

THERAPEUTIC POTENTIAL OF EGFR DERIVED PEPTIDES IN BREAST CANCER

by

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LIST OF ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
ADCC	antibody-dependent cellular cytotoxicity
AR	amphiregulin
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
bFGF	basic fibroblast growth factor
BTC	betacellulin
CaM	calmodulin
Cbl	Casitas B-lineage lymphoma
CoxII	cytochrome oxidase subunit II
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
Egr1	early growth response factor 1
EPR	epiregulin
Eps15	epidermal growth factor receptor substrate 15
ER	estrogen receptor
ER	endoplasmic reticulum

FBS	fetal bovine serum
FDA	Food and Drug Administration
HB-EGF	heparin-binding EGF
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HMGB1	high mobility group box 1
HNSCC	head and neck squamous cell carcinoma
Hsp	heat shock protein
IF	immunofluorescence
IHC	immunohistochemistry
IL-8	interleukin-8
INM	inner nuclear membrane
iNOS	inducible NO synthase
IP	immunoprecipitation
JAK	Janus kinase
MAPK	mitogen-activated protein kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
mRNA	messenger RNA
MVB	multivesicular bodies
NAC	N-acetyl cysteine
NPC	nuclear pore complex

NRG	neuregulin
NSCLC	non small cell lung cancer
ONM	outer nuclear membrane
ORR	overall response rate
PCNA	proliferating cell nuclear antigen
pI	isoelectric point
PI3K	phosphatidylinositol 3-kinase
PLC	phospholipase C
PNPase	polynucleotide phosphorylase
PTB	phosphotyrosine-binding
PR	progesterone receptor
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
RTK	receptor tyrosine kinase
SGLT1	sodium/glucose cotransporter 1
SH2	Src homology 2
STAT	signal transducers and activators of transcription
TGF- α	transforming growth factor- α
TNF α	tumor necrosis factor α
TKI	tyrosine kinase inhibitor
UIM	ubiquitin interacting motif
VEGF	vascular endothelial growth factor

VEGFR

vascular endothelial growth factor receptor

ABSTRACT

The epidermal growth factor receptor (EGFR) belongs to the erbB family of receptor tyrosine kinases which consists of four members (EGFR, ErbB2, ErbB3 and ErbB4). Upon ligand binding, the EGFR is capable of dimerization with other erbB receptors and propagates signals regulating a diverse array of cellular physiologies, including cell growth, migration and survival. Dysregulation of the EGFR is important for development and progression of different types of cancers, including breast cancer.

Breast cancer is the second leading cause of cancer death in women. EGFR overexpression has been observed in about 15% of all breast cancers. Moreover, in triple negative breast cancer (TNBC), which is a more aggressive type of breast cancer and lacks effective therapies, up to 50% of tumors are found to overexpress EGFR. Targeted therapy against EGFR has been used in TNBC. However, limited efficacy has been observed in TNBC due to intrinsic and acquired resistant mechanisms. In order to overcome this issue, we have developed two novel therapeutic peptides derived from the nuclear localization signal (NLS) sequence and juxtamembrane domain of EGFR and investigated their efficacy in regard to inhibiting EGFR translocation and activation in

TNBC.

EGFR has been found to translocate into the nucleus and nuclear EGFR can affect gene transcription, cell proliferation, stress response and DNA repair through interacting with different components in the nucleus. Importantly, these functions of nuclear EGFR correlate with cancer prognosis and therapeutic resistance. We found that an EGFR NLS-derived peptide (ENLS peptide) could inhibit activated EGFR (pY845) undergoing nuclear translocation. We also showed that this ENLS peptide sensitized breast cancer cells to AG1478 (EGFR tyrosine kinase inhibitor) treatment.

The juxtamembrane domain of EGFR regulates its trafficking to the nucleus and mitochondria, interaction with calmodulin and calcium signaling, and participates in dimerization and activation of EGFR. These non-traditional kinase related functions of EGFR represent a novel target for EGFR therapy. We found that a mimetic peptide of the juxtamembrane domain of EGFR (EJ1 peptide) could effectively inhibit EGFR activation through promoting inactive dimer formation. It could also effectively kill cancer cells through processes of apoptosis and necrosis. Mechanistically, this EJ1 peptide affects membrane integrity thereby leading to calcium influx, disruption of mitochondrial membrane potential and reactive oxygen species (ROS) accumulation. Importantly, EJ1

peptide appeared to be effective in inhibition of tumor growth and metastasis in a transgenic mouse model of breast cancer and showed no observable toxicity.

ErbB3, another member of the erbB family, represents an important driver of the parallel signaling pathway to EGFR as well as a key regulator of PI3K/AKT activity which is important for therapeutic resistance. ErbB3 has been shown to interact with MUC1. The interaction between MUC1 and EGFR promotes EGFR stability through recycling of receptors. We found that MUC1 expression also affected ErbB3 activity and stability through ErbB3/EGFR/MUC1 complex formation.

In conclusion, we demonstrated that two EGFR-derived peptides, working through novel strategies, represent a new foundation of effective therapeutic agents to breast cancer. ErbB3/EGFR/MUC1 complex formation under MUC1 expression also represents a druggable target for ErbB3 activity and stability.

CHAPTER 1 – INTRODUCTION

Breast cancer represents the second leading cause of cancer death in women. With the advances in early diagnosis and targeted therapies, the mortality and morbidity of breast cancer patients have decreased significantly since 1990 (2.2% death rate decrease per year between 1990-2007, [1]). However, the basal epithelial-like group of breast cancer (clinically referred as triple negative breast cancer, ER(-), PR(-), and HER2(-)) still harbors a poor prognosis compared with other groups of breast cancers; one of the possible reasons is the lack of specific targeted therapies. Interestingly, the basal epithelial-like group of breast cancer was found to overexpress EGFR in more than 50% of cases. In addition, aberrant localization of EGFR was found to be related to therapeutic resistance. Thus, EGFR represents a new therapeutic target for this group of patients. Therefore, we investigated two novel strategies to target both EGFR activity and trafficking. We have provided evidence to support these two novel strategies as potential therapeutics for breast cancer patients.

I. Breast Cancer

Breast cancer is the most common form of cancer and the second leading cause of cancer death in women. In 2013, it is estimated that there will be 232,340 new cases of invasive breast cancer to be diagnosed and 40,030 breast cancer deaths to be seen among women in the US [2]. The prognosis depends on the stage and classification of the disease. According to the Surveillance, Epidemiology, and End Results (SEER) Summary Stage system, breast cancer can be separated into three stages: local, regional and distant. Local-stage tumors are cancers confined to the breast. Regional-stage tumors have spread to surrounding tissue or nearby lymph nodes. Distant-stage cancers have metastasized to distant organs. The five year survival rate is 99% for localized breast cancer, 84% for regional disease, and 23% for distant-stage disease [3].

Traditionally, breast cancers have long been classified according to their morphological characteristics, histological type, and grade (aggressiveness). Recently, gene expression analysis using DNA microarray technology has identified additional breast cancer subtypes that were not apparent with traditional histopathological methods. Based on gene expression profiles, breast cancer can be classified into five main groups [4, 5]. They are luminal A group, luminal B group, HER2-enriched group, basal

epithelial-like group, and normal breast-like group (Table 1). This classification has been shown to be of clinical relevance for disease prognosis, incidence of relapse rate, and sensitivity to therapies.

A. Luminal A breast cancer

Most breast cancers originate from the luminal cells that line the mammary ducts. Luminal A is the most common type of breast cancer and accounts for 50-60% of breast cancers. This type of breast cancer tends to express both estrogen receptor (ER) and progesterone receptor (PR) with low to zero HER2 and proliferation-associated gene expression. Patients with luminal A breast cancer have an excellent prognosis with a 10-year survival rate of 70% and a 15-year relapse rate of 27.8% [6].

B. Luminal B breast cancer

Luminal B makes up 10-20% of total breast cancers. This type of breast cancer also expresses ER and PR. In contrast to luminal A, luminal B breast cancers are more likely to express high levels of proliferation-related genes. As such, luminal B breast cancers have a worse prognosis compared to luminal A, with a 10-year survival rate of 54.4% and a 15-year relapse rate of 42.9% [6].

C. HER2-enriched breast cancer

The HER2-enriched group is composed of tumors that are preferentially ER-negative and express high levels of HER2 and genes located to the HER2 amplicon. This group of cancer accounts for 10-15% of total breast cancers and has a poor prognosis, even though anti-HER2 treatment has substantially improved survival rates. Its 10-year survival rate is 48.1% and 15-year relapse rate is 51.4% [6].

D. Basal epithelial-like breast cancer

The basal epithelial-like group represents 10-20% of all breast cancers. This group of cancers is characterized by a lack or low levels of expression of ER and PR, and by frequent absence of HER2 overexpression, high levels of expression of proliferation-related genes, and expression of genes usually found in basal and myoepithelial cells of the breast, including cytokeratins 5/6 and 17, and the EGFR. Clinically, this group and the normal breast-like group are classified as triple negative breast cancers because they lack expression of ER, PR and HER2. Basal epithelial-like breast cancers tend to have a poor prognosis with a 10-year survival rate of 52.6% and 15-year relapse rate of 43.1% [6].

E. Normal breast-like breast cancer

Normal breast-like tumors express genes similar to those expressed by normal breast cells. Only 5-10% of total breast cancers belong to this group. The normal breast-like tumors are usually ER-, PR- and HER2- as well as cytokeratin 5- and EGFR-, which distinguish them from basal epithelial-like tumors. Patients with this type of breast cancer have an intermediate prognosis with a 10-year survival rate of 62.6% and 15-year relapse rate of 35.1% [6].

Table 1. Features of the gene expression profiling for defined molecular subtypes of breast cancer.

Molecular Subtype	Frequency	ER/PR/HER2	EGFR	Genes of Proliferation	Prognosis
Luminal A	50-60%	ER+:91-100% PR+:70-74% HER2+:8-11%	-	Low	Excellent
Luminal B	10-20%	ER+:91-100% PR+:41-53% HER2+:15-24%	-	High	Intermediate/ Poor
HER2-enriched	10-15%	ER+:29-59% PR+:25-30% HER2+:66-71%	+/-	High	Poor
Basal epithelial-like	10-20%	ER+:0-19% PR+:6-13% HER2+:9-13%	+	High	Poor
Normal breast-like	5-10%	ER+:44-100% PR+:22-63% HER2+:0-13%	-	Low/ Intermediate	Intermediate

ER: estrogen receptor. PR: progesterone receptor. +: positive. -: negative. Modified from [7]

II. Treatment

The treatment options for breast cancer are similar to those for other cancers, including surgery, radiation therapy, chemotherapy and targeted therapy. Usually, a multimodality approach is required for treatment, depending on the stage, the histological and pathological features, and the molecular profiling subtypes of the disease. Surgery is

still the primary treatment for operable tumors and can be combined with pre-operative chemotherapy for women who desire breast conservation surgery, even though pre-operative chemotherapy does not affect disease outcome as compared to post-operative chemotherapy [8]. Radiation therapy is usually employed after surgery. The main goal of radiation therapy is to eradicate residual disease thereby reducing local recurrence. In order to systemically control the disease and to reduce metastasis, chemotherapy and targeted therapy can be used based on the stage and molecular features of the disease. Chemotherapy usually involves drugs that less selectively kill hyper-proliferative cells in the body whereas targeted therapy acts more selectively on tumor cells.

Hormonal therapy is one of the targeted therapies for breast cancer. There are two classes of hormonal agents for breast cancer: selective ER modulators, e.g. tamoxifen, and aromatase inhibitors, e.g. letrozole. Patients with ER positive breast cancers such as luminal A and luminal B groups of breast cancer mostly benefit from hormonal therapy. For ER positive breast cancer, allocation to about five years of adjuvant tamoxifen reduces the annual breast cancer death rate by 31% [9]. Letrozole in ER positive postmenopausal women who received tamoxifen for five years have a significant

increase in disease free survival when compared to placebo-receiving women [10].

Besides ER, HER2 is another molecular target amplified in 20% of breast cancers and plays an important role in tumorigenesis and progression in these tumors. Trastuzumab (Herceptin[®]) is the first antibody drug against HER2-overexpressing breast cancer. Trastuzumab has been shown to prolong the overall survival of patients with HER2-overexpressing metastatic breast cancer [11]. Adjuvant trastuzumab after chemotherapy also reduced the one-year relapse rate by 46-52% [12, 13]. Triple negative breast cancer patients have a poor prognosis which could be due to lack of ER and PR for hormonal therapy, as well as lack of HER2 for anti-HER2 antibody therapy. Importantly, triple negative breast cancers express EGFR, which also belongs to the erbB family of receptor tyrosine kinases as HER2. Thus, anti-EGFR therapies could be an alternative option for triple negative breast cancers.

III. EGFR

The epidermal growth factor receptor (EGFR/ErbB1/HER1) is a 170 kDa type I transmembrane protein. It belongs to a receptor tyrosine kinase (RTK) subfamily that has four members, including EGFR, ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4) [14]. All of the four receptors have a similar structure which contains an extracellular

ligand-binding domain, a transmembrane domain, a juxtamembrane domain, a cytoplasmic tyrosine kinase-containing domain and a carboxy-terminal tail containing tyrosine phosphorylation sites. Of which, juxtamembrane domain is believed to regulate various functional aspects of erbB receptor, including control of the tyrosine kinase activity, and receptor trafficking, as well as mediating interaction with second messengers such as calmodulin [15, 16]. ErbB2 does not bind to a known ligand but instead functions as a preferred co-receptor for the other three receptors [17]. ErbB3 has a defective kinase domain thus its activation depends on the dimerization with other erbB receptors [18].

After ligand binding, receptors can either form a homodimer or heterodimer. Dimerization of receptors brings the intracellular tyrosine kinase domains in proximity and forms an asymmetric dimer. The juxtamembrane segment from the receiver then makes contact with the C-terminal lobe of the activator kinase domain [19], leading to autophosphorylation of tyrosine residues on the C-terminal tail (Figure 1.1). Phosphorylated tyrosine residues, in turn, allow for docking of second messenger proteins, which contain Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domain motifs, and activating multiple downstream pathways involved in cell proliferation, survival and motility (Figure 1.2). Major pathways associated with erbB

signaling include the Ras/mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, and the phospholipase C γ (PLC γ) pathway. A different dimer formation represents the mechanism by which receptors within this family can regulate a diverse array of cell signals.

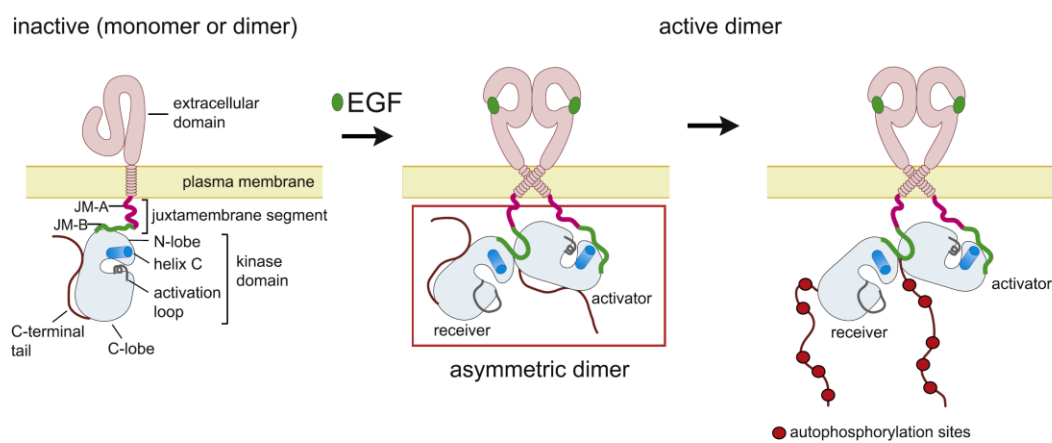


Figure 1.1. Structural model of EGFR activation.

Activation of EGFR by EGF results in a receptor dimerization which brings the intracellular tyrosine kinase domain in proximity with an asymmetric fashion. The juxtamembrane segment from the receiver (green line) makes contact with the C-terminal lobe of the activator kinase domain, leading to autophosphorylation of tyrosine residues on C-terminal tail (red dots). The illustration is adapted from [20].

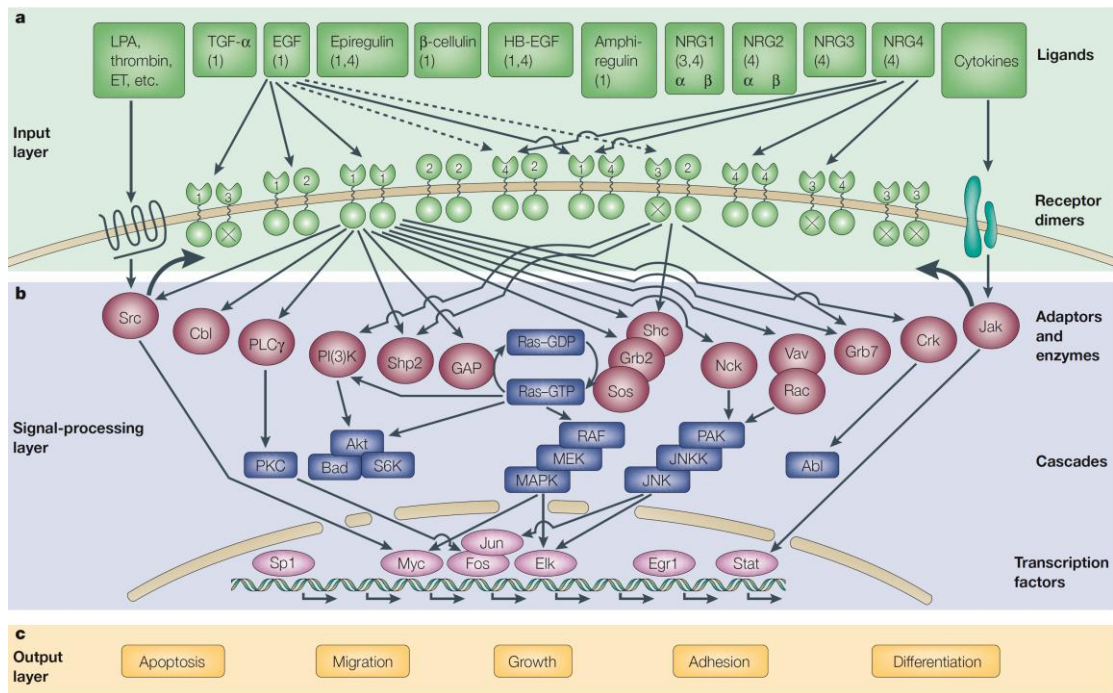


Figure 1.2. The erbB signaling network.

Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity erbB receptors. Dimerization of receptors leads to activation of multiple downstream pathways, as shown in the signal-processing layer, which in turn up-regulate different transcription factors in the nucleus. At the end, different cellular responses are elicited as the output layer. This illustration is adapted from [21].

IV. Ligands of EGFR

ErbB family members are activated by extracellular ligand binding. This EGF family of ligands can be divided into three groups. The first group of ligands includes EGF, transforming growth factor α (TGF α) and amphiregulin (AR), all three of which bind specifically to EGFR. The second group of ligands includes betacellulin (BTC),

heparin-binding EGF (HB-EGF) and epiregulin (EPR), which bind both EGFR and ErbB4. The third group is composed of neuregulins (NRGs) and can be further divided into two subgroups based on their ability to bind ErbB3 and ErbB4 (NRG1 and NRG2) or only ErbB4 (NRG3 and NRG4) (Figure 1.2, reviewed in [22]). These ligands are synthesized as transmembrane precursors that can be cleaved by cell surface proteases and released into the extracellular environment. The main proteases involved in cleavage of the EGF family of ligands belong to the metalloproteinase family, in particular the ADAM (a disintegrin and metalloprotease) family of metalloproteinases [23].

Following the release of ligands from the membrane, ligand binding to erbB receptors induces a conformational change which in turn promotes dimerization [24]. For example, in the absence of EGF, interactions between subdomains II and IV of the extracellular domain of EGFR maintain the extracellular domain in an “off” status and prevent dimerization. When EGF binds to domains I and III, it changes the conformation of the extracellular domain of EGFR and turns it to an “on” status, which exposes domain II and allows the protruding arm of this domain to participate in dimerization (Figure 1.3). Dimerization of receptors then leads to activation of downstream pathways and regulates cell proliferation, migration, differentiation and survival (Figure 1.2, reviewed in [22]).

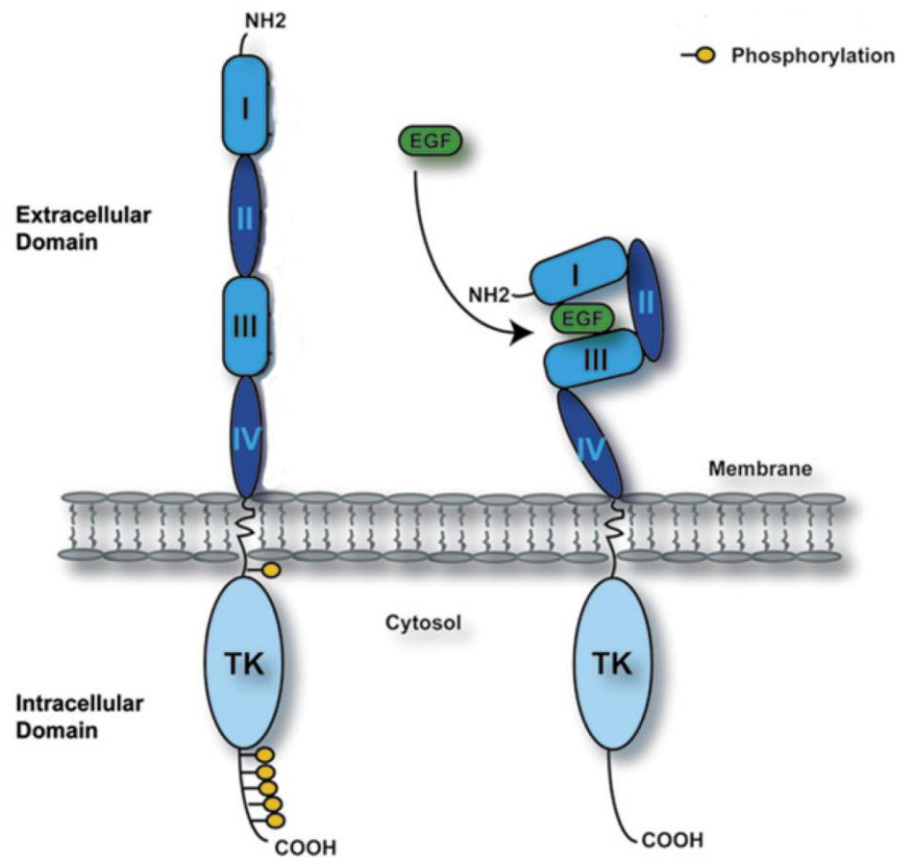


Figure 1.3. EGFR extracellular domain structure.

The extracellular domain is composed of four subdomains designated I, II, III and IV. The domains I, II and III form a ligand-binding pocket, where a ligand is docked between the domains I and III. This illustration is adapted from [25].

V. EGFR Internalization and Degradation

Since EGFR regulates various important cellular physiologies, it is not surprising that EGFR activity is tightly regulated. Degradation of the activated receptors is an

important mechanism by which the cells can control the extent of signaling. Binding of ligands leads to dimerization of the receptor and transphosphorylation of certain tyrosine residues on the C-terminal domain (reviewed in [26]). One of these tyrosine residues (pY1045) then acts as a docking site for the Cbl (Casitas B-lineage lymphoma) protein, which along with E2 ligase, binds EGFR and facilitates its ubiquitination [27]. EGFR ubiquitination promotes the recruitment of Eps15 (epidermal growth factor receptor substrate 15) through its ubiquitin interacting motif (UIM) and mediates translocation of EGFR to a clathrin-coated pit which initiates internalization [28]. A clathrin-coated vesicle is then formed and subsequently the clathrin coat is removed to produce an early endosome where sorting for receptor recycling or degradation occurs. The early endosome then matures to form multivesicular bodies (MVB). EGFR destined for degradation is then internalized into the MVB lumen, fused with lysosomes and degraded [29] (Figure 1.4).

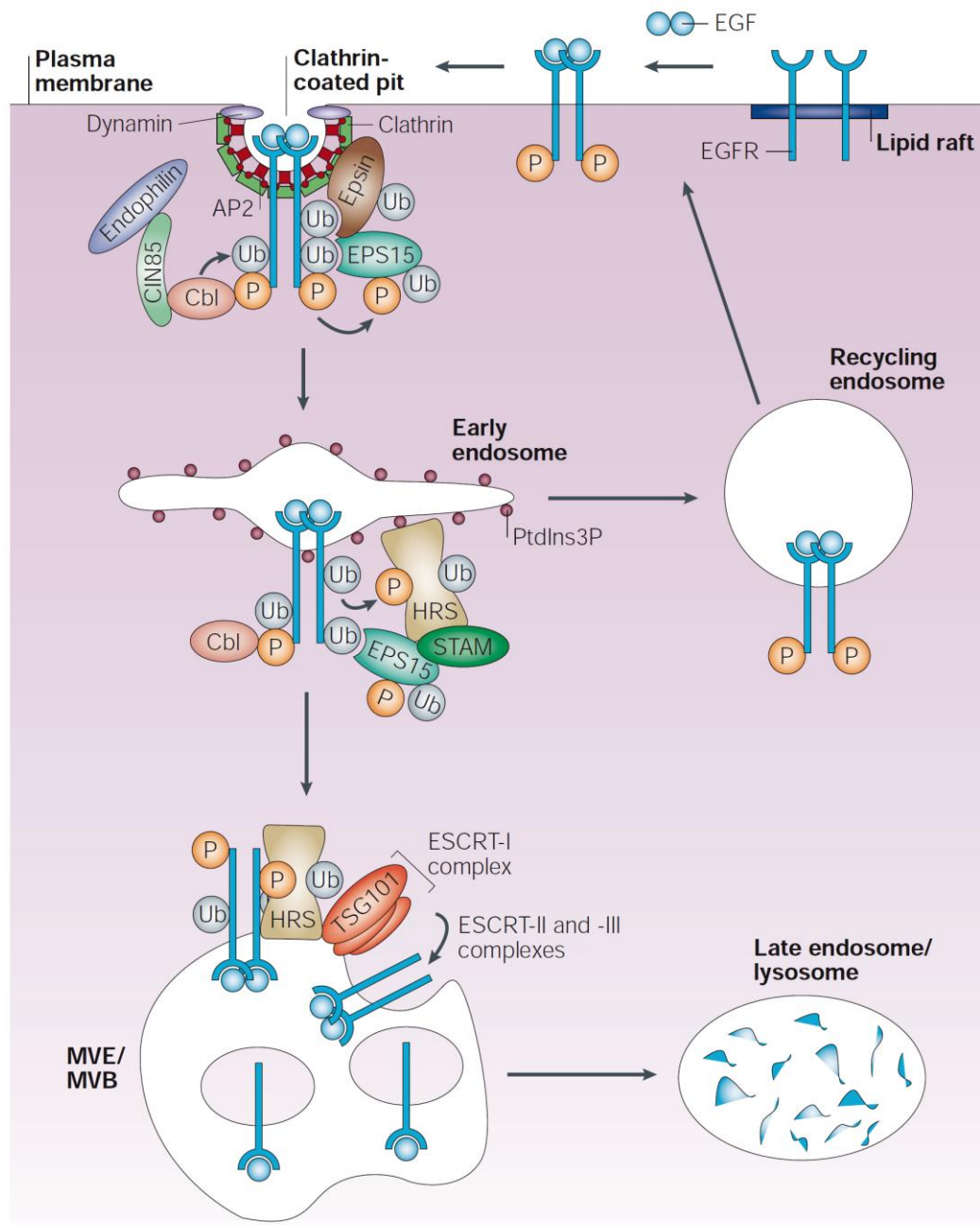


Figure 1.4. Model of EGFR trafficking.

