signaling proteins downstream of EGFR. For example, MDA-MB-468 and BT549 cells have lost PTEN expression and MDA-MB-231 cells contain an activating K-Ras mutation (Hollestelle et al., 2007). Conversely, in SUM159, HCC1937, SUM229, and BT20 breast cancer cell lines, knocking down EGFR expression significantly decreased proliferation, suggesting that EGFR protein expression is at least in part required for the growth of these cell lines (Fig. 3.3C, * = $p<0.05$).

Previous studies have shown that EGFR localization can modulate EGFR signaling (Lin et al., 2001; Chen and Resh, 2002; Zhuang et al., 2002; Boerner et al., 2004; Li et al., 2006). Thus, to determine if the localization of EGFR was mediating the response of cells to EGFR TKIs, immunostaining and confocal microscopy were performed. Cells were stained with Alexa-Fluor 488-labeled EGFR antibodies (Fig. 3.4; green) and DAPI as a nuclear dye (blue). In two EGFR TKI sensitive cell lines (SKBr3 and SUM1315), EGFR localized entirely within intracellular compartments and the cytosol. However, in two other EGFR TKI sensitive cell lines (SUM149 and HCC1954), as well as all four EGFR TKI resistant cell lines, EGFR localized both within intracellular regions and at the plasma membrane. Interestingly, EGFR staining was not always contiguous around the membrane. The patchy nature of the staining, most prominent in SUM159 cells (Fig. 3.4; arrows), suggested that EGFR may localize to lipid rafts (Harder et al., 1998b; Grossmann et al., 2006). EGFR has

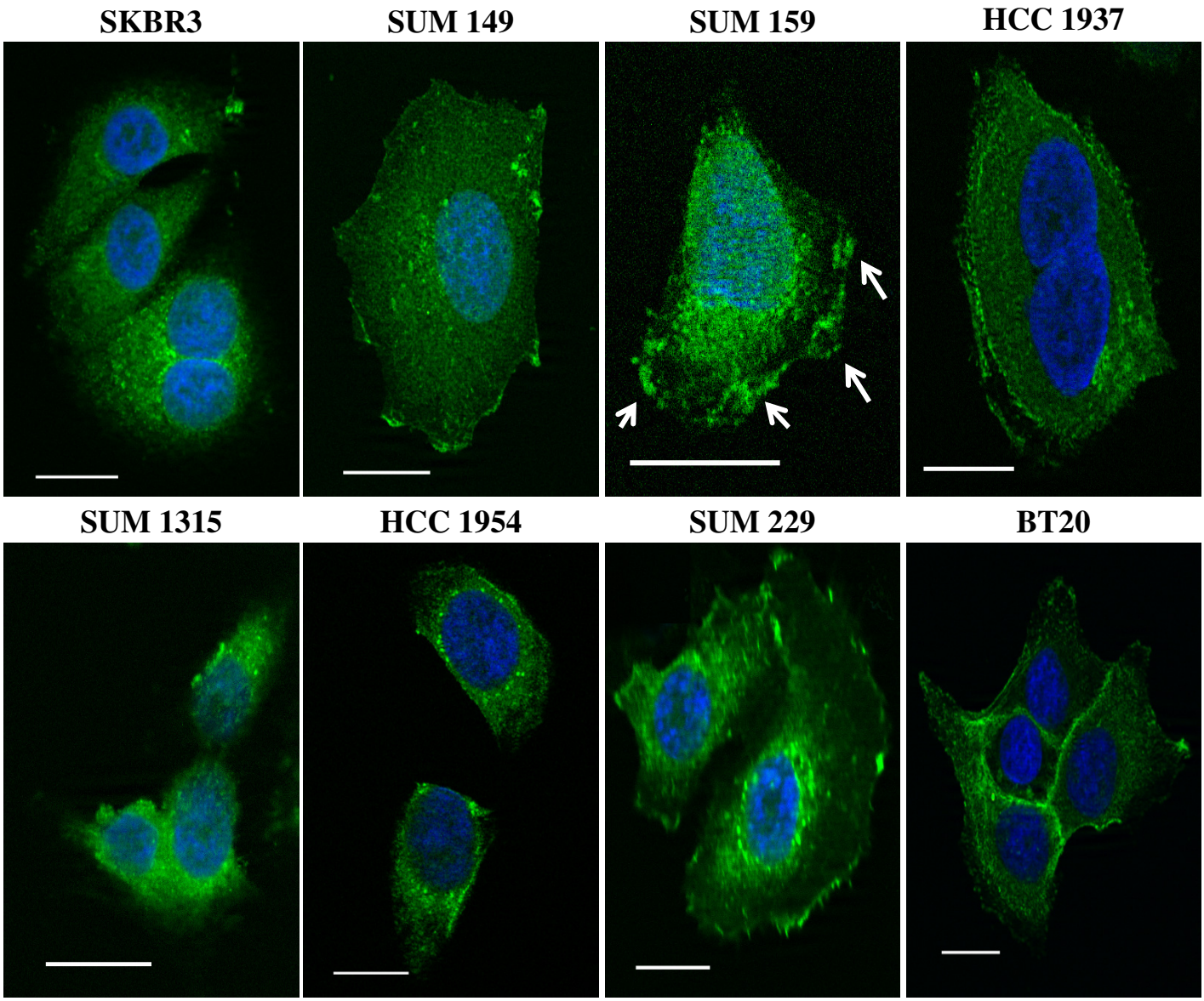


Figure 3.4: EGFR is localized to the plasma membrane in breast cancer cell lines. For each cell line, two hundred thousand cells were plated onto coverslips and cultured under normal growth conditions for 48 h. Cells were fixed, permeabilized, and blocked with 20% goat serum. EGFR was detected with Alexa-fluor 488 labeled EGFR antibody (green) and nuclei were identified with DAPI (blue). Imaging was performed using Zeiss Axioplan2 apotome microscope fitted with a 63X 1.25 oil immersion lens at the Microscopy and Imaging Resources Laboratory (Wayne State University, Detroit, MI). Arrows indicate patchy EGFR staining. Scale bars represent a distance of 50 µm.

been shown to localize within lipid rafts in Hela and CHO cells (Macdonald and Pike, 2005; Pike, 2005). In order to determine if EGFR was localized to lipid rafts in breast cancer cells, we used two methods of identifying these structures: biochemical raft isolation and confocal microscopy. First, a detergent-free Opti-Prep gradient was used to isolate lipid rafts (adapted from (Macdonald and Pike, 2005)). Flotillin, a membrane protein found both within and outside of lipid rafts, was used to show presence of membrane components within all fractions, while transferrin receptor was used as a marker for non-raft containing fractions. These markers, along with dot blotting for the lipid raft specific glycosphingolipid GM-1 (Fig. 3.5A) indicated fractions 1-7 as lipid raft fractions. When these fractions were immunoblotted using EGFR antibodies, EGFR localization to the lipid raft fractions was most prominent in the EGFR TKI resistant cell lines (Fig. 3.5A). As SKBR3 and SUM1315 cell lines showed solely intracellular EGFR staining, these cell lines were excluded from lipid raft analyses. Quantification of the percent of total EGFR that was present in the lipid raft fractions found that the four EGFR TKI resistant breast cancer cell lines contained significantly more EGFR within lipid rafts as compared to the average EGFR content within lipid rafts of two EGFR TKI sensitive cell lines, SUM149 and HCC1954 (Fig. 3.5B, * = $p < 0.05$). Taken together, these data suggest that elevated EGFR localization to lipid rafts may correlate with resistance to EGFR TKI-induced growth inhibition.

While lipid rafts are predominately found within the plasma membrane, there is evidence that they are also present in endosomes, lysosomes, and mitochondria (Galbiati et al., 2001). To determine if EGFR localized specifically within plasma membrane lipid rafts, we used immunofluorescent staining under non-permeabilizing conditions. Cholera toxin subunit B binds specifically to GM-1 and was used to detect localization of lipid rafts and EGFR was

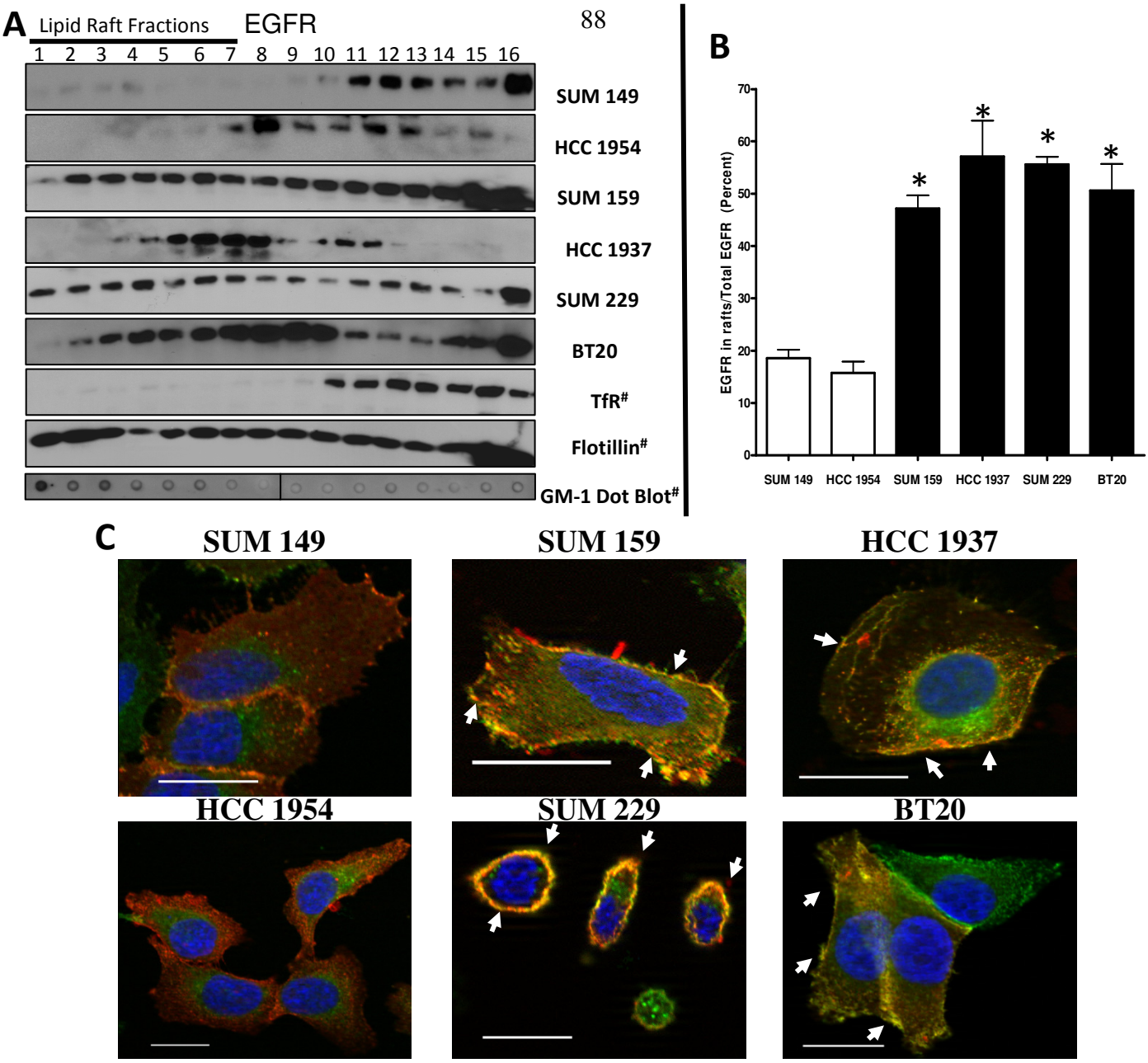


Figure 3.5: EGFR localization to lipid rafts correlates with EGFR TKI resistance (A) One half million cells were plated, cultured 72 h, detergent-free lysis was performed and lipid rafts were separated by ultracentrifugation [23]. Western blotting was performed with EGFR, transferrin receptor, and flotillin antibodies. Fractions were dot blotted for GM-1 utilizing cholera toxin subunit B-HRP. Fractions 1-7 indicate lipid raft fractions. # indicates that the blots are representative. (B) Densitometry was performed on western blots from A. Bars represent the percent of EGFR in lipid raft fractions (1-7) as compared to the total amount of EGFR present (1-14) from at least three independent experiments. Statistical analyses were performed utilizing a student's t-test, * = $p<0.05$ compared to SUM149 and HCC1954 cells. (C) Two hundred thousand cells were plated onto coverslips and cultured for 48 h. Coverslips containing cells were then incubated with Alexa-fluor 594 labeled cholera toxin subunit B (red) for 10 min on ice. Following incubation, cells were fixed, blocked in 20% goat serum, and incubated with immunofluorescent EGFR antibodies (extracellular domain epitope, green) and nuclei were stained with DAPI (blue). Imaging was performed using Zeiss Axioplan2 apotome microscope fitted with a 63X 1.25 oil immersion lens at the Microscopy and Imaging Resources Laboratory (Wayne State University, Detroit, MI). Arrows indicate areas of co-localization. Scale bars represent a distance of 50 µm.

detected as described above. In the EGFR TKI resistant cell lines (SUM159, HCC1937, SUM229, and BT20), EGFR (green) co-localized (yellow/orange) with GM-1 (red) at the plasma membrane (Fig. 3.5C; arrows). In contrast, in the EGFR TKI sensitive cell lines (SUM149 and HCC1954), EGFR and GM-1 did not co-localize (Fig. 3.5C). These data suggested that EGFR localizes within plasma membrane lipid rafts in breast cancer cells that are resistant to EGFR TKI-induced growth inhibition.

