EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) ACTIVATION BY GASTRIN RELEASING PEPTIDE (GRP) IN HEAD AND NECK CANCER: MECHANISMS AND CLINICAL IMPLICATIONS

by

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EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) ACTIVATION BY GASTRIN RELEASING PEPTIDE (GRP) IN HEAD AND NECK CANCER: MECHANISMS AND CLINICAL IMPLICATIONS

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Head and neck squamous cell carcinomas (HNSCC) are characterized by upregulation of the epidermal growth factor receptor (EGFR). We previously reported that a gastrin-releasing peptide/gastrin-releasing peptide receptor (GRP/GRPR) autocrine growth pathway is activated early in HNSCC carcinogenesis. GRP can induce rapid phosphorylation of EGFR as well as p42/44 MAPK activation, in part via extracellular release of transforming growth factor α (TGF-α) by matrix metalloproteinases (MMP). Src family kinases have been reported to be activated by G-protein-coupled receptors (GPCRs) followed by downstream EGFR and MAPK activation. To further elucidate the mechanism of activation of EGFR by GRP in HNSCC, we investigated the role of Src family kinases. Blockade of Src family kinases using three different Src-specific tyrosine kinase inhibitors (A-419259, PP2 or PD0180970) decreased GRP-induced EGFR phosphorylation as well as MAPK activation. GRP also failed to induce MAPK activation in dominant-negative c-Src transfected HNSCC cells. Invasion and growth assays demonstrated that c-Src was required for GRP-induced proliferation or invasion of HNSCC cells. In addition to TGF-α release, GRP induced amphiregulin, but not EGF, secretion into HNSCC cell culture medium, an effect that was blocked by the MMP inhibitor, Marimastat. TGF-α and Amphiregulin secretion by GRP stimulation was also inhibited by blockade of Src family kinases.

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Further investigation showed that TNF-α converting enzyme (TACE) underwent Src-dependent phosphorylation and translocation to the plasma membrane in a complex with c-Src and the p85 subunit of PI-3 kinase, where it regulated amphiregulin release. In addition, we identified that PDK1 kinase, a downstream target of PI-3 kinase, directly phosphorylated TACE. Knockdown of PDK1 augmented the anti-tumor effects of the EGFR inhibitor erlotinib. These findings implicate PDK1 as a new target in HNSCC and suggest that therapeutic strategies that block PDK1 may improve the clinical response to EGFR inhibitors.

Combined targeting of GRPR and EGFR pathway also showed enhanced anti-tumor efficacy by inhibiting cancer cell proliferation, invasion and promoting apoptosis. Overall, these findings show the promises and benefits of combination therapy when targeting EGFR and GRPR pathways in head and neck cancer.

FORWARD

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PREFACE

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List of Abbreviations

ADAM	A disintegrin and metalloproteinase
AR	Amphiregulin
BLP	Bombesin-like peptide
DN	Dominant negative
DOK	Downstream of kinase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immuno Sorbent Assay
ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GFP	Green fluorescence protein
GI	Gastrointestinal
GPCR	G-protein-coupled receptor
GRPR	Gastrin releasing peptide receptor
GRP	Gastrin releasing peptide
GST	Glutathione s transferase
HB-EGF	Heparin binding EGF
HEK	Human embryonic kidney
HNSCC	Head and neck squamous cell carcinoma
IPTG	isopropyl-1-thio-ß-D-galactopyranoside
LPA	Lysophosphatidic acid
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSCLC	Non small cell lung cancer

OD	Optical density
PBS	Phosphate-buffer saline
PDK	3-phosphoinositide-dependent protein kinase
PI-3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
RTK	Receptor tyrosine kinase
Ser	Serine
SH2	Src homology 2
SH3	Src homology 3
siRNA	Small interference RNA
STAT	Signal transducer and activators of transcription
TACE	TNF-α converting enzyme
TGF-α	Transforming growth factor α
Thr	Threonine
TKI	Tyrosine kinase inhibitor
TMPS	Triple membrane passing signaling
TPA	12-O-tetradecanoylphorbol-13-acetate
TUNEL	TdT-mediated dUTP-biotin nick end labeling
Tyr	Tyrosine

1. INTRODUCTION

1.1. General Introduction

1.1.1. Cancer

Cancer is defined as a group of diseases characterized by unlimited cell growth, which ultimately leads to evasion from homeostasis regulation and invasion into adjacent or distant organ systems. Without appropriate treatment, it can result in high mortality. In 2005, it is estimated that there will be more than 1.3 million new cancer cases worldwide[1]. In the United States, there are expected to be 1500 deaths per day owing to cancer[2]. It is the second leading causes of death exceeded only by heart diseases[3]. While the death rate of heart diseases decreased by 52%, the death rate of cancer remained almost the same in the past 3 decades (1). In addition, despite more advanced technology to detect cancer early and better therapy options to treat cancer, the 5 year survival rate only increased by 14% compared to the gains noted 30 years ago[4]. Cancer also causes serious economic burdens in the world. In 2004, nearly 200 billion dollars were spent on cancer treatment[5]. In September of 2005, 92 U.S senators sent a letter to President Bush supporting of the National Cancer Institute's goal of curing cancer by 2015[6]. The work herein is an effort trying to understand the mechanism of carcinognesis by using head and neck cancer as a model system. In addition, the efficacy of combination therapy

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[1, 2, 3, 4] The American Cancer Society, Cancer Statistics and Figures 2005 (www.cancer.org)

5 The National Cancer Institute (www.cancer.gov)

6 http://feinstein.senate.gov/05releases/r-cancer2015.htm

in head and neck cancer is explored, which could facilitate the rational design of drug therapy and benefit cancer patients.

1.1.2. Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) accounts for 90% of head and neck cancers (2). HNSCC is the 6th most common cancer worldwide. There are approximately 40,000 new cases and 12,000 new deaths annually in the United States (3). In India, more than 50% of newly diagnosed cancers are the squamous cell carcinoma of the oral cavity (4). The major risk factors include tobacco usage and alcohol consumption. Currently, standard treatment for head and neck cancer is surgery followed by chemoradiation. Despite a near 60% 5 year survival rate for primary HNSCC, most HNSCC patients die of a secondary aerodigestive tract cancer. The 5 year survival rate for the secondary cancer remains below 25% (5). Furthermore, there is little evidence of an improvement in the 5 year survival rates over past several decades (6). Although the critical pathways leading to HNSCC remain largely unknown, the epidermal growth factor receptor (EGFR) pathway likely plays a major role. 95% of HNSCC tumors overexpress EGFR when compared to normal mucosa (7). However, targeting EGFR with either tyrosine kinase inhibitor or monoclonal antibodies has had limited anti-tumor effects in head and neck cancer patients when these agents are delivered as monotherapy (8, 9).