Binding of EGF leads to dimerization of the receptors and transphosphorylation of tyrosine 1045 (pY1045), then acts as a docking site for the Cbl protein, which binds and facilitates the ubiquitination of EGFR. EGFR ubiquitination promotes the recruitment of Eps15 and mediates translocation of EGFR to a clathrin-coated pit which initiates internalization. A clathrin-coated vesicle is then formed and subsequently the clathrin

coat is removed to produce an early endosome. The early endosome then matures to form MVB. EGFR destined for degradation is then internalized into the MVB lumen, fused with lysosomes and degraded. P, phosphate; Ub, ubiquitin. This illustration is adapted from [30].

Deregulation of the degradation pathway can lead to over-activation of EGFR signaling. Both inhibition of endocytosis and promotion of recycling have been reported in several circumstances [31-34]. They can be a result of different ligand binding, different heterodimer formation or of interaction with other proteins. Binding of EGF or TGF- α to EGFR has a similar affinity at pH 7.4. However, these two ligands have substantially different pH sensitivities at lower pH levels, which is a characteristic of endosomes. These different pH sensitivities are due to their difference in pI (isoelectric point, pH 6.2 for TGF- α vs. pH 4.3 for EGF). TGF- α is dissociated from EGFR in acidic conditions which leads to recycling of EGFR [31]. EGFR/ErbB2 heterodimer can also alter its degradation. Overexpression of ErbB2 causes constitutive activation of ErbB2 as well as of its interaction partner, EGFR. Endocytosis of EGFR is not affected by overexpression of ErbB2. On the other hand, degradation of EGFR is strongly inhibited due to a preferential recycling pathway instead of the lysosomal targeting pathway for EGFR/ErbB2 heterodimers [32]. Aberrant protein-protein interaction also affects EGFR

trafficking. For example, Src overexpression has been observed in many different types of cancers, and Src/EGFR interaction induces EGFR activation and redistributes EGFR into recycling endosomes [33]. MUC1/EGFR interaction also potentiates EGFR activation and inhibits EGFR degradation through promoting recycling of EGFR [34]. Over-activation of EGFR due to alteration in receptor trafficking can ultimately lead to tumor formation and progression.

VI. EGFR in Breast Cancer

EGFR can be oncogenic by a variety of mechanisms, including over-expression or mutation of receptors, over-expression of ligands, defective down-regulatory mechanisms, and co-expression of other erbB family members (reviewed in [22]).

A. Overexpression of EGFR

EGFR over-expression has been observed in about 15% of unselected breast cancers. In triple negative breast cancer, up to 50% of tumors are found to overexpress EGFR [35]. This overexpression can be a result of several different events including gene amplification, up-regulated EGFR transcription or translation. EGFR gene amplification which results in increased protein expression has been found in approximately 6% of

breast cancers [36]. Another important inducer for EGFR overexpression is hypoxia, which occurs during development of cancers and is correlated with cancer progression. Hypoxia has been observed to modulate EGFR expression through transcriptional and translational regulation. Hypoxia-induced early growth response factor 1 (Egr1) protein up-regulates EGFR transcription by binding to EGFR promoter and in turn enhances EGFR expression [37]. In addition, activation of hypoxia-inducible factor 2 α (HIF2 α) protein leads to up-regulation of EGFR mRNA translation [38]. Although overexpression of EGFR seems to be a frequent phenomenon in triple negative breast cancers, EGFR mutation is not. Only about 11% of triple negative breast cancers were positive for EGFR mutations in a recent analysis [39].

B. Overexpression of ligands

The concept that EGFR ligands could be oncogenic was first demonstrated in a mouse model engineered to overexpress TGF- α [40]. TGF- α overexpression in a post-lactational mammary gland induces secretory mammary adenocarcinomas. The underlying mechanism could be that ligand-dependent EGFR activation results in a positive feedback autocrine loop, which further induces the expression and/or activation of ligands. Other EGFR ligands, such as amphiregulin, have also been shown to promote

proliferation, invasion and migration of normal and neoplastic mammary epithelial cells [41]. It is not surprising that up-regulation of ligands mRNA can be detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in 80-96% and by immunohistochemistry (IHC) in 53-67% of breast cancers [42, 43].

C. Defective down-regulatory mechanism

A defective down-regulatory mechanism is seen in activating mutations of EGFR, such as point mutation L858R (substitution of leucine to arginine at position 858) or EGFRvIII mutation. The L858R mutation results from an amino acid substitution at position 858 within the EGFR activation loop. EGFR L858R mutation leads to defective ubiquitination and impaired degradation of EGFR [44, 45]. EGFRvIII has an in-frame deletion of the extracellular domain and is unable to bind ligands; however, the receptor is constitutively active. The constitutive activity is because the degradation of EGFRvIII is inhibited. Ubiquitination of EGFRvIII is less effective which leads to inefficient internalization and impaired trafficking to lysosomes [46]. Other than activating mutations in EGFR, aberrant protein-protein interaction can also cause defective degradation of EGFR; EGFR/MUC1 interaction is one of the examples [34].

D. Co-expression of other erbB family members

Lastly, cross-talk between EGFR and other erbBs serves as another mechanism for oncogenic activation of EGFR. ErbB2 has no known ligand and ErbB3 harbors a defective kinase domain which implies the necessity of heterodimerization of these receptors to effectively transduce extracellular signals. Co-overexpression of multiple erbB receptors has been found in different cancers, including oral, brain and breast cancers [47-49]. The EGFR-ErbB2 heterodimer appears to be the most common and the most potent inducer of cell transformation [50]. The enhanced EGFR-ErbB2 signaling could be due to impaired endocytosis of ErbB2 and thus a sustained signaling from the receptors [51]. In addition, EGFR-ErbB3 heterodimers have been shown to play an important role in mediating resistance to EGFR-targeted therapy through their predominant engagement with PI3K/AKT survival signaling [52, 53].

EGFR can gain its oncogenic activity through a variety of mechanisms; all of these can be demonstrated in breast cancers which suggests targeting EGFR can be an excellent therapy for those EGFR-dependent or EGFR-addicted breast cancers.

VII. EGFR-Targeted Therapy

The finding that EGFR is activated in a variety of cancers and plays critical roles in tumor initiation and progression prompts the development of EGFR-targeted therapeutics. Currently, there are two types of strategies focused on blocking EGFR signaling, including anti-receptor antibodies and small molecule tyrosine kinase inhibitors (TKI).

A. Anti-EGFR antibodies

Anti-EGFR antibodies bind the extracellular domain of the receptor. In one of the very first studies, anti-EGFR monoclonal antibody treatment resulted in more than 75% of growth reduction in a xenograft tumor model of athymic mice [54]. Interestingly, neither the expression level of EGFR in tumor cells nor the capability of competing with EGF for receptor binding of the monoclonal antibody is a determinant of anti-proliferative activity *in vivo*, suggesting that some host animal responses may be involved in the antitumor effect. As an example of anti-receptor antibodies, cetuximab is a chimeric IgG1 anti-EGFR monoclonal antibody derived from the murine anti-EGFR monoclonal antibody M225. Cetuximab was the first anti-EGFR antibody approved by the FDA in February of 2004 for the treatment of colorectal cancer. It is also considered for the treatment of EGFR-overexpressing breast cancer patients including the triple

negative cases that overexpress EGFR [55]. A phase II study on metastatic breast cancer patients was recently done to evaluate the combination of cetuximab with irinotecan and carboplatin, two standard chemotherapeutic agents used for breast cancer therapy. The preliminary result showed an improved overall response rate (ORR) with the addition of cetuximab over irinotecan and carboplatin alone (39% vs. 19%) [56]. Mechanistically, cetuximab competitively binds to the accessible extracellular subdomain III of EGFR with high affinity preventing EGFR ligand binding and promoting receptor dimerization, endocytosis and degradation [57]. Cetuximab also induces cell-cycle arrest through upregulation of p27^{kip1} [58]. In addition, cetuximab has been shown to inhibit angiogenesis through reduction of angiogenic factors, such as interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) [59, 60]. Lastly, cetuximab also works by mediating antibody-dependent cellular cytotoxicity (ADCC) [61, 62]. From different clinical trial results, cetuximab seems to be a promising agent for the treatment of breast cancer, especially for the subgroup of patients with aggressive triple-negative tumors that overexpress EGFR. Selection of patients who will benefit from targeted therapy will be the key to improve the clinical outcome of EGFR-targeting agents.

B. Tyrosine kinase inhibitors

The second type of anti-EGFR therapeutics is small molecule TKIs. This type of inhibitor specifically targets the ATP-binding pocket of EGFR and inhibits the EGFR phosphorylation and downstream cascade. One of the advantages for small molecule TKIs over anti-EGFR monoclonal antibodies is that they are orally bioavailable and well tolerated. Gefitinib, a reversible inhibitor of EGFR tyrosine kinase, is the first anti-EGFR TKI approved by the FDA in May of 2003. It was approved for the treatment of refractory non small cell lung cancer (NSCLC) as an accelerated-track drug based on tumor response rates in a phase III trial [63]. However, when survival data became available for the trial, gefitinib failed to show benefit and was subsequently placed on restricted-use status. Further investigation showed activating mutations in the EGFR dictate responsiveness of NSCLC to gefitinib [64]. Meanwhile, a second EGFR-TKI, erlotinib, which is a reversible inhibitor of wild type EGFR and EGFRvIII mutant, completed phase III trials. In contrast to gefitinib, erlotinib showed a 2-month improvement in median survival compared to placebo when used as monotherapy in previously-treated NSCLC. Based on these data, erlotinib was approved by the FDA in November of 2004 for advanced NSCLC [65]. Further investigation showed an increased

responsiveness to erlotinib of never-smokers and patients with EGFR mutation [66, 67]. Noteworthy is that somatic mutations in EGFR are found in 10% to 15% of Caucasian and in 30% to 40% of Asian NSCLC patients [68-70]. EGFR mutations associated with increased response to gefitinib and erlotinib are found to be predominantly of two types: 45% are deletions involving at least 12 nucleotides in exon 19, eliminating a conserved LREA motif (corresponding to amino acid residues leucine, arginine, glutamic acid and alanine located at 747-750), and 40% are single point mutations in exon 21 (L858R) [71]. Unfortunately, neither gefitinib nor erlotinib showed success in the treatment of breast cancer. One possible reason seems to be the lack of activating mutations in breast cancer.

Among EGFR-TKIs, lapatinib is the only one to receive FDA approval for the treatment of metastatic breast cancer. It is a dual inhibitor of the tyrosine kinase domain of both EGFR and ErbB2. Lapatinib was found to be an active and well-tolerated oral dual TKI for the treatment of ErbB2-overexpressing breast cancer [72]. It is also active in trastuzumab refractory, ErbB2-overexpressing metastatic breast cancer patients [73]. Hence, lapatinib has potential as a successful targeted therapy for erbB2 positive breast cancer patients. There are two more EGFR-TKI, canertinib and neratinib, currently under clinical evaluation. Both of them are irreversible pan-erbB inhibitors forming covalent

binding to the ATP-binding site. Since they both nonspecifically inhibit all four erbB receptors, they hold the potential for a broader range of action. For example, neratinib also inhibits the growth of cultured cells that contain resistance-associated EGFR mutations [74].

From the experience of these two types of anti-EGFR therapeutics, it is clear that only a small portion of patients with certain types of EGFR mutations will be responsive to these anti-EGFR drugs. Discouragingly, a majority of patients either do not respond to the treatment at all (intrinsic resistance) or respond to the initial treatment but relapse quickly and ultimately develop drug resistance in 6 to 12 months (acquired resistance).

VIII. Resistance Mechanism to EGFR-Targeted Therapy

Resistance is a significant issue impairing the use of anti-EGFR therapies. As mentioned above, resistance to EGFR-targeted therapy can be intrinsic or acquired in nature. Intrinsic, or primary, resistance to EGFR inhibitors presents as rapidly progressing disease despite treatment. Acquired, or secondary, resistance typically appears in responding patients within 12 months of initiation of therapy. It is still controversial in some cases regarding whether acquired resistance arises from the selection of resistant

subclones that are present before the initial therapy, or whether it develops *de novo* in response to the treatment [75, 76]. So far, multiple mechanisms have been shown to contribute to resistance to EGFR-targeted therapies, including EGFR mutations, activating downstream signaling, alternative activating parallel signaling, and translocation of EGFR.

A. Intrinsic resistance

Intrinsic resistance to EGFR-targeted therapy is likely the result of lack of EGFR-dependency or presence of redundant pathways. The best examples of intrinsic resistance to EGFR-targeted therapy are KRAS mutation [77, 78] and Met/ErbB2/VEGF/VEGFR overexpression [79-83]. EGFR controls different downstream signaling, including Ras/Raf/MAPK and PI3K/AKT pathways and regulates cell proliferation and survival. A constitutively active downstream effector will render the cancer cells independent of EGFR activity. One of the most frequent mutations is KRAS mutation. The frequency of point mutations at codons 12 and 13 of the KRAS gene was detected in a variety of cancers, including 75% of adenocarcinomas of the pancreas, 40% of adenomas and carcinomas of the colon and rectum, 30% of carcinomas of the bile duct, 25% of carcinomas of the lung, and 5% in breast [84]. Thirty metastatic colorectal cancer

patients treated by cetuximab were screened for KRAS mutation. As a result, 43% of them were found to have KRAS mutation and none of them responded to cetuximab [77]. A different study determining the association of KRAS mutation and sensitivity to EGFR TKI in 60 lung adenocarcinomas also concluded that mutation in KRAS was associated with a lack of sensitivity to both gefitinib and erlotinib [78]. Both studies suggest KRAS mutations are associated with intrinsic resistance to EGFR-targeted agents and can be used as a biomarker to select candidates for EGFR-targeted therapy.

PI3K/AKT pathway regulates cell survival and thus is important for cancer cell maintenance. Activation of parallel pathways that can up-regulate PI3K/AKT activity also leads to resistance to EGFR inhibitors. Different receptor tyrosine kinases have been found to compensate down-regulation of PI3K/AKT activity in the treatment of EGFR inhibitors. Well characterized examples include Met and its ligand, hepatocyte growth factor (HGF), as well as ErbB2, VEGFR and its ligand, VEGF. Met was found to cooperate with c-Src to phosphorylate EGFR in an EGFR kinase-independent manner which leads to insensitivity to EGFR TKI in some breast cancer cell lines [79]. Another study showed that the Met ligand, HGF, as well as tumor-stromal interactions can both play important roles in resistance to EGFR TKI. Specifically, fibroblast-secreted HGF

activated Met and led to EGFR/Met crosstalk and resistance to EGFR TKI in triple negative breast cancer [80]. In addition to Met signaling, the activation level of ErbB2 correlated with intrinsic resistance to gefitinib in head and neck squamous cell carcinoma (HNSCC) and may have potential as a predictive biomarker and as a therapeutic target for combination therapy in treatment of HNSCC with gefitinib [81]. VEGFR-1 expression also contributes to resistance to EGFR-targeted therapy in different human cancer cells [82]. VEGF overexpression was found to correlate with resistance to cetuximab in metastatic colorectal cancer and can be a useful predictive biomarker for cetuximab treatment [83].

B. Acquired resistance

Acquired resistance is also a significant hurdle in the clinical use of EGFR-targeted agents. EGFR secondary mutation (EGFR T790M, substitution of threonine to methionine at position 790 of EGFR) and Met amplification are the two best-studied mechanisms among other acquired resistance mechanisms and account for ~60% to 70% of all known causes of acquired resistance to gefitinib or erlotinib.

Uncovered in clinical trials of erlotinib in NSCLC, EGFR T790M mutation is a common mechanism for acquired resistance. This T790M mutation results in an increase

in the ATP binding affinity and thus reduces the potency of any ATP-competitive kinase inhibitor. The irreversible inhibitors, such as canertinib and neratinib, overcome this resistance simply through covalent binding [85].

Met amplification is also identified as an acquired resistance mechanism to EGFR TKI and accounts for ~20% of the cases [52]. Other mechanisms of acquired resistance are also discovered, such as up-regulation of ErbB2 and ErbB3 activities [53, 86], and changes of subcellular localization of EGFR to nucleus [87, 88] or cytosol [89].

IX. Kinase-Independent EGFR Function

Current strategies to target EGFR mainly focus on EGFR kinase activities. However, a recent study showed that a kinase-dead mutant of EGFR was able to inhibit autophagic cell death by maintaining intracellular glucose levels through interaction and stabilization of the sodium/glucose cotransporter 1 (SGLT1) in cancer cells [90]. This finding indicates an EGFR oncogenic function that is independent of its kinase activity. In addition, a myriad of separate, non-traditional kinase related functions of the EGFR receptor have also been demonstrated in the past two decades. These functions include the ability to translocate to the nucleus and act as transcriptional co-factors, as well as

participate in DNA damage repair and replicative pathways, involvement in calcium signaling, and ability to traffic to mitochondria, where EGFR can interact directly with cytochrome oxidase subunit II (CoxII) to affect cellular ATP levels and apoptosis [91-94]. These non-traditional kinase related functions of EGFR along with the kinase-independent function of EGFR [90] suggest that in order to control EGFR-dependent tumors, solely targeting kinase activity may not be enough.

X. EGFR Juxtamembrane Domain

Curiously, the highly conserved juxtamembrane domain of the erbB receptors, composed of amino acids 645-682 of EGFR and located just c-terminal of the transmembrane domain, has been shown to be involved in all of these non-traditional kinase related processes.

The N-terminal portion of the EGFR juxtamembrane domain contains a tripartite sequence of three clusters of basic amino acids that promotes EGFR nuclear translocation [95] (Figure 1.5). Following EGFR internalization, the receptor is trafficked to the endoplasmic reticulum (ER) where it associates with Sec61 β , a component of the Sec61 translocon, and is then retrotranslocated from the ER to the cytoplasm [96] (Figure 1.6).

In the cytoplasm, the EGFR then interacts with Importin $\alpha 1/\beta 1$ through its nuclear localization signal sequence (NLS, RRRHIVRKRTLRR) [97, 98] (Figure 1.5) and translocates to the nucleus through the nuclear pore complex (NPC) (Figure 1.6). Prior work from our lab and that of others has shown that once there, EGFR is able to interact with the promoters of several genes, including Cyclin D, b-myb, Cox2, iNOS (inducible NO synthase), and BCRP (breast cancer resistance protein) to up-regulate their transcription, thereby effecting proliferation, stress response, and resistance to chemotherapeutics [99-103]. In addition, nuclear localized EGFR can interact with DNA-PKs (DNA-dependent protein kinases) and PCNA (proliferating cell nuclear antigen) to enhance double strand DNA repair in response to ionizing radiation and to promote DNA replication [104, 105]. Importantly, the presence of nuclear EGFR has been shown to correlate with increased aggressiveness and a higher rate of recurrence in several cancer types [106-108].

Along with its involvement in nuclear trafficking, this same juxtamembrane region is important for EGFR trafficking to the mitochondria as its deletion reduces ligand-mediated EGFR/mitochondrial co-localization [109] (Figure 1.5). Additionally, the influence of the transmembrane domain in this process has been demonstrated, as only

the combination of transmembrane domain amino acids, 622-644, and the juxtamembrane amino acids, 645-666, was able to direct an eGFP (enhanced green fluorescent protein) fusion protein to the mitochondria. Once inside the mitochondria, EGFR can interact directly with and phosphorylate CoxII, causing a reduction in its activity and resulting in a temporary decrease in cellular ATP levels [109].

In addition to its effects on EGFR subcellular localization, the juxtamembrane domain of the receptor is integral to receptor dimerization. This domain is responsible for both maintaining the inactive conformation of an EGFR monomer through electrostatic interactions with the plasma membrane, as well as facilitating the interaction of two EGFR receptors in an active dimer formation [110]. It has been proposed that during ligand-mediated activation, the helical N-terminal portion (amino acids 645-663) of this domain participates in the formation of a stabilizing anti-parallel dimer, while the C-terminal portion (amino acids 664-682) of the domain from the “receiver” receptor forms a clamp to engage the C-terminal lobe of the kinase domain from the “activator” receptor in an asymmetric dimerization (Figure 1.1). The specific interactions of both portions of the juxtamembrane domain are necessary for activation to occur [20].

Dimerization of erbB receptors can be further modulated by calcium influx, another

function regulated by the juxtamembrane domain. Upon ligand binding, an induction of cytosolic Ca^{2+} is quickly observed, and this Ca^{2+} influx is likely a Ras-mediated phenomenon depending on interactions of the receptor kinase domain with $\text{PLC}\gamma$ [111]. The resultant elevation of free Ca^{2+} levels in the cytoplasm induces activation of calmodulin (CaM) and leads to the association of $\text{Ca}^{2+}/\text{CaM}$ and the juxtamembrane domain (Figure 1.5). This interaction was shown to increase the rate at which the juxtamembrane domain of EGFR dissociates from the plasma membrane and affects both the trafficking and kinase activity of EGFR [16, 110, 112].

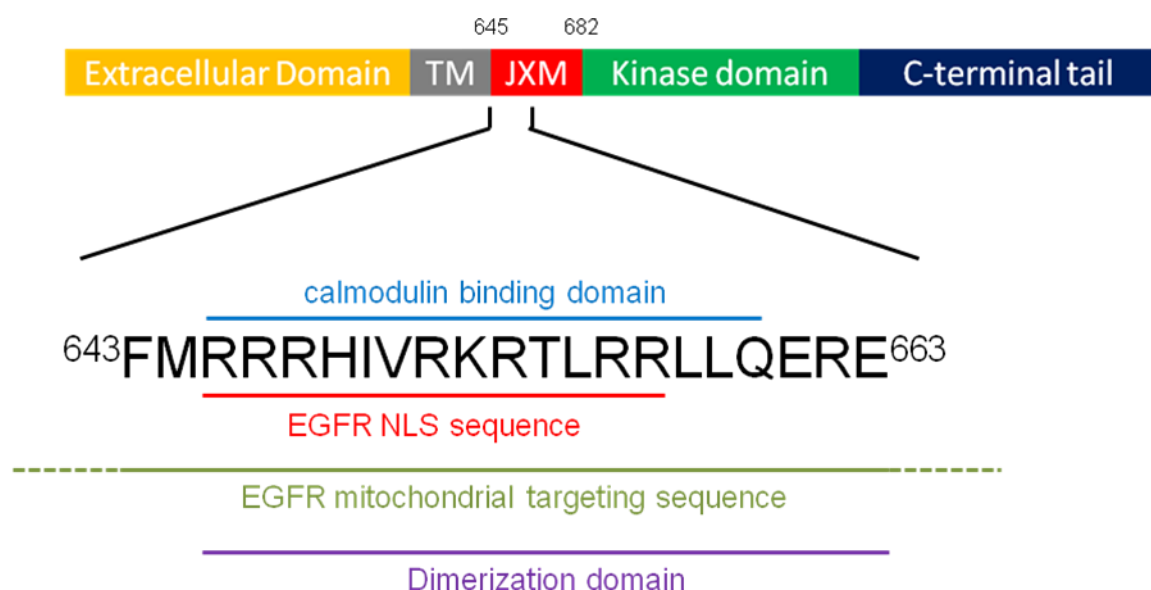


Figure 1.5. Schematic model of EGFR juxtamembrane domain.

Different EGFR domains are shown and juxtamembrane domain (JXM) is emphasized. Sequences involved in different functions are highlighted.

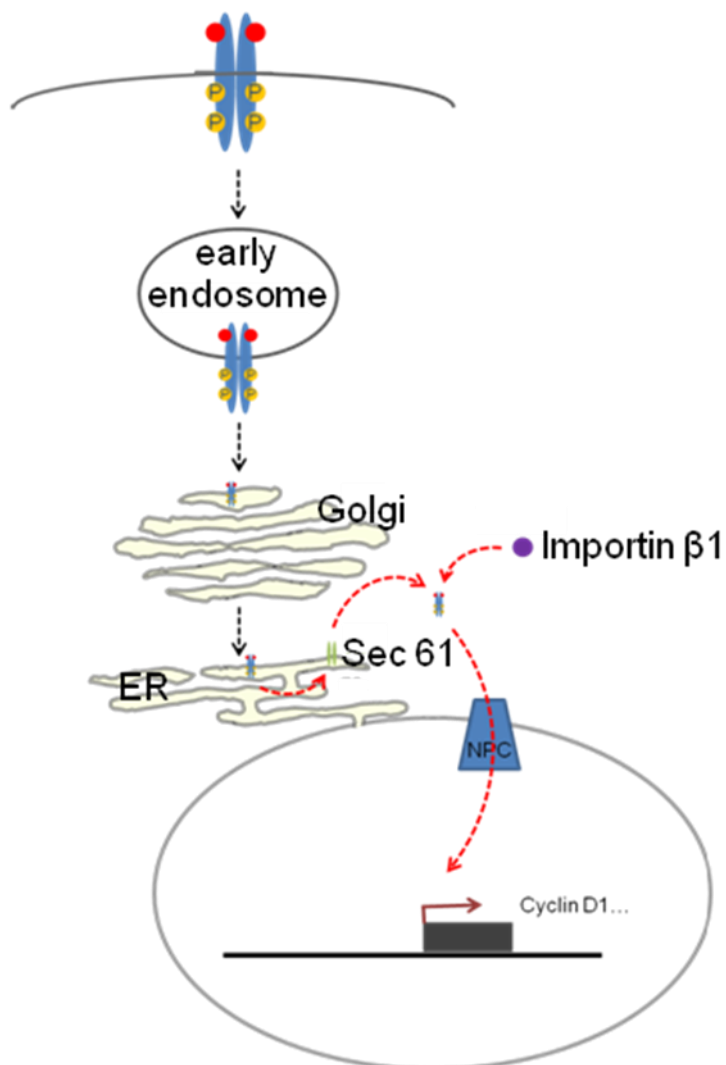


Figure 1.6. Schematic model of nuclear translocation of EGFR.

Following EGFR internalization, the receptor is trafficked through Golgi to the ER where it associates with Sec61 and retrotranslocated to the cytoplasm. In the cytoplasm, the EGFR interacts with Importin β 1 through its NLS sequence and translocates to the nucleus through the NPC.