B. Disruption of lipid rafts sensitizes breast cancer cells to EGFR inhibitors

Cholesterol is the primary structural component of lipid rafts [reviewed in (Barenholz, 2002)], thus, to determine if the presence of EGFR in lipid rafts mediates cellular response to EGFR TKIs, we pharmacologically depleted cholesterol from the cells. HMG CoA-reductase inhibitors lovastatin and atorvastatin were used to reduce lipid raft cholesterol content [reviewed in (Simons and Toomre, 2000)]. The Amplex Red cholesterol assay, which determines total cellular cholesterol content by measuring the amount of H_2O_2 produced by the reaction of cholesterol in the sample with cholesterol oxidase and cholesterol esterase enzymes, was utilized to determine the ability of these drugs to reduce cellular cholesterol (Fig. 3.6). Methyl-beta cyclodextrin (MBCD), a cytotoxic cholesterol sequestering agent, reduced cholesterol by 41.5% +/- 8.1%, and was therefore used as a positive control for these experiments. Seventy-two hours of treatment with the HMG CoA reductase inhibitors lovastatin and atorvastatin resulted in depletion of cholesterol content, with a reduction of 59.0% +/-12.4% at 1.0 μM lovastatin and a reduction of 49.6% +/-10.3% at 1.0 μM atorvastatin (Fig. 3.6). Importantly, gefitinib treatment had no effect on cholesterol content of these cells, and did not alter the ability of lovastatin to reduce total

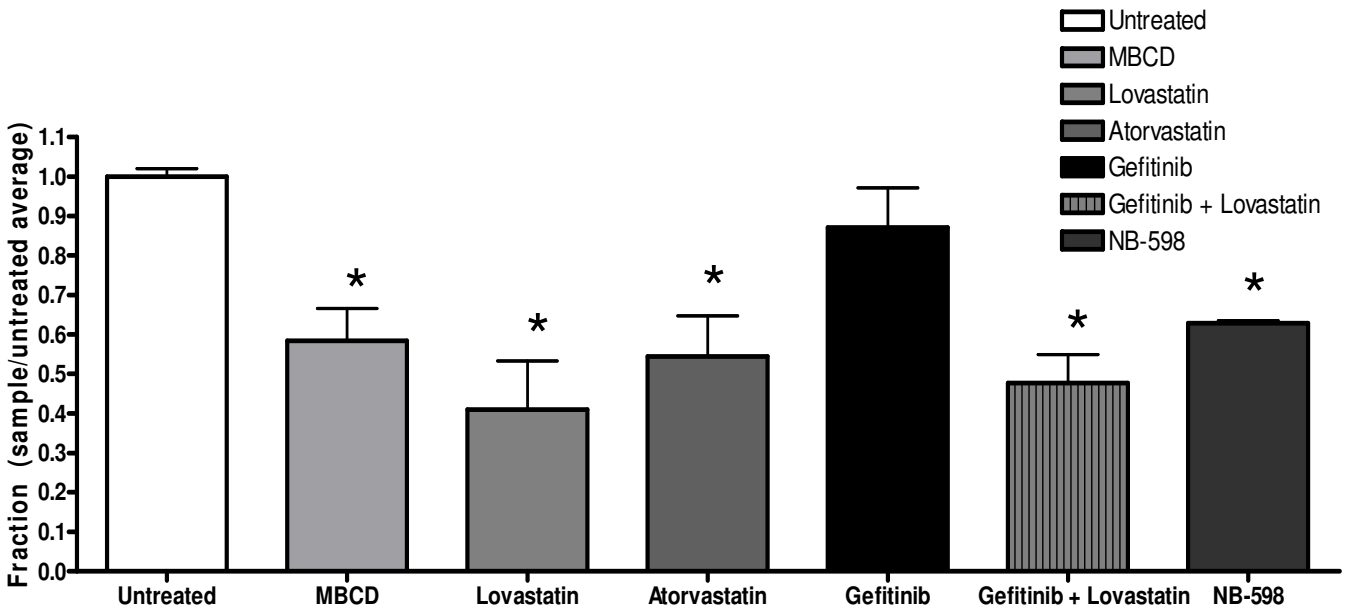


Figure 3.6: MBCD, lovastatin, atorvastatin, and NB-598 reduce cholesterol in breast cancer cells. Fifty thousand cells were plated into 6-well plates and treated with 1 mM MBCD (1 h), 1 µM lovastatin (72 h), 1 µM atorvastatin (72 h), 1.0 µM NB-598 (72 h), 1 µM gefitinib (1 h), or a combination of 1 µM gefitinib (1 h) and 1 µM lovastatin (72 h). Lysis was followed by protein quantification and cholesterol was measured using the Amplex Red cholesterol assay kit. Absorbance was converted to µg cholesterol/mL utilizing a cholesterol standard curve, and then samples were normalized to protein concentration for a final value in µg cholesterol/µg protein. Bars represent fraction of cholesterol with untreated samples as 1 (µg cholesterol/µg protein sample)/(µg cholesterol/µg protein untreated). Experiments were repeated at least three times. Error bars represent the standard error of the mean. Statistical analyses were performed utilizing Student's t-test, * = $p < 0.05$ compared to untreated.

cellular cholesterol (Fig. 3.6). The levels of cholesterol reduction produced by the statins are comparable with published results (Sethy-Coraci et al., 2005; Ehehalt et al., 2008).

To determine if lovastatin has the ability to sensitize breast cancer cells to gefitinib, cell counting assays were used to measure proliferation. Cells were treated every other day with the drugs and counted on days 1, 4, and 8 (Fig. 3.7). As described previously, the four EGFR TKI resistant cell lines continued to proliferate in the presence of gefitinib. Interestingly, lovastatin was able to significantly reduce proliferation in the presence of gefitinib when compared to gefitinib or lovastatin treatment alone (Fig. 3.7; * = $p<0.0001$, ♦). Taken together, these data suggested that treatment with lovastatin sensitizes EGFR TKI resistant cell lines to gefitinib.

In order to determine if the effects of lovastatin and gefitinib were synergistic in EGFR TKI resistant breast cancer cells, cell viability assays were performed. Briefly, cells were treated for 72 h with the combination of lovastatin and gefitinib prior to performing tetrazolium-based cell viability assays. It can be noted that the IC_{50} values for cell viability analyses were much higher than doses found to be effective in cellular proliferation assays. While proliferation assays allow for the measurement of the number of cells over time, cell viability assays indicate the metabolic activity of the cell population. The IC_{50} of gefitinib was calculated at various doses of lovastatin, and then isobolograms were generated (Fig. 3.8). An additive interaction in SUM149 and HCC1954 cells was calculated from these assays (Fig. 3.7, points on the line). In contrast, synergistic effects were seen in all four EGFR TKI resistant cell lines (Fig. 3.8, points below the line). Combination index (CI) values were calculated based on the IC_{50} values (Table 3.2). These values were significantly lower than one in all of the EGFR TKI resistant cell lines. These results suggested that the

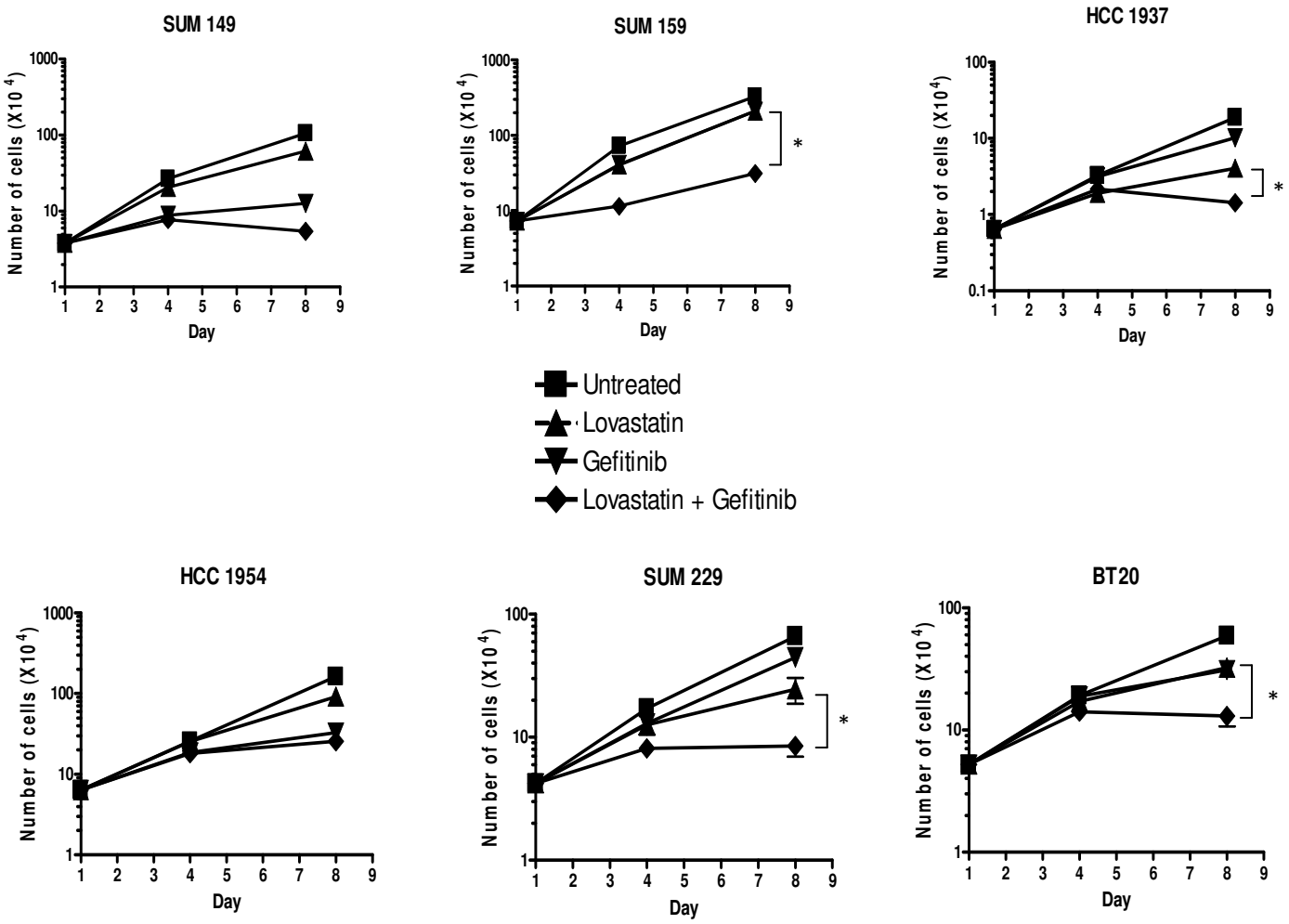


Figure 3.7: Lovastatin sensitizes EGFR TKI resistant breast cancer cells to gefitinib. Thirty five thousand cells were plated into 6-well plates and treated for eight days with lovastatin and/or gefitinib (HCC1954, SUM149 and SUM159 cell lines were treated with 1 μM of both lovastatin and gefitinib, while HCC1937, SUM229, and BT20 cells were treated with 5 μM lovastatin and 1 μM gefitinib). Cell number was determined on days 1, 4, and 8 using a coulter counter. Experiments were repeated at least three times and counts were averaged. Error bars represent the standard error of the mean. Statistical analyses were performed utilizing Student's t-test, * = $p < 0.0001$.

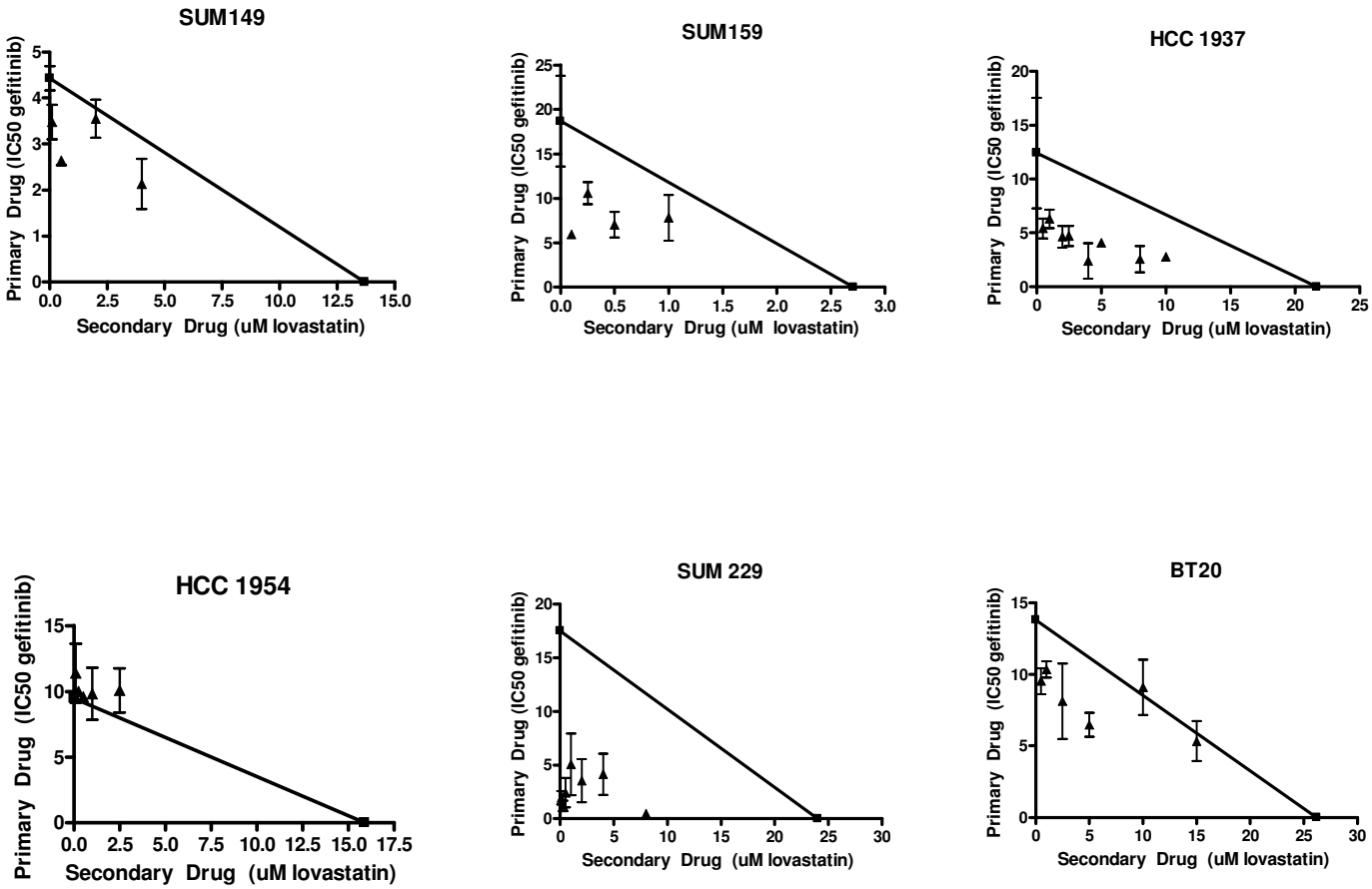


Figure 3.8: Lovastatin and gefitinib act synergistically to reduce breast cancer cell viability. Two thousand cells were plated onto 96-well plates and treated for 72 h with varying doses of gefitinib and lovastatin alone and in combination. Values were normalized and then plotted. Non-linear regression (sigmoidal-dose response) curves were generated and IC50s were calculated and plotted on isobolograms. Experiments were repeated at least three times.