1.2. EGFR in Cancer

1.2.1. EGFR family

Epidermal growth factor receptor (EGFR), also known as ErbB1, is a 170-kDa transmembrane protein. EGFR is mainly composed of an extracellular ligand binding, a

transmembrane domain, an intracellular protein kinase domain and SH2 domain binding sequences at the c-terminus (10). Six different EGFR ligands have been identified so far, including EGF, transforming growth factor α (TGF-α), amphiregulin, heparin binding EGF (HB-EGF), β-cellulin and epiregulin (10). In addition to EGFR, there are three other ErbB family members identified: ErbB2, ErbB3 and ErbB4. They share a similar structure with EGFR and exert their effects through homo or hetero-dimerization.

Ligand binding to EGFR family receptors induces the formation of homo and heterodimers, which lead to intracellular kinase activation. Upon activation, EGFR cytoplasmic tail tyrosine residues are phosphorylated, which then serve as the docking sites for downstream molecules, especially SH2 domain containing proteins. For example, EGF induces GRB2 interaction with EGFR, which leads to recruitment of SOS, Ras, Raf and activation of mitogen-activated protein kinase (MAPK). Phosphorylation of EGFR also leads to PI-3 kinase/Akt activation by the interaction between the PI-3 kinase p85 subunit and EGFR. Signal transducer and activator of transcription 3 or 5 (STAT 3/5) have been reported to be activated by interaction with EGFR on tyrosine residue 1086 and 1068 (11). In addition, the non-receptor tyrosine kinase Src can also be activated by binding with EGFR c-terminal sequences in the cytoplasm (12). Figure 1 depicts a representation of ErbB family signaling pathways.

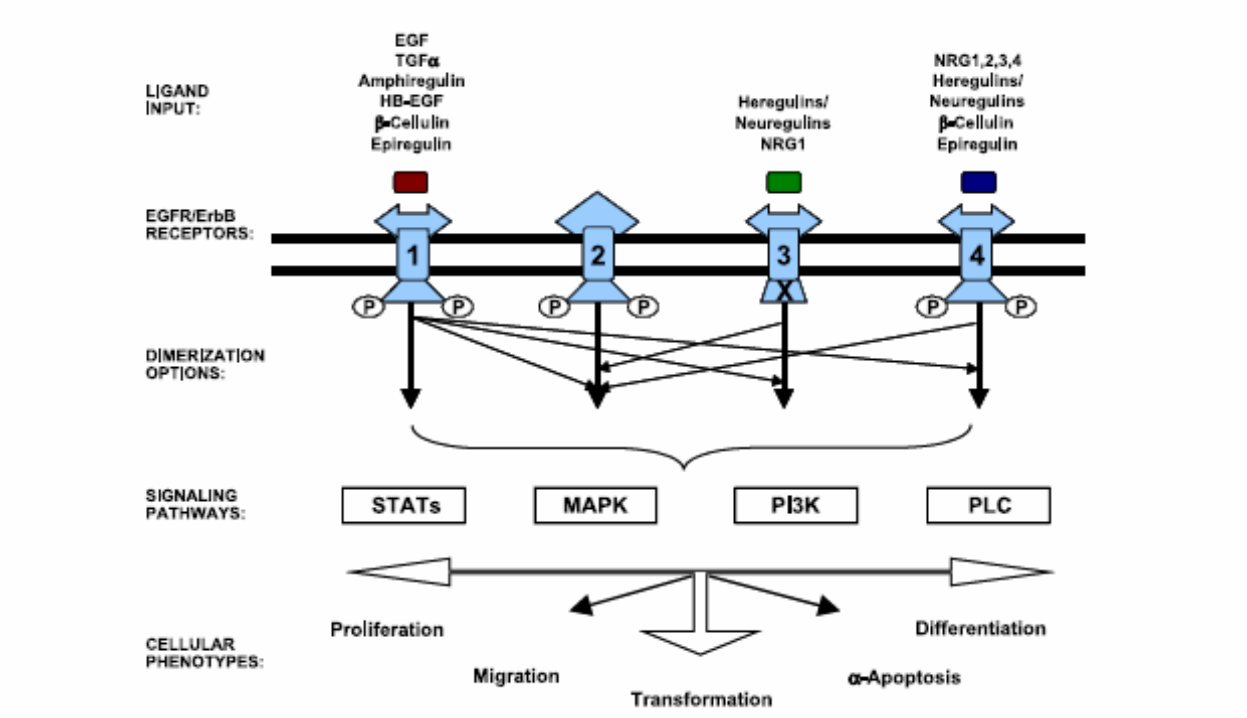


Figure 1: The diversity of the EGFR signaling network.

The EGFR transduction cascade is a highly complex network, consisting of signaling options based on multiple layers. The ligand input and receptor engagement occurs in the extracellular layer. Receptor-specific ligands for ErbB1, ErbB3, and ErbB4 have been identified as shown. No direct ligands for ErbB2 have been isolated to date. At the cell surface, receptor engagement leads to tyrosine phosphorylation and several receptor dimerization options (depicted by arrows: thick arrow denotes homodimerization and thin arrow denotes heterodimerization; the "X" in ErbB3 represents absence of the intrinsic tyrosine kinase activity). The selective activation of well-characterized signaling transduction pathways (shown in boxes), depends on the various arrangements of ligand-ErbB engagement, tyrosine phosphorylation, and subsequent receptor dimerization combinations beneath the cell surface. Finally, the output layer

includes a variety of cell responses (shown in bold). Reprinted from Pharmacology&Therapeutics 102 (2004): 37-46 with permission from Elsevier. Jennifer R Grandis and John S Sok, Signaling through the epidermal growth factor receptor during the development of malignancy.