XI. Statement of the Problem

The EGFR and its family members, including ErbB2 and ErbB3, have been shown

to actively contribute to the pathogenesis and progression of breast cancer [113-116]. This evidence led to the development of different anti-EGFR and anti-ErbB2 agents, and some of them have undergone clinical trials, including trastuzumab and lapatinib being FDA-approved as monotherapy for breast cancer. However, clinical benefit obtained from these drugs as monotherapy is usually limited. Even patients who have responded to initial treatment are likely to develop resistance within one year. These observations clearly indicate that intrinsic and acquired resistance to targeted therapy, especially EGFR-targeted therapy, is a common phenomenon in breast cancer. Moreover, although response of NSCLC patients to gefitinib/erlotinib is directly related to the occurrence of specific activating mutations in the EGFR, breast cancer patients cannot benefit from this observation because such EGFR mutations are rare in breast carcinomas. Importantly, the triple-negative subtype of breast cancer lacks effective therapies due to loss of expression of ER, PR and ErbB2. Among these tumors, 50% of them actually overexpress EGFR. Targeting EGFR through a novel therapeutic strategy to improve responsiveness and overcome resistance mechanisms in breast cancer is critical in order to improve the outcome of triple negative breast cancer patients.

EGFR juxtamembrane domain contains important sequences for its activation,

dimerization, regulation of trafficking, and interaction with calmodulin. All of these functions of EGFR are also important for its oncogenic activity as well as for drug resistance. From the success of inhibitory peptide derived from MUC1 to interrupt EGFR/MUC1 interaction and attenuate proliferation and migration in breast cancer cells [117], it was hypothesized that targeting the nuclear localization domain or juxtamembrane domain of EGFR will inhibit oncogenic activities of EGFR and circumvent resistant mechanisms to conventional targeted therapy. ErbB3, another member in the erbB family, is also overexpressed in breast cancers and plays important roles in therapeutic resistance to EGFR targeted therapy. MUC1, a transmembrane mucin, has been shown to interact with all four erbB receptors and inhibit EGFR degradation [34, 118]. It was hypothesized that ErbB3/MUC1 interaction will alter ErbB3 signaling through affecting ErbB3 trafficking and/or degradation. To test these hypotheses, three different approaches were undertaken to answer three specific questions. These questions are the following:

1. Could the peptide derived from EGFR nuclear localization signal sequence inhibit EGFR nuclear translocation and prevent nuclear EGFR-dependent radiation resistance, chemoresistance or EGFR-targeted therapy resistance?

2. Could the peptide derived from EGFR juxtamembrane domain inhibit EGFR oncogenic activity and suppress tumor growth?

3. Does MUC1 expression affect ErbB3 activities?

CHAPTER 2 - A POTENTIAL DRUG DERIVED FROM EGFR NLS SEQUENCE

Note: The work presented in this chapter has not been published. All experiments were performed by Hsin-Yuan Su.

I. Introduction

EGFR represents an important anti-cancer therapeutic target and many EGFR-targeted therapies have been developed in hope to replace or work synergistically with non-targeted chemotherapy and radiation therapy. Resistance to chemotherapy and radiation therapy has also been linked to EGFR expression, activity, and its nuclear translocation [119-121]. Nuclear translocation of EGFR starts at the plasma membrane after ligand binding and receptor activation. Following EGFR internalization, the receptor is trafficked to the endoplasmic reticulum (ER) where it associates with Sec61 β , a component of the Sec61 translocon, and is then retrotranslocated from the ER to the cytoplasm [96]. In the cytoplasm, the hydrophobic regions of EGFR interact with Hsp70 (heat shock protein 70kDa) to prevent protein aggregation. EGFR then interacts with Importin α 1/ β 1 through its nuclear localization signal sequence (NLS,

RRRHIVRKRTLRR) [97, 98] and translocates to the nucleus through the nuclear pore complex (NPC) (Figure 2.1).

Recently, another model of nuclear transport of EGFR was proposed by Hung's group, namely the integral trafficking from the ER to the nuclear envelope transport (INTERNET) pathway [122]. In this model, membrane-associated EGFR interacts with importin β all the way from the endocytic vesicle to the nucleus and travels from the ER/ONM (outer nuclear membrane) to the INM (inner nuclear membrane) via the NPCs. When utilizing this pathway, EGFR remains embedded in the membrane from the cell surface to the nucleus envelope in the entire trafficking process. In both trafficking models, EGFR/Importin $\alpha 1/\beta 1$ interaction is critical for EGFR nuclear translocation.

Studies showed that both ionizing radiation and cisplatin were able to induce EGFR nuclear translocation, and the nuclear EGFR was able to regulate repair of double-strand DNA breaks through both activation of DNA-PK and inhibition of polynucleotide phosphorylase (PNPase) activity. Therefore, it was hypothesized that targeting EGFR could overcome chemoresistance or radiation resistance. However, targeting EGFR through antibodies against the extracellular ligand binding domain or small molecules against the intracellular kinase domain has demonstrated a limited efficacy in breast

cancer patients. In addition, nuclear EGFR was found to contribute to resistance to cetuximab [87] and gefitinib [88]. Therefore, specifically targeting EGFR nuclear translocation can be an alternative strategy to circumvent the nuclear EGFR-dependent resistance.

Based on these findings and notions, it was hypothesized that inhibiting EGFR/Importin $\alpha 1/\beta 1$ interaction by an EGFR NLS mimetic peptide could prevent nuclear translocation of EGFR and thereby prohibit the emergence of therapeutic resistance (Figure 2.1).

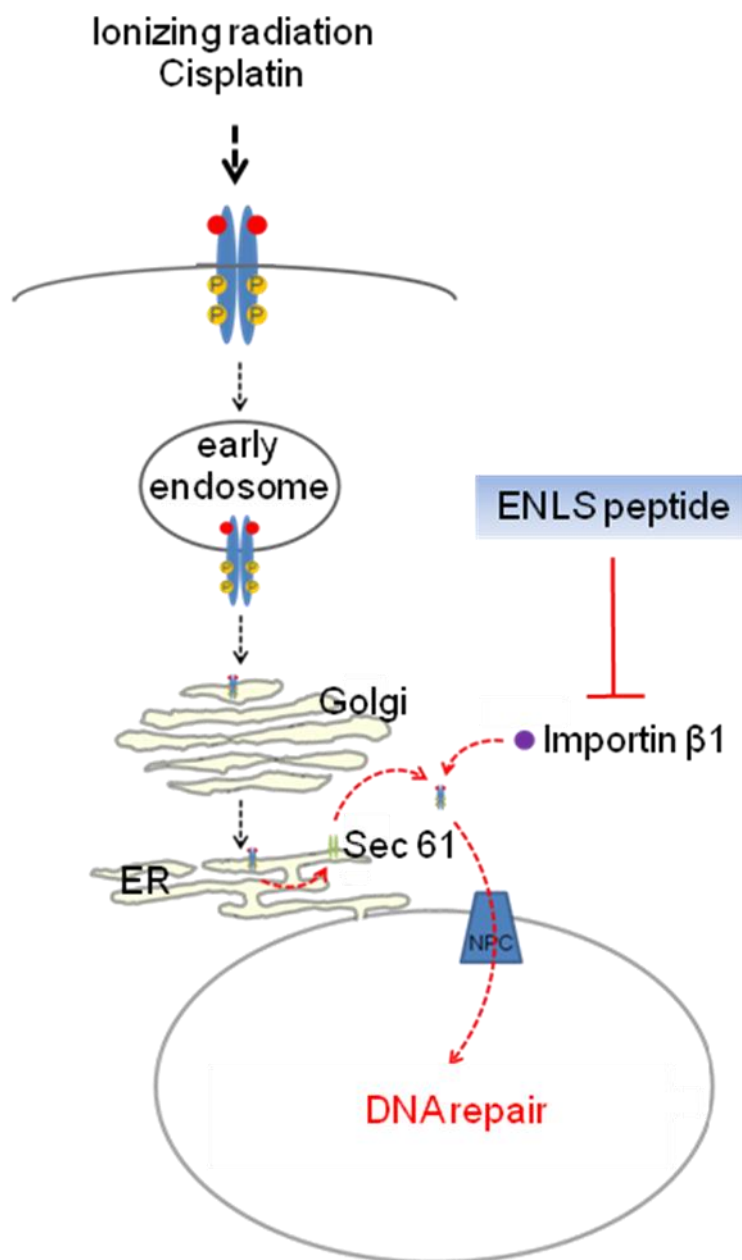


Figure 2.1. Schematic working model of ENLS peptide.

Ionizing radiation and cisplatin can induce nuclear translocation of EGFR and this nuclear EGFR is able to regulate DNA repair for double-strand breaks, which ultimately leads to therapeutic resistance. ENLS peptide designed to prevent EGFR binding to Importin $\beta 1$ could potentially inhibit nuclear translocation of EGFR.

II. Materials and Methods

Cell culture MDA-MB-468 cells were obtained from American Type Culture Collection and were grown in RPMI (Mediatech, Inc., Manassas, VA) and supplemented with 5% fetal bovine serum (FBS, PAA, Piscataway, NJ) in 37°C incubator with 5% CO₂.

Compounds and reagents EGFR 1005 antibody was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). p-EGFR (pY845) antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA). β -actin and laminB1 antibodies were purchased from Sigma-Aldrich (St. Louis, MO). AG1478 and MTT reagent (thiazolyl blue tetrazolium bromide) were also purchased from Sigma-Aldrich (St. Louis, MO). EGF was purchased from Invitrogen Life Technologies Inc. (Carlsbad, CA).

Peptide Synthesis ENLS and CP polypeptides were synthesized by GenScript (Scotch Plains, NJ) and delivered lyophilized. The peptides were resuspended at a concentration of 2mM in water and stored at 4°C in single-use aliquots.

Differential Detergent Fractionation The differential detergent fractionation protocol

was modified from [123]. Briefly, cells were collected in an eppendorf tube and resuspended in digitonin lysis buffer. The cytosolic fraction was collected from the supernatant after centrifugation at $400 \times g$ for 7.5 minutes. The pellet was then resuspended in Triton X-100 lysis buffer and then subjected to centrifugation for 7.5 minutes at $5100 \times g$. The supernatant was stored as the membrane fraction and the pellet was further resuspended in Tween 40/ DOC lysis buffer. After brief sonication, nuclear fraction was collected from the supernatant after centrifugation for 10 minutes at $6900 \times g$.

Western blotting Following treatments, cells were harvested and lysed in lysis buffer consisting of 20mM Tris, pH 7.5, 150mM NaCl, 1% NP-40, and 5mM EDTA, pH 8.0, along with protease and phosphatase inhibitors. Protein concentration was determined using Bicinchoninic Acid assay (Thermo Fisher Scientific Inc., Waltham, MA). Lysates were then separated using SDS-PAGE before being transferred to PVDF membranes (EMD Millipore Inc., Billerica, MA) and immunoblotted using indicated antibodies.

Cell viability assay Cells were analyzed by MTT following manufacturer's instructions

(Sigma) and analyzed using a U-Quant Spectrophotometer (Bio-TEK Instruments, Inc.).

III. Results

A. Design and synthesis of the ENLS peptides

EGFR NLS sequence was shown to contain three clusters of basic amino acids and all three clusters of basic amino acids were required for efficient nuclear translocation of EGFR [98]. The NLS sequence is recognized by a group of proteins called Importins. EGFR NLS sequence has been shown to interact with Importin $\alpha 1/\beta 1$ and this protein-protein interaction is critical for nuclear transport of EGFR [97]. Therefore, a 13-amino acid peptide was synthesized in tandem with a protein transduction domain (PTD4, [124]) to help peptides penetrate cells to be used as a decoy to interrupt EGFR/Importin $\alpha 1/\beta 1$ interaction. Basic amino acids (R or K) at each of the three clusters were replaced with acidic amino acids (D) and this peptide was used as the control peptide (CP). As shown in Figure 2.2, these peptides are hereafter referred to as ENLS (EGFR Nuclear Localization Signal) or CP (Control Peptide) peptides.

EGFR NLS sequence: RRRHIVRKRTLRR

ENLS	NH ₂ -YARAAARQARAR <u>RRRHIVRKRTLRR</u> -COOH
CP	NH ₂ -YARAAARQARAR <u>DRHIVRDRTLRD</u> -COOH

Figure 2.2. ENLS peptide design.

Peptides were synthesized according to EGFR NLS sequence. The control peptide was EGFR NLS sequence with 3 amino acid mutations at each of the three clusters of basic amino acids. Mutated amino acids are shown in red. In order to allow peptides get into cells, both peptides were synthesized in tandem with a protein transduction domain (PTD4, [124]).

B. ENLS peptide significantly inhibits activated EGFR to translocate into the nucleus

In order to test whether the ENLS peptide could affect nuclear translocation of EGFR, a time course experiment was done first to determine the nuclear translocation of EGFR after EGF stimulation (data not shown). As phosphorylated EGFR at tyrosine 845 (pY845) had been used to demonstrate nuclear localization of activated EGFR [125], this same phospho-EGFR was used in our experiments. In a triple- negative breast cancer cell line, MDA-MB-468, phosphorylated EGFR (pY845) was detected in the nucleus at 30 minutes after EGF stimulation. The same time point was used to determine whether ENLS peptide affected nuclear translocation of phosphorylated EGFR. As shown in the left panel of Figure 2.3, EGFR activation was not affected either by water or CP

treatment. In addition, it was not affected by ENLS peptide up to 20 μ M. The decrease of phospho-EGFR (pY845) in 50 μ M of ENLS treatment could be due to a nonspecific cytotoxic effect of the peptide. In nuclear fraction, phosphorylated EGFR levels were the same for water and CP treated samples. However, the phosphorylated EGFR level was significantly less in the ENLS peptide treated group. A concentration of 10 μ M of ENLS was able to decrease the phosphorylated EGFR level in the nuclear fraction (Figure 2.3, right panel). Since EGFR/importin α 1/ β 1 interaction is important for EGFR nuclear translocation, interruption of EGFR/importin β 1 interaction was then determined by immunoprecipitation (IP). Both anti-EGFR and anti-importin β 1 antibodies were first used for immunoprecipitation to detect EGFR/importin β 1 interaction from cell lysates collected 15 minutes after EGF-stimulation. However, no obvious interaction between the two could be detected. As a consequence, a direct evidence showing that decreased phospho-EGFR (pY845) in the nucleus was due to interruption of EGFR/importin β 1 interaction by ENLS peptide could not be provided. Nevertheless, data presented in Figure 2.3 still indicated that the ENLS peptide could inhibit nuclear translocation of phosphorylated EGFR (pY845) without affecting EGFR activation.

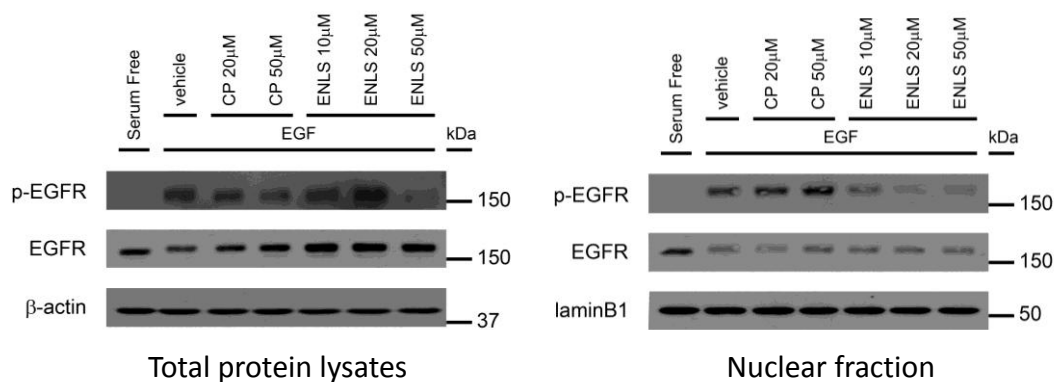


Figure 2.3. ENLS peptide affects phosphorylated EGFR (pY845) to translocate into the nucleus but not EGFR activation.

MDA-MB-468 cells were serum-starved overnight and pretreated with different concentration of control peptide or ENLS peptide for 30 minutes then followed by 100ng/ml of EGF for 10 min on ice. Cells were then incubated with water (vehicle), different concentration of CP or ENLS peptide in serum free medium for 30 minutes at 37°C and lysed. Total protein lysates and nuclear fraction of proteins were separated by SDS-PAGE and immunoblotted with antibodies as indicated.

C. ENLS peptide does not affect cell viability of MDA-MB-468 cells

As mentioned earlier, nuclear EGFR can also affect cell cycle progression through transactivational regulation of Cyclin D1 expression [126]. We next investigated whether inhibiting nuclear translocation of activated EGFR could affect Cyclin D1-regulated cell proliferation. MDA-MB-468 cells were used because overexpression of EGFR as well as nuclear localization of EGFR were both found in this cell line. After three days of treatment with either the control peptide or the ENLS peptide, viable cells were

quantified by MTT assay. Surprisingly, as shown in Figure 2.4, the ENLS peptide did not affect cell viability compared to the control peptide. This result indicated that inhibiting nuclear EGFR with 20 μ M ENLS peptides was not enough to affect cell viability in MDA-MB-468 cells.

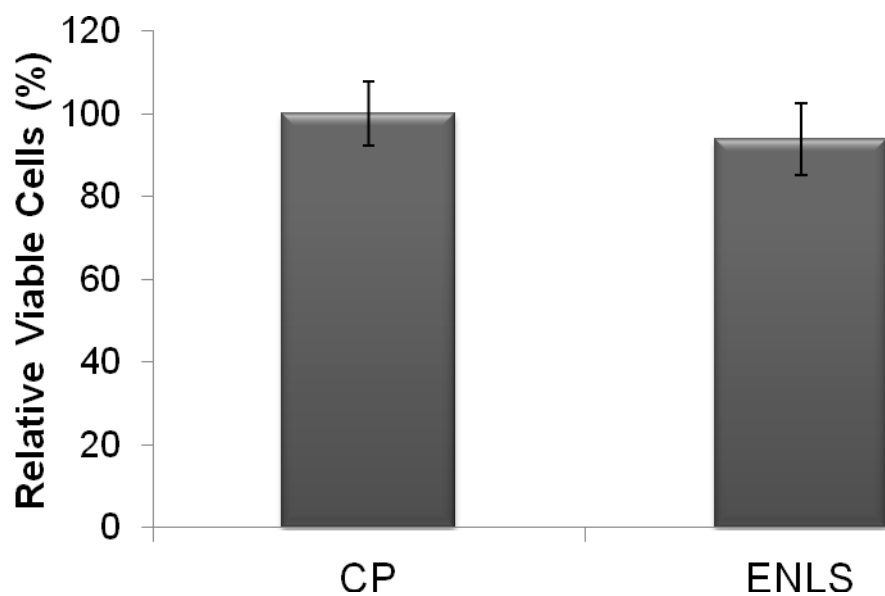


Figure 2.4. ENLS peptide does not affect cell viability of MDA-MB-468 cells.

MDA-MB-468 cells were seeded in a 24-well plate (1×10^4 cells/well) and treated with 20 μ M control peptide (CP) or 20 μ M ENLS peptide (ENLS) for 3 days. A MTT assay was done to quantify viable cells at day 3. CP-treated cells at day 3 is set as 100%. Values are the means of 3 independent determinations \pm SD.

D. ENLS peptide does not affect radiosensitivity of MDA-MB-468 cells

Nuclear EGFR also regulates DNA double-strand break repair through activation of DNA-PK and PCNA, and therefore has been linked to chemoresistance to DNA-damaging agents, such as cisplatin, and radiation resistance. We next determined whether ENLS peptide affected radiosensitivity in MDA-MB-468 cells by inhibiting nuclear translocation of activated EGFR. Four Gy ionizing radiation (IR) has been demonstrated to increase EGFR translocation into the nucleus of MDA-MB-468 cells [121]. Thus, MDA-MB-468 cells were pre-treated with water, 20 μ M CP or ENLS for 1 hour then irradiated with 4Gy IR and allowed to grow for 3 days. Viable cells were then quantified by MTT assay. As shown in Figure 2.5, radiation caused a ~50% decrease in viable cells across three different treatments. However, no differences between each treatment could be detected. This result indicated that ENLS peptide was not able to affect radiosensitivity in MDA-MB-468 cells, even though it was able to prevent phospho-EGFR (pY845) translocation to the nucleus.

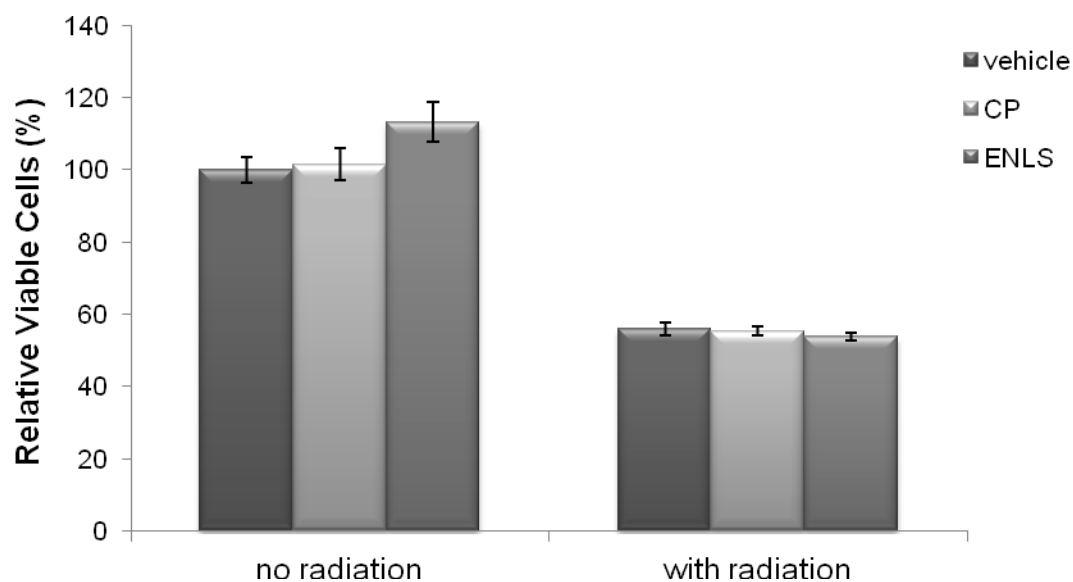


Figure 2.5. ENLS peptide does not affect radiosensitivity of MDA-MB-468 cells.

MDA-MB-468 cells were seeded in a 24-well plate (1×10^4 cells/well) and pre-treated with vehicle (water), 20 μ M control peptide (CP) or ENLS peptide (ENLS) 1 hour before radiation (4Gy). A MTT assay was done to quantify viable cells at day 3. Cells of vehicle/no radiation treated group at day 3 are set as 100%. Result is shown as one representative of two independent experiments. Error bars, SD.

E. ENLS peptide sensitizes AG1478-resistant cells

Lastly, nuclear EGFR is also important for acquired resistance to EGFR-targeted therapy, including both monoclonal antibodies and tyrosine kinase inhibitors. Huang *et al.* demonstrated a possible mechanism that nuclear EGFR-mediated breast cancer resistant protein (BCRP/ABCG2) expression might contribute at least in part to the acquired

resistance to gefitinib [88]. In this study, the authors found an increase of nuclear EGFR in the gefitinib resistant-MDA-MB-468 cells. Thus, we tested the effects of the ENLS peptide in this TKI-resistant cell line model and investigated whether inhibition of nuclear translocation of phospho-EGFR affected drug sensitivity of an established resistant cell line. We first established AG1478-resistant clones of MDA-MB-468 by culturing and selecting them with increasing concentrations of AG1478 from 0.1 μ M to 10 μ M over a period of three months. Cells that grew in the presence of 10 μ M AG1478 were then treated with vehicle, CP or ENLS, in the presence or absence of 10 μ M AG1478, and cell survival was measured after 3 days. As shown in Figure 2.6, removal of AG1478 could still promote cell growth in these AG1478-resistant cells. Without AG1478, there were no differences in CP and ENLS treated cells, similar to the results observed in Figure 2.4. With AG1478, interestingly, a statistically significant difference was detected between CP- and ENLS-treated cells. ENLS peptide by itself did not affect cell viability of MDA-MB-468 cells; in this case it could turn AG1478-insensitive MDA-MB-468 cells into AG1478-sensitive cells (Figure 2.6). This indicated that inhibition of nuclear EGFR could possibly overcome resistance to AG1478. However, the increased sensitivity only caused ~10% cell death when CP and ENLS treatments were compared.

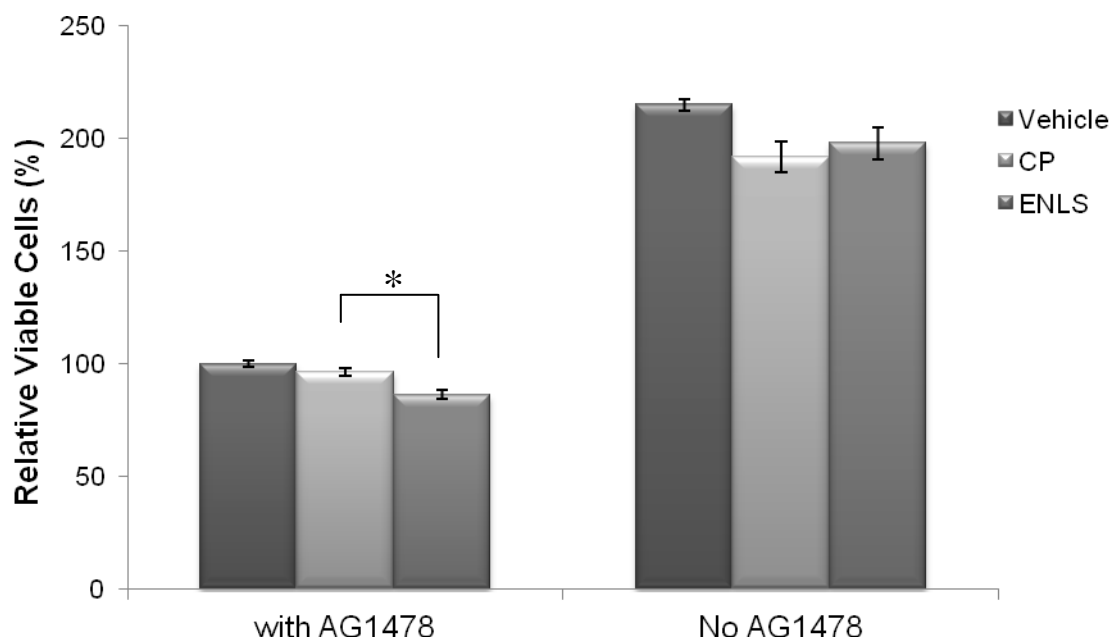


Figure 2.6. ENLS peptide sensitizes AG1478-resistant MDA-MB-468 cells.

AG1478-resistant cells were selected and grown with an escalated AG1478 concentration to 10 μ M. These AG1478-resistant cells were seeded in a 24-well plate (1 \times 10⁴ cells/well) and treated with water (Vehicle), 20 μ M control peptide (CP) or ENLS peptide (ENLS) in the presence or absence of 10 μ M AG1478 for 3 days. A MTT assay was done to quantify viable cells at day 3. CP/AG1478-treated cells at day 3 are set as 100%. Result is shown as one representative of three independent experiments. *, P < 0.05, Student's t-test. Error bars, SD.