Cell Line	Lovastatin Dose	Combination Index (CI-value)	p-value (compared to 1)
SUM159	**0.25 μM**	**0.659 +/-0.066**	**0.0262**
HCC1937	**1.0 μM**	**0.554 +/-0.069**	**0.0124**
SUM229	**1.0 μM**	**0.331 +/- 0.164**	**0.0269**
BT20	**5.0 μM**	**0.695 +/- 0.065**	**0.0348**

Table 3.2: Effects of lovastatin and gefitinib are synergistic in EGFR TKI resistant cell lines. The combination index (CI-value) was calculated as follows: (IC50 gefitinib at X dose of lovastatin)/(IC50 gefitinib alone) + (dose of lovastatin)/(IC50 lovastatin alone). p-values were calculated as a difference between CI-value and one. p-values, calculated by student's t-test, less than 0.05 were considered significant.

combinatorial inhibition of lipid raft structure and EGFR-kinase activity resulted in a synergistic decrease in cell viability when EGFR is localized to lipid rafts. Therefore, the use of lovastatin and gefitinib in combination may effectively decrease viability and proliferation of breast cancers that contain EGFR within lipid rafts.

Statin drugs work by inhibiting HMG-CoA reductase. In addition to cholesterol biosynthesis, this enzyme also regulates isoprenoid synthesis. Therefore, in order to determine if the synergistic effect between lovastatin and gefitinib is mediated by cholesterol depletion, the drug NB-598 was used. NB-598 is a squalene monooxygenase inhibitor (Horie et al., 1990), and therefore inhibits cholesterol biosynthesis but not isoprenoid synthesis. First, to determine if NB-598 effectively inhibited cholesterol biosynthesis, SUM159 cells were treated with NB-598 for 72 h prior to assaying cholesterol esterase activity (Fig. 3.6). NB-598 treatment reduced cholesterol by 37.1% +/- 0.59%, suggesting that NB-598 depleted cholesterol to a comparable level as lovastatin. Therefore, we utilized NB-598 to determine if inhibiting cholesterol biosynthesis in the absence of altering isoprenoid synthesis has the ability to sensitize cells to gefitinib. EGFR TKI resistant breast cancer cells were treated with variable doses of NB-598 alone, or in combination with gefitinib. Cell viability assays were used to determine the IC_{50} of gefitinib at variable doses of NB-598. As shown in Figure 3.9, the effects of gefitinib and NB-598 were synergistic. These data suggest that cholesterol depletion is sufficient to sensitize EGFR TKI resistant cells to gefitinib.

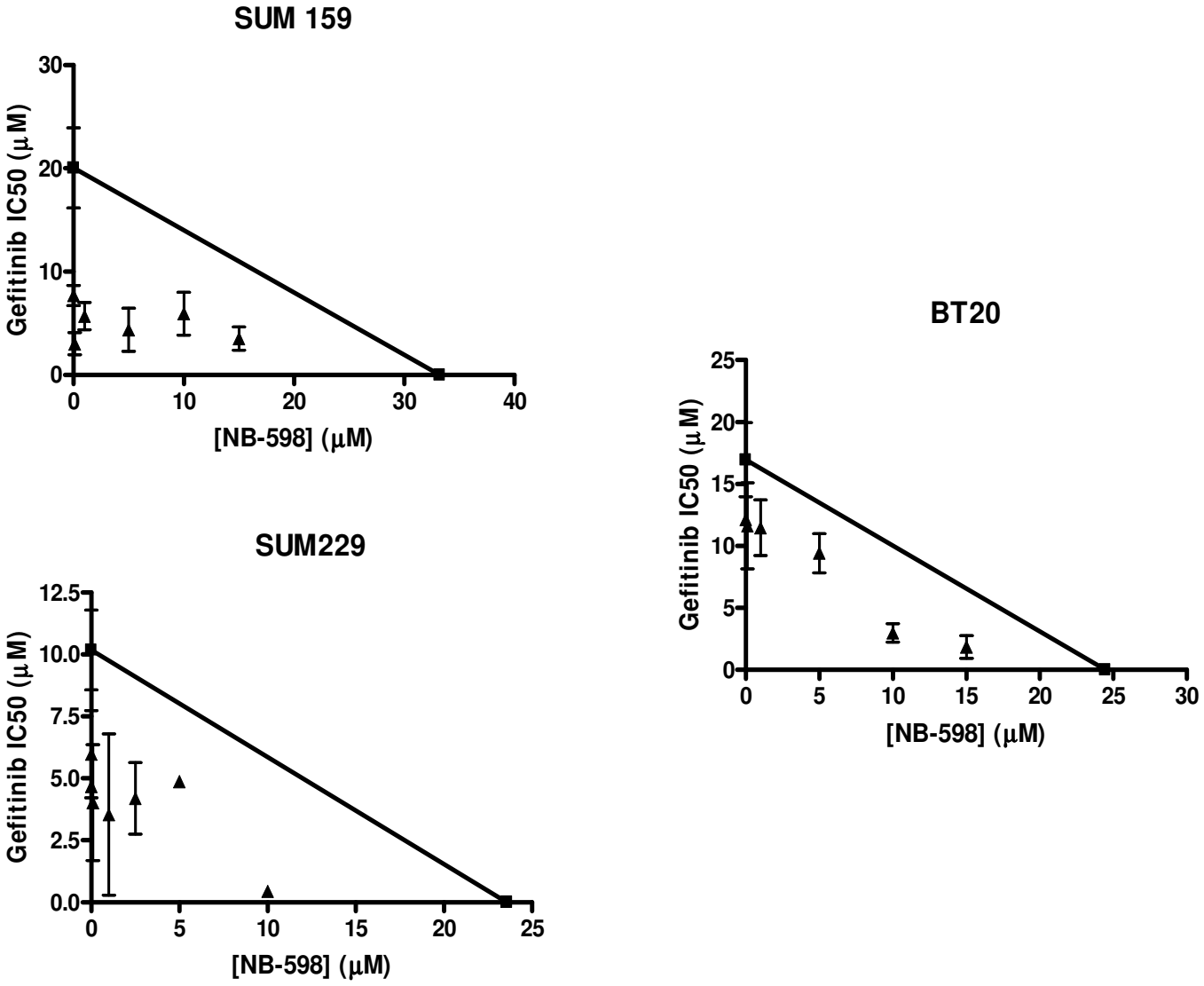


Figure 3.9: The effects of NB-598 and gefitinib are synergistic. Two thousand cells were plated onto 96-well plates and treated for 72 h with varying doses of gefitinib and NB-598 alone and in combination. Values were normalized and then plotted. Non-linear regression (sigmoidal-dose response) curves were generated, and IC50s were calculated and plotted on an isobologram. Experiments were repeated at least three times.

C. Akt phosphorylation is abrogated with lipid raft disruption

Resistance to EGFR TKIs suggests that inhibiting the EGFR kinase activity is insufficient to turn off growth and survival signaling in these cells. Localization of EGFR to lipid rafts has variable effects on signaling pathways downstream of EGFR (Chen and Resh, 2002; Li et al., 2006), thus we determined what effect depletion of cholesterol had on EGFR signaling in EGFR TKI resistant cells as compared to EGFR TKI sensitive cells. As discussed further below, BT20 cells contain a PIK3CA mutation, and the HCC1937 cell line has loss of PTEN expression, therefore, and lovastatin does not effect the phosphorylation of Akt in these cell lines (data not shown). Thus, two EGFR TKI resistant cell lines (SUM159 and SUM229) and one EGFR TKI sensitive cell line (SUM149) were treated with lovastatin and gefitinib alone or in combination and immunoblotting was performed to determine the phosphorylation of Akt and MAPK. Gefitinib treatment resulted in a reduction of MAPK phosphorylation in both the sensitive SUM149 cell line and two gefitinib resistant cell lines (SUM159 and SUM229). In contrast, Akt phosphorylation was inhibited in the EGFR TKI sensitive cell line yet persisted in the presence of gefitinib in EGFR TKI resistant cell lines (Fig. 3.10, lane 3). This phosphorylation persisted even after 72 h treatment with gefitinib (data not shown). When treated with lovastatin, alone or in combination with gefitinib, Akt phosphorylation was abrogated (Fig. 3.10, lanes 2 and 4). These data suggested that, co-treatment of cells with lovastatin and gefitinib was able to inhibit two major EGFR signaling pathways. Thus, we propose that lipid rafts may provide a platform whereby EGFR may functionally interact with other proteins to activate Akt and modulate the response to EGFR TKIs.

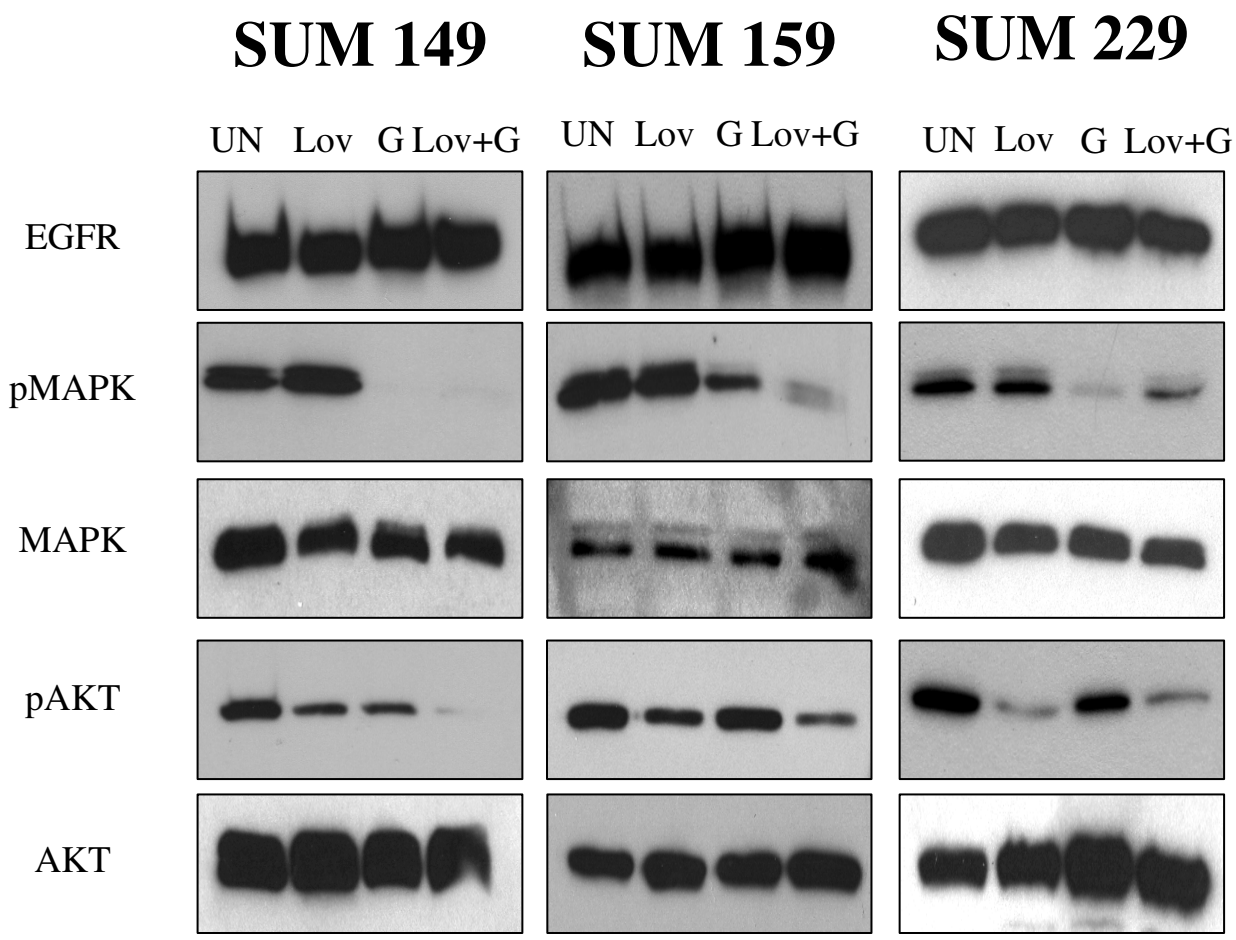


Figure 3.10: Lovastatin inhibits Akt phosphorylation in EGFR TKI resistant cell lines. One million cells were plated and allowed to grow for 48 h. Cells were then treated with 1 μM (SUM 149 and SUM 159) or 5 μM lovastatin (SUM 229) for 72 h (LOV) and/or 1 μM gefitinib (G) for 1 h. Lysates were prepared and separated by SDS-PAGE. Immunoblotting using EGFR, Akt, MAPK, phospho-Akt, and phospho-MAPK antibodies was performed as described.

3.3 Discussion

We have provided evidence describing a role for lipid rafts in resistance to EGFR TKI-induced growth inhibition using four EGFR expressing breast cancer cell lines which continue to proliferate in the presence of gefitinib, an EGFR TKI. We have shown that seven of thirteen EGFR-expressing breast cancer cell lines are resistant to EGFR TKI-induced growth inhibition, and that four of those cell lines retain the requirement of EGFR protein expression for growth. Also, we have provided evidence that EGFR localization to lipid rafts correlates with EGFR TKI resistance. Further, lovastatin, a HMG CoA reductase inhibitor, as well as NB-598, a squalene monooxygenase inhibitor, reduced cholesterol biosynthesis in the EGFR TKI resistant breast cancer cells. In addition, lovastatin sensitized EGFR TKI resistant breast cancer cells to gefitinib-induced growth inhibition. Importantly, this sensitization of EGFR TKI growth resistant cells to gefitinib was determined to be synergistic for both lovastatin and NB-598. Our data suggests that Akt phosphorylation persists in the presence of EGFR kinase inhibition, and that lovastatin abrogated this phosphorylation, thus sensitizing the cells to EGFR kinase inhibition.