1.2.2. EGFR and cancer

Following activation of MAPK, Akt and STATs, these molecules translocate into the nucleus and regulation proto-oncogene transcription, such as fos, jun and myc, which enable cancer cells to proliferate, survive, invade/metastasize. EGFR was the first oncogene to be directly linked to human cancers (13, 14). EGFR overexpression has been reported in a variety of premalignant lesions as well as epithelial malignancies, including lung, prostate, gastric, breast, colon, pancreatic and head and neck cancer (10). The mechanism of EGFR overexpression is mainly due to increased transcriptional activity (15-17). In addition to transcriptional activation, gene amplification can also lead to EGFR overexpression. The best example is in human gliomas cancer cells where an in frame deletion in the EGFR extracellular domain leads to a constitutively active receptor EGFRvIII that is capable of ligand independent signaling (18). In addition to gliomas, lung, breast, ovary and head and neck cancer were found to contain this EGFRvIII mutant (19).

EGFR overexpression can induce its activation and downstream signaling leading to carcinogenesis. In addition, EGFR can also be activated by autocrine production of its ligands in cancer, such as TGF-α, amphiregulin and HB-EGF. EGFR can also be activated by other signaling pathways through crosstalk, such as G-protein-coupled receptor (GPCR) pathways, which will be discussed in detail in the following section.

1.2.3. EGFR as a therapeutic target for cancer

Since EGFR is overexpressed in most solid tumors and EGFR activation can also induce downstream signaling leading to cancer progression, EGFR targeting strategies have been extensively studied for cancer therapy. Among them, EGFR monoclonal antibodies and tyrosine kinase inhibitors are the most well developed. Erbitux/Cetuximab/C225 is the most extensively studied EGFR monoclonal antibody. C225 binds with the EGFR ligand binding domain and prevents EGFR activation. The binding between C225 and EGFR is irreversible, which is followed by EGFR internalization and degradation. C225 has been approved by the FDA to treat refractory colorectal cancer patients and an indication for HNSCC patients in combination with radiation is currently under review (20, 21). In combination with radiation and chemotherapy, C225 achieves a higher anti-tumor efficacy. Of 15 patients with head and neck cancer, 13 displayed complete response and 2 of them had partial response (22).

In addition to EGFR monoclonal antibodies, several different EGFR tyrosine kinase inhibitors have been used to treat cancer patients. These inhibitors compete with ATP binding to the tyrosine kinase domain of EGFR, which inhibits EGFR activity and blocks downstream signaling (23). Gefitinib/Iressa/ZD1839 (Astra Zeneca) has been approved by the FDA to treat non-small-cell lung cancer (NSCLC). Gefitinib belongs to the 4-anilinoquinazoline class of compounds, which has been shown to inhibit cell proliferation in a variety of cancer cell lines *in vitro*, including breast, lung, ovarian and head and neck cancer (14). When combined with other other chemotherapy drugs, gefitinib shows a synergistic cell killing pattern. Furthermore, gefitinib can also inhibit tumor growth in nude mice bearing different xenografts (14). Another tyrosine kinase inhibitor, OSI-774/Erlotinib/Tarceva (OSI Pharmaceutical) has also been

approved by the FDA to treat NSCLC. Table I depicts the current EGFR targeting strategies in clinical trials.

Table 1. Overview of Clinical EGFR Targeting Strategies

Category	Inhibitor	Status	Route	Cancer types
Monoclonal Ab	C225	Approval	i.v	NSCLC
		Phase III	i.v	Pancreatic, HNSCC, Colon
		Phase II	i.v	Breast, renal and prostate
	ABX-EGF	Phase II	i.v	Metastatic kidney cancer
TKI	Gefitinib	Approval	Oral	NSCLC
		Phase III	Oral	HNSCC
		Phase II	Oral	Colon, Breast, GI, prostate
	Erlotinib	Approval	Oral	NSCLC
		Phase III	Oral	Pancreatic,
		Phase II	Oral	Ovarian,HNSCC, brain, breast, renal, colon
Ligand conjugated Toxin	TP-38	Phase I	Intratumoral	Malignant brain tumor
Gene therapy	EGFR antisense	Phase I	Intratumoral	HNSCC

Despite promising results in preclinical models with these inhibitors, the responses to these drugs in cancer patients remain relatively low, below 20% percent (24, 25). In addition, EGFR expression levels in the tumor do not appear correlate with the clinical response to these inhibitors (20). Recently, EGFR tyrosine kinase domain mutations have been found to correlate with NSCLC responses to gefitinib treatment (26, 27). Somatic mutations in ATP-binding region of EGFR could stabilize the interaction between EGFR and ATP or a tyrosine kinase inhibitor.

As a result of this stabilized interaction, much lower doses of gefitinib are needed to inhibit receptor activity (26). In addition, because mutant EGFR selectively active Akt and STAT anti-apoptotic pathways, treatment with gefitinib or erlotinib can induce rapid tumor cell death (28).

Although EGFR mutations account for some percentage of patients who responded to EGFR tyrosine kinase inhibitor treatment in NSCLC, these mutations are exceedingly rare in other cancers, including HNSCC. The mechanism that contributes to resistance of gefitinib or erlotinib treatment in other cancers remains largely unknown. Recent evidence suggests that constitutive activation of EGFR by other signaling pathways may contribute to the response to EGFR targeting strategies.

1.2.4. EGFR transactivation in cancer

EGFR is phosphorylated in response to EGFR ligand binding. Constitutively secreted mature EGFR ligand binding will stimulate EGFR signaling. For the past decade, extensive research has been done to elucidate the mechanism of constitutive EGFR signaling by other pathways. Among them, G-protein-coupled receptors (GPCRs) are among the most well characterized. Ullrich's group first reported that GPCR ligands, such as endothelin-1, LPA and thrombin, can induce EGFR phosphorylation in Rat-1 fibroblast (29). The same group discovered that another GPCR ligand, bombesin, can induce EGFR phosphorylation in cancer cells (30). Further investigation showed that GPCR ligand induced EGFR phosphorylation is through EGFR ligand release and metalloproteinase activity dependent, particularly a disintegrin and metalloproteinase (ADAM) and matrix metalloproteinases (MMPs) (30-33). Following EGFR phosphorylation, downstream MAPK pathways are activated. GPCRs transmit mitogenic

signals through intracellular and extracellular molecules, potentially including Src, PI-3 kinase, PKC and ADAM/MMPs.