IV. Discussion

In cancer cells, EGFR has been shown to translocate into the nucleus where it can act as a transcriptional co-activator to regulate target gene expression, including Cyclin D, b-myb, COX2, iNOS, and BCRP [88, 127, 128]. Nuclear EGFR is also able to regulate

DNA double-strand break repair through both activation of DNA-PK and inhibition of PNPase, and increased DNA repair has been linked to therapeutic resistance [119, 121, 129]. In this study, we set out to determine whether a peptide derived from EGFR NLS sequence could inhibit nuclear translocation of EGFR and overcome nuclear EGFR-dependent therapeutic resistance. We found that the ENLS peptide could effectively inhibit phospho-EGFR (pY845) to translocate into the nucleus without affecting EGFR activation (Figure 2.3). However, by itself, 20 μ M ENLS peptide was neither able to affect cell viability of MDA-MB-468 cells (Figure 2.4) nor able to change radiosensitivity of MDA-MB-468 cells (Figure 2.5). Even though ENLS peptide demonstrated an ability to sensitize AG1478-resistant cells to AG1478, the effect was observed in only 10% of the cells (Figure 2.6).

In Figure 2.3, we found that EGF stimulation only increased phospho-EGFR in the nucleus but not total EGFR. This finding was consistent with what Lin *et al.* reported [126]. Therefore, ENLS peptide could only affect translocation of phospho-EGFR. The fact that EGFR activation was not affected by ENLS peptide further supported that only trafficking of EGFR was inhibited. Interaction between EGFR and Importin β 1 was first demonstrated in A431 cells and the interaction was only slightly increased after EGF

stimulation [97]. An optimization for this immunoprecipitation experiment is needed to be able to directly determine the mechanisms of action of ENLS peptide.

The lack of obvious biological output shown in Figure 2.4 and Figure 2.5 could be due to the high expression level of EGFR in MDA-MB-468 cells. The high expression level of EGFR could drive a strong signal through canonical cytosolic signaling pathways which could mask or easily compensate for the nuclear effect of EGFR. The stability of the peptide could also be an issue affecting the biological effect of this peptide. Modification of peptides with N-terminal acetylation and C-terminal amide/PEGylation might increase stability of peptides inside cells [130-132]. Replacement with D-amino acids could also increase peptide stability [132]. Besides being used as a strategy for improving stability, peptide modification may be rationally designed to achieve better efficacy. For example, Dittmann *et al.* used a phospho-peptide (Ac-RKRpTLRRLK) to inhibit radiation-induced nuclear shuttling of EGFR, based on the finding that phosphorylation of threonine 654 within NLS sequence of EGFR was critical for nuclear translocation of EGFR [133]. The efficacy of modified peptides warrants further investigation.

Nuclear EGFR contributes to resistance to cetuximab [87] and gefitinib [88]. We

found that inhibition of nuclear phospho-EGFR could sensitize AG1478-resistant MDA-MB-468 cells to AG1478 again. However, this sensitization only affected 10% of the cells. This finding indicated that there were other possible mechanisms involved in AG1478-resistance, at least in this case.

So far, we provide evidence to support the possibility of overcoming therapeutic resistance with a peptide derived from EGFR NLS sequence to inhibit nuclear translocation of EGFR. A further investigation is definitely warranted to explore this as a combination therapy in the future.

CHAPTER 3 –A POTENTIAL DRUG DERIVED FROM JUXTAMEMBRANE

DOMAIN OF EGFR

Note: The work presented in this chapter has already been combined with more data from Matt Hart and submitted to *Molecular Therapy*. Experiments in Figure 3.2, Figure 3.2, Figure 3.4a, Figure 3.8, Figure 3.14, Figure 3.17, Figure 3.21, Figure 3.22, Figure 3.23 and Figure 3.24 were performed by Derrick Broka. Experiments in Figure 3.4b, Figure 3.4c and Figure 3.18 were performed by Matt Hart.

I. Introduction

EGFR belongs to the family of receptor tyrosine kinases based on its function as both “receptor” and “tyrosine kinase”. Upon ligand binding, a conformational change leads to transactivation of the receptor dimers and propagates signals downstream of the pathway, ultimately controlling cell proliferation, migration and survival (reviewed in [22]). Aberrant activation of EGFR results in tumor formation and progression. First attempts to target EGFR were based mainly on the most important characteristics of EGFR, which are the “receptor” and the “tyrosine kinase”. Yet, both the anti-EGFR antibody against “receptor” and TKI against “tyrosine kinase” showed somewhat unsuccessful results as treatments for breast cancer patients. A different approach may be

needed as an EGFR-targeted therapy.

Interestingly, the juxtamembrane domain of EGFR controls various non-traditional kinase related functions of the receptor, including trafficking to the nucleus/mitochondria, involvement in calcium signaling and regulation/stabilization of activation/inactivation structure of EGFR [15, 20, 94, 134, 135]. Disruption of this important domain of EGFR would affect multiple aspects of EGFR biology, all of which contribute to tumorigenesis. Therefore, It was hypothesized that targeting the juxtamembrane domain of EGFR by peptide inhibitors could inhibit oncogenic activities of EGFR.

II. Materials and Methods

Cell culture All cell lines were obtained from American Type Culture Collection (ATCC). MDA-MB-468, MDA-MB-231, T47D breast cancer cell lines were grown in RPMI (Mediatech, Inc., Manassas, VA) and supplemented with 10% (5% for 468 cells) FBS (PAA, Piscataway, NJ). BT20 cells were grown in Modified Eagles Medium (MEM, ATCC, Manassas, VA) supplemented with 10% FBS. MCF10A cells were grown in DMEM F12 (Invitrogen Life Technologies Inc., Carlsbad, CA), supplemented with 5% Horse Serum, 20ng/ml EGF, 0.5µg/ml Hydrocortisone, 100ng/ml Cholera-Toxin,

10 μ g/ml Insulin, and 1% Penicillin/Streptomycin (Mediatech, Inc., Manassas, VA) as described in [136]. All lines were grown under 5% CO₂.

Compounds and reagents EGFR 1005 antibody and ML-7 were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). EGFR Ab-13 was obtained from NeoMarkers (Fremont, CA) and the following antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA): p-EGFR (pY845), LC3B, PARP, p-AKT (pS473), AKT, p42/44 MAPK (ERK1/2), HMGB1, p-p38 (pT180/Y182), and p38. dp-ERK and β -actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-4 calcium assay kit and Vybrant apoptosis assay kit were purchased from Invitrogen Life Technologies Inc. (Carlsbad, CA). 3-MA was obtained from Calbiochem (Billerica, MA). Carbonyl cyanide 3-chlorophenyl- hydrazone (CCCP) was purchased from Sigma-Aldrich (St. Louis, MO). Y-27632 was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). W-13 hydrochloride was purchased from Tocris Bioscience (Minneapolis, MN).

Western blotting Following treatments, cells were harvested and lysed in lysis buffer consisting of 20mM Tris, pH 7.5, 150mM NaCl, 1% NP-40, and 5mM EDTA, pH 8.0,

along with protease and phosphatase inhibitors. Protein concentration was determined using Bicinchoninic Acid assay (Thermo Fisher Scientific Inc., Waltham, MA). Lysates were then separated using SDS-PAGE before being transferred to PVDF membranes (EMD Millipore Inc., Billerica, MA) and immunoblotted using indicated antibodies.

Crosslinking/dimerization assay MDA-MB-468 cells were plated in 100mm dish with 3×10^6 cells. Cells were pre-treated with water (vehicle), 20 μ M control peptide (CP) or 20 μ M EJ1 (EJ1) in serum-free medium for 10 minutes on ice and then stimulated with 100ng/ml EGF and respective treatments for another 10 minutes on ice and then washed with PBS twice. Proteins were crosslinked by 3 μ M DMS (Thermo Fisher Scientific Inc., Waltham, MA) for 30 minutes on ice and then the cells were lysed.

Annexin V/propidium iodide staining (Apoptosis assay) MDA-MB-468 cells were treated with 20 μ M CP or EJ1 in complete medium for indicated times. Following treatment, cells were then trypsinized and collected in eppendorf tubes and stained with Vybrant apoptosis assay kit (Invitrogen Life Technologies Inc., Carlsbad, CA) following the manufacturer's protocol to assess the percentage of non-stained, Annexin V only, PI

only, or Annexin V plus PI double-stained cells. Cells were then sorted by a FACScan flow cytometer (BD Biosciences, San Jose, CA) and analyzed by Cellquest Pro 4.0 software.

Mitochondrial morphology change MDA-MB-468 cells were treated in complete media with 200nM MitoTracker Red CMXRos (Molecular Probes Life Technologies Inc., Carlsbad, CA) along with 5µg/ml Hoechst 33342 (Invitrogen Life Technologies Inc., Carlsbad, CA) nuclear stain for 15 minutes. Media was then removed and fresh media containing 20µM CP or EJ1 were added. Images were taken on an Olympus IX71 microscope and deconvolved using softWoRx 4.0 image analysis software (Applied Precision, Issaquah, WA) at the Imaging Shared Service in the Arizona Cancer Center (AZCC). Images were brightened using Adobe Photoshop.

Cell growth/viability assay Cells were plated in 96 well plates with 2×10^3 cells or in 24 well plates with 1×10^4 cells. On the following day (day 0) treatment (water, CP or EJ1) began and was changed every other day if not specified. On the final day of treatment, media were removed and cells were incubated in 0.5-1mg/ml MTT reagent

(Sigma-Aldrich, St. Louis, MO) for 2-3 hours at 37°C. Following this incubation, media and MTT were removed and formazan crystals were dissolved in 100µl DMSO/well for 96 well plates or 600µl DMSO/well for 24 well plates. Absorbance was read at 540nm using a U-Quant Spectrophotometer (Bio-TEK Instruments Inc., Winooski, VT).

Calcium assay Following treatment in 96 well plates, Fluo-4 calcium assay kit was used to stain cells following manufacture's protocol. Fluorescence intensity was measured by fluorescent plate reader using excitation/emission wavelengths set at 494 nm and 516 nm respectively.

HMGB1 release assay Medium from treated cells was harvested, spun at 800g for 5 minutes and supernatant was filtered (0.45 µm filter) (EMD Millipore Inc., Billerica, MA). Proteins were precipitated with 0.02% DOC (deoxycholate) and 10% TCA (trichloroacetic acid) and lysed in 2x SDS buffer for immunoblots as referenced from [137].

Measurement of intracellular ROS formation The generation of intracellular ROS was

determined using a fluorescein-labeled dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Invitrogen Life Technologies Inc., Carlsbad, CA). The non-fluorescent dye permeates cells easily and is hydrolyzed to 2',7'-dichlorofluorescein (DCF) upon interaction with intracellular ROS. Cells were first labeled with 20 μ M DCFH-DA for 30 min at 37°C. Then, cells were treated as indicated and washed twice with ice-cold PBS and harvested by trypsin. Then, the cells were immediately analyzed by a FACScan flow cytometer at the Flow Cytometry Shared Service at the AZCC and excitation/emission wavelengths were set at 488 nm and 525 nm respectively [138].

Mitochondrial membrane potential Cells were stained with 2 μ M 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) (Molecular Probes Life Technologies Inc., Carlsbad, CA) in serum-free media for 15 minutes and washed twice with PBS after staining. Cells were then treated as indicated. Fluorescence was determined with a plate reader at wavelengths of 514 nm (excitation) and 529 and 590 nm (emission). The ratio of green and red fluorescence signals serves as a parameter for the mitochondrial membrane potential independent of the mitochondrial mass.

MMTV-PyMT mouse experiments Female MMTV-PyMT mice were palpated weekly until tumors >5.0 mm in diameter. At this point, animals were placed on study, and injected daily for 21 days or until total tumor burden reached >10% of initial body weight, an individual tumor was over 2 cm in average diameter, or a tumor ulcerated through the skin. Tumors were measured on average every 5 days and size was calculated using the formula: $(x \times x) \times (y/2)$, where x and y are separate horizontal and vertical measurements (*i.e.* length and width), respectively.

The number of metastases to the lungs was assessed in control (6 mice) and EJ1 (7 mice) treated mice. Lungs from these mice were fixed, sectioned (10 μ m thickness) and stained with Haematoxylin and Eosin. Metastatic foci of 5 individual sections spanning 200 μ m/mouse lung were then counted, and each section was included as a separate “n” (*i.e.* control n=30 and EJ1 n=35).

Statistical Analysis All statistics were performed in Excel (Microsoft). Test implemented was two-tailed Student’s t-test.

III. Results

A. EGFR juxtamembrane peptide reduces cell viability

The juxtamembrane domain of EGFR contains sequences responsible for receptor dimerization, calmodulin binding, nuclear localization, and mitochondrial localization (Figure 1.5). Therefore, we set out to determine if blocking the function of the juxtamembrane domain of EGFR would result in an effective, EGFR-dependent cancer therapeutic. To do this, we created cell-penetrating peptides to act as dominant-negative “decoys”, thereby inhibiting endogenous juxtamembrane domain interactions. Peptides specific for juxtamembrane subdomains were synthesized downstream of the Protein Transduction Domain-4 (PTD4, [139]) (Figure 3.1). Next, the effect of peptide treatment on cell viability was analyzed on the breast cancer cell line MDA-MB-468 by MTT analysis after three days of treatment (Figure 3.2). We found that the amino acid region between hEGFR⁶⁴³⁻⁶⁶³ (EJ1, >90% reduction) demonstrated optimal reduction in viability, and partial reduction was also obtained with sub-sequences within EJ1, including EJ2 (hEGFR⁶⁴³⁻⁶⁵⁵, ~30% reduction), EJ3 (hEGFR⁶⁴⁹⁻⁶⁶³, ~60% reduction) and EJ5 (hEGFR⁶⁵³⁻⁶⁶³, ~40% reduction) (Figure 3.2).

	<i>Sequence</i>	<i>Change</i>	<i>Name</i>
hEGFR ⁶⁴³⁻⁶³	PTD4-FMRRRHIURKRTLRRLLQERE•••••		EJ1
hEGFR ⁶⁴³⁻⁵⁵	PTD4-FMRRRHIURKRTL•••••	-RRLLQERE	EJ2
hEGFR ⁶⁴⁹⁻⁶³	PTD4-•••••IURKRTLRRLLQERE•••••	FMRRRH-	EJ3
hEGFR ⁶⁴⁵⁻⁵⁷	PTD4-••RRRHIURKRTLRR•••••	FM-LLQERE	EJ4
hEGFR ⁶⁵³⁻⁶³	PTD4-•••••RTLRRLLQERE•••••	FMRRRHIVRK-	EJ5
hEGFR ⁶⁵⁸⁻⁶⁹	PTD4-•••••LLQERELVEPLT	JXM-A C-terminal	EJ6
hEGFR ^{658-69LVΔ4xA}	PTD4-•••••AAQEREAAEPLT	658-69 L&V to A	EJ7
hEGFR ^{s643-63}	PTD4-FRMHRIRURTKLRLRLRQERE•••••	Scramble 643-63	EJ8
hEGFR ^{643-63RKΔ8xA}	PTD4-FMAAAHIUAAATLAALLQERE•••••	Scramble 643-63 R&K to A	EJ9
hEGFR ^{s643-55}	PTD4-FRMHRIRURTKLR•••••	Scramble 643-55	EJ10
hEGFR ^{s649-63}	PTD4-•••••RURTKLRLRLRQERE•••••	Scramble 649-63	EJ11
hEGFR ^{645-57RKΔ3xD}	PTD4-••RDRHIURDRTLRLD•••••	645-57 R&K to D	EJ12
hEGFR ^{643-63RKΔ3xD}	PTD4-FMRDRHIURDRTLRLD•••••	643-63 R&K to D	EJ13
hEGFR ^{643-63RKΔ3xQ}	PTD4-FMRQRHIURQRTLRLD•••••	643-63 R&K to Q	EJ14

Figure 3.1. Peptide design and nomenclature.

The amino acid number of EGFR is shown in the left column, which corresponds to the specific amino acids shown in the middle column (sequence). Peptides were designated EJ1-14, as indicated in the right column. Using EJ1 as the parental sequence, changes in EJ2-14 are denoted in the second column from the right.

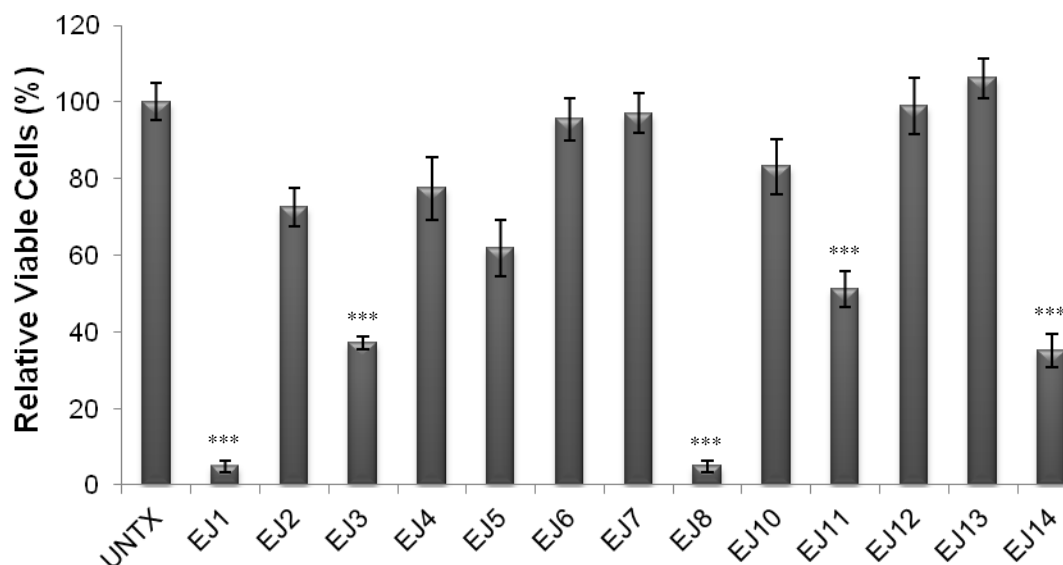


Figure 3.2. Juxtamembrane domain peptides reduce cell viability.

MDA-MB-468 cells were treated daily with 20 μ M of different peptides or left untouched for three days, and cell viability was determined by MTT assay. ***, $p < 0.001$, Student's t-test. Error bars, SD.

Interestingly, scrambling of the amino acids of EJ1 resulted in no loss of efficacy (EJ8), indicating charge may be important. To test the role of charge of the peptide, one of the basic amino acids (R or K) in each of the three basic clusters of EJ1 was substituted with an acidic amino acid (D; EJ13 hereafter referred to as control peptide, CP) and this completely ablated the effects on viability (Figure 3.2). Note that replacement of the eight arginines and lysines with alanines resulted in an insoluble peptide (EJ9). Substituting those same basic amino acids with polar amino acids (Q; EJ14)

instead only marginally blocked the anti-proliferative effects of EJ1 (Figure 3.2).

Together, these results strongly implicate charged residues in the function of EJ1.

To determine if either the minimal nuclear localization sequence (EJ4), or the minimal basolateral domain (EJ6) was responsible for the anti-proliferative effects of EJ1, peptides of these subdomains were created. No anti-proliferative effect was observed for either peptide, implicating the calmodulin and dimerization domains as essential for cell death (Figure 3.2). After determining the optimal peptide concentration in MDA-MB-468 cells (Figure 3.3, $IC_{50}=6.14\pm 0.79\mu M$), EJ1 was tested for its ability to affect cell viability in additional breast cancer cell lines including MDA-MB-231 (Figure 3.4a), and T47D (Figure 3.4b), and the immortalized breast epithelial cell line MCF10A (Figure 3.4c). Due to limited availability of the peptides, we only determined IC_{50} for MDA-MB-468 cells. In analyzing the effects of EJ1 in these lines, we found that its effects range from a minimum of 27% reduction in MCF10A cell viability (Figure 3.4c), to a maximum of 75% reduction in that of T47D cells over a three day treatment period (Figure 3.4b). Analysis of the erbB expression profile (including EGFR, ErbB2 and ErbB3) in these cell lines demonstrated expression of at least two of the three erbB receptors in each of the lines (Figure 3.5).

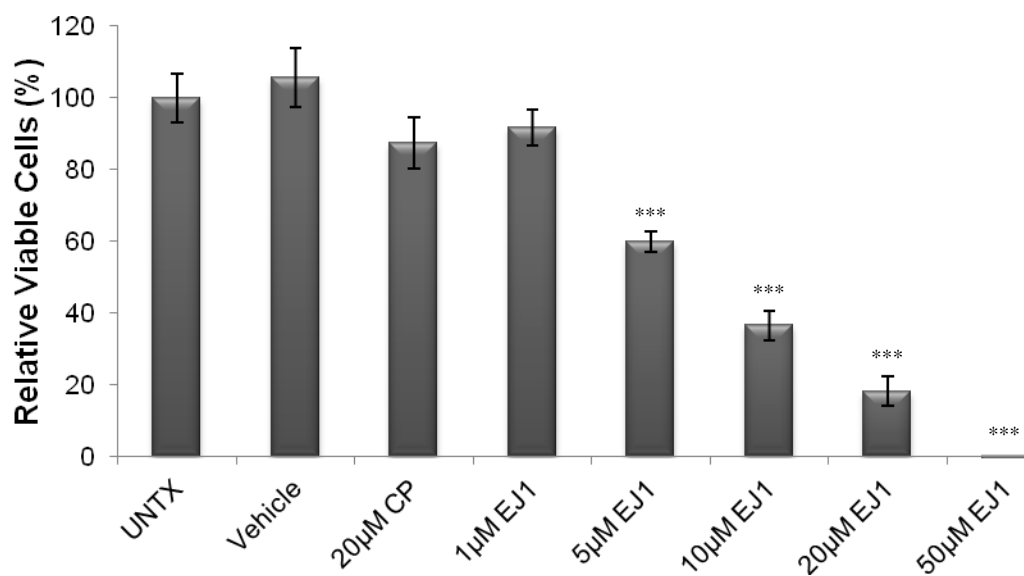


Figure 3.3. Dose-dependent response of EJ1 peptide in MDA-MB-468 cells.

MDA-MB-468 cells were treated daily with a different concentration of EJ1 peptide or left untouched (UNTX) for three days and cell viability was determined by MTT assay. The UNTX group of cells was set as 100%. ***, $p < 0.001$, Student's t-test. Error bars, SD.

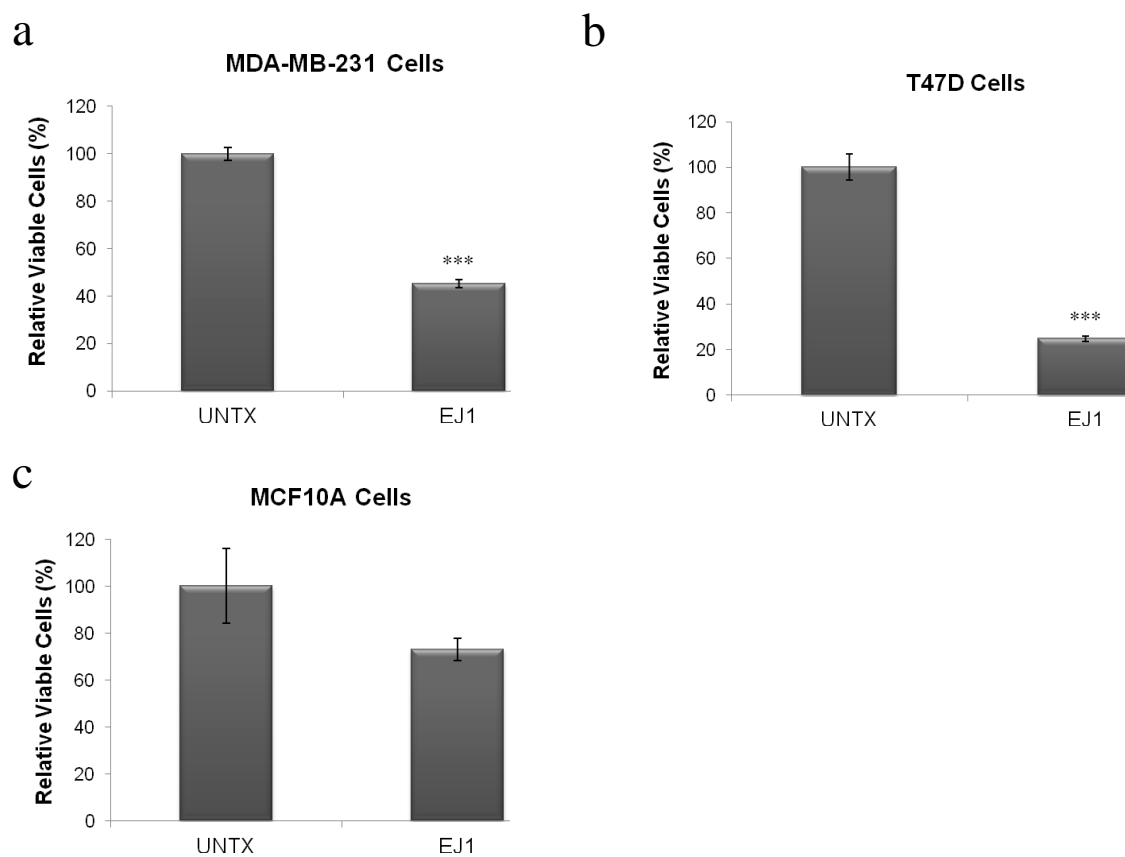


Figure 3.4. EJ1 peptide affects cell viability in different cell lines.

MDA-MB-231 (a), T47D (b), and MCF10A (c) cells were treated daily with 20 μ M of EJ1 peptide or left untouched (UNTX) for three days, and cell viability was determined by MTT assay. ***, $p < 0.001$, Student's t-test. Error bars, SD.

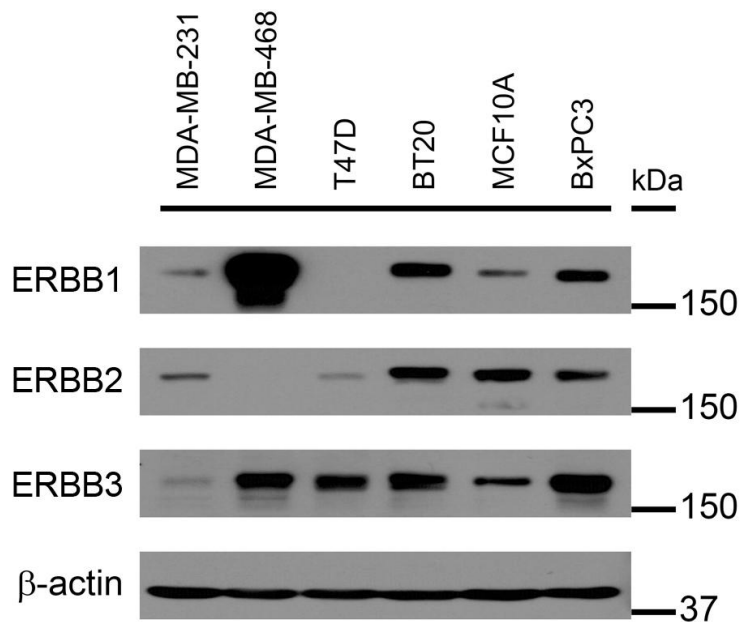


Figure 3.5. EGFR, ErbB2 and ErbB3 expression in different cell lines.