Overexpression of EGFR is one mechanism by which EGFR contributes to cancer progression. In fact, overexpression of EGFR occurs in glioblastomas, breast, prostate, ovary, liver, bladder, esophagus, larynx, stomach, colon, and lung cancers (Khazaie et al., 1993). This fairly ubiquitous overexpression suggests that EGFR may be an attractive target for cancer therapeutics. Inhibitors of EGFR kinase activity show clinical efficacy in lung, pancreatic, colorectal, and head and neck cancers (Baker, 2004; Cohen et al., 2005; Giusti et al., 2008; Sobrero et al., 2008), however they have proven ineffective in the treatment of breast cancers (Blagosklonny and Darzynkiewicz, 2003; Twombly, 2005). Herein, we have

provided evidence that breast cancer cell lines differ in their responses to these EGFR TKIs (Table 3.1). Seven of thirteen breast cancer cell lines were found to be resistant to EGFR TKI-induced growth inhibition using cellular proliferation assays (Table 3.1). Specifically, SUM159, SUM229, BT20, BT549, HCC1937, MDA-MB231, and MDA-MB468 cell lines continued to proliferate in the presence of 1 μM gefitinib (Table 3.1).

A number of mechanisms have been suggested for resistance to EGFR TKI-induced growth inhibition including EGFR independence, mutations in EGFR and alterations in downstream signaling pathways. We have shown that three of seven EGFR TKI resistant breast cancer cell lines grow independently of EGFR protein expression, while four retain the requirement of EGFR expression for their proliferation (Fig. 3.3A-C). Mutations of EGFR, such as the VIII or T790M, have been implicated in glioblastomas and non-small cell lung cancers; however, these mutations are rare in breast tumors (Bianco et al., 2005). We have sequenced EGFR in the cell lines we used for our studies and no EGFR mutations were present (R. Haddad, personal communication).

Failure to inhibit Akt signaling, due to mutation or loss of PTEN, constitutive activation of PI3K, or overexpression of Akt, has also been shown to be a mechanism of resistance to EGFR TKI-induced growth inhibition (Cheng et al., 1992; Lu et al., 1999; Hollestelle et al., 2007). Of the cell lines that retain the requirement of EGFR protein expression for growth, but are EGFR TKI resistant, one has a PIK3CA mutation (BT20), and one has loss of PTEN expression (HCC1937) suggesting that the PI3K/Akt pathway may be important in the tumorigenicity of these cell lines (Hollestelle et al., 2007). Indeed, Akt phosphorylation persists in the absence of EGFR kinase activity in these two cell lines and lovastatin had no effect on Akt phosphorylation (data not shown). The two other EGFR TKI resistant cell lines

(SUM159 and SUM229) do not contain genetic mutations in the Akt pathway, yet retain Akt phosphorylation in the presence of gefitinib (Fig. 3.10, lane 3). Lovastatin treatment was sufficient to abrogate this phosphorylation, suggesting that lipid rafts play a role in the regulation of Akt phosphorylation in EGFR TKI resistant cells (Fig. 3.10, lanes 2 and 4). Specifically, we suggest that lipid rafts provide a platform for EGFR protein-dependent, EGFR kinase-independent activation of Akt signaling. However, as EGFR signaling is mediated by many more proteins than addressed here, it is possible that other pathways may also be downstream of EGFR-kinase independent, lipid raft-dependent activation. Nevertheless, localization of EGFR to lipid rafts is an important factor in the resistance of breast cancer cells to EGFR TKI-induced growth inhibition.

Lipid rafts have been suggested to play a functional role in cancer cell drug resistance. Depletion of lipid rafts through inhibition of fatty acid synthase (FAS) has been found to overcome trastuzumab resistance in breast cancer (Menendez et al., 2005). Specifically Her2/Neu co-localizes with lipid rafts in breast cancer cells, and the lipid environment of Her2/Neu-overexpressing cells influences the dimerization properties and signaling functions of Her2/Neu (Menendez et al., 2005). Furthermore, preclinical data suggest that lipid raft depletion via statins can decrease cell growth and sensitize cells to apoptotic stimuli in a number of cancer models including melanoma, prostate, and HER2-overexpressing breast cancers (Li et al., 2006; Glynn et al., 2008; Herrero-Martin and Lopez-Rivas, 2008). Epidemiologic data regarding the use of statins as singular agents in breast cancer are mixed (Beck et al., 2003; Cauley et al., 2003; Kwan et al., 2008). The apparent *in vitro* benefit of combining statins with other therapies suggests that statins may have a greater clinical benefit when utilized as a part of combinatorial therapies (Katz, 2005). In that regard, we

have shown that cholesterol depletion synergizes with gefitinib in four EGFR TKI resistant breast cancer cell lines (Fig. 3.8 and 3.9, Table 3.2). Specifically, co-treatment of these cell lines with lovastatin and gefitinib significantly reduces cell proliferation compared to either drug alone (Fig. 3.7). Also, when CI-values were determined for the combination of lovastatin and gefitinib, all four cell lines resistant to EGFR TKI-induced growth inhibition showed synergy (Table 3.2). Thus, in breast cancer cells resistant to EGFR TKI-induced growth inhibition, EGFR is commonly localized to lipid rafts, and our data indicate that this localization plays a functional role in such resistance.

Our data suggest that the synergistic mechanism between lovastatin and gefitinib in breast cancer cells is due to depletion of cholesterol and thereby depletion of lipid rafts. However, it is important to note that while statin use has been a common method to deplete cells of lipid raft structure for many years, the mechanism of action of statin drugs is not solely through the reduction of cholesterol. Statin treatment and consequent reduction of HMG-CoA reductase activity also inhibits protein prenylation. Indeed, previous studies have demonstrated that lovastatin can potentiate the effects of gefitinib (and *vice versa*) in squamous cell carcinoma, non-small cell lung cancer, colon carcinoma, and glioblastoma cell lines due to decreased protein prenylation (Mantha et al., 2003; Mantha et al., 2005; Cemeus et al., 2008; Park et al., 2009; Zhao et al., 2010). Specifically, in 2003 Mantha and colleagues combined gefitinib and lovastatin in head and neck cancer cell lines and found a synergistic interaction between these drugs due, at least in part, to protein prenylation (Mantha et al., 2003). This group later showed a synergistic interaction with this drug pairing in cervical and non-small cell lung cancers in addition to recapitulating their findings in head and neck cancer. In that manuscript, the effects of lovastatin are completely

attributed to protein prenylation (Mantha et al., 2005). Further, researchers have described such an interaction between lovastatin and gefitinib in glioblastoma and non-small cell lung cancer, again attributing their effect to protein prenylation (Cemeus et al., 2008; Park et al., 2009). Most recently, Zhao and colleagues have proposed that EGFR dimerization is inhibited by treatment with lovastatin, an effect dependent on aberrant prenylation of RhoA (Zhao et al., 2010). While all of these groups show a functional interaction between lovastatin and gefitinib, they do not link EGFR localization to lipid rafts as a mechanism of this effect. We cannot completely eliminate the possibility that protein prenylation may play a role in the synergistic effect between lovastatin and gefitinib; however, we have clearly shown a role for the cholesterol lowering effect of lovastatin in such synergy, as the squalene monooxygenase inhibitor, NB-598 (which inhibits cholesterol biosynthesis downstream of the prenylation branches) was sufficient to sensitize EGFR TKI resistant breast cancer cells to gefitinib (Figs. 3.8 and 3.9). Taken together, these results suggest that the effects of lovastatin treatment in our study are due to cholesterol modulation and subsequent lipid raft impairment rather than decreased protein prenylation.

Here, we have shown that EGFR localizes to lipid rafts in EGFR expressing, EGFR TKI resistant, breast cancer cell lines. We have provided evidence that reducing cholesterol biosynthesis sensitizes these EGFR TKI resistant cells to the EGFR TKI gefitinib. We have demonstrated that cholesterol reducing drugs and gefitinib act synergistically to decrease cell viability in breast cancer cells that are resistant to EGFR TKI-induced growth inhibition. We have also shown evidence to suggest that cholesterol depletion, not protein prenylation, results in a synergistic effect with gefitinib in these cells. Mechanistically, while gefitinib effectively reduced MAPK phosphorylation in EGFR TKI resistant cell lines, Akt

phosphorylation persisted. Lovastatin was sufficient to abrogate this phosphorylation of Akt. As EGFR kinase activity is completely inhibited by gefitinib treatment in these cells (Mueller et al., 2008), we hypothesize that lipid rafts provide a platform by which EGFR interacts with other proteins to activate EGFR kinase-independent signaling pathways including the Akt pathway. Thus, as both statin drugs and gefitinib are well tolerated and approved for use in patients, the work herein provides rationale for further exploration of the combination of these drugs in breast cancers that are resistant to EGFR TKI-induced growth inhibition.

Chapter 4

4.1 Introduction

Breast cancer is divided into molecular sub-types that are defined by distinct genetic signatures (Perou et al., 2000; Sorlie et al., 2001). While targeted therapeutics have been approved for two of these sub-types (ER+ and HER2+ breast cancers), the basal-type of breast cancer has no currently approved targeted therapeutic. Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is over-expressed in 60% of basal-type breast cancers (Livasy et al., 2006; Siziopikou and Cobleigh, 2007). Such over-expression correlates with poor clinical outcome [reviewed in (Jardines et al., 1993)]. As such, EGFR is an attractive therapeutic target in breast cancer. Unfortunately, while EGFR-targeted therapeutics have shown efficacy in estrogen-receptor positive breast cancers (Polychronis et al., 2005; Guix et al., 2008; Cristofanilli et al., 2010), basal-type breast cancers, which are estrogen receptor-negative, fail to respond to EGFR inhibitors (Blagosklonny and Darzynkiewicz, 2003).

Our lab has recently described a mechanism of intrinsic resistance of breast cancer cells to EGFR tyrosine kinase inhibition (chapter 3), where EGFR localization to lipid rafts promotes EGFR-kinase independent survival signaling. To further characterize the mechanism by which lipid rafts mediate EGFR TKI resistance, we sought to identify other proteins that associate with EGFR within lipid rafts. c-Src, a non-receptor tyrosine kinase, is over-expressed in many cancer types, including breast cancer (Biscardi et al., 2000; Irby and Yeatman, 2000), where it enhances EGFR-dependent cellular functions, including DNA synthesis, protein tyrosine phosphorylation, cellular transformation, and tumor formation in

nude mice (Luttrell et al., 1988; Wilson et al., 1989; Wilson and Parsons, 1990; Chang et al., 1995; Maa et al., 1995; Boerner et al., 2005; Dimri et al., 2007). Recent data from our laboratory has described a role for c-Src in c-Met-dependent intrinsic resistance of a basal-type breast cancer cell line to EGFR inhibitors (Mueller et al., 2008). c-Src is over-expressed in many of the same cancer types as EGFR, including approximately 70% of breast cancers, and is known to be co-over-expressed with EGFR in a subset of breast tumors (Maa et al., 1995). Additionally, EGFR and c-Src co-overexpression in mouse fibroblasts and human mammary epithelial cells results in synergistic increases in tumorigenesis (Maa et al., 1995; Belsches et al., 1997; Ware et al., 1997; Biscardi et al., 1999a; Biscardi et al., 1999b; Wilde et al., 1999; Dimri et al., 2007). Furthermore, interaction between EGFR and c-Src results in EGFR kinase-independent signaling in breast cancer cell lines (Mueller et al., 2008).

In addition to its interaction with EGFR, the Src family of tyrosine kinases is well known to localize within lipid rafts (Liu et al., 1997; Furuchi and Anderson, 1998; Davy et al., 2000). c-Src, the prototypical member of this family, interacts with lipid rafts through N-terminal myristoylation and basic residues within the unique domain (Sigal et al., 1994). Localization of c-Src within lipid rafts has been described in neuronal, hematopoietic, and cervical and lung cancer cell lines (Shenoy-Scaria et al., 1993; Mukherjee et al., 2003; Kasai et al., 2005; Arcaro et al., 2007). Lipid rafts mediate c-Src signaling as well, including c-Src-dependent activation of PI3K/Akt signaling in human small cell lung cancer (SCLC) cell lines (Arcaro et al., 2007).

The data herein shows that c-Src co-localizes and co-associates with EGFR in lipid rafts in SUM159 breast cancer cells. Co-inhibition of c-Src and EGFR kinase activities resulted in a synergistic decrease in cell viability. c-Src kinase inhibition abrogated EGFR-kinase

independent Akt phosphorylation, and was additive in combination with lovastatin, results that suggest that these inhibitors work on the same pathway. Thus, c-Src is a portion of the mechanism by which lipid rafts mediate EGFR kinase-independent signaling in SUM159 cells.

4.2 Results

A. c-Src localizes to lipid rafts in SUM159 cells

In chapter 3, we show that in SUM159 cells, an EGFR TKI resistant breast cancer cell line, EGFR localized to lipid rafts . Also, depletion of lipid rafts using cholesterol inhibitors sensitized SUM159 cells to the EGFR TKI gefitinib (chapter 3). To determine the mechanism by which EGFR localization to lipid rafts mediates EGFR TKI resistance, we wanted to identify other signaling components present in lipid rafts. EGFR and c-Src have been shown to functionally interact and c-Src associates with lipid rafts (Maa et al., 1995; Furuchi and Anderson, 1998; Davy et al., 2000; Liu et al., 2007); therefore, we hypothesized that c-Src co-localized with EGFR in lipid rafts of SUM159 cells. Using biochemical raft isolation techniques described previously, density fractions were collected from SUM159 and SUM149 (a breast cancer cell line that does not express EGFR in lipid rafts) cell lines. Fractions 1-7 were determined to be lipid raft fractions due to absence of transferrin receptor, presence of flotillin protein (Fig. 4.1A), and the lipid raft specific ganglioside GM1 (data not shown) (Wolf et al., 1998). Interestingly, in SUM159 cells, significantly more c-Src was present in fractions 1-7 than in SUM149 cells when quantified by densitometry (Fig. 4.1B; p-value < 0.05).