Intracellularly, Src family kinases were reported to associate with G proteins and be directly activated by G_{α} subunits in fibroblasts as well as in a cell free system (34). Activation of c-Src kinase has also been shown to be an early event in $G_{\beta\gamma}$-mediated MAPK activation by GPCRs in fibroblasts (35). In colon and gastric cancer cell lines, Src family kinases have been reported to be activated by GPCR ligands followed by downstream EGFR and MAPK phosphorylation (31, 36). In addition, Src family kinases directly associate with EGFR and mediate phosphorylation of tyrosine residues Y845 and Y1101 on the EGFR (37, 38). Notably, Src family kinases have been shown to act downstream of EGFR and activate MAPK by GPCR ligands, indicating that the recruitment of Src by activated EGFR could contribute to the Ras signaling pathway (39-41).

In addition to Src family kinases, PI-3 kinase has been indicated to contribute to GPCR ligand induced MAPK pathways through EGFR dependent or independent pathways. Alpha2 adrenoceptor mediated vasoconstriction is mainly through a signaling cascade of activation of PI-3 kinase, calcium influx followed by phosphorylation of EGFR and MAPK (42). In contrast, there are some reports showing that PI-3 kinase contributed to GPCR ligand induced MAPK activation, but not EGFR phosphorylation. In ovarian cancer cells, PI-3 kinase inhibitors abrogated endothelin-induced MAPK phosphorylation, but have no effects on endothelin induced EGFR and Shc phosphorylation (43). In prostate cancer cells, PI-3 kinase was shown to mediate neurotensin induced MAPK phosphorylation, but not EGFR phosphorylation (44).

Extracellularly, ADAM/MMP plays an important role in mediating GPCR ligand induced EGFR proligand release followed by downstream EGFR and MAPK phosphorylation. ADAM

family members share sequence homology with MMP family members. They both contain a key metalloproteinase domain, which plays a major role in cleaving EGFR ligands. MMP2 and MMP9 were shown to mediate estradiol induced EGFR ligand release and downstream MAPK activation (33). ADAM17 has been indicated to mediate LPA induced EGFR phosphorylation in head and neck cancer cells (45). ADAM10 has been shown to mediate LPA or bombesin induced EGFR activation in Cos-7 fibroblast (46).

Although ADAM/MMP members are shown to mediate EGFR ligand release, the mechanism of GPCR induced ADAM/MMP activation remains largely unknown. Since GPCR ligands potentially induce intracellular Src, PKC, PI-3 kinase activation, these molecules may provide a link between GPCR and ADAM/MMP activation. Previous research showed that ADAM17/TACE activity is important for TPA-induced EGFR ligand shedding in cultured cells, indicating the involvement of PKC in the activation of ADAM17 (47-49). PKC has been reported to mediated TPA induced ADAM9 phosphorylation and contribute to HB-EGF release (50). Recent research suggested that since ADAM family members contain proline rich motifs on the cytoplasmic domain, Src SH3 domain can interact with ADAMs on the cytoplasmic domain(51). In mouse fibroblast, Src has been shown to interact with ADAM12 (52). This interaction is dependent on Src kinase activity and potentially contributes to ADAM family member activation. For instance, ADAM15 undergoes tyrosine phosphorylation on the cytoplasmic domain in a Src kinase dependent manner (53). The mechanism underlying ADAM/MMP activation awaits further investigation. Figure 2 depicts EGFR and GPCR crosstalk signaling pathways.

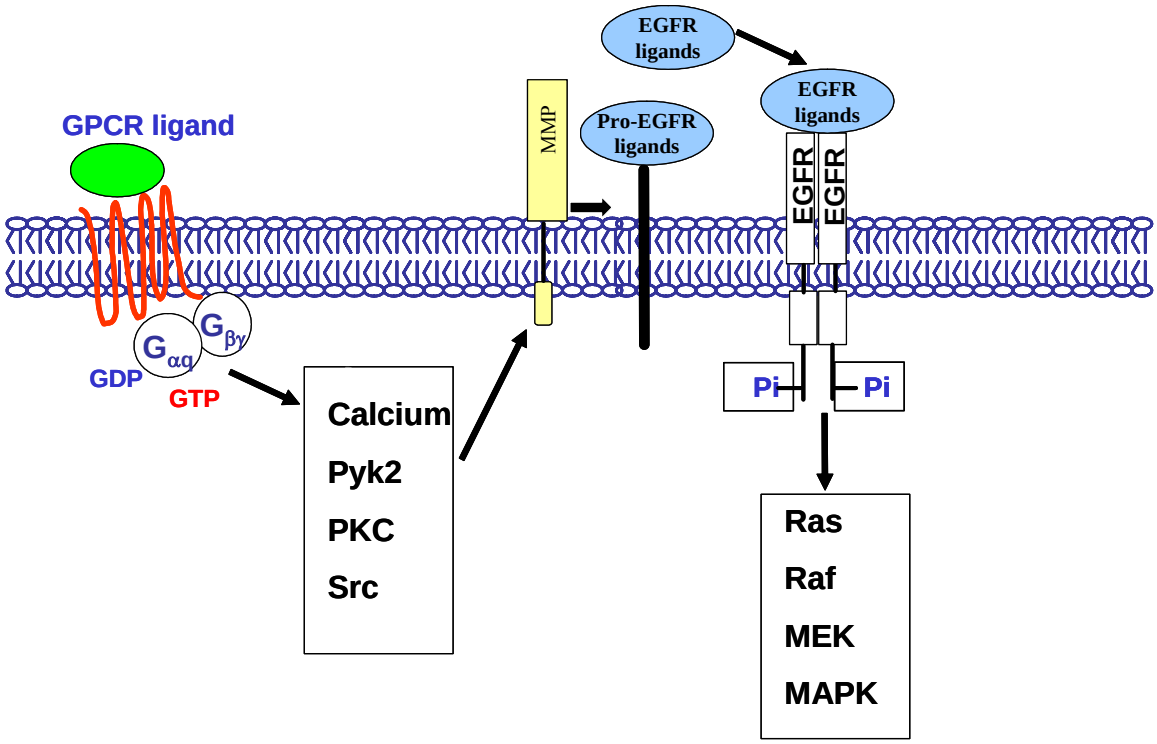


Figure 2: Potential mechanism of GPCR and EGFR crosstalk.