EGFR, ErbB2 and ErbB3 expression were determined in breast cancer cell lines (MDA-MB-231, MDA-MB-468, T47D, and BT20), an immortalized breast epithelial cell line (MCF10A), and a pancreatic cell line (BxPC3). Forty micrograms of cell lysates from each cell line were loaded on SDS-PAGE and immunoblotted with antibodies as indicated.

B. EJ1 inhibits EGFR activation through promoting inactive dimers

To determine whether EJ1 was affecting EGFR activity, we first treated MDA-MB-468 with EJ1, CP or a vehicle in the presence or absence of EGF to activate EGFR (Figure 3.6). We found that EJ1 significantly suppressed EGF-induced phosphorylation of EGFR (pY845). This suppression also affected downstream signaling partners, resulting in a reduction of p-AKT, dp-ERK (Figure 3.6 and data not shown).

Interestingly, treatment with EJ1 also resulted in a loss of total protein for AKT, p38, and p42/44 MAPK (Figure 3.6 and data not shown). In addition, an increase of the activated stress response kinase, p38, was observed upon EJ1 treatment.

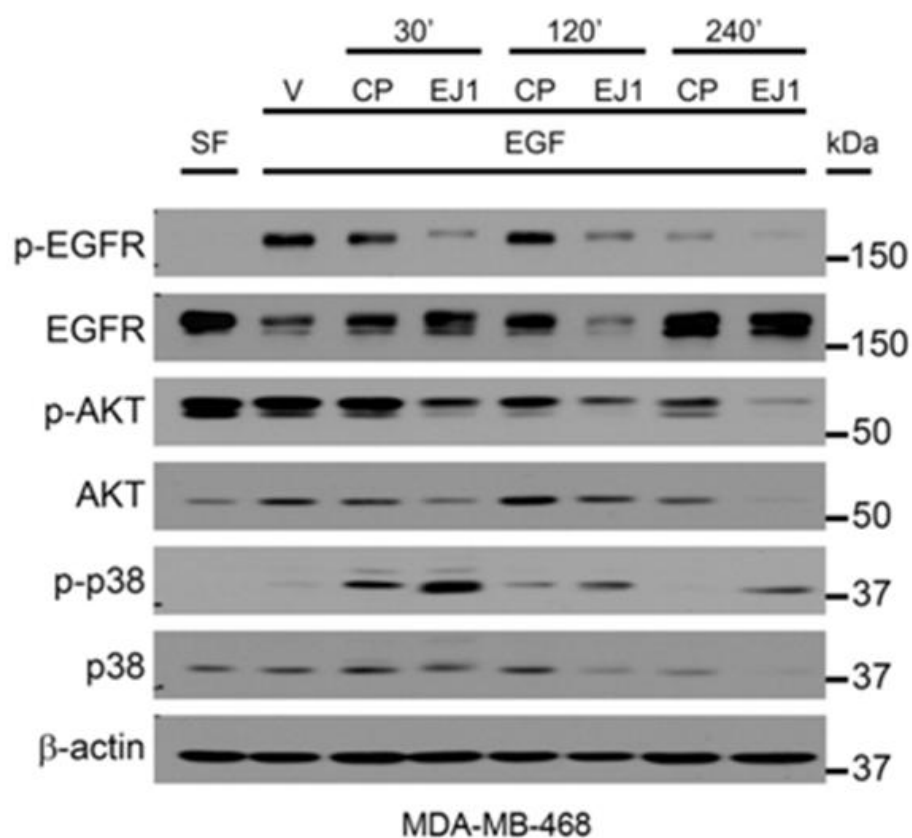


Figure 3.6. EJ1 peptide inhibits EGFR activation.

MDA-MB-468 cells were serum-starved overnight and treated with 100ng/ml EGF for 10 minutes on ice and then incubated with water (V), 20μM CP (CP) or EJ1 (EJ1) in serum-free medium for the indicated times at 37°C and lysed. Protein lysates were separated by SDS-PAGE and immunoblotted with antibodies as indicated.

As the EJ1 peptide mimics the dimerization domain of EGFR, we next evaluated the ability of EJ1 to block dimerization. To evaluate the effects of EJ1 on EGFR homodimers, MDA-MB-468 cells were treated with EGF and EJ1 or controls in the presence of a non-cleavable cross-linker. Surprisingly, we found that EJ1 induced the formation of EGFR dimers (Figure 3.7). Together, these results indicate that EJ1 inhibits EGFR activation and promotes the dimerization of inactive EGFR receptors.

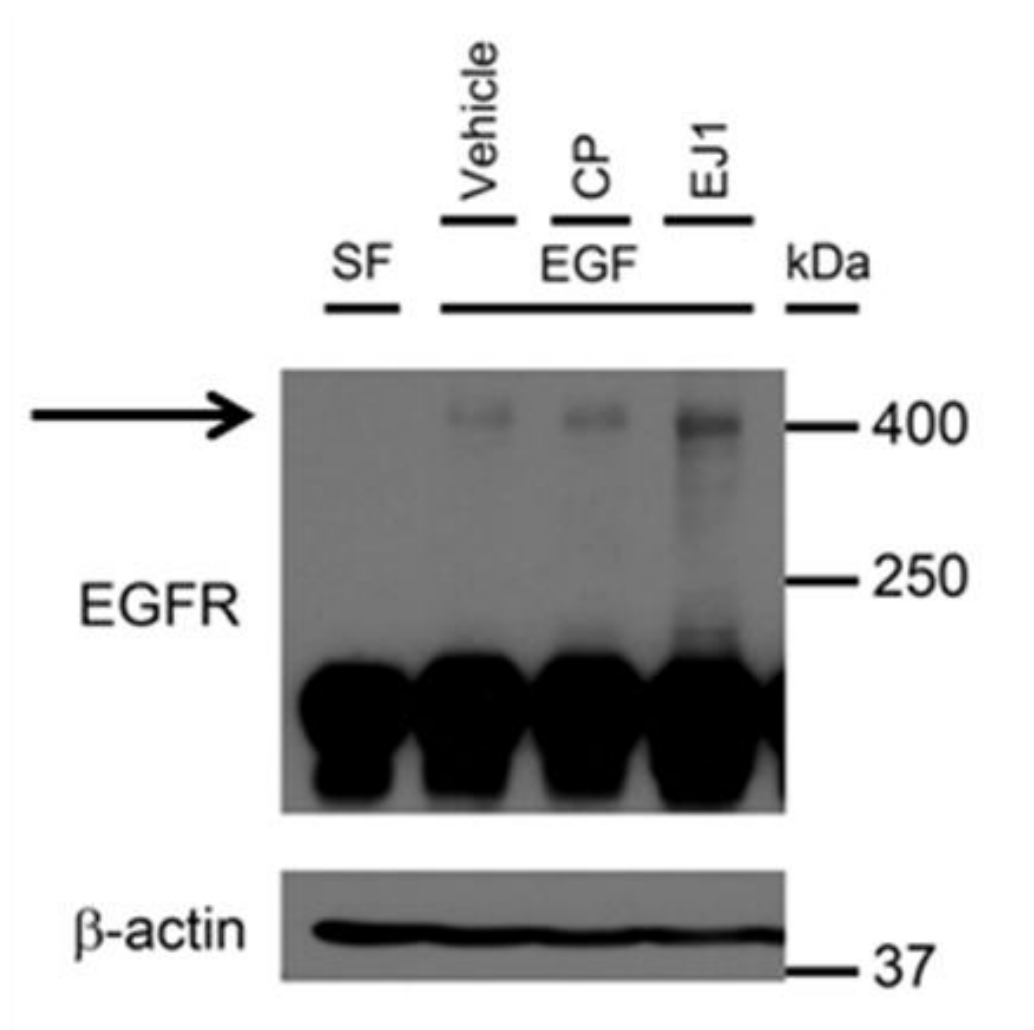


Figure 3.7. EJ1 peptide promotes EGFR homodimer formation.

MDA-MB-468 cells were serum-starved overnight and pre-treated with water (Vehicle), 20 μ M CP (CP) or EJ1 (EJ1) in serum-free medium for 10 minutes on ice. Cells were then stimulated with 100ng/ml of EGF and respective treatment for another 10 minutes on ice and then washed with PBS twice. Proteins were crosslinked by 3 μ M DMS for 30 minutes on ice and then lysed. Protein lysates were separated by SDS-PAGE and immunoblotted with antibodies as indicated. Arrow indicates EGFR homodimers.

C. EJ1 affects cell survival through apoptosis/necrosis

In order to understand how EJ1 peptide affected cell viability, MDA-MB-468 cells were evaluated for cleaved PARP (apoptosis marker) and LC3B (autophagy marker [140]) expression (Figure 3.8). An induction of the cleavage of PARP as well as a conversion of LC3B-I (upper band) to LC3B-II (lower band) were both observed upon EJ1 treatment compared to controls. Autophagy has been linked with cell death as well as with the cell survival mechanism of cancer cells (reviewed in [141]). To determine whether EJ1-induced autophagy was a mechanism of cell death, we measured cell viability upon a co-treatment of EJ1 and 3-MA, an autophagy inhibitor via the inhibition of type III PI3K [142] (Figure 3.9). We found that the inhibition of autophagy results in more dead cells upon EJ1 treatment, which indicated autophagy in this case was actually a pro-survival signal for cells. To further test if apoptosis was the only mechanism of cell death caused by EJ1, we performed a flow cytometry to quantitatively determine propidium iodide (PI) staining and annexin V binding in EJ1-treated cells. As shown in Figure 3.10 and Figure 3.11, to our surprise, we observed only 6% and 2% of cells stained with annexin V alone after 6 hours and 24 hours of EJ1 treatment, respectively. For annexin V/PI double-stained cells, we also observed only 10% and 9% of cells after 6 hours and 24

hours of EJ1 treatment respectively. Of note, there were 4% and 2% of cells stained with annexin V alone and there were 6% and 5% of cells double-stained with annexin V/PI after 6 hours and 24 hours of CP treatment, respectively. Both the percentage of cells undergoing apoptosis and the differences between EJ1 and CP treatment indicated that apoptosis might not be the only mechanism of EJ1-induced cell death.

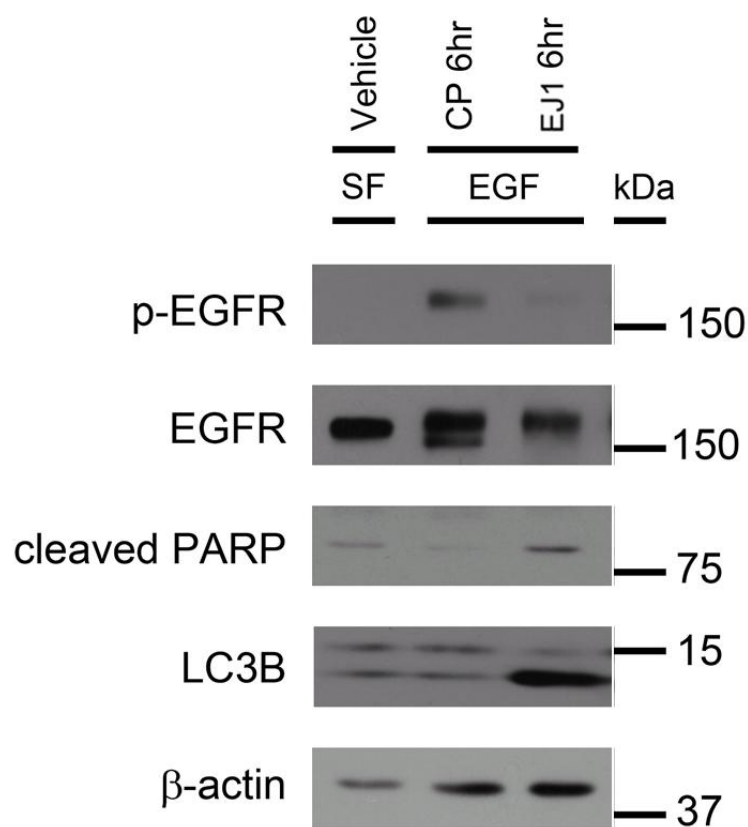


Figure 3.8. EJ1 peptide induces apoptosis and autophagy.

MDA-MB-468 cells were serum-starved overnight and treated with 100ng/ml EGF for 10 minutes on ice, then washed with PBS to remove unbound ligands. Cells were then incubated with water (Vehicle), 20 μ M CP or 20 μ M EJ1 in serum-free medium at 37°C for 6 hours and lysed. Protein lysates were separated by SDS-PAGE and immunoblotted with antibodies as indicated.

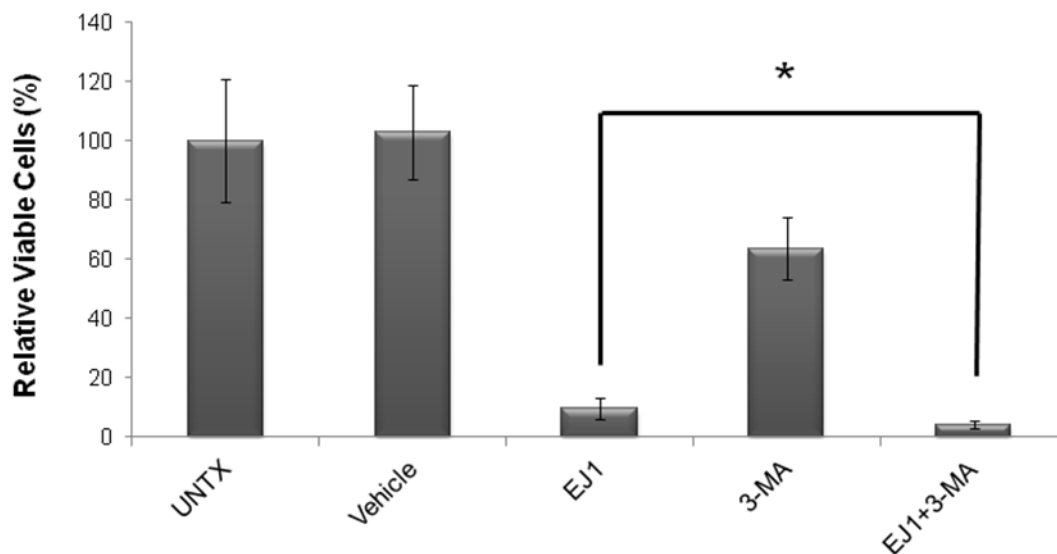


Figure 3.9. Inhibition of autophagy causes more cell death upon EJ1 treatment.

MDA-MB-468 cells were cultured in a 96-well plate (2×10^3 cells/well) and treated with either water (Vehicle), 20 μ M EJ1, 1mM 3-MA or EJ1 in combination with 3-MA in complete medium for seven days. A MTT assay was done to quantify viable cells at day 3. Y-axis is expressed as percentage change of each treatment to untreated cells (UNTX). *, $P < 0.05$, Student's t-test. Error bars, SD.

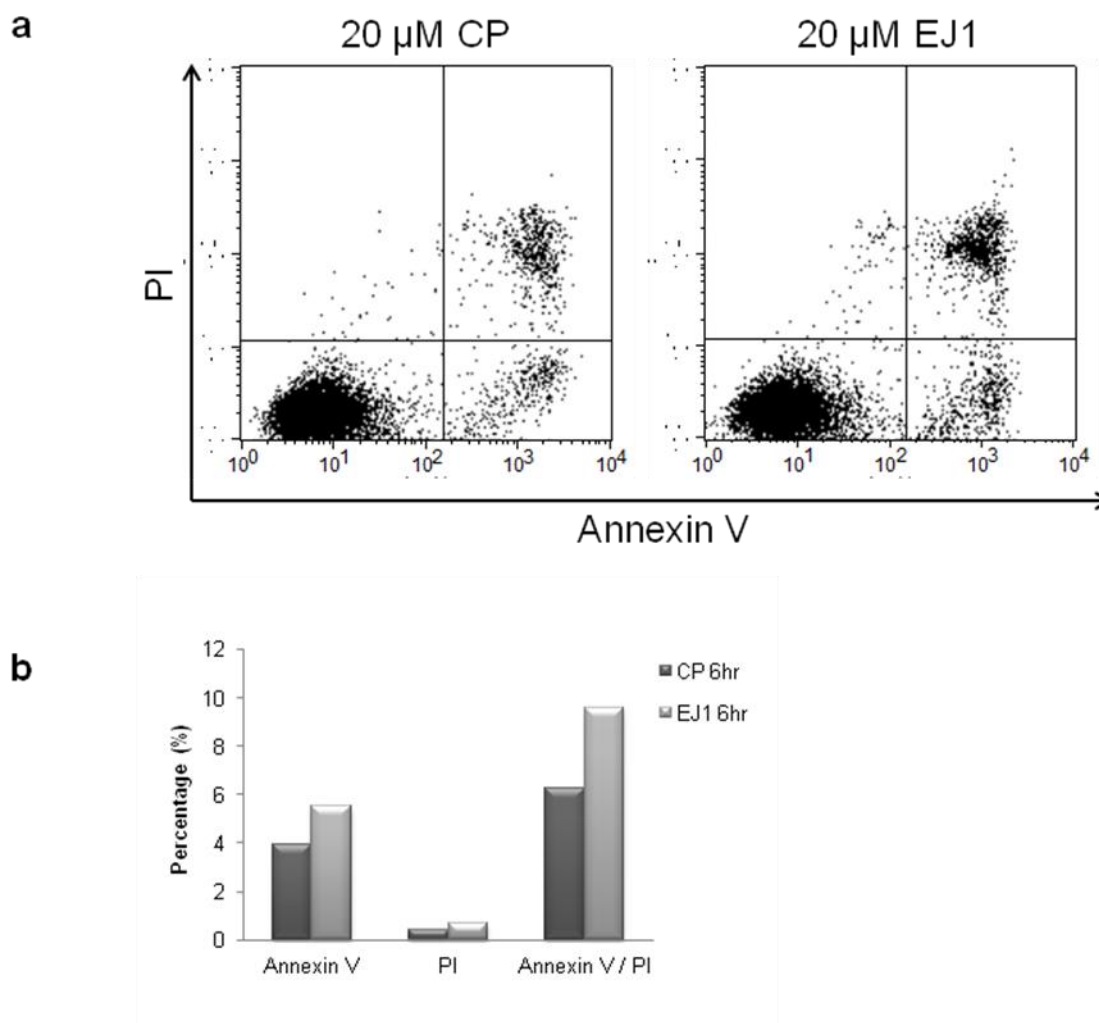


Figure 3.10. EJ1 peptide induces a minor fraction of cells to undergo apoptosis after 6 hours of treatment.

(a) MDA-MB-468 cells were treated with 20 μ M CP or EJ1 in complete medium for 6 hours at 37 $^{\circ}$ C and then stained with propidium iodide (PI) and annexin V-FITC. Cells were then sorted by a FACScan flow cytometer (BD Biosciences) and analyzed by Cellquest Pro 4.0 software. The result is shown as flow cytometry dot plots with annexin V staining in x axis and PI in y axis. (b) The result is shown as histogram to represent percentage of cells in each quadrant.

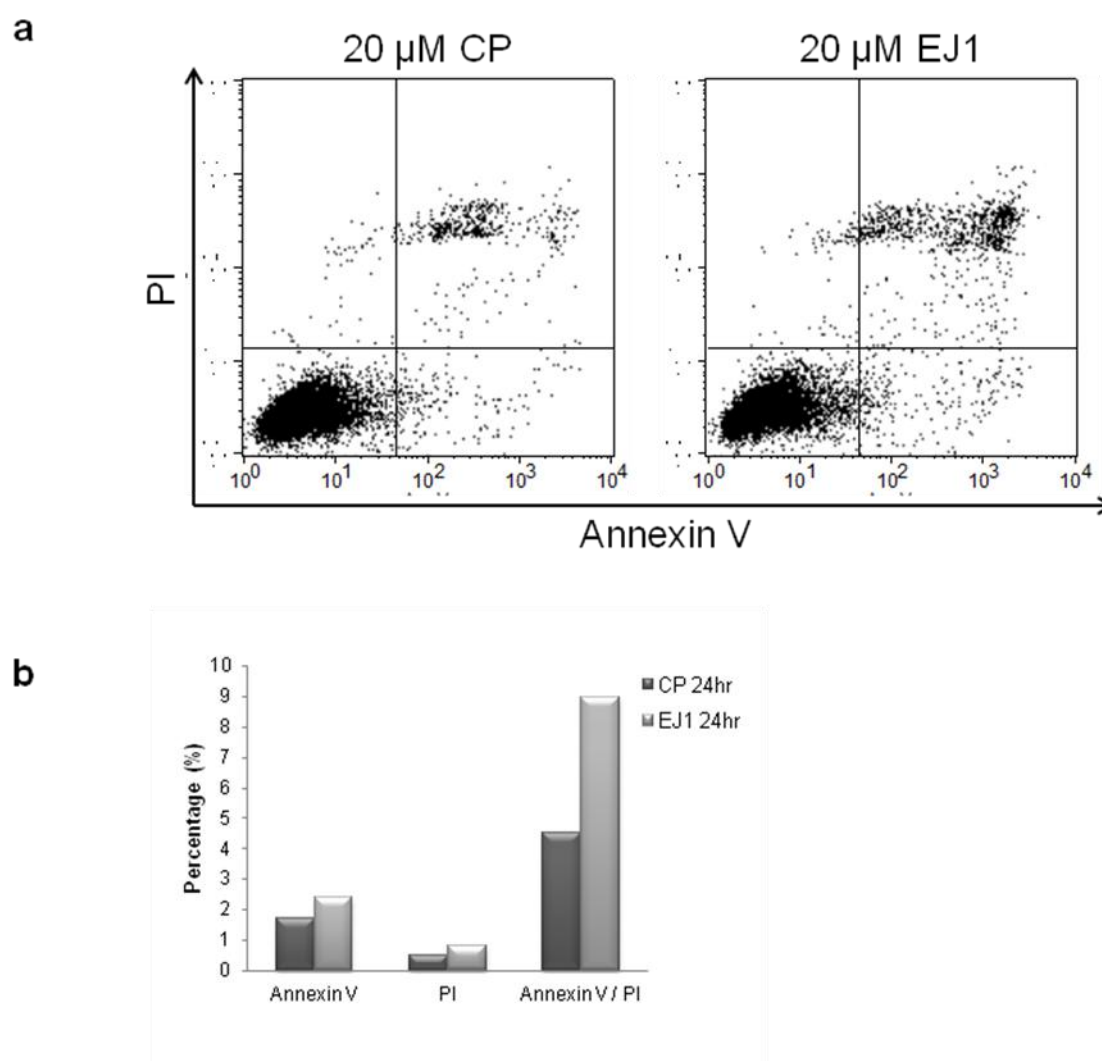


Figure 3.11. EJ1 peptide induces a minor fraction of cells to undergo apoptosis after 24 hours of treatment.

(a) MDA-MB-468 cells were treated with 20 μ M CP or EJ1 in complete medium for 24 hours at 37°C and then stained with propidium iodide (PI) and annexin V-FITC. Cells were then sorted by a FACScan flow cytometer (BD Biosciences) and analyzed by Cellquest Pro 4.0 software. The result is shown as flow cytometry dot plots with annexin V staining in x axis and PI in y axis. (b) The result is shown as histogram to represent percentage of cells in each quadrant.

To further explore the mechanism of cell death induced by the EJ1 peptide, cell morphology was examined following EJ1 treatment. We found that by 15 minutes, the EJ1 peptide induced the formation of large membrane protrusions or blebs (Figure 3.12f, arrows) and by 60 minutes of treatment, cells had formed large intracellular vacuoles (Figure 3.12g, arrowheads). After 16 hours, most EJ1-treated cells had died (Figure 3.12h). To investigate the vacuoles shown in Figure 3.12g, transmission electron microscopy (TEM) was done (Figure 3.13). MDA-MB-468 cells were treated with 20 μ M EJ1 and evaluated at several timepoints by TEM (Figure 3.13a-d). By 30 minutes, double-membrane structures (Figure 3.13c', arrowheads) filled with organelle debris (Figure 3.13c', filled arrows) and electron dense deposits (Figure 3.13c', open arrows) were observed in EJ1-treated cells. Electron-dense deposits in the swollen mitochondria usually suggest an increase of Ca²⁺ influx [143] or even a calcium overload [144]. A possible mechanism of massive Ca²⁺ influx is loss of membrane integrity. To test this possibility, plasma membrane integrity was determined by a calcein-AM leakage assay. In live cells, nonfluorescent calcein-AM can cross the membrane and be converted to a green-fluorescent calcein (after acetoxymethyl ester hydrolysis by intracellular esterases), becoming membrane impermeable. If the plasma membrane integrity is compromised,

the calcein dye will be released from the cells and can then be detected in the media. Therefore, MDA-MB-468 cells were treated with calcein-AM for 30 minutes, followed by treatment with vehicle, CP, EJ1, or Triton X-100 as a positive control for 15 minutes (Figure 3.14). EJ1 treatment resulted in a significant increase of calcein signal in the media, and the same results were seen with T47D, BT20 and MDA-MB-231 cells (data not shown), indicating the plasma membrane integrity was breached by EJ1. To further understand whether the observed membrane damage caused an increase of intracellular calcium concentration, which in turn resulted in swollen mitochondria with electron-dense deposits, a calcium assay was done to measure intracellular calcium concentration after EJ1 treatment (Figure 3.15). Not surprisingly, a significant increase of intracellular calcium concentration was observed after EJ1 treatment. These results indicate that the EJ1 peptide affects plasma membrane integrity, leading in turn to a rapid increase of intracellular calcium concentration. This calcium overload may further cause mitochondrial swelling and intracellular vacuoles formation [145].

Loss of plasma membrane integrity is one of the main characteristics of necrosis. In order to understand if EJ1-induced cell death was necrosis-related, culture media from EJ1-treated MDA-MB-468 cells were collected and evaluated for the release of the

nuclear protein HMGB1 (high mobility group box 1), an indicator of necrosis [137] (Figure 3.16). We found detectable HMGB1 in EJ1- but not control-treated cell media. Interestingly, we also found AKT and p38 presented in EJ1-treated cell media (Figure 3.16), which was consistent with our previous findings (Figure 3.6) that EJ1 resulted in loss of cellular AKT and p38. Taken together, these data indicate that EJ1 causes cell death through both apoptosis and necrosis.

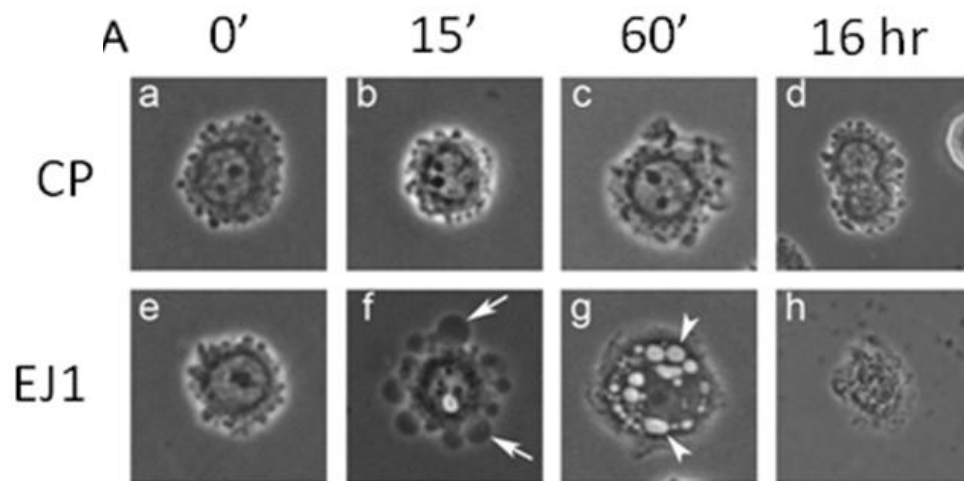


Figure 3.12. EJ1 causes membrane dynamic change and intracellular vesicle formation.