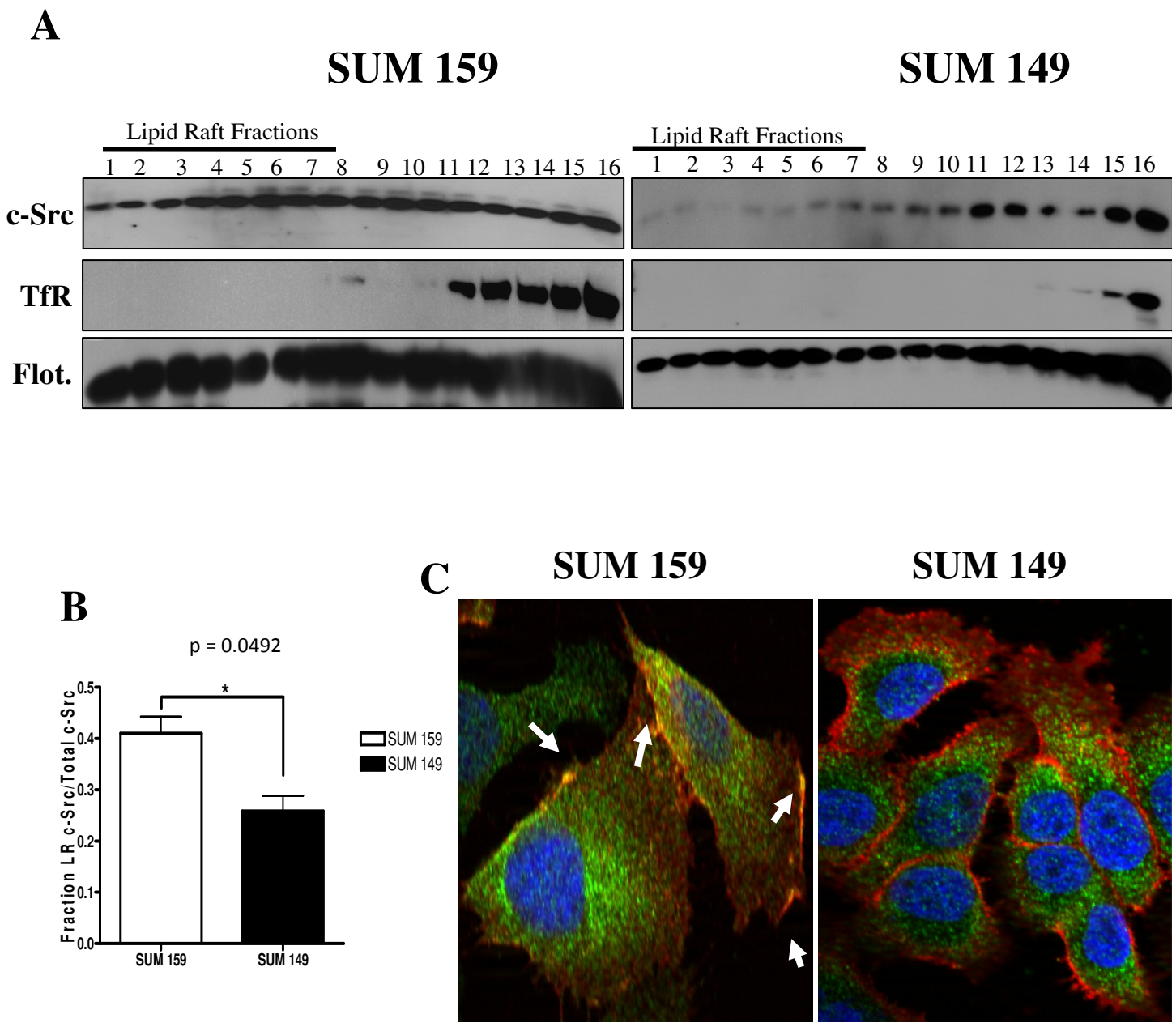


Figure 4.1: c-Src localizes within lipid rafts in SUM159 breast cancer cells. (A) SUM159 or SUM149 cells were plated, cultured for 72 h. Lipid rafts were separated by ultracentrifugation and immunoblotting was performed for c-Src, transferrin receptor and flotillin. Lanes numbered 1-7 indicate lipid raft fractions (Macdonald and Pike, 2005). (B) Densitometry was performed on c-Src immunoblots from A. Bars represent the percent of total c-Src in lipid raft fractions (1-7) compared to the total c-Src (fractions 1-14). Fractions 15 and 16 were excluded as pellet fractions. Experiments were repeated at least three times. p-value was calculated by comparing the percent of c-Src in lipid rafts in SUM159 cells compared to SUM159 cells using the student's t-test. (C) Two hundred thousand cells were plated onto coverslips and cultured under normal growth conditions for 48 h. Coverslips containing cells were then incubated with Alexa-fluor 594 labeled cholera toxin subunit B (red) to stain lipid rafts, fixed, permeabilized, blocked, and then stained for c-Src utilizing 2-17 and an alexa-fluor 488 labeled secondary antibody (green). DAPI was used to stain the nucleus (blue). Imaging was performed using Zeiss Axioplan2 apotome microscope fitted with 63X 1.25 oil immersion lens at the Microscopy and Imaging Resources Laboratory (Wayne State University, Detroit, MI). Arrows represent lipid raft localized c-Src.

To determine if c-Src localizes to plasma membrane lipid rafts, SUM159 cells were co-immunostained with Alexafluor labeled cholera toxin subunit B (red) which binds to GM1, and with c-Src antibodies (green; Fig. 4.1C). Co-localization between GM1 and c-Src occurred along the membrane of SUM159 cells (yellow, arrows), while staining of c-Src alone (green) was seen intracellularly. No co-localization was observed between GM1 and c-Src in SUM149 cells. Taken together, these data further confirm the association of c-Src with lipid rafts and define the localization of this association to be at the plasma membrane.

B. EGFR and c-Src co-localize in SUM159 cells

Although EGFR and c-Src proteins both localize to lipid rafts in SUM159, it was unknown if the proteins co-associate within the same lipid raft domain. Thus, to determine if c-Src and EGFR co-localize, SUM159 and SUM149 breast cancer cells were immunostained with Alexafluor labeled EGFR (green) and c-Src (red) antibodies (Fig. 4.2). The merged image shows co-localization of EGFR and c-Src (yellow) intracellularly in both cell lines, however; co-localization in patchy areas of the membrane (yellow, arrows) occurs solely in SUM159 cells. These results suggest that c-Src and EGFR co-localize in both SUM159 and SUM149 cells, but only co-localize at the plasma membrane in SUM159 cells.

Physical interaction between EGFR and c-Src has been shown in a number of cell types (Maa et al., 1995; Tice et al., 1999). Activation of EGFR increases c-Src catalytic activity (Sato et al., 1995a; Sato et al., 1995b; Weernink and Rijksen, 1995), and c-Src can, in turn, phosphorylate EGFR, leading to increased activity of downstream pathways that promote mitogenesis (Maa et al., 1995; Biscardi et al., 1999a; Tice et al., 1999). In order to determine if EGFR and c-Src physically interact in breast cancer cell lines, co-immunoprecipitation was

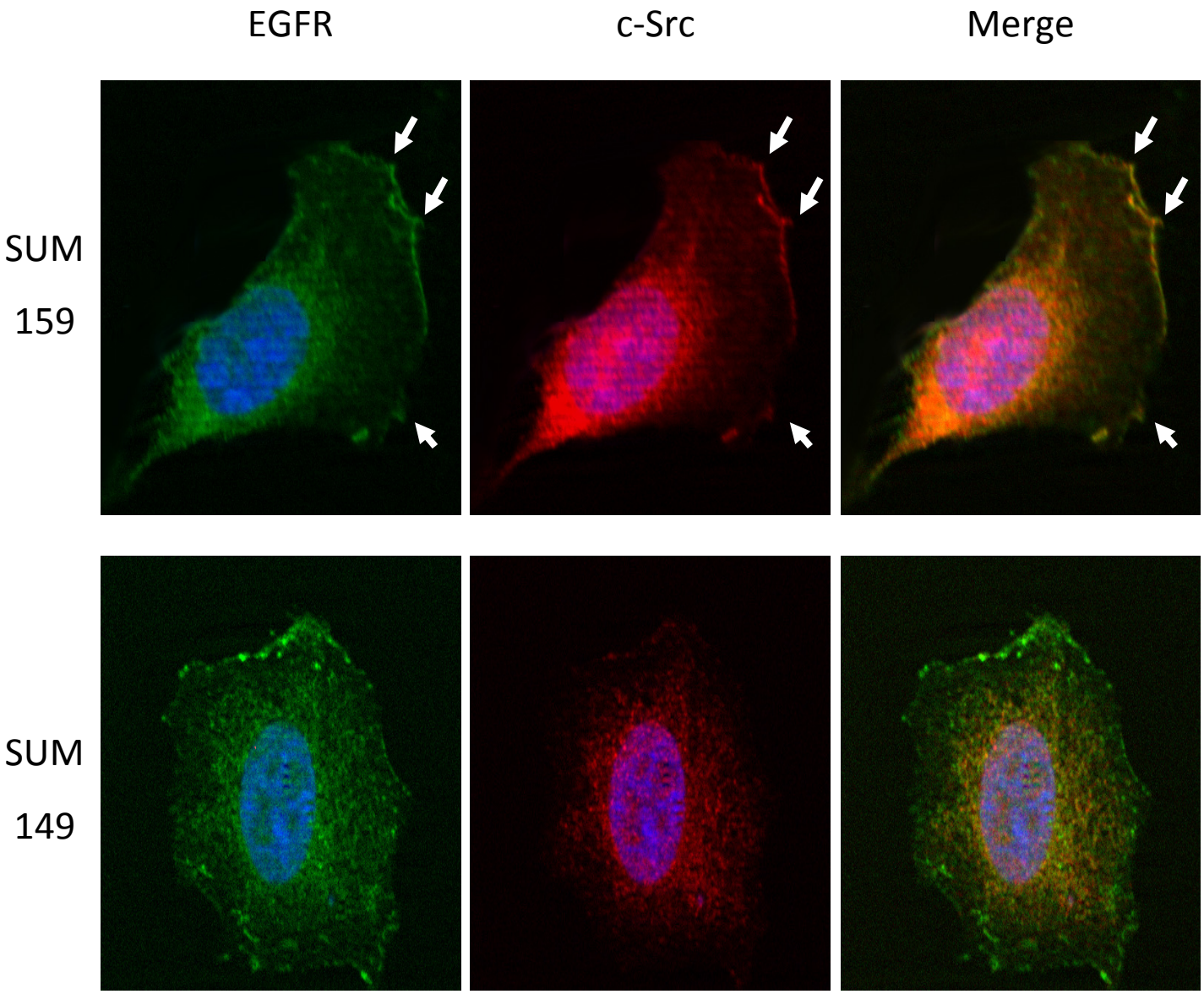


Figure 4.2: EGFR and c-Src co-localize in SUM159 and SUM149 cells. Two hundred thousand cells were plated onto coverslips and cultured under normal growth conditions for 48 h. Coverslips containing cells were blocked, and then incubated with fluorphore-labelled antibodies (EGFR-488: green; Src-594: red). The nucleus was stained with DAPI (blue). Imaging was performed using Zeiss Axioplan2 apotome microscope fitted with 63X 1.25 oil immersion lens at the Microscopy and Imaging Resources Laboratory (Wayne State University, Detroit, MI). Arrows indicate areas of co-localization (yellow).

performed. Whole cell lysate (WCL) was utilized as a positive immunoblotting control (Fig. 4.3A and B, lane 1), while mouse immunoglobulin immunoprecipitation was performed as a negative immunoprecipitation control (IgG, Fig. 4.3A and B lane 2). We have previously shown that c-Src does not co-immunoprecipitate with EGFR from SUM149 cells (Mueller et al., 2010). However, in SUM159 cells, when EGFR was pulled down from whole cell lysates, c-Src co-immunoprecipitated (Fig. 4.3A, lane 3). When the reverse immunoprecipitation was performed, EGFR co-immunoprecipitated with c-Src (Fig. 4.3B, lane 3). These data support the co-localization results demonstrating that EGFR and c-Src are localized to similar regions in SUM159 cells.

The immunofluoresence data from the SUM159 cells suggests that EGFR and c-Src co-associate at the plasma membrane, and even more specifically within lipid rafts, in the SUM159 cells. Therefore, biochemical raft isolation was performed and equal sample volumes from fractions 1-7 were pooled and EGFR or c-Src was immunoprecipitated. Samples were then subjected to SDS-PAGE followed by immunoblotting for EGFR and c-Src. When EGFR was pulled down from lipid raft fractions of SUM159 cells, c-Src co-associated (Fig. 4.3C, lane 3). Low levels of EGFR were also precipitated from non-lipid raft fractions (Fig. 4.3C, lane 4). When the reverse precipitation was performed, EGFR and c-Src were again both found immunoprecipitated from lipid raft fractions (Fig. 4.3C, lane 5). These data suggest that EGFR and c-Src physically interact within the lipid rafts of SUM159 cells.