Upon GPCR ligand binding with receptors, GPCR will transactivate EGFR and leads to downstream MAPK activation. Intracellularly, this process involves different mediators such as Src-family kinases, calcium (Ca^{2+}), Pyk2 and protein kinase C (PKC), depending on the cell type. Extracellulaly, transactivation of EGFR generally requires pro-EGFR ligand cleavage by MMP members (54).

1.2.5. EGFR and HNSCC

Overexpression of EGFR is characteristic of many epithelial malignancies, including HNSCC (55). Cumulative evidence suggests that upregulation of EGFR occurs early in HNSCC carcinogenesis, primarily as a result of activated gene transcription (56). Elevation of EGFR in

HNSCC is accompanied by increased levels of its ligand, transforming growth factor alpha (TGF-α), implicating an autocrine regulatory pathway in this tumor system. In HNSCC tumors, the expression level of EGFR in the tumor was a predictor of decreased survival, independent of nodal status (N-stage) (7). Abrogation of EGFR *in vitro* or *in vivo* inhibited HNSCC proliferation without affecting the growth of normal mucosal epithelial cells (7, 56, 57). The critical importance of EGFR in HNSCC is demonstrated by the remarkable results of phase I clinical trials where treatment with a monoclonal antibody (C225) directed against EGFR in combination with either cisplatin or radiotherapy, resulted in a response rate of nearly 100% (22, 58). Phase II clinical trials with the EGFR tyrosine kinase inhibitor Erlotinib showed partial response (around 5%) and stabilized tumor progression (around 40%) in 115 head and neck cancer patients (59). A phase I study using EGFR antisense gene therapy is presently underway at our institution to examine the safety and biologic effects of this approach in HNSCC patients (60).

1.3. GRPR in Cancer

1.3.1. GRPR

Gastrin releasing peptide receptor (GRPR) is a member of the family of G-protein-coupled receptors (GPCRs). These transmembrane receptors exhibit a common structural motif consisting of seven membrane-spanning regions that, when activated, undergo a conformational change resulting in the unmasking of G-protein binding sites in the intracellular loops. The complexity of GPCRs is illustrated by the 17 distinct mammalian G-protein α subunits that have been identified and categorized according to sequence similarity into 4 families. Multiple intracellular pathways have been proposed that mediate the proliferative effect of GPCRs. GRPR has been shown to be primarily a Gαq-coupled GPCR (61). Upon ligand binding, the exchange

of GDP for GTP in the G protein a subunit occurs. This leads to dissociation of the βγ complexes from the Gα subunit, followed by intracellular signaling events initiated by both Gα and βγ complexes.

GRP is a bombesin family ligand. Bombesin was first purified from the European frog, while the bombesin-like ligands GRP and neuromedin B were purified from mammalian tissues (62). Expression of bombesin-like peptide (BLP) ligands and receptors has been reported in several cancers including glioblastomas, ovary, colon, prostate, kidney, breast, and lung cancer, including NSCLC (63-68). Although classically associated with cells of neuroendocrine origin, several transformed cell types, including airway epithelial cells, have been shown to respond to BLPs (69, 70). Immortalized human bronchial epithelial cells engineered to overexpress GRPR, demonstrated calcium influx and proliferation in response to exogenous bombesin (71). In an animal model of chemical-induced oral cancer, a bombesin antagonist has been shown to prevent the formation of premalignant mucosal lesions (72).

1.3.2. GRPR in head and neck cancer

Female smokers appear to have a higher risk for the development of aerodigestive tract cancers (including lung and HNSCC) compared with men who have a similar exposure to tobacco (73, 74). The gene encoding the GRPR, which mediates the proliferative effects of bombesin-like peptides, is located on the X chromosome, and has been shown to escape X inactivation in somatic cell hybrids produced from fibroblasts and lymphoblasts of normal women (75). We previously reported that GRPR was overexpressed in both HNSCC tumors (6 fold) and adjacent normal mucosa (4 fold) from HNSCC patients compared with levels in control mucosa from individuals without cancer (76). GRPR/GRP was also shown to be overexpressed

(5 fold) in HNSCC cancer cells compared with normal cells. More importantly, GRP was found to stimulate cancer cell proliferation *in vitro* and tumor growth *in vivo,* effects that were blocked by GRP neutralizing antibody 2A11. When dividing patients into two different groups according to GRPR expression level, higher GRPR expression group was found to have a decreased survival rate compared to the HNSCC patients whose tumors expressed lower levels of GRPR (76).

1.4. Statement of Problems and Hypothesis

HNSCC are characterized by upregulation of EGFR (8). While promising results in the preclinical setting have been observed by targeting EGFR expression or activation using EGFR monoclonal antibodies or EGFR tyrosine kinase inhibitors, limited anti-tumor effects have been observed when these agents have been administered to cancer patients (9). These cumulative results suggest that EGFR-independent pathways contribute to tumor growth. The Grandis lab previously reported that gastrin-releasing peptide (GRP) and gastrin-releasing peptide receptor (GRPR) are overexpressed in HNSCC cell lines as well as in tumor and normal mucosa from HNSCC patients when compared to control cells and tissues (76). Further research demonstrated that the mitogenic effects of GRP are mediated by phosphorylation of EGFR followed by stimulation of the MAPK pathway (77). However, the mechanism of GRP induced EGFR activation in HNSCC remains largely unknown. This thesis will test the hypothesis that **a GRPR autocrine pathway contributes to HNSCC carcinogenesis via integration with EGFR mitogenic signaling.** Integration of GRPR and EGFR signaling in cancer cells suggests that treatment regimens designed to target both receptor pathways may be efficacious. HNSCC is frequently fatal despite surgery, radiation and chemotherapy (78). Innovative therapies that

specifically target growth pathways utilized by the tumor cells are required to effectively treat this disease.

2. SRC FAMILY KINASES MEDIATE EGFR LIGAND CLEAVAGE, PROLIFERATION AND INVASION OF HEAD AND NECK CANCER

2.1. Introduction

Overexpression of EGFR has been reported in variety of human cancers such as those derived from the breast, lung, colon, prostate, brain, ovarian and head and neck (8, 79-81). This increased expression of EGFR is generally accompanied by upregulation of EGFR ligands, implicating an autocrine regulatory pathway (82, 83). While promising results in the preclinical setting have been observed by targeting EGFR expression or activation in tumor cells using EGFR monoclonal antibodies or EGFR tyrosine kinase inhibitors, limited anti-tumor effects have been observed when these agents have been administered to cancer patients (84, 85). These cumulative results suggest that alternative routes of EGFR activation and/or EGFR-independent pathways contribute to tumor growth.