MDA-MB-468 cells were treated with either 20 μ M CP (a-d) or 20 μ M EJ1 (e-h) in complete medium at 37°C for 0 minute (a and e), 15 minutes (b and f), 60 minutes (c and g) or 16 hours (d and h). Images represent the bright-field images. Arrows indicate membrane blebbing. Arrowheads indicate intracellular vacuoles.

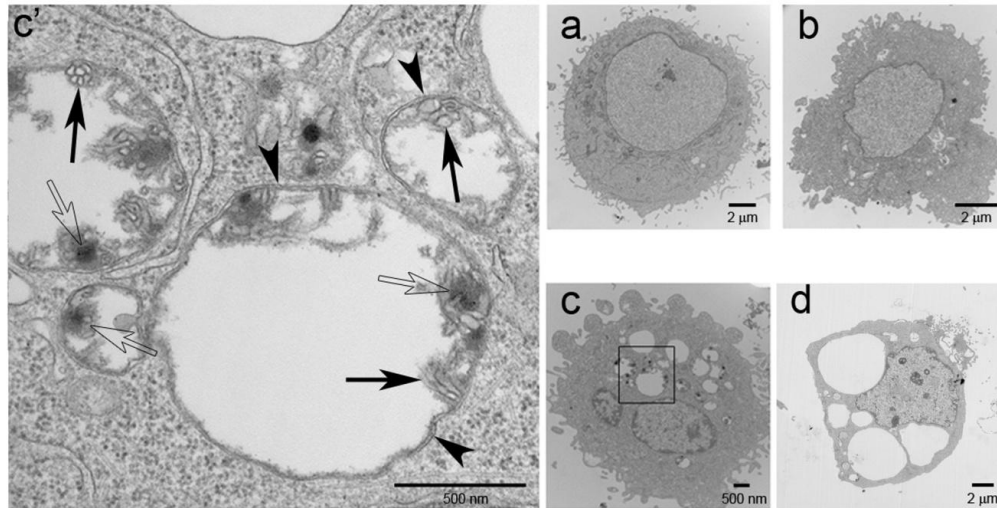


Figure 3.13. EJ1 causes double-membrane-vacuole formation.

MDA-MB-468 cells were treated with 20 μ M EJ1 in complete medium at 37°C for 0 minute (a), 5 minutes (b), 30 minutes (c and c') or 2 hours (d). Cells were then prepared for TEM. Magnification is represented as scale bars indicated. Filled arrows indicate organelle debris. Open arrows indicate electron-dense deposits. Arrowheads indicate double-membrane structures.

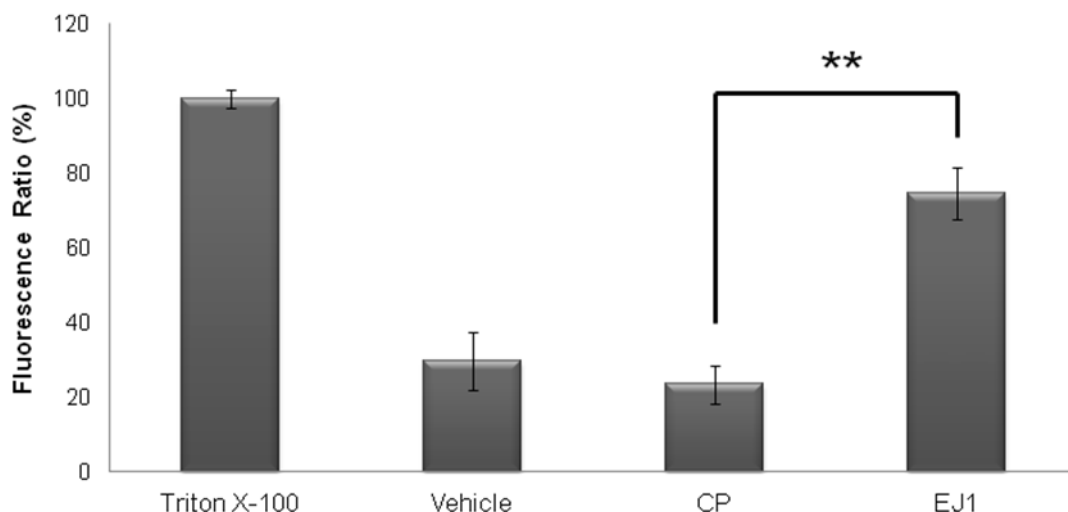


Figure 3.14. EJ1 treatment affects membrane integrity.

MDA-MB-468 cells were cultured in a 24-well plate (5×10^4 cells/well) and loaded with $1\mu\text{M}$ Calcein-AM in complete medium at 37°C for 30 minutes. Cells were then treated with 1% Triton X-100, water (Vehicle), $20\mu\text{M}$ CP or EJ1 in PBS with 5% FBS at 37°C for 15 minutes. Media were then carefully transferred to a new plate and fluorescent signal (Ex/Em 490/515 nm) was measured by a plate reader. Y-axis is expressed as percentage of fluorescent intensity for each treatment compared to Triton X-100 treated cells. **, $P < 0.01$, Student's t-test. Values are the means of three independent determinations \pm SD.

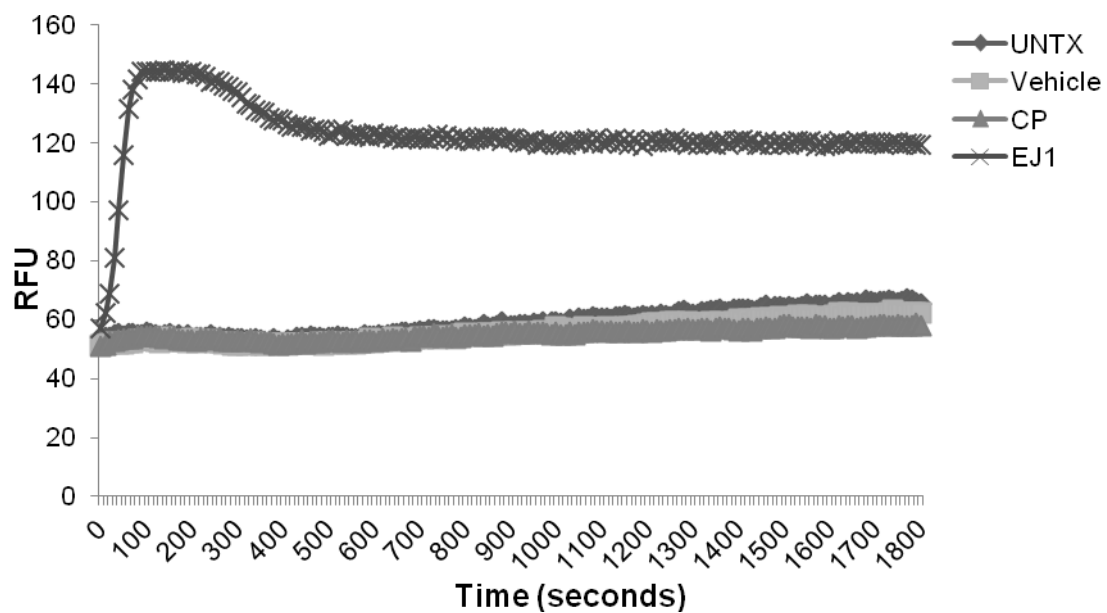


Figure 3.15. EJ1 causes an increase of intracellular calcium concentration.

MDA-MB-468 cells were seeded in 96 well plates and treated with water (Vehicle), 20 μ M CP or EJ1 and measurement of intracellular calcium concentration was done following manufacturer's protocol as described in Materials and Methods section.

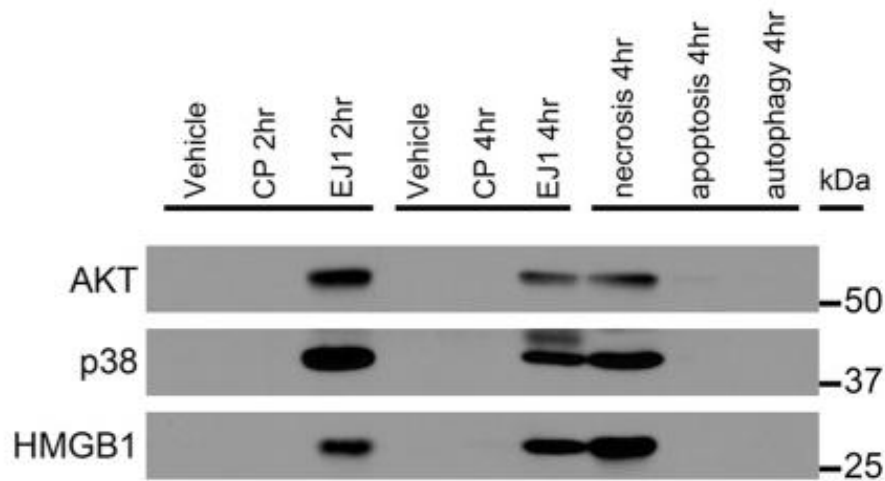


Figure 3.16. EJ1 causes necrosis.

MDA-MB-468 cells were treated with water (Vehicle), 20 μ M CP, 20 μ M EJ1, 2 μ M ionomycin and 50 μ M CCCP as necrosis inducers (necrosis), 10 ng/ml human tumor necrosis factor α and 35 μ M cycloheximide as apoptosis inducers (apoptosis), or 100nM rapamycin as an autophagy inducer (autophagy) for indicated times. Following treatments, media were collected and processed as described in [137]. Proteins from media were then separated in SDS-PAGE and immunoblotted with antibodies as indicated.

D. EJ1 causes membrane dynamic change through affecting Ca²⁺/CaM downstream

MLCK signaling

EJ1 induces a rapid membrane dynamic change (Figure 3.12f) and a rapid increase of intracellular calcium concentration (Figure 3.15). Some speculation occurred regarding whether these two events were correlated. Binding of Ca²⁺ to CaM leads to activation of Ca²⁺/CaM-regulated downstream signaling, which controls many different cellular events

such as membrane dynamics, cell survival, mitochondrial function, motility, and exocytosis [146-148]. Myosin light chain kinase (MLCK) activity is responsible for membrane dynamic regulation [149]. Once MLCK is activated by Ca^{2+} /CaM it can then phosphorylate myosin light chain (MLC) and regulate actinomyosin reorganization during membrane blebbing. To investigate whether EJ1-induced membrane blebbing was via the MLCK pathway, MDA-MB-468 cells were treated with vehicle, CP, or EJ1 alone (Figure 3.17a-c) or EJ1 in combination with the calmodulin inhibitor W-13 or the MLC phosphorylation inhibitors ML-7 and Y-27632 (Figure 3.17d-f). We found that both the calmodulin inhibitor W-13 and the MLC phosphorylation inhibitors ML-7 and Y-27632 completely inhibited EJ1-induced membrane blebbing. To determine if these effects on membrane blebbing were related to cell survival, the inhibitors were used in conjunction with EJ1 in a MTT assay. To perform this MTT, cells were evaluated after only one day of treatment due to long term cytotoxicity of ML-7. After one day of treatment, both Y-27632 and ML-7 were able to significantly reduce the effects of EJ1 on cell viability (Figure 3.18). Taken together, EJ1 affects membrane integrity which leads to calcium influx and activation of MLCK pathway which is integral to the EJ1 mediated reduction in cell survival.

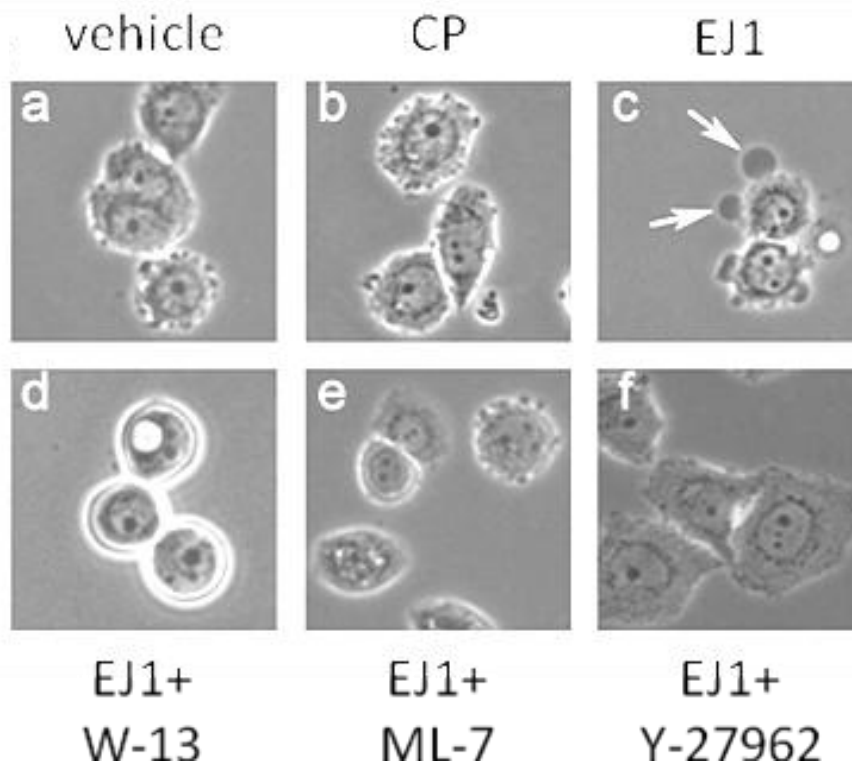


Figure 3.17. EJ1 induces membrane blebbing through activation of Ca^{2+} /CaM and its downstream effector, MLCK.

MDA-MB-468 cells were pre-treated with 50 μM W-13 (d), 10 μM ML-7 (e) or 10 μM Y-27632 (f) in complete medium at 37°C for 30 minutes, then treated with either water (vehicle) alone (a), 20 μM CP alone (b), or 20 μM EJ1 alone (c) or in combination with W-13 (d), ML-7 (e) and Y-27632 (f) in complete medium at 37°C for 15 minutes. Images represent the bright field images. Arrows indicates membrane blebbing.

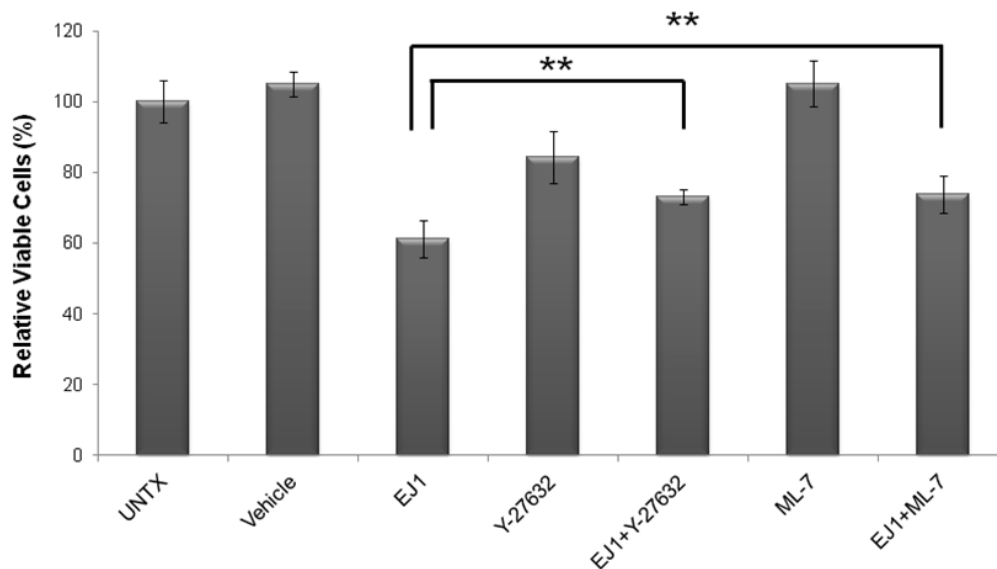


Figure 3.18. MLC phosphorylation inhibitors can rescue EJ1-induced cell death.

MDA-MB-468 cells were cultured in a 96-well plate (2×10^3 cells/well) and treated with either water, 20 μ M EJ1 alone, 10 μ M Y-27632 alone, 10 μ M ML-7 alone or EJ1 in combination with Y-27632 or ML-7 in complete medium for one day. A MTT assay was done to quantify viable cells after one day. Y-axis is expressed as percentage change of each treatment to untreated cells (UNTX). **, $P < 0.01$, Student's t-test. Error bars, SD.

E. EJ1 causes mitochondrial disruption and reactive oxygen species (ROS) generation

During the evaluation of intracellular vesicles created by EJ1 treatment (Figure 3.12 and Figure 3.13), what observed appeared to be damaged mitochondria (Figure 3.13c', filled arrows). To further explore the effects of EJ1 treatment on mitochondria, MDA-MB-468 cells were labeled with Mitotracker, treated with either EJ1 or CP and

then imaged (Figure 3.19a-d). Mitochondria appeared enlarged and rounded within five minutes of EJ1 treatment (Figure 3.19d', arrowheads). To determine if the mitochondrial membrane was damaged during this process, cells were treated with JC-1 dye, a reporter of mitochondrial membrane potential. MDA-MB-468 cells were labeled with JC-1 for 15 minutes and then treated for two hours with CP, EJ1 or carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a compound disrupting mitochondrial integrity, as a positive control for mitochondrial damage. A significant loss of mitochondrial membrane potential was observed with EJ1 treatment (Figure 3.20). Overall, these results imply that EJ1-induced loss of membrane integrity and calcium influx may cause mitochondrial swelling and loss of mitochondrial membrane potential.

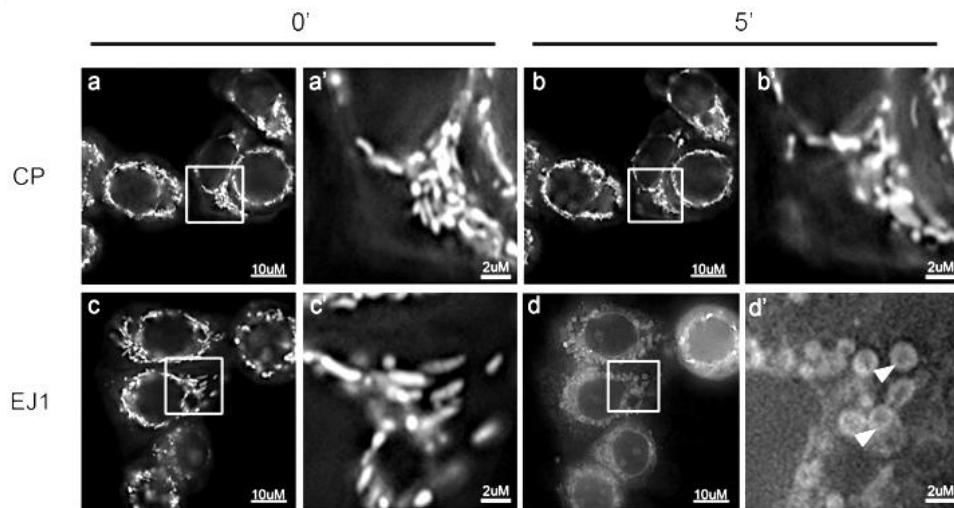


Figure 3.19. EJ1 causes mitochondrial swelling.

MDA-MB-468 cells were incubated with 200nM MitoTracker Red CMXRos and 5 $\mu\text{g}/\text{ml}$ Hoechst 33342 nuclear stain, followed by either 20 μM CP (a-b') or 20 μM EJ1 (c-d'), and imaged at 0 minute (a, a' and c, c') or at five minutes (b, b' and d, d'). Arrowhead indicates enlarged mitochondria.

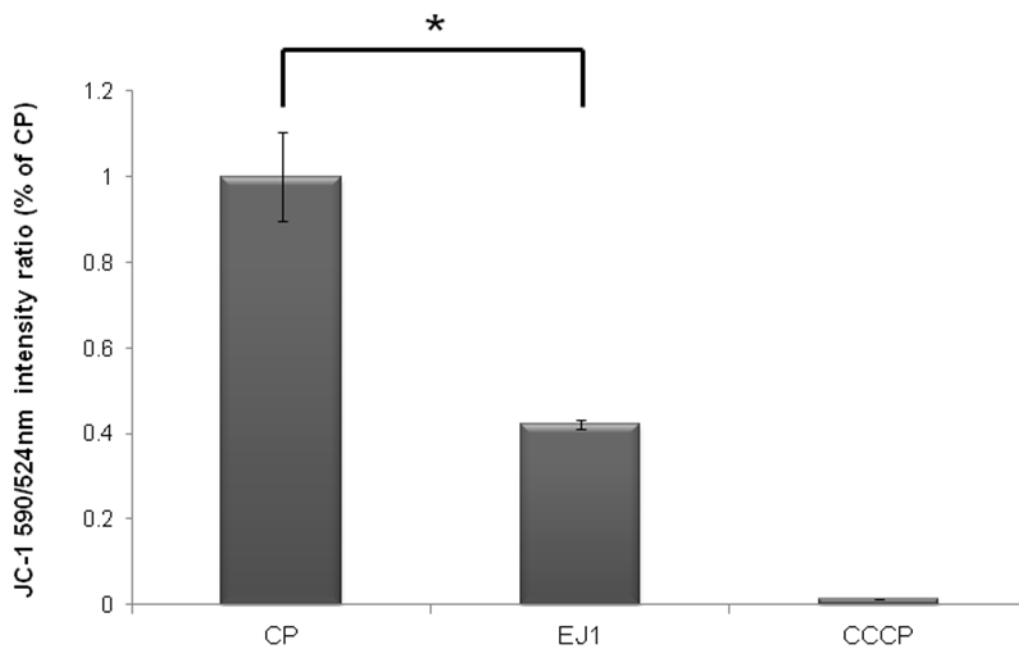


Figure 3.20. EJ1 treatment disrupts mitochondrial membrane potential.

MDA-MB-468 cells were serum-starved overnight and first stained with $1\mu\text{M}$ JC-1 in serum-free medium for 15 min at 37°C and then incubated with $20\mu\text{M}$ CP, $20\mu\text{M}$ EJ1 or $50\mu\text{M}$ CCCP in serum-free medium for 2 hr at 37°C . Following the treatments, cells were trypsinized and collected. After being washed twice with PBS, the cell pellets were re-suspended in PBS and transferred to a 96-well plate. Fluorescent signals were then detected at 514/529 nm and 514/590 nm by a plate reader. Results were calculated as the ratio of the 514/590 nm to 514/529 nm and normalized to the CP-treated samples. *, $P < 0.05$, Student's t-test. Values are the means of 3 independent determinations \pm SD.

Loss of mitochondrial membrane potential is frequently linked to an accumulation of ROS within the cells (reviewed in [150]). To measure intracellular ROS levels, DCFH-DA, which becomes fluorescent DCFH in the presence of ROS, was used to measure ROS levels in MDA-MB-468 cells. Cells were treated with either CP, N-acetyl

cysteine (NAC, a ROS scavenger which reduces intracellular ROS levels), EJ1 or NAC + EJ1 (Figure 3.21). While EJ1 treatment increased intracellular ROS levels as indicated by DCFH fluorescence, co-treatment with NAC significantly reduced EJ1-induced ROS levels. It was then to be determined whether decreasing ROS would prevent EJ1-induced cell death. We found that NAC could significantly inhibit EJ1-induced cell death (Figure 3.22). These data demonstrate that EJ1 causes cell death at least in part through loss of mitochondrial function and ROS accumulation.

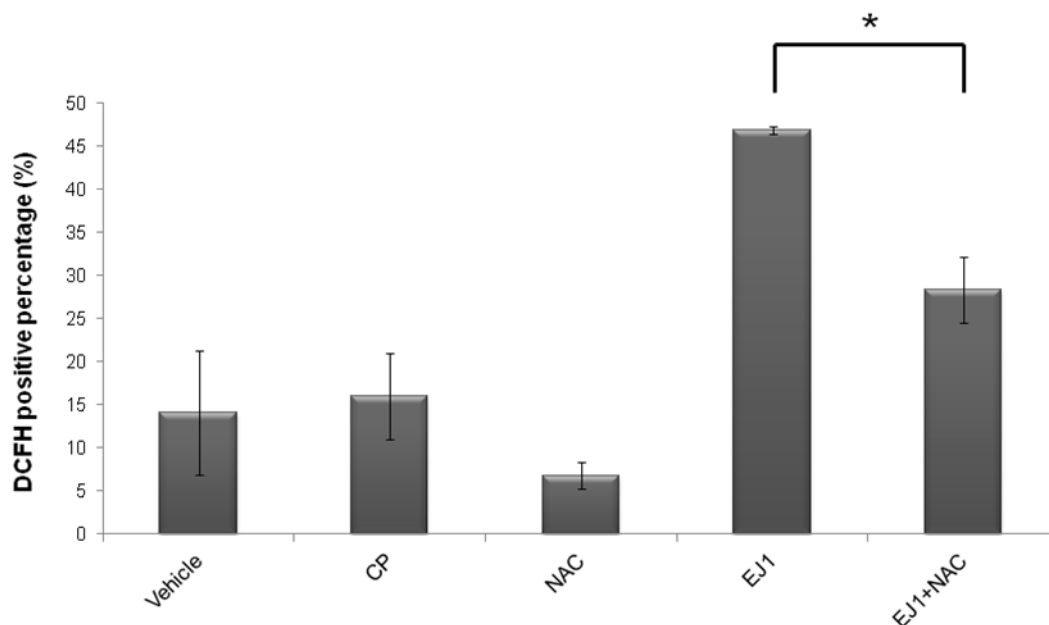


Figure 3.21. EJ1 causes accumulation of ROS.

MDA-MB-468 cells were stained with 10 μ M DCFH in complete medium for 30 minutes at 37°C and then incubated with water (Vehicle), 20 μ M CP, 0.5mM NAC, 20 μ M

EJ1 or EJ1 in combination with NAC in complete medium for 1 hr at 37°C. Cells were then sorted by a FACScan flow cytometer (BD Biosciences) and analyzed by Cellquest Pro 4.0 software. The results are expressed as the percentage of green fluorescent cells. *, $P < 0.05$, Student's t-test. Values are the means of three independent determinations \pm SD.