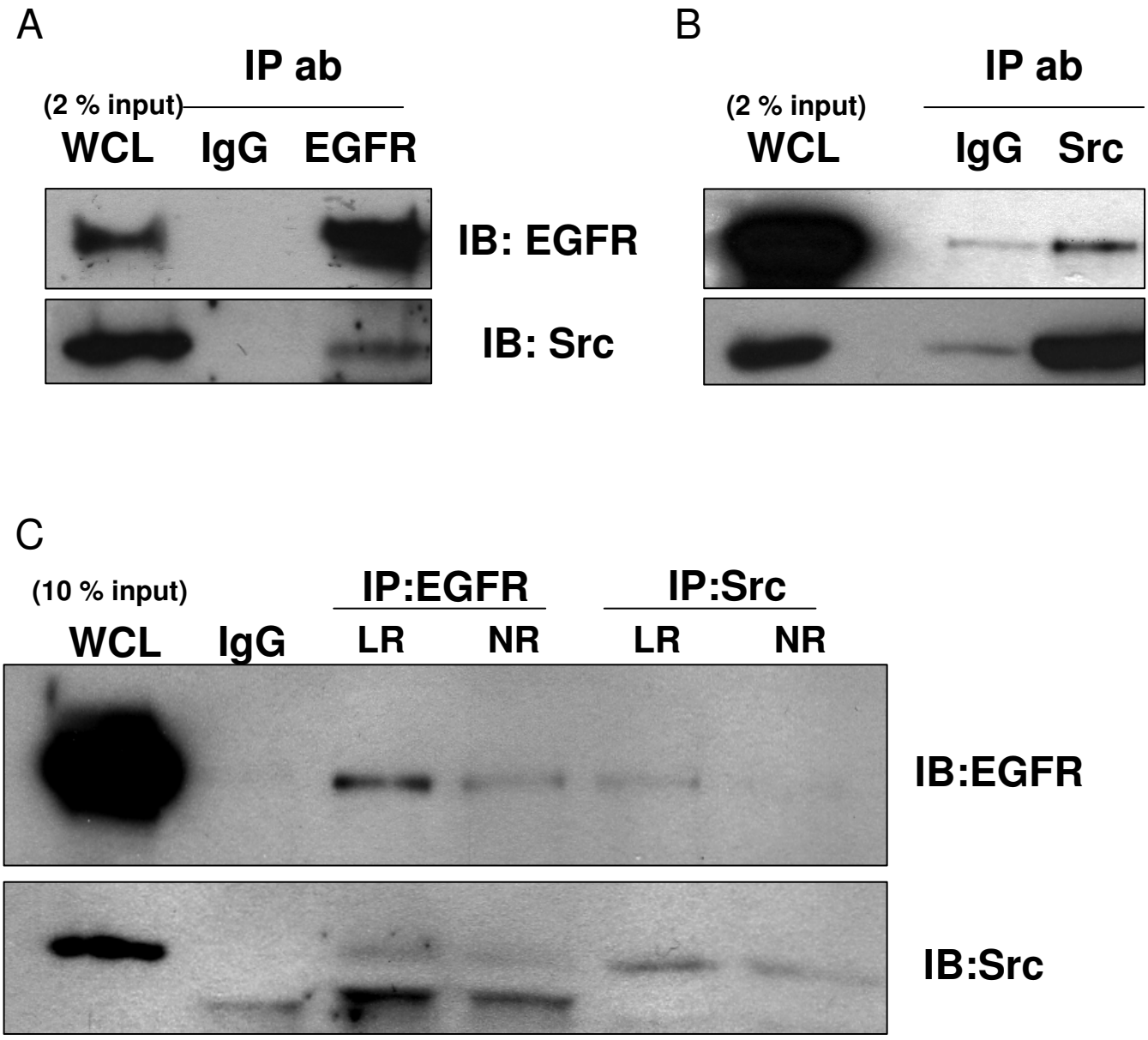
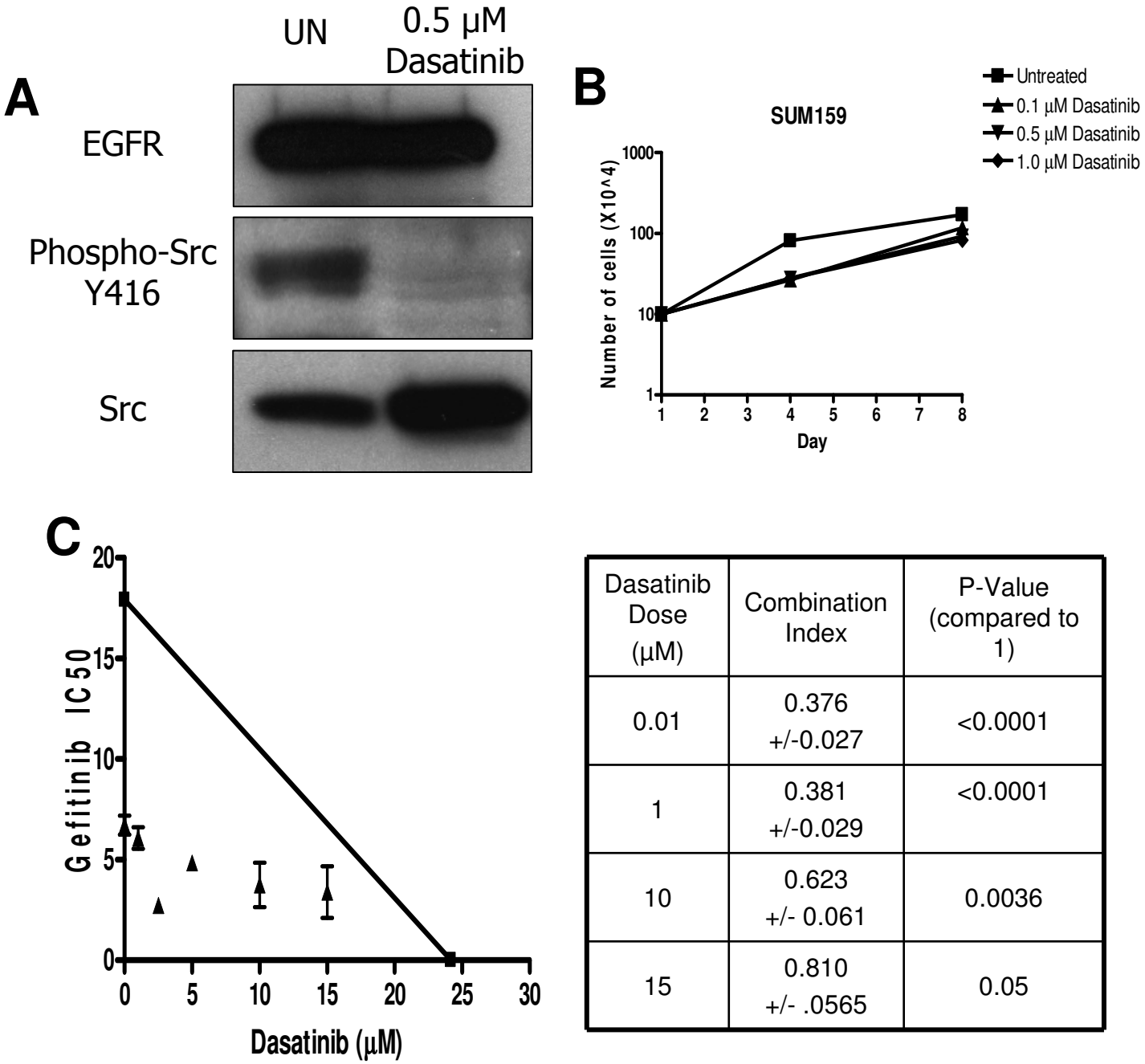


Figure 4.3: EGFR and c-Src co-associate within lipid rafts in SUM159 cells. (A and B) SUM159 cells were plated, allowed to grow for 48 h, and then lysed. Five hundred micrograms of protein was immunoprecipitated with EGFR antibodies (A) or c-Src antibodies (B), then subjected to SDS-PAGE and immunoblotting for EGFR and c-Src. Ten micrograms (2% input) of whole cell lysate (WCL) was utilized as a positive control and mouse IgG immunoprecipitation was performed as a negative control (IgG). (C) Lipid rafts were isolated from SUM159 cells by biochemical raft isolation as described. Two hundred microliters of each fraction (fractions 1-7 (LR), and fractions 8-14 (NR)) were pooled, and EGFR or c-Src was immunoprecipitated. Immunoblotting was then performed for EGFR and c-Src. Ten micrograms of whole cell lysate (WCL, 10% input) was utitilized as a positive control, while immunoprecipitation with IgG was utilized as a negative control. Immunoblots were performed at least three times.

C. EGFR and c-Src kinase inhibitors synergistically inhibit cell viability when both EGFR and c-Src are localized to lipid rafts

Small molecule tyrosine kinase inhibitors of c-Src are currently approved and in use in cancer treatment. Specifically, dasatinib, a c-Src TKI, was approved in 2006 for the treatment of leukemia (Brave et al., 2008), and is currently in clinical trials in a number of solid tumor types including breast cancer (Laird et al., 2003; Kim et al., 2010). Tyrosine phosphorylation of c-Src on tyrosine 416 is required for activation of c-Src kinase (Sun et al., 1998; Boerner et al., 2004). Thus, to determine if dasatinib inhibits c-Src kinase activity in our model system, phosphorylation of c-Src on tyrosine 416 was measured. Cells were treated with 0.5 μM dasatinib for 2 h and lysates were immunoblotted with phospho-tyrosine 416 specific antibodies. As shown in figure 4.4A, phosphorylation of c-Src on tyrosine 416 was abrogated with dasatinib treatment.

Preclinically, dasatinib treatment results in decreased cell growth in some breast cancer cell lines (Laird et al., 2003; Kim et al., 2010). To determine if inhibition of c-Src kinase activity was sufficient to decrease SUM159 cell growth, cellular proliferation assays were performed. SUM159 cells were treated with increasing doses of dasatinib and proliferation was measured by cell counting on days 1, 4, and 8. As seen in figure 4.4B, SUM159 cells continued to proliferate in the presence of all doses of dasatinib tested. As mentioned previously, SUM159 cells also continue to proliferate in the presence of the EGFR TKI gefitinib. Therefore, to determine if concomitant treatment with dasatinib and gefitinib could alter cell viability of SUM159 cells, MTS analyses were performed. Cells were treated with dasatinib and gefitinib alone, or in combination for 72 h. Cell viability was used to calculate



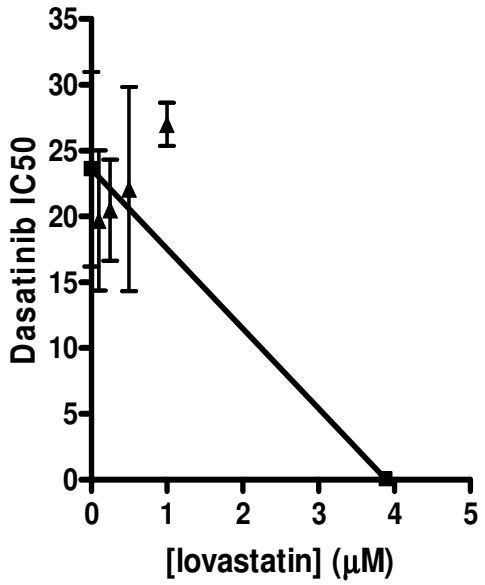
Dasatinib Dose (μM)	Combination Index	P-Value (compared to 1)
0.01	0.376 +/-0.027	<0.0001
1	0.381 +/-0.029	<0.0001
10	0.623 +/- 0.061	0.0036
15	0.810 +/- .0565	0.05

Figure 4.4: Dasatinib sensitizes SUM159 cells to gefitinib. (A) SUM159 cells were treated with the indicated dose of dasatinib and lysates were subjected to SDS-PAGE followed by immunoblotting for EGFR, c-Src and phospho-Y416 c-Src. (B) Cells were plated in 35mm plates and grown for eight days. Treatment with the indicated doses of dasatinib occurred every other day and cell counts were performed on days 1, 4, and 8. (C) Cells were plated in a 96-well plate and treated with variable doses of dasatinib and gefitinib for 72 h. MTS analyses was then performed. The IC_{50} of gefitinib at each dose of dasatinib was calculated and plotted on an isobologram. IC_{50} values below the line denote a synergistic interaction between the two drugs, while on the line indicates an additive interaction. The combination index (CI-value) was calculated as follows: (IC_{50} gefitinib at X dose of dasatinib)/(IC_{50} gefitinib alone) + (dose of dasatinib)/(IC_{50} dasatinib alone). CI-values below one denote a synergistic interaction between the two drugs, CI-values equal to one indicate an additive interaction, and CI-values above one designate an antagonistic interaction. All experiments were performed at least three times. p-values were calculated as a difference between CI-values and one utilizing the student's t-test.

IC_{50} values and isobolograms were generated to determine drug synergy (Fig. 4.4C). The IC_{50} of gefitinib alone was plotted on the y-axis and the IC_{50} of dasatinib alone was plotted on the x-axis. A line was drawn between them. This line represents the dose pairing that suggests an additive relationship between two drugs. The IC_{50} of gefitinib was calculated at various doses of dasatinib and these data were plotted on the graph. IC_{50} points that fall on the line are considered additive, while below the line is considered synergistic. When these points were plotted, the IC_{50} of gefitinib at each dose of dasatinib fell below the line of additivity indicating that the effects of gefitinib and dasatinib are synergistic. Combination index (CI) values were also determined for co-treatment of dasatinib and gefitinib. At all doses of dasatinib, the CI-value was significantly below 1 ($p<0.05$), which indicates that gefitinib and dasatinib have a synergistic interaction to reduce the viability of SUM159 breast cancer cells.

D. Inhibition of c-Src kinase activity and depletion of lipid rafts is additive

Previous data have indicated that lovastatin, a cholesterol biosynthesis inhibitor, could synergize with gefitinib (chapter 3). This is hypothesized to be due to depletion of lipid rafts after cholesterol inhibition. The data presented so far indicate that c-Src localizes to these lipid raft microdomains in SUM159 cells, where it co-associates with EGFR (Figs. 4.1-4.3). Therefore, we hypothesized that localization of c-Src to lipid rafts is a mechanism by which lipid rafts mediate EGFR TKI resistance in SUM159 cells. If this is the case, then treatment of SUM159 cells with dasatinib and lovastatin would not be synergistic, as they would inhibit the same pathway. Thus, SUM159 cells were treated with dasatinib in combination with lovastatin and cell viability analyses were performed. An isobologram was then created as previously described. As shown in figure 4.5, the effect of dasatinib and lovastatin co-



Lovastatin Dose (μM)	Combination Index	P-Value (compared to 1)
0.1	0.862 +/- 0.227	0.574
0.25	0.933 +/- 0.163	0.701
0.5	1.065 +/- 0.329	0.854

Figure 4.5: Dasatinib and lovastatin are additive in SUM159 cells. Cells were placed in a 96-well plate and treated with variable doses of dasatinib and lovastatin for 72 h. MTS analyses were then performed. The IC_{50} of dasatinib at each dose of lovastatin was calculated and plotted on an isobologram. IC_{50} values below the line denote a synergistic interaction between the two drugs, while on the line indicates an additive interaction. The combination index (CI-value) was calculated as follows: (IC_{50} dasatinib at X dose of lovasatin)/(IC_{50} dasatinib alone) + (dose of lovastatin)/(IC_{50} lovastatin alone). CI-values below one denote a synergistic interaction between the two drugs, CI-values equal to one indicate an additive interaction, and CI-values above one designate an antagonistic interaction. All experiments were performed at least three times. p-values were calculated as a difference between CI-values and one utilizing the student's t-test.

treatment of SUM159 cells was additive, as the IC_{50} of dasatinib at varying doses of lovastatin fell along the line of additivity. When CI-values were calculated, the CI-values for the combination of dasatinib and lovastatin were not significantly different from one, indicating, again, that these two drugs act additively. This additive interaction suggests that dasatinib and lovastatin act on the same pathway.