Cumulative evidence demonstrates that EGFR can be activated by GPCRs in diverse cell types including fibroblasts, and smooth muscle cells, in addition to tumor cells (30-32, 39, 86). The primary mechanism of GPCR-mediated EGFR activation involves proteolytic release of EGFR ligand(s) (30, 32, 33, 86). However, the specific EGFR ligand released by GPCRs appears to be both cell type and GPCR ligand-specific. Heparin-binding EGF-like growth factor (HB-EGF) has been shown to be the primary EGFR ligand involved in the EGFR activation by GPCRs in COS-7, HEK-293 and breast cancer cells (30, 33), while TGF-α has been implicated in colon epithelial cells and HNSCC (31, 86, 87). Amphiregulin has also been shown to play a role in GPCR-EGFR transactivation by LPA or carbachol treatment in HNSCC (32). We previously reported that GRP and its receptor, GRPR participate in an autocrine growth pathway

in HNSCC where GRPR levels in the primary tumor are correlated with survival (76). In contrast, the biological significance of the other GPCR ligands studied to date in EGFR activation in HNSCC remains undetermined.

G proteins transduce signals from GPCRs to EGFR and MAPK via unknown intermediate molecules. Emerging evidence has shown that Src family kinases are associated with G proteins and are directly activated by G_{α} subunits in fibroblasts (88). Activation of c-Src kinase has also been shown to be an early event in $G_{\beta\gamma}$-mediated MAPK activation by GPCRs in fibroblasts (35). Src family kinases have been reported to be activated by GPCR ligands, followed by downstream activation of EGFR and MAPK in the colon cancer cell line Caco-2, gastric epithelial cells RGM1, COS-7 fibroblasts and GT1-7 neuronal cells (31, 35, 89). Src family kinases directly associate with EGFR and mediate phosphorylation of tyrosine residues Y845 and Y1101 on the EGFR (37, 38). The precise mechanism of GPCR-mediated EGFR activation by Src family kinases is incompletely understood.

The present study was undertaken to test the hypothesis that Src kinases mediate EGFR activation by GRP via cleavage of EGFR proligands. Here we show that blockade of Src kinases using either pharmacological inhibitors or dominant-negative mutants decreased GRP-induced EGFR phosphorylation and MAPK activation by inhibiting TACE-mediated release of TGF-α and Amphiregulin into HNSCC cell culture medium. Physical interaction between TACE and c-Src contributed to GRP-mediated MAPK activation. Further investigation demonstrated the importance of Src family kinases in mediating GRP induced growth and invasion in HNSCC cells. These results demonstrate a novel role for Src kinases in mediating EGFR proligand release in response to GPCR ligands.

2.2. Materials and Methods

2.2.1. Chemicals and reagents

Human gastrin-releasing peptide (GRP) was obtained from Sigma-Aldrich Corporation (St. Louis, MO). Human recombinant epidermal grown factor (EGF) was obtained from Oncogene Research Products (Boston, MA). Antibodies against epidermal growth factor receptor (monoclonal antibody) were obtained from the Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY). The Src-specific tyrosine kinase inhibitor, PD0180970 was from Parke-Davis Pharmaceutical Company (Ann Arbor, MI) and A-419259 was a kind gift from Abbott Bioresearch Center (Worcester, MA) and PP2 was obtained from Calbiochem Corporation (San Diego, CA). Antibodies against p42/44 MAPK and phosphorylated p42/44 MAPK were from New England Biolabs (Beverly, MA). Amphiregulin neutralizing antibody and monoclonal TACE antibody were purchased from R&D systems (Minneapolis, MN). The EGFR blocking antibody C225 was a kind gift from Imclone Systems Incorporated (New York, NY). Antibody against the activation loop of Src (PY418) was purchased from Biosource international (Camarillo, CA93012). Rabbit anti-TACE antibody was purchased from Chemicon international (Temecula, CA92590). The matrix metalloproteinase inhibitor Marimastat was obtained from British Biotech, Oxford, England and Calbiochem-Novabiochem Corporation (San Diego, CA) respectively. The TGF-α ELISA kit was purchased from Oncogene research products (San Diego, CA) and the Amphiregulin and EGF ELISA kits were purchased from R&D systems (Minneapolis, MN).

2.2.2. Cell culture

All HNSCC cell lines (1483, PCI-37a) were of human origin. Cells were maintained in DMEM with 12 % heat inactivated fetal calf serum (Invitrogen, Carlsbad, CA) at 37°C with 5 % CO_2. Primary cell cultures were generated from murine embryonic fibroblasts derived from EGFR knockout mice (Jackson laboratories), and their corresponding wild-type littermates (CD1 background) obtained at age E16.5 days. Genotyping was determined using the appropriate primers. Following genotyping, tissues were harvested and cultures generated by mincing organs and incubating in trypsin-EDTA (Invitrogen Corporation, Carlsbad, CA) for 15 min at 37°C followed by centrifugation and resuspension in cell dissociation solution (Sigma-Aldrich Corporation, St. Louis, MO) for 40 min at 37°C, and then plating in petridishes pretreated with 0.05mg/ml collagen in 0.2 N acetic acid. Media was changed twice a week and cells were trypsinized very 7-14 days and passaged. Primary cell cultures were maintained in DMEM containing 20% heat inactivated fetal calf serum at 37°C with 5% CO_2.

2.2.3. Expression and purification of GST-Dok substrate

To create a substrate for in vitro Src kinase assays, we expressed a portion of the p62 Ras GAP-associated protein Dok as a GST fusion protein (90). The coding region of murine Dok residues Ser 309 to Leu 429 was amplified by PCR and cloned downstream and in-frame of GST in the baculovirus transfer vector, pVL-GST (91). This region of Dok contains multiple consensus sequences for tyrosine phosphorylation. The resulting pVL-GST-Dok vector was used to create a recombinant baculovirus using Baculogold DNA and the manufacturer's protocol (BD-Pharmingen). The GST-Dok fusion protein was expressed in Sf-9 insect cells and purified in one step using glutathione-agarose beads. The protein was eluted from the beads with

free glutathione and dialyzed against 50 mM Hepes buffer (pH 7.4) containing 10% glycerol. The final protein ran as a single band of the expected molecular mass following analysis by SDS-PAGE and Coomassie staining.