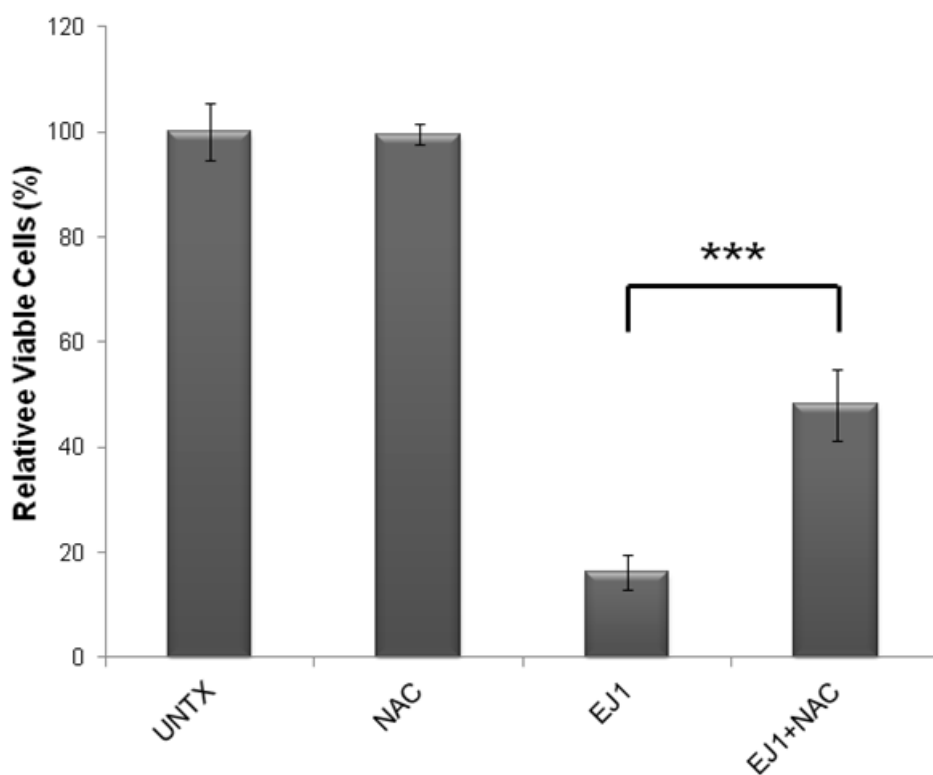


Figure 3.22. Inhibition of ROS reduces EJ1-induced cell death.

MDA-MB-468 cells were cultured in a 96-well plate (2×10^3 cells/well) and treated with either 0.5mM NAC alone, 20 μ M EJ1 alone, or NAC in combination with EJ1 in complete medium for 3 days. A MTT assay was done to quantify viable cells at day 3. Y-axis is expressed as a percentage change of each treatment to untreated cells (UNTX). ***, $P < 0.001$, Student's t-test. Error bars, SD.

So far, the effect of EJ1 was shown to be partially inhibited by both inhibitors of MLC phosphorylation (ML-7 and Y-27632) and an inhibitor of ROS (NAC) (Figure 3.18 and Figure 3.22). To determine if a combination of these two classes of drugs could effectively block EJ1-induced cell death, cells were treated with the inhibitors (alone or in combination) in the presence or absence of EJ1 (Figure 3.23). Virtually all of the cell death induction from EJ1 was eliminated by blocking both MLC phosphorylation and ROS accumulation, indicating that these two pathways account for a majority of the mechanism by which EJ1 kills cells.

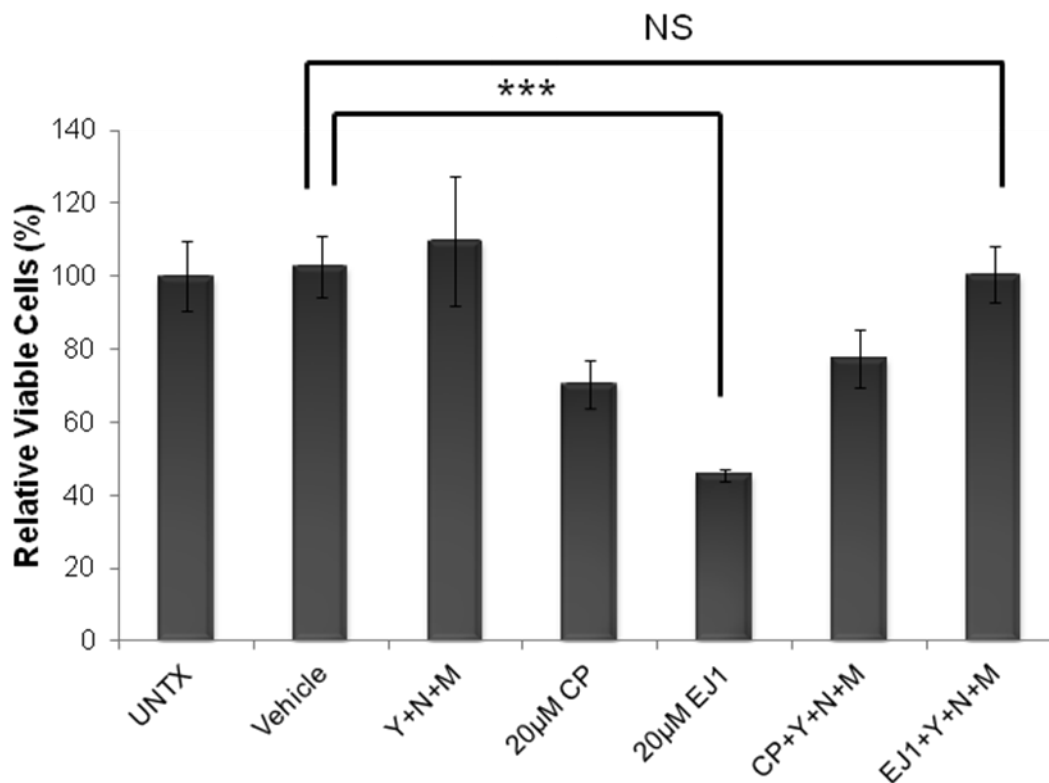


Figure 3.23. The mechanisms of EJ1-induced cell death are MLCK-regulated membrane blebbing and accumulation of ROS.

MDA-MB-468 cells were treated with either water (Vehicle), 20µM CP, 20µM EJ1 alone, or in combination with Y-27632 (Y), ML-7 (M) and NAC (N) and MTT assays were performed to quantify viable cells after one day. ***, $p < 0.001$, NS, no statistical significance, Student's t-test. Error bars, SD.

F. EJ1 reduces tumor growth and metastasis in mouse models of breast cancer

We next set out to determine whether EJ1 would function as an anti-tumor therapy *in vivo*. MMTV-pyMT murine model of breast cancer was used, which develops synchronous, multifocal mammary tumors in all ten mammary glands with a multistep

progression that resembles human disease [151, 152]. To determine dosage, animals were injected daily (intraperitoneal, *i.p.*) with 5, 10, 20 or 40 mg/kg body weight EJ1 or vehicle (PBS). Twenty mg/kg body weight appeared to provide the optimal tumor regression (data not shown), and this dose was then given to tumor-bearing MMTV-pyMT mice for either EJ1 (n=6) or CP (n=3). Three additional mice were given PBS (n=3). We found that both the average tumor size (~37.1% reduction in average tumor size) and the growth rate of tumors were significantly reduced by treatment with EJ1 compared to CP or PBS (12.1 mm³/day in control-treated versus 7.8 mm³/day in EJ1-treated; Figure 3.24 and data not shown). Many EJ1-treated tumors were also necrotic in appearance compared to controls (Figure 3.24, insets). Importantly, no toxicity from this dose of EJ1 was observed (weight loss, grooming, behavior, or gross changes to organs upon necropsy).

One thing to be noted is that MMTV-pyMT mice display highly penetrant secondary metastases to the lungs [153]. Since EGFR expression and activity have been correlated with tumor metastasis in different types of cancers [154-157], lung metastases were evaluated to determine whether the EJ1 peptide also affected metastasis in this mouse model of breast cancer. We found that on average, the lungs of EJ1-treated mice had

significantly less metastatic foci than did comparable CP-treated mice as assessed by bright field microscopic analysis and H&E assessment of tissue architecture (Figure 3.25 and insets).

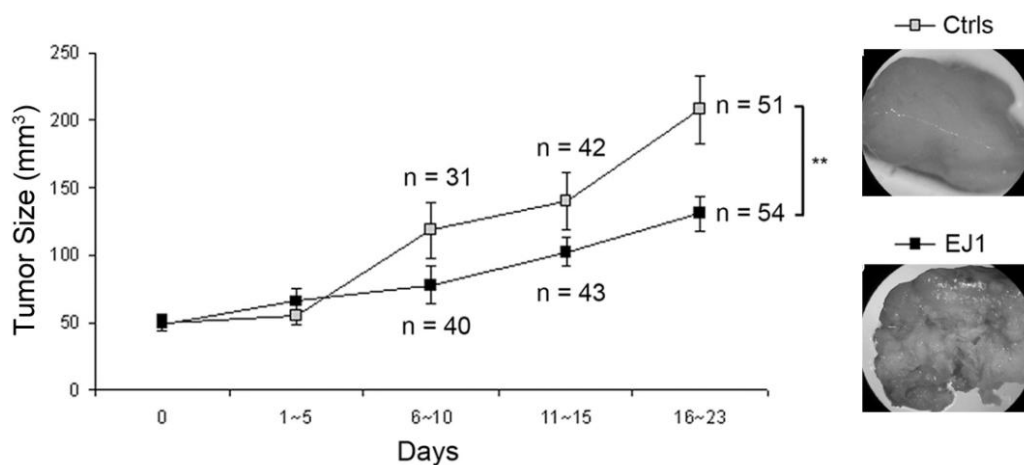


Figure 3.24. EJ1 reduces tumor growth in a MMTV-PyMT mouse model.

Tumor-bearing MMTV-pyMT mice were injected with 20 mg/kg body weight (i.p.) EJ1 (filled squares) or control peptides (open squares) for 21 days and tumors were measured every five days. **, $p < 0.01$, Student's t-test. Error bars, SE. Representative images of control- and EJ1-treated tumors are shown.

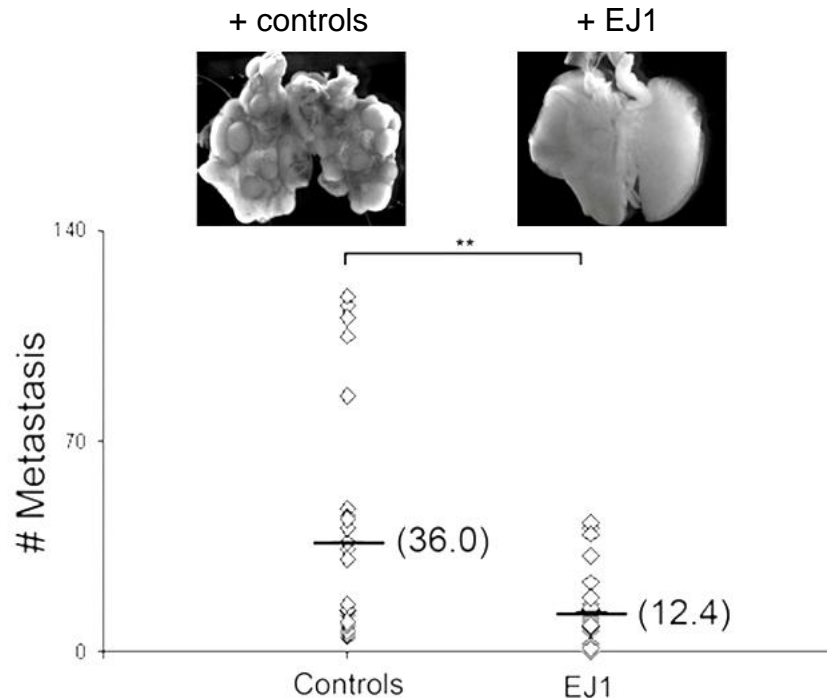


Figure 3.25. EJ1 reduces lung metastasis in a MMTV-PyMT mouse model.

Tumors metastasized to the lungs were evaluated by embedding the lungs and enumerating microscopic metastasis by H&E. Horizontal line represents average number of lung metastasis foci in each mouse. **, $p < 0.01$, Student's t-test.

IV. Discussion

In recent years, the essential regulatory role of the juxtamembrane domain of the EGFR has been investigated and hypothesized (reviewed in [158]). In the present study, we set out to determine whether this domain could be targeted by an EGFR-dependent anti-cancer therapeutic. We found that a peptide composed of a 21 amino acid portion of

the juxtamembrane domain (EJ1, FMRRRHIVRKRTLRLLLQERE) could effectively kill breast cancer cell lines. A similar peptide (TE-64562, RRRHIVRKRTLRLLLQER) was used to demonstrate inhibition of EGFR signaling and anti-cancer activity by another group [159]. We discovered that this peptide (EJ1) promotes the formation of inactive EGFR dimers, resulting in the activation of MLCK signaling, a downstream effector of Ca^{2+} /calmodulin regulated signaling. The results of this selective signaling were membrane blebbing and cell death. In addition, EJ1 can affect mitochondrial membrane potential involving the generation of ROS and induction of apoptosis/necrosis. Finally, these effects appear to be tumor-specific, as injection of EJ1 into an immune-competent transgenic mouse model of breast cancer resulted in an inhibition of tumor growth and metastasis without any gross toxicity.

Previous studies have shown that the helical transmembrane domain and anti-parallel dimeric interaction of the helical juxtamembrane domain A (amino acids 645-663) are both likely important for functional dimerization of the EGFR, as well as for EGFR activation [20, 158, 160-163]. Two studies using an EGFR transmembrane fragment or ErbB2 transmembrane peptide both demonstrated an inhibition of receptor dimerization and activation [164, 165]. We have demonstrated that treatment of breast

cancer cells with EJ1 results in a dramatic reduction in the EGF-mediated phosphorylation of EGFR through the promotion of dimer formation. Our data suggest that EJ1 could affect EGFR activity by altering the structural interaction of endogenous EGFR dimers. The ability of an EGFR inhibitor to promote the formation of inactive dimers is not unprecedented. Several groups have demonstrated that the EGFR kinase inhibitors AG1478, AG1517 and ZD1839 promote receptor dimerization, while at the same time impairing kinase activity [166-168].

MLCK-mediated phosphorylation of the myosin light chain is one way by which actin cytoskeleton reorganization is regulated, and it is known to be involved in membrane blebbing [149, 169]. Membrane blebbing induced by EJ1 peptide indicates that myosin light chain phosphorylation is upregulated (Figure 3.12). Complete inhibition of membrane blebbing by pre-treatment of calmodulin inhibitor (W-13), MLCK inhibitor (ML-7) or ROCK-1 inhibitor (Y27632), which also phosphorylates the myosin light chain, further confirms that EJ1 induced membrane blebbing is controlled by phosphorylation of the myosin light chain through both MLCK and ROCK-1 (Figure 3.17). In addition, phosphorylation of the myosin light chain has been correlated with both cell survival and cell death under different conditions [170-173]. Under the

circumstances of EJ1 treatment, MLCK and ROCK-1 inhibitors partially rescue EJ1-induced cell death, indicating induction of phosphorylation of MLC by EJ1 treatment is a regulator of membrane dynamics as well as of cell death.

We found that EJ1 treated cells were filled with vacuoles and TEM showed those vacuoles were double membrane structures (Figure 3.13c', arrowheads). It was speculated that these vacuoles were swelled mitochondria and organelle debris inside the vacuoles were cisternae remnants (Figure 3.12c' filled arrows). How could the EJ1 peptide cause mitochondrial swelling? One explanation is mitochondrial permeability transition (MPT). The opening of high conductance permeability transition pores in the mitochondrial inner membrane causes MPT and that leads to mitochondrial depolarization and uncoupling of oxidative phosphorylation, which will eventually result in depletion of ATP and cell death [174]. Loss of mitochondrial membrane potential is one of the measurable events of MPT. As shown in Figure 3.20, a significant loss of mitochondrial membrane potential was seen in EJ1 treated cells.

There are multiple compounds that can act on permeability transition pore complex to induce MPT. Other agents that increase cytosolic Ca^{2+} or stimulate ROS generation can also trigger MPT [175]. Interestingly, we found that EJ1 treatment caused an

immediately increase of intracellular calcium concentration (Figure 3.15) and accumulation of ROS (Figure 3.21). This implies that the EJ1 peptide causes calcium influx and overloads which leads to MPT and loss of mitochondrial membrane potential as well as to cell death. MPT induced cell death can be apoptosis or necrosis depending on ATP levels of the cells [176]. We found both types of cell death occurring in cells with EJ1 treatment (Figure 3.8, Figure 3.10, Figure 3.11, and Figure 3.16).

Mitochondria control cell growth by regulating energy production and also guard cell death by modulating the translocation of pro-apoptotic proteins from the mitochondrial intermembrane space to the cytosol. Furthermore, mitochondria also play a major part in multiple forms of non-apoptotic cell death and, in particular, in necrosis. As mitochondria are key regulators of cell death, it is not surprising that many of the mitochondrial functions are often altered in cancer, and mitochondrial targeted therapy could be a promising strategy to kill cancer cells. Our results indicate that the EJ1 peptide induces mitochondrial swelling (Figure 3.19) and loss of mitochondrial membrane potential (Figure 3.20). This implies that the EJ1 peptide could be a potential mitochondrial-targeted therapeutic which warrants further investigation.

In conclusion, EJ1 represents a novel EGFR therapeutic, targeting multiple activities

of the EGFR receptor in a tumor-specific manner. In addition, EJ1 targets cells in an EGFR-independent manner via its ability to disrupt membrane integrity in a tumor-specific manner. Together, these effects result in a highly tumor-specific anti-cancer therapeutic that may have significant clinical utility.

CHAPTER 4 – EFFECTS OF MUC1 EXPRESSION ON ERBB3 ACTIVITY

Note: The work presented in this chapter has not been published. All experiments were performed by Hsin-Yuan Su.

I. Introduction

MUC1, a transmembrane mucin, is heavily glycosylated and normally found on the apical surface of glandular epithelia [177]. The expression of MUC1 is aberrantly regulated in more than 90% of breast carcinomas and metastases and its expression has been linked to cell transformation *in vitro* and *in vivo* [178-180]. One mechanism by which MUC1 can drive transformation is through cancer-dependent protein-protein interaction. This has been shown in a WAP-TGF α transgenic mouse model, where the expression of MUC1 dramatically affects EGFR-dependent mammary gland transformation and cancer progression [181]. In addition, the interaction between MUC1 and EGFR inhibits ligand-induced ubiquitination and degradation of EGFR in human breast cancer cell lines [34]. This indicates that expression of MUC1 may promote oncogenic transformation through the inhibition of EGFR degradation. Additionally,

MUC1 physically associates with all four erbB receptors and the interaction between MUC1 and the receptor tyrosine kinase EGFR results in an increase in MAP kinase activation [182]. The deregulation of erbB signaling frequently occurs during breast cancer initiation and progression, making it important to understand the effects of MUC1 expression on other erbB receptors.

The erbB receptors belong to a receptor tyrosine kinase superfamily. Reviewed in [183], there are four members in this family: EGFR, erbB2/neu, erbB3 and erbB4. All members share a similar structure which contains an extracellular ligand-binding domain, a single membrane-spanning domain, a juxtamembrane domain, a cytoplasmic tyrosine-kinase-containing domain, and a carboxy-terminal tail. Ligands for erbB family receptors comprise more than 11 different members, and upon binding to erbB receptors they induce the formation of receptor homo- or heterodimers. Dimerization leads to activation of the receptor and initiation of different downstream signaling pathways, such as MAPK, PI3K/AKT, JAK/STAT and PLC γ . These different signals can promote cell proliferation, migration and survival, resulting in breast cancer development and metastasis [183]. EGFR has been shown to be either overexpressed or overactivated in different types of human cancers, including breast cancer. The development of

EGFR-targeting antibodies or tyrosine kinase inhibitors has earned great success *in vitro* and *in vivo*, reviewed in [184, 185]. However, these agents show very limited clinical anti-tumor activity in breast cancer [186]. Recent studies have shown that EGFR therapeutic resistance can be linked to ErbB3 expression [53].

ErbB3 receptors are highly expressed in more than 30% of invasive breast cancers and are correlated to poor prognosis by many studies [187, 188]. Within the c-terminal regulatory domain of ErbB3, there are six binding sites for the p85 regulatory subunit of PI3K. Accordingly, the ErbB3 receptor is considered as a key regulator of PI3K activity [189-191]. The PI3K/AKT pathway mediates survival signals that are required for tumor maintenance as well as therapeutic resistance (reviewed in [192]). MUC1 can dramatically alter EGFR-dependent breast cancer progression, and can also interact with ErbB3 [182]. Therefore, it was hypothesized that MUC1 expression could alter ErbB3 signaling through affecting ErbB3 trafficking and/or degradation.

II. Materials and Methods

Western Blotting and *Cell Viability Assay* are the same as described in chapter 2.

Transfection with siRNA Transfection with siRNA was performed as in [87]. The MUC1 siRNA targets the extracellular domain (5'-AAGACTGATGCCAGTAGC- ACT-3') [160]. Additionally, the control siRNA has no known mammalian gene targets (5'-AATTCTCCGAACGTGTCACGT-3') (Qiagen Sciences Inc., German- town, MD). The transfections were performed with Lipofectamine 2000 (Invitrogen Life Technologies Inc., Carlsbad, CA) following the manufacturer's instructions.

Immunoprecipitation BT20 cells were transfected with either MUC1 siRNA or control siRNA. After 48 hours, cells were serum starved overnight and then treated with/without 5ng/ml of NRG1 β for 10 minutes on ice. Protein lysates were collected and immunoprecipitation with ErbB3 antibodies was performed as described by Pochampalli *et al.*, 2007 [34]. Immunoprecipitants were separated by SDS-PAGE and immunoblotted with EGFR, ErbB3 and MUC1 antibodies.

Wound Healing 2D Migration Assay BT20 cells were transiently transfected with either MUC1 siRNA (MUC1) or control siRNA (control). After 24 hours, cells were trypsinized and re-plated in 24 well plates at 2×10^4 cells per well to get a confluent monolayer. Then

the cells were serum-starved overnight. A p200 pipet tip was used to create a scratch in the cell monolayer. Cells were then washed with PBS and refreshed with serum-free (SF) medium containing different concentrations of NRG1 β (NRG) as indicated. Pictures were then taken at different time points and the distance between the closest margins was measured.

III. Results

A. MUC1 expression affects NRG1 β -induced ErbB3 degradation and downstream signaling

ErbB receptors, including EGFR and ErbB3, are typically endocytosed upon ligand binding, trafficked to the lysosome and degraded [193]. MUC1 is known to prevent the ligand-induced degradation of EGFR [34]. Therefore, we investigated whether it has a similar effect on ErbB3. BT20 breast cancer cells were used to determine the effect of MUC1 expression on ErbB3 stability. After transient transfection of either control siRNA or MUC1 siRNA to knockdown MUC1 expression, NRG1 β (neuregulin-1 β , an ErbB3 ligand) was used to induce receptor internalization. We found that MUC1 expression inhibited ligand-induced ErbB3 down-regulation at one- and two-hour time points, when

ErbB3 receptors began to be degraded in this cell line [194, 195]. Importantly, this effect was only seen after ligand stimulation, indicating that this is a ligand-mediated event (Figure 4.1, top 2 panels). A similar experiment was done in MDA-MB-231 cells in which pCMV-MUC1 plasmid was transfected into cells to overexpress MUC1. Similarly, MUC1 expression inhibited ligand-induced ErbB3 down-regulation of both phosphorylated (pY1289 [196]) and total protein levels (Figure 4.2, top 2 panels).

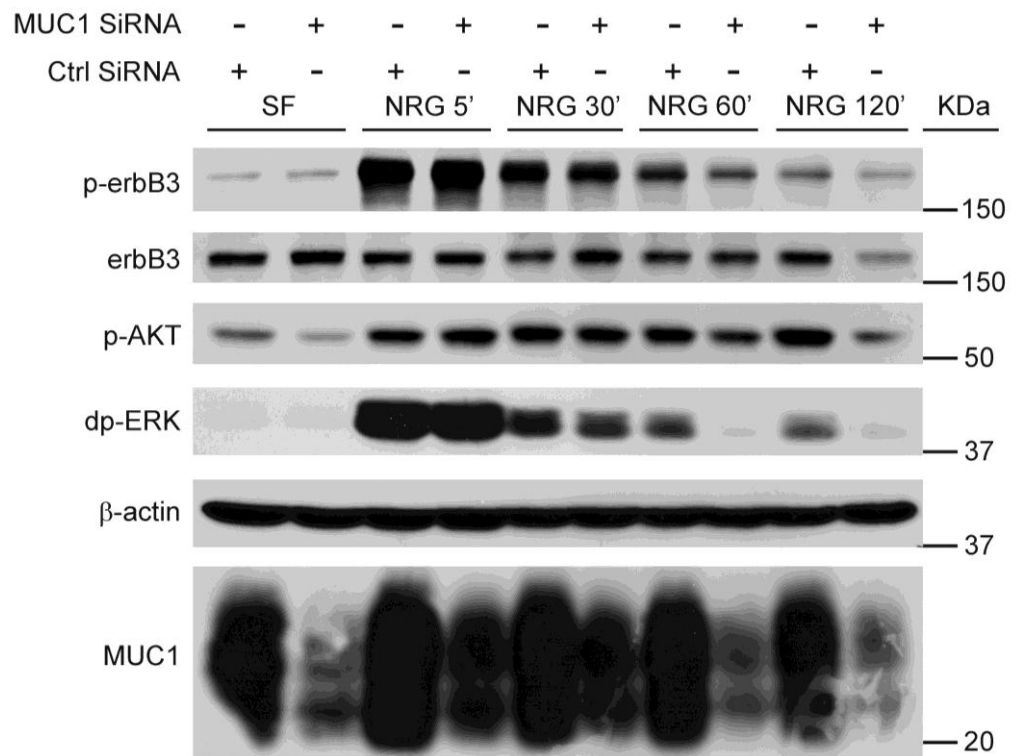


Figure 4.1. MUC1 inhibits the degradation of NRG1 β -stimulated ErbB3 and sustains downstream signaling in BT20 cells.

BT20 cells were transfected with either MUC1 siRNA or control siRNA, and treated with 5ng/ml NRG1 β for ten minutes on ice. Following treatments, endocytosis was allowed for the indicated times before lysis. Protein levels were determined as indicated.

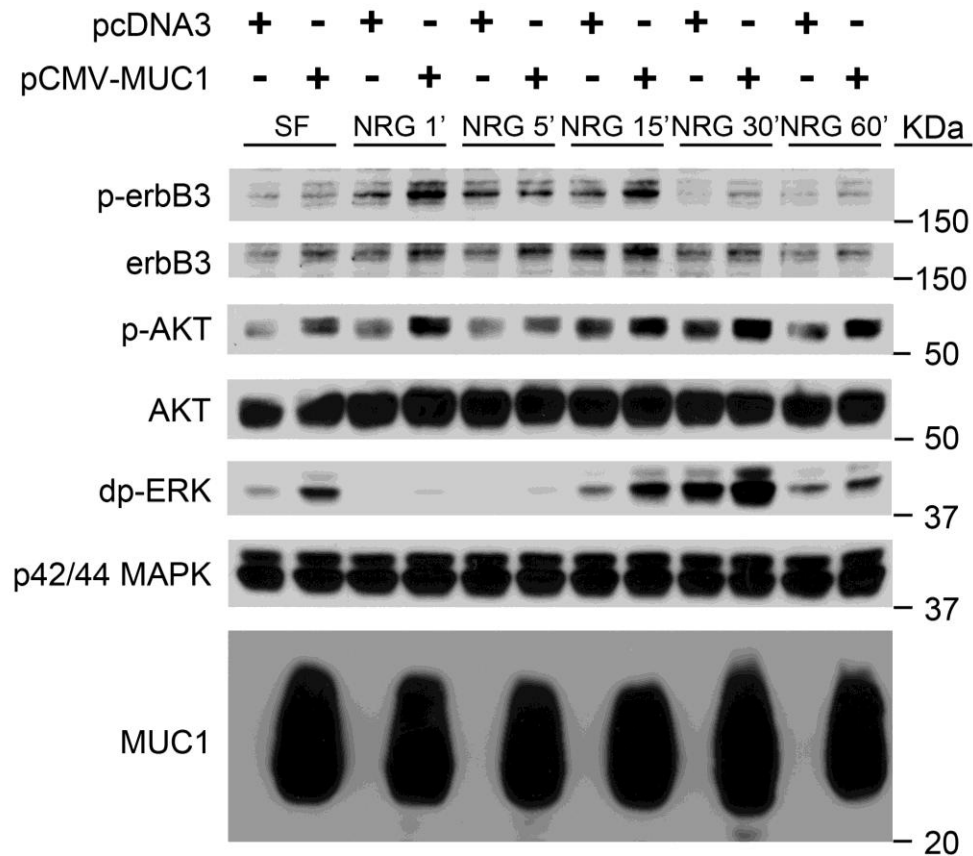


Figure 4.2. MUC1 inhibits the degradation of NRG1 β -stimulated ErbB3 and sustains downstream signaling in MDA-MB-231 cells.