E. c-Src kinase activity mediates EGFR kinase-independent Akt phosphorylation

In 2007, Arcaro and colleagues found that lipid raft associated c-Src was critical for the activation of the PI3K/Akt signaling pathway in SCLC cell lines (Arcaro et al., 2007). Their results suggested that lipid rafts provided a platform for interaction between c-Src and PI3K. To determine if lipid rafts may also provide such a platform in SUM159 breast cancer cells, biochemical raft isolation was performed followed by immunoblotting for p110α (Fig. 4.6A), the catalytic subunit of PI3K [reviewed in (Vanhaesebroeck et al., 2010)]. While the SUM149 cells contained little p110α in lipid rafts, there was a significant amount of p110α in the lipid raft fractions of SUM159 cells. Thus, it is possible that PI3K signaling occurs downstream of the EGFR and c-Src co-association in lipid rafts within the SUM159 cells.

We have previously shown that depletion of lipid rafts results in decreased EGFR-kinase independent Akt phosphorylation (chapter 3). c-Src is known to be a downstream mediator of EGFR signaling pathways, including Akt and MAPK (Stover et al., 1995; Biscardi et al., 1999a; Tice et al., 1999). Thus, to determine if c-Src mediates EGFR kinase-independent signaling in SUM159 cells, lysates were immunoblotted for phosphorylation of Akt and MAPK following treatment with gefitinib or dasatinib alone, or in combination (Fig. 4.6B).

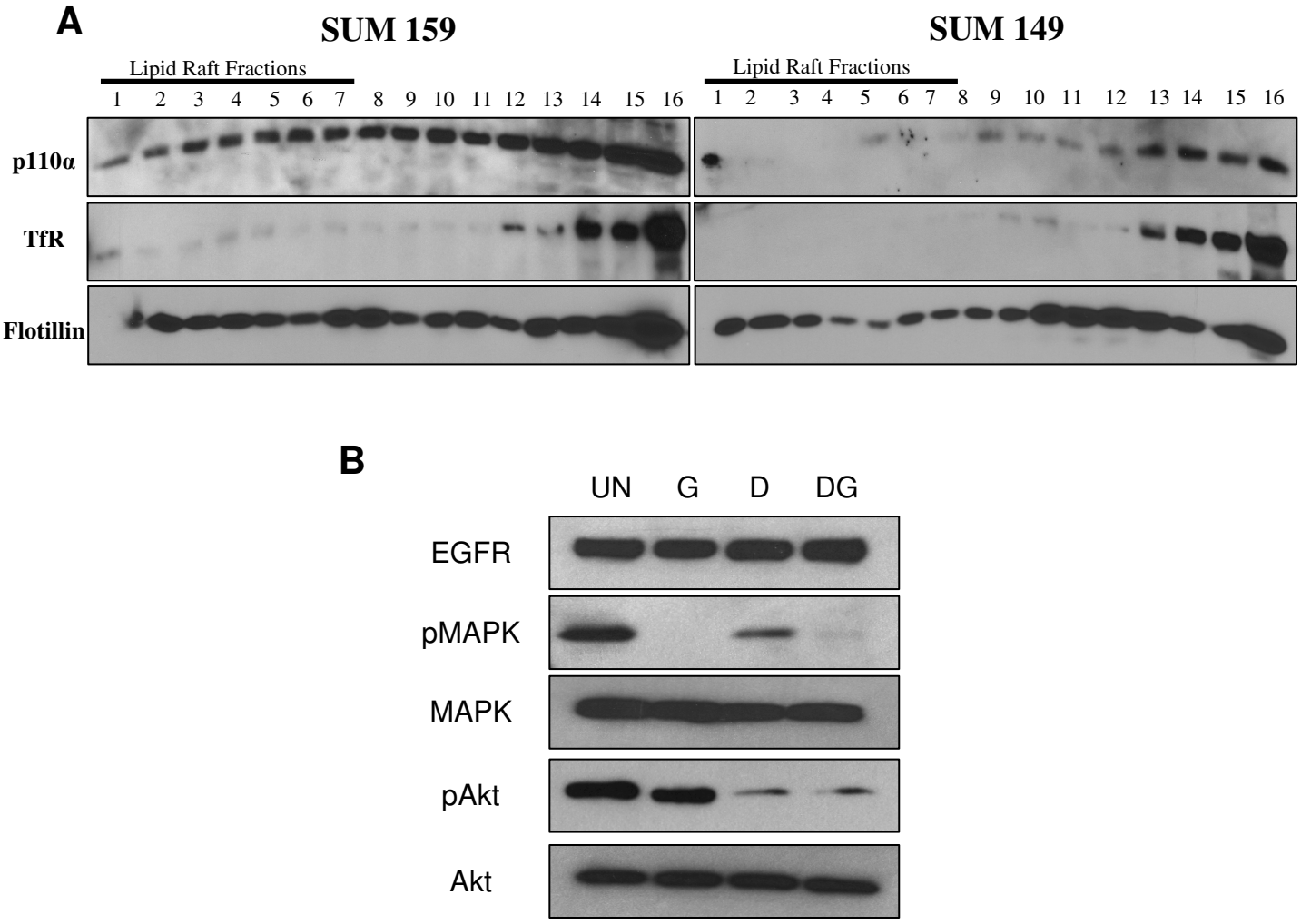


Figure 4.6: Phosphorylation of Akt occurs through a c-Src/p110α co-localization to lipid rafts. (A) Biochemical raft isolation was performed as described on SUM159 and SUM149 cells. Fractions were separated by SDS-PAGE and immunblotting for p110α, transferrin receptor, and flotillin was performed. (B) Whole cell lysates (right) were collected from SUM159 cells treated with 1.0 μM gefitinib (G) and 1.0 μM dasatinib (D) alone or in combination (DG). Expression and phosphorylation of Akt and MAPK, as well as expression of EGFR was determined by immunoblotting. Immunoblots were repeated at least three times.

As shown previously, gefitinib effectively abrogates MAPK phosphorylation in SUM159 cells, but Akt phosphorylation persists (Figure 4.6B, lane 2, and Irwin et Al., under review). c-Src kinase inhibition by dasatinib treatment resulted in a small decrease of MAPK phosphorylation, and was sufficient to decrease Akt phosphorylation in SUM159 cells (Fig. 4.6B, lane 3). Co-treatment with gefitinib and dasatinib resulted in no additional decrease in MAPK or Akt phosphorylation. These data suggest that c-Src mediates EGFR kinase-independent Akt phosphorylation in SUM159 cells.

4.3 Discussion

This study provides evidence that c-Src plays a role in lipid raft-dependent EGFR TKI resistance of SUM159 cells. Specifically, we have shown that c-Src localizes to lipid rafts in the SUM159 cells, where it co-localizes and co-associates with EGFR. The effects of inhibiting both EGFR and c-Src tyrosine kinase activities was synergistic in these cells. Also, we have demonstrated that dasatinib and lovastatin treatments were additive, suggesting that they inhibit members of the same biological pathway. Lastly, we have shown that a catalytic subunit of PI3K (p110α) co-localizes with EGFR and c-Src to lipid rafts in SUM159 cells and that inhibition of both EGFR and c-Src kinase activities decreased both Akt and MAPK phosphorylation. Taken together, these data have further characterized a mechanism for lipid rafts mediating EGFR TKI resistance by showing that c-Src is active and present in lipid rafts where it has the ability to regulate PI3K/Akt survival signals.

Src family kinases have been described as being capable of localizing to lipid rafts (Liu et al., 1997; Furuchi and Anderson, 1998; Davy et al., 2000). While other family members are likely to associate preferentially to lipid rafts through their double lipid modification, c-Src is

only singly lipid modified (Paige et al., 1993; Koegl et al., 1994; Shenoy-Scaria et al., 1994; Robbins et al., 1995; Yurchak and Sefton, 1995). However, c-Src localization to lipid raft still occurs through interactions between the lipid environment and basic residues within the unique domain of c-Src (Sigal et al., 1994). c-Src has been shown to localize to lipid rafts in neuronal (Mukherjee et al., 2003; Kasai et al., 2005), hematopoietic (Stoddart et al., 2002), and madin-darby canin kidney cells (Shenoy-Scaria et al., 1994), as well as skeletal myoblasts (Smythe et al., 2003), murine fibroblasts (Robbins et al., 1995), and cervical and lung cancer cell lines (Shenoy-Scaria et al., 1994; Arcaro et al., 2007). Specifically, in breast cancer cells, Hitosugi and colleagues have shown that adhesion and growth is inhibited by lipid raft specific knockdown of c-Src (Hitosugi et al., 2007), suggesting that lipid rafts promote c-Src dependent cellular signaling to pathways of cellular adhesion and growth. We have shown that c-Src localizes to lipid rafts in SUM159 breast cancer cells (Fig. 4.1). Thus, it is possible that c-Src may mediate mitogenic signaling in these cells.

We have identified an interaction between EGFR and c-Src within lipid raft membrane microdomains in breast cancer cells (Figs. 4.1-4.3). Physical interaction between these two proteins has been noted previously. Specifically, Maa and colleagues showed an initial physical interaction between EGFR and c-Src in a murine fibroblast model which was engineered to over-express both EGFR and c-Src (Maa et al., 1995). In this model system, c-Src potentiated EGFR-mediated transformation through direct physical interaction with EGFR. c-Src was also co-immunoprecipitated as part of a complex between EGFR and c-Met in SUM229 breast cancer cells (Mueller et al., 2008; Mueller et al., 2010). This interaction was described to mediate EGFR TKI resistance of SUM229 cells. We have shown that EGFR and c-Src physically associate in SUM159 breast cancer cells (Fig. 4.3A

and B). More specifically, we have shown that a physical interaction occurs between these two proteins within lipid rafts (Fig. 4.3C). While previous studies have revealed that these two proteins are both capable of localizing to lipid rafts in the same cell line (Hur et al., 2004; Kasai et al., 2005; Jeon et al., 2010), the actual physical association within these lipid raft domains has not been previously shown.

Our data indicate the concomitant inhibition of c-Src and EGFR kinase activities may result in decreased cell viability of breast cancer cell lines. In particular, we have shown that while SUM159 cells are resistant to inhibition of either EGFR (chapter 3) and c-Src kinase activities (Fig. 4.4B), co-treatment with dasatinib and gefitinib resulted in synergistic decreases in cell viability (Fig. 4.4C). It is not surprising that co-treatment resulted in decreased cellular viability in breast cancer cells. First, c-Src is over-expressed in many of the same cancer types as EGFR, including approximately 70% of breast cancers, and is known to be co-over-expressed with EGFR in a subset of breast tumors (Maa et al., 1995). Additionally, EGFR and c-Src co-overexpression in mouse fibroblasts and human mammary epithelial cells results in synergistic increases in tumorigenesis (Maa et al., 1995; Belsches et al., 1997; Ware et al., 1997; Biscardi et al., 1999a; Biscardi et al., 1999b; Wilde et al., 1999; Dimri et al., 2007). Thus, it stands to reason that these two proteins together may play an important role in mitogenic signaling in breast cancer cells. Also, data shown in other cancer types suggest that dual inhibition of EGFR and c-Src kinase activities decreased cancer cell viability. In particular, Johns and colleagues have demonstrated that down regulation of c-Src expression improved the response of gliomas to EGFR monoclonal antibodies (Johns et al., 2007). Also, decreased c-Src kinase activity results in increased sensitivity to EGFR inhibitors in head and neck cancer and epidermoid carcinoma cell lines (Koppikar et al.,

2008; Andersen et al., 2009). A recent Phase I/II study has also been published utilizing dasatinib in combination with the EGFR TKI erlotinib in non-small cell lung cancer (Haura et al., 2010). Not only was this combination tolerable in patients, but disease control was observed as well. Thus, our data suggest that combinatorial inhibition of EGFR and c-Src kinase activities may result in the decrease of the viability of breast cancer cells.

It is thought that lipid rafts play a dual role in c-Src signaling. Lipid rafts are also important in mediation of the c-Src dependent activation of PI3K/Akt signaling in human SCLC cell lines (Arcaro et al., 2007) and in the activation of FAK to promote early contact signaling in cells (Baillat et al., 2008). Also, c-Src trafficking and co-localization with EGFR promotes EGFR-ligand independent MAPK signaling (Donepudi and Resh, 2008). However, lipid rafts also provide a platform for Csk binding protien (Cbp) to bring Csk, an endogenous inhibitor of c-Src, in close proximity to its substrate, which, in turn, results in down regulation of c-Src activity (Torgersen et al., 2001). We have shown evidence that lipid rafts promote c-Src-dependent signaling in SUM159 breast cancer cells. In particular, we suggest that lipid raft localized c-Src plays a role in EGFR-kinase independent signaling seen in these cells. Inhibition of c-Src kinase activity with dasatinib results in abrogation of EGFR-kinase independent Akt phosphorylation (Fig. 4.6B).

Previous studies have suggested that lipid rafts also mediate EGFR-kinase independent Akt phosphorylation in this cell line (chapter 3). We have utilized synergy analyses to determine if lipid raft localized c-Src is responsible for Akt phosphorylation in these cells. Specifically, we hypothesized that if c-Src was mediating the effects seen downstream of lipid rafts in these cells, that concomitant inhibition of c-Src kinase activity and cholesterol biosynthesis (which results in reduction of lipid raft levels), would not be synergistic.

Indeed, co-treatment with dasatinib and lovastatin was additive in SUM159 cells (Fig. 4.5), suggesting that these two inhibitors are working together to down-regulate the same pathway. Thus, these data together suggest that lipid raft localized c-Src mediates EGFR-kinase independent Akt phosphorylation in SUM159 cells. Furthermore, the co-localization of p110α, EGFR, and c-Src within lipid rafts in SUM159 cells (Fig. 4.6A) suggests that lipid rafts provide a platform for interaction between these molecules, leading to EGFR kinase-independent activation of PI3K/Akt signaling.