2.2.4. *In vitro* kinase assay

After treatment with GRP or EGF, cells were washed three times with cold PBS, lysed with lysis buffer (10mM Tris HCl, pH 7.6, 50mM $Na_4P_2O_7$, 50mM NaF, 1mM NaV_3O_4, 1% TritonX-100 and 1X protease inhibitor cocktail tablet that included an inhibitor of protein tyrosine phosphatases{Roche, Germany}), scraped off the plate, and passed through a 26 and a half gauge needle 3-4 times. The lysate was then centrifuged at 4 °C, 14000 rpm for 10 mins. Supernatant was collected for protein quantitation. Protein quantitation was performed using the Protein Assay Solution (BioRad Laboratories, Hercules, CA) and bovine serum albumin of known concentration as the standard. Anti- c-Src, c-Yes, Fyn, or Lyn antisera (Santa Cruz Biotechnology, Santa Cruz, CA) were used to immunoprecipitate Src family proteins. Forty microliters of protein G agarose beads (Invitrogen, Carlsbad, CA) were added to the lysate and incubated overnight at 4 °C with gentle agitation. The beads were collected by centrifugation at 4 °C, 14000 rpm for 1 min. The beads were resuspended and washed with lysis buffer 3 times. Immunoprecipitates were washed twice with kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$). Kinase buffer containing 1 μg of the tyrosine kinase substrates GST-Dok and 5 μCi of [γ-^{32}P] ATP (3,000 Ci/mmol; NEN Life Science Products) were added, and the reactions were incubated for 15 min at 30 °C. Reactions were stopped by adding SDS-PAGE sample buffer and heating to 95 °C for 5 min. Radiolabeled substrates was visualized by autoradiography.

2.2.5. Transfection of HNSCC cells with dominant negative c-Src

1483 cells were transfected with a pUSEamp vector (Upstate Biotechnology, Inc. Lake Placid, NY) containing mutant c-Src [K296R/528F] cDNA using Lipofectamine (GIBCO Laboratories, Grand Island, NY) according to the manufacturer's recommendations. Stably transfected clones were selected for resistance to the neomycin analogue, G418 (800 μg/ml, Gibco BRL) as described previously (92). Dominant-negative c-Src was generated by mutation of K296 to R, which rendered the kinase domain incapable of binding ATP.

2.2.6. Cell treatments

HNSCC cells were plated at a density of 2 x 10^5 cells/ml in 10 cm^2 plates. Twenty-four hours after plating, cells were serum-starved for 72 h in serum-free DMEM. During serum starvation the media was changed every 24 h. For the experiments with inhibitors cells were pretreated with either 6 μg/ml of C225, 20 μM Marimastat, 100 nM A-419259, 5 μM PP2, 500 nM PD0180970 or 15 μg/ml Amphiregulin neutralizing antibody. Control wells were treated with vehicle (water) or DMSO for 2 hrs before the addition of growth factors. After pretreatment where required, 400 nM GRP or 10 ng/ml EGF was added to the cells. At selected time points after growth factor stimulation, cells were washed three times with cold PBS, lysed with lysis buffer (10mM Tris HCl, pH 7.6, 50mM $Na_4P_2O_7$, 50mM NaF, 1mM NaV_3O_4, 1% TritonX-100 and 1X protease inhibitor cocktail tablet that included a broad spectrum potent inhibitor of protein tyrosine phosphatases{Roche, Germany}), scraped off the plate, and passed through a 26 and a half gauge needle 3-4 times. The lysate was then centrifuged at 4 °C, 14000 rpm for 10 mins. Supernatant was collected for protein quantitation. Protein quantitation was performed

using the Protein Assay Solution (BioRad Laboratories, Hercules, CA) and bovine serum albumin of known concentration as the standard.

2.2.7. Western blotting and immunoprecipitation

For immunoprecipitation**,** 100 μg of total protein was incubated for 2 h at 4 °C with 3 μg of anti-EGFR antibody (Upstate Biotechnology, Lake Placid, NY) or with 7.5 μl of anti- c-Src, c-Yes, Fyn, or Lyn antisera (Santa Cruz Biotechnology, Santa Cruz, CA) with gentle agitation. Forty microliters of protein agarose G beads (Invitrogen, Carlsbad, CA) were added to the lysate and incubated overnight at 4 °C with gentle agitation. The beads were collected by centrifugation at 4 °C, 14000 rpm for 1 min. The beads were resuspended and washed with lysis buffer 3 times. The beads were resuspended in 20 μl of 2x loading dye and boiled for 5 mins at 95 °C followed by western blotting. The immunoprecipitated proteins were then resolved on an 8 % SDS-PAGE gel. After being transferred onto a Protran membrane, the membrane was blocked in 5% milk and blotted with the anti-phosphotyrosine antibody PY99 (Santa Cruz Biotechnology, California) or PY20 (Transduction Laboratories, Inc.) at 1:1000 in Blotto solution (0.6 % dry milk powder, 0.9 % NaCl, 0.5 % Tween 20 and 50 mM Tris, pH 7.4). After washing three times with Blotto solution, the membrane was then incubated with the secondary antibody (Goat anti-rabbit/mouse IgG-HRP conjugate, Bio-Rad Laboratories, Hercules CA) for 1 h and washed 3 times for 10 mins. The membrane was developed with Luminol Reagent (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) by autoradiography. Blots were stripped in Restore Western Blot Stripping buffer (Pierce, Rockford, IL) for 15 mins at room temperature, blocked for 1 h and reprobed with EGFR antibody (Transduction Laboratories, Lexington, KY) at 1:1000 or with anti- c-Src, c-Yes, Fyn, or Lyn antisera (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours.

2.2.8. *In vitro* invasion of HNSCC cells defective in c-Src

The *in vitro* ability of HNSCC cells to invade on GRP stimulation in the presence and absence of c-Src was measured using Matrigel-coated modified Boyden inserts with a pore size of 8 μm (Becton Dickenson/Biocoat, Bedford, MA). Cells were plated at a density of 2×10^4 cells/well in DMEM with or without GRP (400 nM)/ 5% serum in the insert. The lower well contained 10% FBS with or without 400 nM GRP. After 48 h of treatment at 37°C in a 5% CO2 incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientifics, USA) according to the manufacturer's instructions. Invading cells in 4 representative fields were counted using light microscopy at 400X magnification. Mean ± SEM was calculated from 2 independent experiments.