MDA-MB-231 cells stably expressing empty vector (pcDNA3) or MUC1 (pCMV-MUC1) were treated with 5ng/ml NRG1 β for ten minutes on ice. Following treatments, endocytosis was allowed for the indicated times before lysis. Protein levels were determined as indicated.

Activation of erbB receptors leads to different downstream signal transduction events [183]. For ErbB3, PI3K/AKT is the prominent effector pathway regulated by the receptor activity [197]. Thus, AKT activation was determined. As a result, p-AKT (pS473)

level was sustained by MUC1 expression in both BT20 and MDA-MB-231 cells (Figure 4.1 and Figure 4.2). MAP kinase signaling pathway, another important pathway downstream of ErbB3, was also investigated. Similarly, the status of ERK (extracellular signal-regulated kinase) phosphorylation was sustained by MUC1 expression in both cell lines (Figure 4.1 and Figure 4.2). These results demonstrate that MUC1 expression has a profound effect on NRG1 β -induced ErbB3 degradation, and on downstream PI3K/AKT and MAP kinase signaling pathways.

B. MUC1 expression facilitates EGFR/ErbB3/MUC1 complex formation

ErbB receptors form homo- or heterodimers after ligand binding, and different dimerization leads to different combinations of downstream signaling. BT20 cells were used to determine whether MUC1 expression affects dimer formation. After transient transfection of either control siRNA or MUC1 siRNA to knockdown MUC1 expression, cells were treated with NRG1 β to induce receptor dimerization. Complex formation was determined by immunoprecipitation with the ErbB3 antibody. EGFR formed a complex with ErbB3 only in the presence of MUC1 and NRG1 β stimulation. In addition, MUC1 formed a complex with EGFR and ErbB3 only in the presence of NRG1 β stimulation

(Figure 4.3). These results demonstrate that MUC1 expression facilitates an EGFR/ErbB3/MUC1 complex formation.

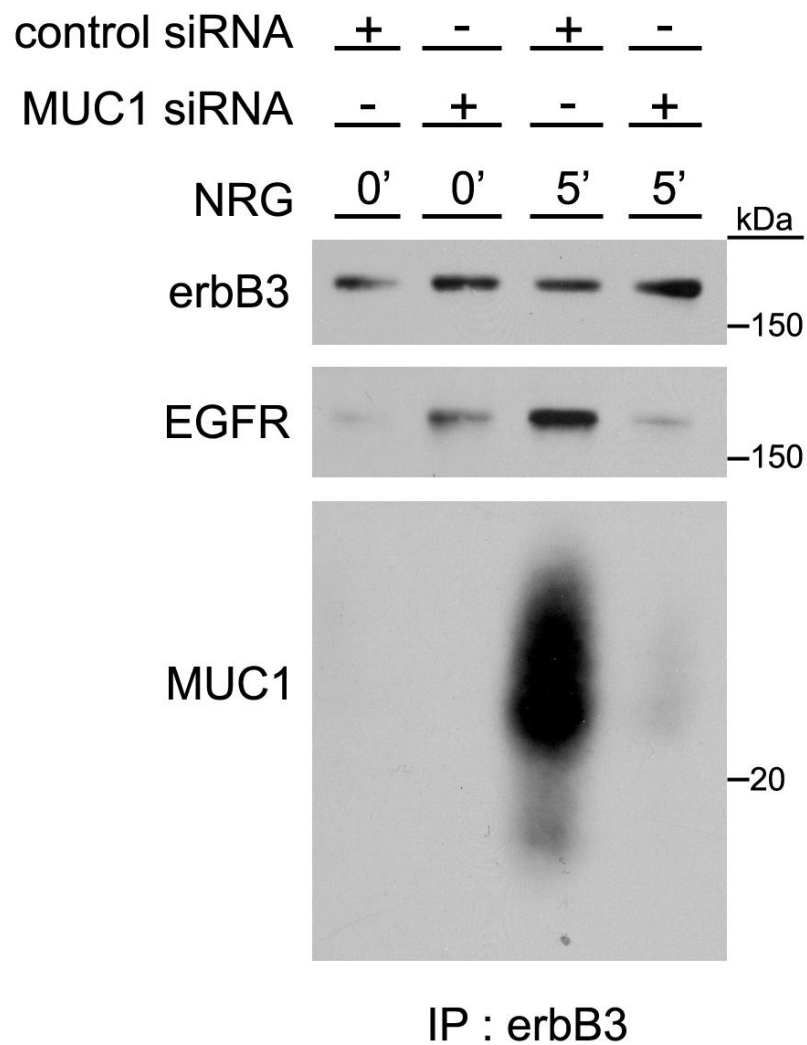


Figure 4.3. MUC1 expression facilitates EGFR/ErbB3/MUC1 complex formation.

BT20 cells were transiently transfected with either MUC1 siRNA or control siRNA to knockdown MUC1 expression. Cells were then treated with 5ng/ml NRG1 β to induce receptor dimerization. We then determined complex formation by immunoprecipitation

with the ErbB3 antibody and protein levels were determined as indicated.

C. Knockdown of MUC1 expression promotes both NRG1 β -dependent and NRG1 β -independent cell survival and migration

Both the PI3K/AKT and the MAP kinase pathways are important regulators for cell proliferation, migration and survival. Since MUC1 expression affected both PI3K/AKT and MAPK signaling activities, cell survival and migration were next used to determine the effect of MUC1 expression. Cell survival was determined in BT20 cells with/without MUC1. As shown in Figure 4.4, contrary to our expectations, the survival rates were 30%~50% higher in cells without MUC1 than in cells expressing MUC1, with/without NRG1 β at 5 or 10ng/ml. Next, wound healing 2D migration assay was used to investigate whether MUC1 expression affects cell motility. Again, cells without MUC1 unexpectedly moved faster than cells expressing MUC1, regardless of the presence of NRG1 β (Figure 4.5).

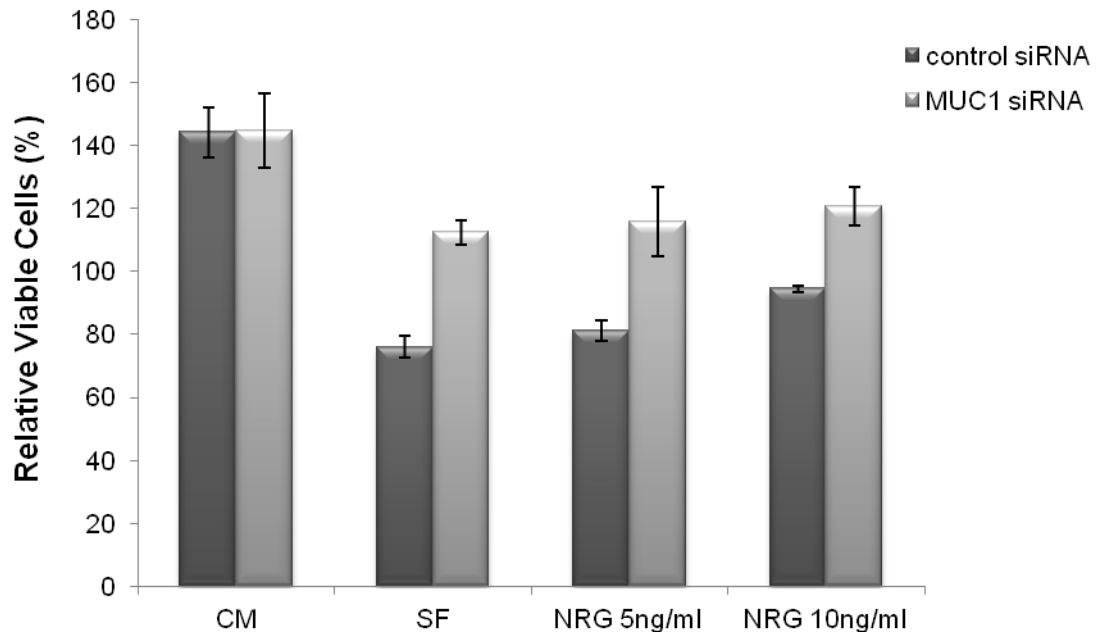


Figure 4.4. Knockdown of MUC1 expression promotes both NRG1 β -dependent and NRG1 β -independent cell survival in BT20 cells.

BT20 cells were transiently transfected with either MUC1 siRNA or control siRNA. After being serum-starved overnight (day 0), cells were then subjected to different treatments (as indicated) for 48 hours (day 2). Relative numbers of viable cells were determined by MTT assays on day 0 and day 2 of treatment, and cell survival was assessed by the ratio of day 2 to day 0. CM, complete medium; SF, serum-free medium. Values are the means of three independent determinations \pm SD.

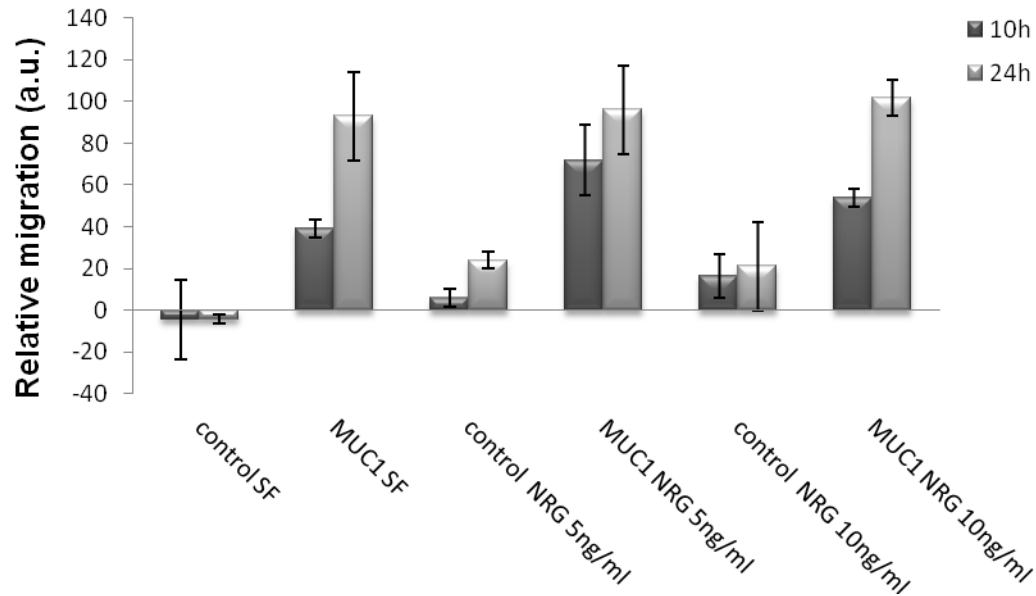


Figure 4.5. Knockdown of MUC1 expression promotes both NRG1 β -dependent and NRG1 β -independent cell migration in BT20 cells.

BT20 cells were transiently transfected with either MUC1 siRNA (MUC1) or control siRNA (control). After the cells were serum-starved overnight, a p200 pipet tip was used to create a scratch in the cell monolayer. Cells were then washed with PBS and refreshed with serum-free (SF) media containing different concentrations of NRG1 β (NRG) as indicated. After indicated times, migration distance was measured between the closest margins of the scratch. Values are the means of three independent determinations \pm SD.

In summary, we found that MUC1 expression affected ErbB3 stability and downstream signaling. This effect could be the result of EGFR/ErbB3/MUC1 complex formation. However, we were not able to demonstrate a direct translation of these

molecular changes into biological effects, such as cell survival and 2D migration.

IV. Discussion

ErbB3 is an important partner for transducing PI3K/AKT signals and is one of the key players involved in resistance to EGFR inhibitors [53]. We found that MUC1 expression sustained ErbB3 activity as well as downstream PI3K/AKT and MAPK activities (Figure 4.1 and Figure 4.2). We also demonstrated that NRG1 β -dependent EGFR/ErbB3/MUC1 complex formation might be the cause of sustained signaling (Figure 4.3). The complex formation might interfere with endocytosis or promote receptor recycling, which need to be determined. Nevertheless, both possible events could result in a sustained signaling, which would lead to cell proliferation, migration and survival.

To our surprise, we did not observe a positive correlation of sustained signaling with cell survival or migration (Figure 4.4 and Figure 4.5). The reasons for these observations are obscure. Normally, serum starvation for two days would result in apoptosis in BT20 cells and that was what control cells showed (Figure 4.4, third column from the left) [198]. However, the lack of apoptosis in MUC1 knockdown cells (Figure 4.4, fourth column from the left) indicated that there might be other signaling pathways involved.

When the p-AKT or dp-ERK level was compared between cells with/without MUC1 in a serum-free condition, a generally higher level of p-AKT or dp-ERK was observed in MUC1-expressing cells (Figure 4.1 and Figure 4.2), which was also in disagreement with the MTT result (Figure 4.4). MUC1 can promote cancer cell survival by modulating apoptosis and autophagy pathways [199, 200]. The only situation where MUC1 promotes cell death is in activated T cells [201]. Since there was only one cell line tested, what we observed could be a cell line specific phenomenon.

Importantly, MUC1 expression sustains NRG1 β -dependent ErbB3 activation through an EGFR/ErbB3/MUC1 complex formation. This complex formation might represent a potential therapeutic target as to inhibit ErbB3 activities.

CHAPTER 5 – CONCLUDING REMARKS

It is predicted that more than 230,000 new cases of invasive breast cancer will be diagnosed and more than 40,000 cases of breast cancer-related death will be seen just in 2013 in the US. Early diagnosis of the diseases has already improved prognosis significantly. Molecular profiling of breast cancers has also increased specificities of targeted therapies. Anti-hormonal therapy and anti-HER2 therapy has significantly improved prognosis of breast cancer patients. However, in a subgroup of breast cancer, basal epithelial-like or clinically referred as triple negative breast cancer, which lacks expression of estrogen receptor, progesterone receptor and HER2 receptor, the anti-hormonal and anti-HER2 therapies are ineffective. Molecular profiling demonstrates that EGFR and cytokeratin 5/6 are usually overexpressed in this type of breast cancer.

EGFR expression and activity are tightly regulated in normal cells. Consequently, dysregulation of EGFR leads to tumor formation and progression. Anti-EGFR drugs therefore are considered as a promising therapy specifically for EGFR-dependent cancers. In triple negative breast cancer, more than 50% of cases overexpress EGFR. Thus, EGFR-targeted therapies were introduced for the treatment of patients with this type of

cancer. Unfortunately, anti-EGFR therapies only achieve a limited efficacy in triple negative breast cancer patients. The reasons are attributed to intrinsic and acquired resistance to EGFR-targeted therapies. Although secondary mutation to EGFR is not frequently seen in breast cancer, compensatory activation of parallel signaling pathways, such as ErbB2, ErbB3 and Met, are often seen in EGFR-targeted refractory breast cancers [52, 79]. These parallel pathways regulate an important survival pathway, PI3K/AKT pathway, to escape cytotoxicity of anti-EGFR drugs. Nuclear localization of EGFR also contributes to therapeutic resistance through up-regulation of DNA repair activity [119-121] or expression of drug efflux proteins (ABCG2) [88]. In order to improve therapeutic efficacy of treatments in patients with triple negative breast cancer, we developed a three-part study to target nuclear translocation of EGFR, to inhibit EGFR activity through a novel approach, and to understand mechanisms involved in ErbB3 over-activation.

I. Nuclear EGFR as a potential therapeutic target

Nuclear EGFR is involved in many aspects of therapeutic resistance. We demonstrate in this dissertation that it is possible to inhibit nuclear translocation of

activated EGFR (pY845) by a peptide derived from the EGFR NLS sequence. Additionally, nuclear translocation of ErbB2 and ErbB3 has been demonstrated and both are involved in tumor progression and therapeutic resistance [202-204]. It is noteworthy that the predicted NLS sequences are highly conserved among EGFR, ErbB2 and ErbB3 [98]. It is therefore important to determine if the ENLS peptide also inhibits nuclear translocation of ErbB2 and ErbB3. Alternatively, peptides based on sequence homology among the three NLS sequences could be synthesized and investigated. Also, due to the nature of the peptide half life, chemical molecules with similar structures could be used instead for improved stability.

The limited biological effects after ENLS peptide treatment could simply indicate that both cytosolic and nuclear activities of EGFR are important. Cytosolic activity of EGFR is usually dominant and regulated by kinase activation to control cell proliferation, motility and survival. When kinase activity is inhibited by either monoclonal antibodies or TKIs, the nuclear activity of EGFR can become dominant to regulate transcription, stress response, and DNA repair. Therefore, much work should be done to determine the efficacy of the ENLS peptide in combination with EGFR-targeted therapy or other tyrosine kinase inhibitors.

II. Juxtamembrane domain of EGFR as a potential therapeutic target

Juxtamembrane domain of EGFR plays important roles in receptor dimerization, activation, trafficking and interaction with calmodulin. However, none of these non-traditional kinase related functions are directly affected by monoclonal antibodies and TKIs. We demonstrate that a peptide derived from juxtamembrane domain of EGFR can effectively inhibit EGFR activation through promoting inactive dimer formation. It can also effectively kill cancer cells through processes of apoptosis and necrosis. Mechanistically, this peptide affects membrane integrity and leads to calcium influx, disruption of mitochondrial membrane potential, and ROS accumulation (Figure 5.1).

Clearly, this peptide possesses EGFR-dependent and EGFR-independent properties. The EGFR-independent property could provide a solution to overcome resistance to EGFR-targeted therapy. In a preliminary experiment, we investigated whether the EJ1 peptide is still effective in AG1478-resistant cells. As shown in Figure 5.2, the EJ1 peptide still affects cell viability of AG1478-resistant cells, although the efficacy is not as good as that observed in parental cells. Further studies need to be done to confirm that this effect is actually through EGFR-independent mechanisms. Importantly, the effect of

EJ1 peptide appears to be cancer-specific *in vitro* (Figure 3.4). In addition, the EJ1 peptide shows no observable toxicity *in vivo* as determined by gross appearance, activity and body weights of treated mice.

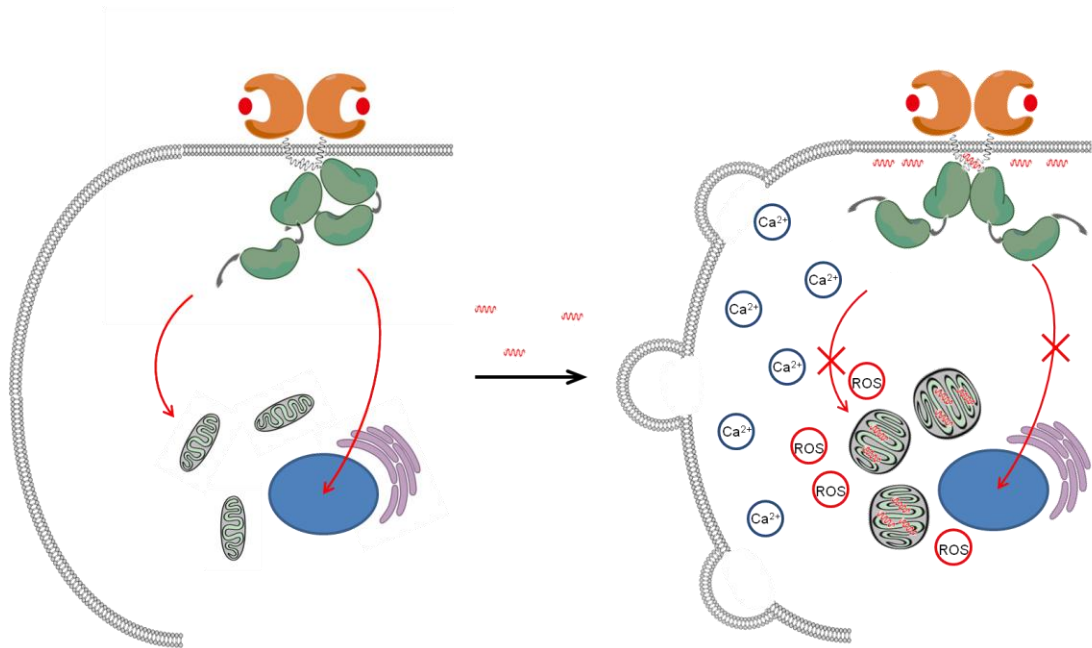


Figure 5.1. Schematic model of EJ1-induced cell death.

Left side of the figure represents the condition before EJ1 peptide treatment. Activation of EGFR induces juxtamembrane domain mediated translocation of the receptor to the nucleus and mitochondria. Right side of the figure represents possible mechanisms by which EJ1 peptide induces cell death. EJ1 inhibits activation of EGFR through promotion of inactive dimer formation. EJ1 peptide affects membrane integrity and leads to calcium influx, activation of MLC-regulated membrane blebbing, disruption of mitochondrial membrane potential, and ROS accumulation which eventually lead to apoptotic and necrotic cell death.

To improve its efficacy and stability, modification of the peptides and screening for chemical mimetics are possible options. Combination therapy also warrants further investigation. The EGFR-independent function of EJ1 could be attributed as a calcium and ROS-dependent function. Even though the definitive mechanism to explain how EJ1 works through calcium and ROS is still lacking, targeting the calcium signaling pathway and ROS as cancer therapy is not unprecedented [205-207]. Simultaneously targeting multiple pathways could provide a better therapeutic efficacy and minimize the likelihood of developing resistance. Therefore, the EJ1 peptide might provide hints for effective combination therapy, such as EGFR inhibitors in combination with ROS inducers.

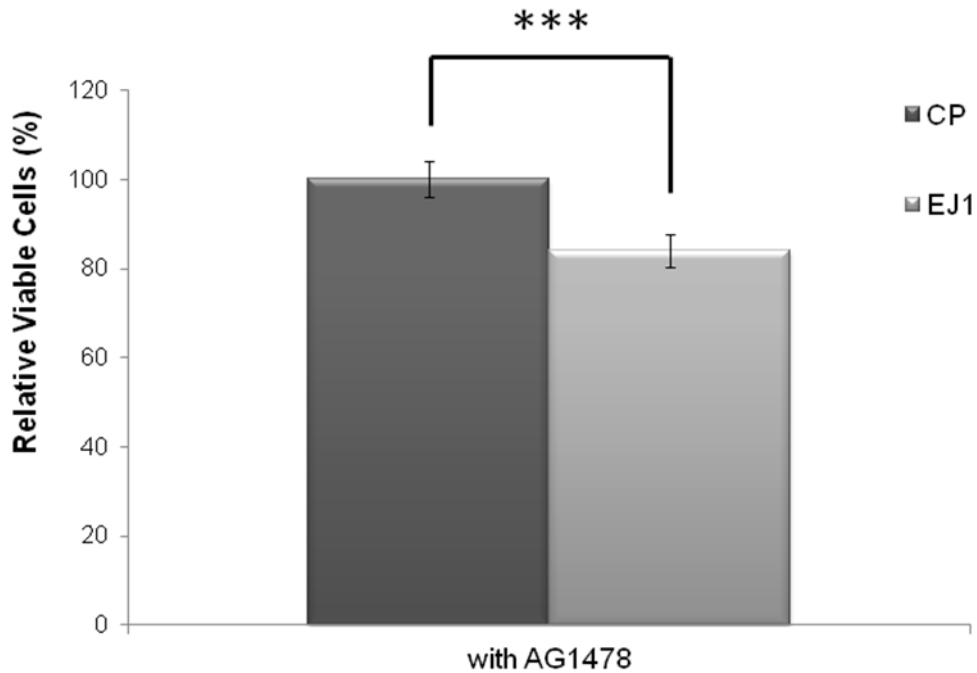


Figure 5.2. EJ1 peptide affects cell viability of AG1478-resistant cells.

AG1478-resistant cells were selected and grown as described earlier. These AG1478-resistant cells were treated with 20 μ M control peptide (CP) or 20 μ M EJ1 peptide (EJ1) in the presence of 10 μ M AG1478 for 3 days. A MTT assay was done to quantify cell viability at day 3. The reading for CP/AG1478-treated cells at day 3 was set as 100%. ***, $p < 0.001$, Student's t-test. Error bars, SD.

III. MUC1 sustains NRG1 β -dependent ErbB3 activities

Crosstalk between different members of erbB receptors through heterodimerization results in a wide array of downstream signaling events. Therefore, it is almost impossible to completely downregulate signaling pathways by solely inhibiting one erbB receptor member. ErbB3 is a kinase-defective receptor whose activation highly depends on its

dimer partner. ErbB3 has been shown to contribute to resistance to EGFR-targeted therapy through membrane expression of ErbB3 and shifting phosphorylation-dephosphorylation equilibrium [53]. MUC1, on the other hand, has been shown to interact with four erbB receptors and was specifically demonstrated to promote EGFR recycling [34]. In the present study, we investigated whether MUC1 expression also affected ErbB3 activity and stability. We show that the MUC1 expression sustains NRG1 β -induced ErbB3 activation and downstream PI3K/AKT as well as MAPK activities. We also demonstrate that MUC1 promotes NRG1 β -dependent EGFR/ErbB3/MUC1 interaction in BT20 breast cancer cells. This result might provide a hint for the development of ErbB3-targeted therapy. Interruption of MUC1/ErbB3 interaction could be a potential therapeutic strategy to inhibit ErbB3 activity.

IV. Conclusions

The work presented in this dissertation provides evidence supporting several novel strategies for targeting EGFR activity and localization as effective therapies. Although more data need to be provided to consolidate our conclusion, this “proof-of-principle” approach has offered a new avenue to further develop cancer-specific inhibitors targeting

protein-protein interactions to act against tumor-addicted proteins. Our work shows that not only can the activity of receptors be targeted, but also localization of active receptors can be manipulated. These results offer unique perspectives on the development of potential novel therapeutics targeting the EGFR. By using this structure-function peptide approach, we can determine whether a certain protein-protein interaction is targetable. Once verified, modification of peptides and screening for chemical molecules with a similar structure can be done to improve peptide stability and efficacy. Because therapeutic resistance is an inevitable result for every single drug, the combination of drugs working through different mechanisms might be the improved way to impede emergence of resistance. Our work also provides insights for effective combination therapy in the future.

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