The results described here suggest a model for the activation of EGFR-dependent signaling pathways independent of EGFR-kinase activity. Specifically, we have shown previously that lipid rafts play a role in such a pathway (Irwin et Al. under review). Here, we have described a role for the non-receptor tyrosine kinase c-Src in the activation of EGFR-kinase independent Akt signaling in the EGFR TKI resistant SUM159 breast cancer cell line. c-Src co-localized with EGFR in plasma membrane lipid rafts in SUM159 cells. This co-localization allowed the interaction between EGFR, c-Src, and PI3K, leading to EGFR-kinase independent Akt phosphorylation. The effects of dasatinib, a small molecule c-Src kinase inhibitor, and gefitinib, an EGFR TKI, were synergistic in these cells. Dasatinib is currently in clinical trials in solid tumors (Laird et al., 2003; Kim et al., 2010), and therefore may be useful in combination with EGFR TKIs for breast cancers that are resistant to EGFR-directed therapeutics alone.

Chapter 5

5. Conclusions

The work herein provides evidence of a role for lipid rafts in EGFR TKI resistance. We have found that EGFR localization to lipid rafts correlates with EGFR TKI resistance. We have shown that depletion of cholesterol through the use of cholesterol biosynthesis inhibitors sensitizes breast cancer cells that are resistant to EGFR TKI-induced growth inhibition. Our data suggest that lipid rafts provide a platform for the interaction of EGFR with other proteins, including c-Src and PI3K, to promote signaling in the absence of EGFR kinase activity (Fig. 5.1). These findings provide rationale for the use of the cholesterol lowering drug lovastatin in combination with EGFR inhibitors in breast cancer.

As mentioned previously, breast cancer is one of the prevailing forms of cancer mortality in American women. Specifically, basal-type breast cancers have the worst clinical prognosis. The approval of targeted therapeutics for the treatment of basal-type breast cancers is of the utmost importance. Unfortunately, there is no currently approved targeted therapeutic for this sub-type of breast cancer, due to the general lack of estrogen, progesterone, and HER2 receptors. The epidermal growth factor receptor, a HER2 family member, is expressed in a large percentage of this sub-type of breast cancer, however; EGFR-targeted therapeutics have had limited success here. Thus, our research provides insight that may allow the use of these targeted agents in breast cancer patients afflicted with basal-type breast cancer.

The work in this dissertation is not without its limitations. Primarily, gefitinib is no longer approved for clinical use in the United States, which may limit the ability to translate

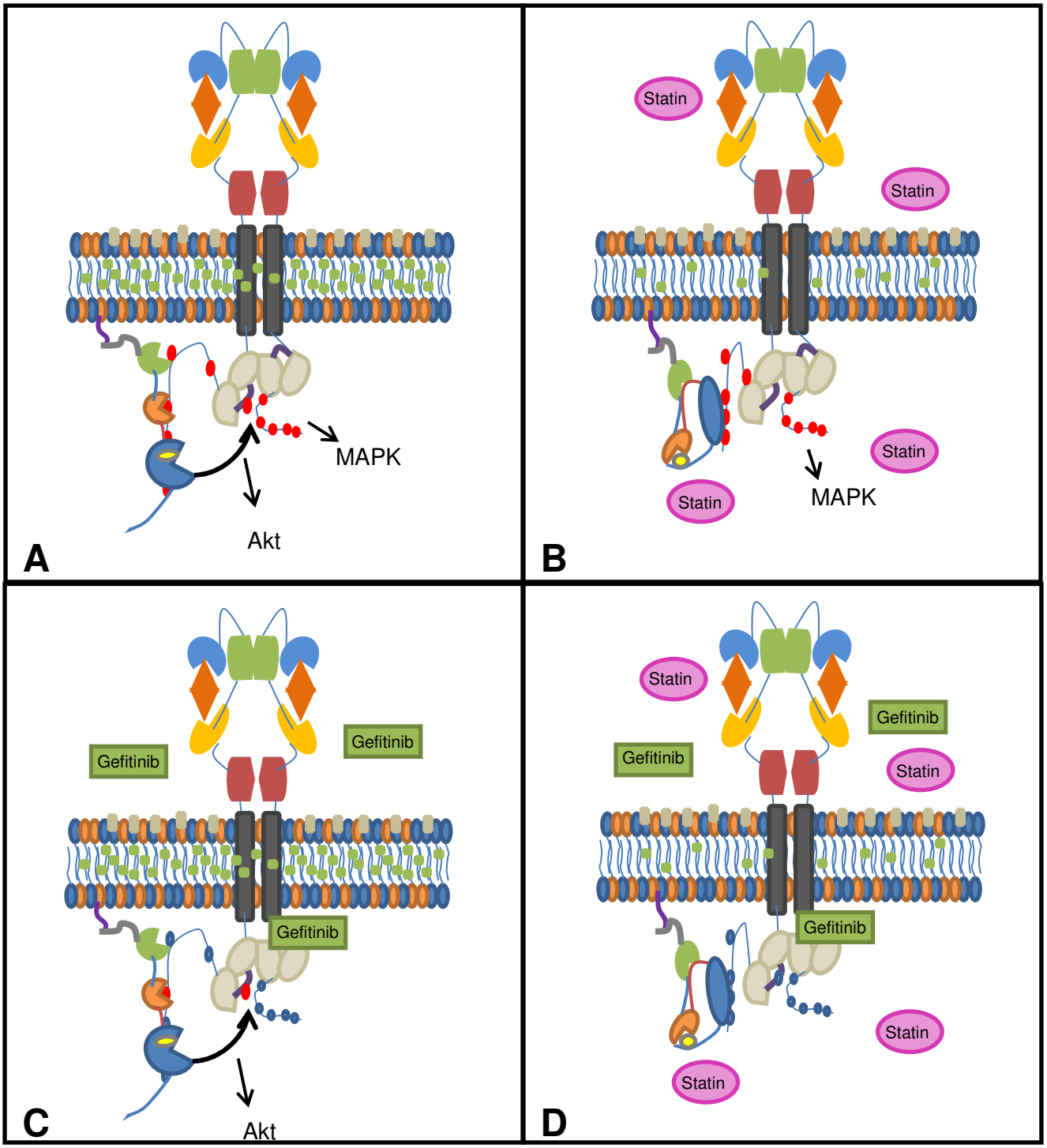


Figure 5.1: Co-localization of EGFR and c-Src to lipid rafts promotes EGFR kinase-independent siganling. (A) Under normal growth conditions, EGFR and c-Src co-localize and co-associate within lipid rafts, and Akt and MAPK are phosphorylated leading to cell survival and proliferation. (B) When cholesterol is depleted (Statin), Akt phosphorylation is decreased, but MAPK remains phosphorylated. (C) When EGFR kinase acitivity is inhibited (gefitinib), EGFR-kinase dependent MAPK phosphorylation is abrogated, however, Akt phosphorylation is maintained (D) When there is cholesterol is depleted in combination with EGFR kinase inhibition, both of these signaling pathways are blocked correlating with an inhibition of cellular proliferation.

this work into the clinical setting. Further studies utilizing other EGFR inhibitors, such as erlotinib, lapatinib, and cetuximab may strengthen the clinical significance of this work. In particular, combination studies looking at proliferation and viability of cells treated with the combination of these other EGFR inhibitors and cholesterol lowering drugs may provide further rationale for the use of these combinations in patients. Also, *in vivo* studies are required to move these results towards the clinical arena. For example, xenograft mouse models can be utilized to determine if the combination of EGFR inhibitors and cholesterol lowering drugs is a viable option to reduce tumor burden *in vivo*. With these types of data, and the knowledge that both EGFR inhibitors and cholesterol lowering drugs are well tolerated in patients, this work can be translated forward into phase I studies to determine proper combinatorial dosing.

EGFR is not the only protein that is known to localize within lipid rafts. Indeed, we have shown two other proteins, c-Src and p110α, that also localize to lipid rafts in basal breast cancers. The EGFR family member HER2 also is capable of localizing within lipid raft membrane microdomains (Menendez et al., 2005). HER2 positive breast cancers can currently be targeted by the monoclonal antibody trastuzumab, however, inevitably, resistance to trastuzumab occurs [reviewed in (Nahta and Esteva, 2006)]. Depletion of lipid rafts through inhibition of fatty acid synthase (FAS) has been found to overcome trastuzumab resistance in breast cancer (Menendez et al., 2005). These results suggest that inhibition of lipid rafts, through a variety of mechanisms, may be important in the resistance to EGFR family member directed therapeutics.

Recent data have implicated the EGFR family in resistance to estrogen receptor-directed therapeutics. In particular, levels of EGFR and HER2 are elevated in MCF7 cell lines that

have acquired resistance to tamoxifen (McClelland et al., 2001; Knowlden et al., 2003). Thus, lapatinib, a dual EGFR/HER2 inhibitor (described earlier), has been used in preclinical studies in an effort to overcome such resistance (Chu et al., 2005). However, our findings, and those of Menedez and colleagues, suggest that there may be resistance to this inhibitor due to elevated levels of lipid rafts in all breast cancer cells. Preclinical studies regarding the localization of EGFR and/or HER2 to lipid rafts in these tamoxifen-resistant cell lines may therefore be useful to determine if cholesterol lowering drugs may be useful in such a setting.

In general, while there is still more work to be done, the data in this dissertation moves the field forward towards a better understanding of the underlying mechanisms of resistance to EGFR tyrosine kinase inhibition in breast cancer. The use of cholesterol lowering drugs in combination with EGFR inhibitors may provide the targeted clinical therapy needed in basal-type breast cancers. It is our hope that the research described here will set the stage for the further study of the localization of proteins to lipid rafts, and how this localization may affect resistance to well developed targeted therapeutics in breast cancer.

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ABSTRACT

A ROLE FOR LIPID RAFTS IN EGFR TKI RESISTANCE IN BREAST CANCER

by

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Advisor: Julie Boerner, PhD

Major: Pharmacology

Degree: Doctor of Philosophy

Breast cancer can be divided into genetic sub-types including luminal, HER2+, and basal-type. With the introduction of targeted therapies against estrogen receptor and HER2 receptor mortality rates of American women with breast cancer have declined. Unfortunately, basal-type breast cancers, which have the worst clinical outcome, do not express estrogen receptor or HER2, and as such, have no targeted therapeutic option. The epidermal growth factor receptor is an attractive target for therapeutics in basal-type breast cancer, as it is over-expressed in 60% of these cases. Also, over-expression of EGFR correlates with poor patient prognosis. Unfortunately, inhibitors of EGFR have shown little clinical efficacy in basal-type breast cancers. We have utilized basal-type breast cancer cell lines to determine a potential mechanism of resistance to EGFR-targeted small molecule tyrosine kinase inhibitors (TKIs). Specifically, we have shown that EGFR localizes to discrete membrane microdomains (lipid rafts) in cell lines that are resistant to EGFR TKI-induced growth inhibition. Depletion of lipid rafts via cholesterol reduction results in sensitivity of EGFR TKI resistant breast cancer cell lines to the EGFR TKI gefitinib.

Importantly, the effects of cholesterol lowering drugs and EGFR TKI in combination were synergistic. We have shown that the non-receptor tyrosine kinase c-Src, which is co-over-expressed with EGFR in a subset of breast tumors, also localizes to lipid rafts in the SUM159 breast cancer cell line. In this model system, c-Src kinase inhibition results in synergistically decreased cell viability in combination with EGFR tyrosine kinase inhibition. c-Src kinase inhibition and cholesterol depletion are additive, results that suggest these two inhibitors work within the same pathway. Indeed, treatment with either cholesterol lowering drugs or c-Src kinase inhibitors results in decreased EGFR-kinase independent Akt phosphorylation. Thus, lipid rafts may provide a platform whereby EGFR and c-Src interact to promote Akt signaling in the absence of EGFR kinase activity. These results suggest, for the first time, that lipid rafts are involved in EGFR-kinase independent signaling, and that depletion of these rafts may work in combination with EGFR tyrosine kinase inhibition to decrease breast cancer cell growth.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

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GRANT FUNDING

NCI T32-CA009531 training grant 2009-2010 (P.I. Larry Matherly, PhD.)

PUBLICATIONS

Under Review

Irwin, M.E., Mueller, KL, Ge, Y, and Boerner, J.L., Depletion of cholesterol from lipid rafts sensitizes breast cancer cells to EGFR inhibitors.

In preparation

Irwin, M.E., Bohin, N., and Boerner, J.L., c-Src mediates EGFR kinase-independent signaling in a breast cancer cell line model.

SELECTED PRESENTATIONS

Posters

Graduate Student Research Day, Wayne State University, Irwin, M.E., Griffin, K.L., and Boerner, J.L., 2007. "EGFR/c-Src localization in breast cancer cells."

Karmanos Cancer Institute Research Day, Irwin, M.E. and Boerner, J.L., 2008. "EGFR/c-Src co-localization to membrane microdomains in breast cancer cells."

AACR Annual Meeting, Irwin, M.E. and Boerner, J.L., 2008. "EGFR/c-Src co-localization to membrane microdomains in breast cancer cells."

AACR Annual Meeting, Irwin, M.E. and Boerner, J.L., 2009, "Lipid raft localization of EGFR as a mechanism for EGFR TKI resistance."

Pharmacology Research Colloquium, Irwin, M.E. and Boerner, J.L., 2009. **"**Lipid raft localization of EGFR as a mechanism for EGFR TKI resistance."

AACR Annual Meeting, Irwin, M.E., Mueller, K.L., and Boerner, J.L., 2010, "Disruption of lipid rafts sensitizes EGFR expressing breast cancer cells to EGFR inhibitors."

Oral Presentations

Michigan Pharmacology Research Colloquium, Irwin, M.E. and Boerner, J.L., 2008. **"**EGFR/c-Src co-localization to membrane microdomains in breast cancer cells."

Michigan Pharmacology Research Colloquium, Irwin, M.E. Mueller, K.L., and Boerner, J.L., 2010. **"**Disruption of lipid rafts sensitizes EGFR expressing breast cancer cells to EGFR inhibitors."

COMMITEES

Graduate Student Research Day organizational committee 2006-2009

HONORS/AWARDS

Third Place – Karmanos Cancer Institute Research Day 2008
Wayne State University Graduate School Professional Travel Award 2009
First Place – Michigan Pharmacology Research Colloquium Oral Presentations 2010