2.2.9. ELISA assay

HNSCC cells were plated at a density of 2 x 10^5 cells/ml in 10 cm^2 plates. Twenty-four hours after plating, cells were serum-starved for 72 h in serum-free DMEM. During serum starvation the media was changed every 24 h. Cells were pretreated with 20 μM Marimastat or 100 nM A-419259 for 2 h at 37°C with 5 % CO_2. Cells were treated with 400 nM GRP for 10 mins. Cell culture media were collected and cell subjected to centrifugation at 1300 rpm for 10 min. Pellets were discarded and the supernatant was tested for levels of TGF-α, Amphiregulin, or EGF as per the manufacturer's instructions.

2.2.10. Statistics

All group differences were tested with the exact Wilcoxon test. The p values from multiple comparisons within the same experiment were adjusted with the Bonferroni procedure.

2.3. Results

2.3.1. GRP induces c-Src kinase activity in HNSCC cells

We have previously shown that GRP stimulates p42/44 MAPK activation via EGFR phosphorylation (87). Further investigation demonstrated that TGF-α induced phosphorylation of Src family kinases including c-Src, Lyn, Fyn and c-Yes in HNSCC cells (92). Src family kinases have previously been reported to mediate EGFR activation by GPCRs in fibroblasts (93, 94). To determine whether GRP can mediate activation of Src family kinases, we treated HNSCC (1483) cells with GRP or EGF. The c-Src and c-Yes immunoprecipitates were incubated *in vitro* with [γ-^{32}P] ATP and a 40-kDa GST-Downstream of kinases (Dok) (90) fusion protein as substrate. As shown in Figure 3A, c-Src immunoprecipitates exhibited both strong autophosphorylation and substrate phosphorylation *in vitro* upon EGF or GRP treatment. However, c-Yes immunoprecipitates showed strong autophosphorylation and substrate phosphorylation upon EGF treatment, but not upon GRP treatment as shown in Figure 3B. Control blots indicated that equivalent amounts of Src family proteins were present in the immunoprecipitates. These results suggest that c-Src can be activated in HNSCC cells by GRP as well as EGFR ligands.

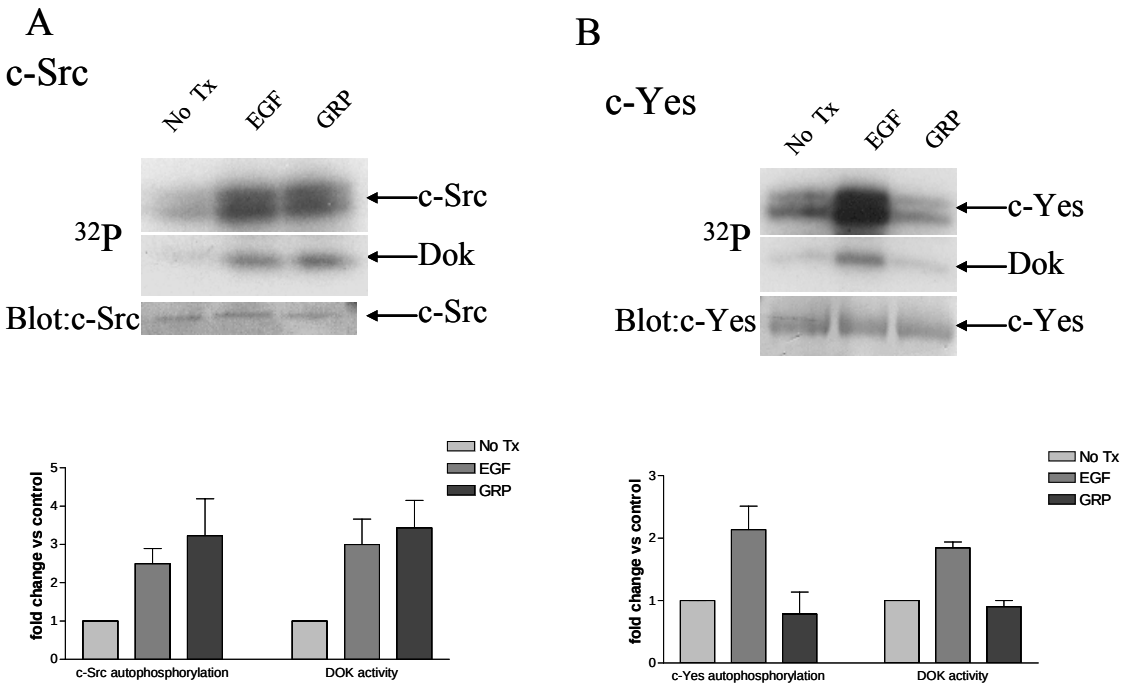


Figure 3: Stimulation of Src family kinase activity by GRP.

Representative HNSCC cells were serum starved for 72 hours then stimulated with GRP (400 nM) or EGF (10 ng/ml) for 10 min, followed by immunoprecipitation with antisera against (A) c-Src, (B) c-Yes. Immunoprecipitates were washed and resuspended in 20 μl of kinase buffer containing [γ-^{32}P] ATP and a GST-Dok fusion protein as substrate. Following incubation, Src family proteins and Dok were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane followed by autoradiography. The position of autophosphorylated Src family proteins and Dok are indicated by arrows (*upper panel and middle panel*). The membranes were probed with anti-c-Src and c-Yes to ensure equivalent recovery of Src family proteins in the immunoprecipitates (*lower panel*). Cumulative results are shown from two independent experiments.

2.3.2. EGFR activity is required for maximum GRP-induced activation of Src family kinase

Both EGF and GRP can activate c-Src kinase. While EGF activates Src family kinases by direct interaction between Src and EGFR (92), the mechanism of c-Src kinase activation by GRP remains unclear. To investigate the role of EGFR in the activation of Src family kinases by GRP, we treated cells derived from EGFR knockout mice or their wild-type littermates with EGF or GRP. As expected, EGF stimulated activation of Src family kinases in EGFR wild-type cells, but not in EGFR knockout cells. GRP induced activation of Src family kinases in EGFR wild-type cells as well as in EGFR knockout cells, but to a lesser degree in the EGFR deficient cells, suggesting that EGFR activity is required for maximum activation of Src family kinase by GRP (Figure 4).

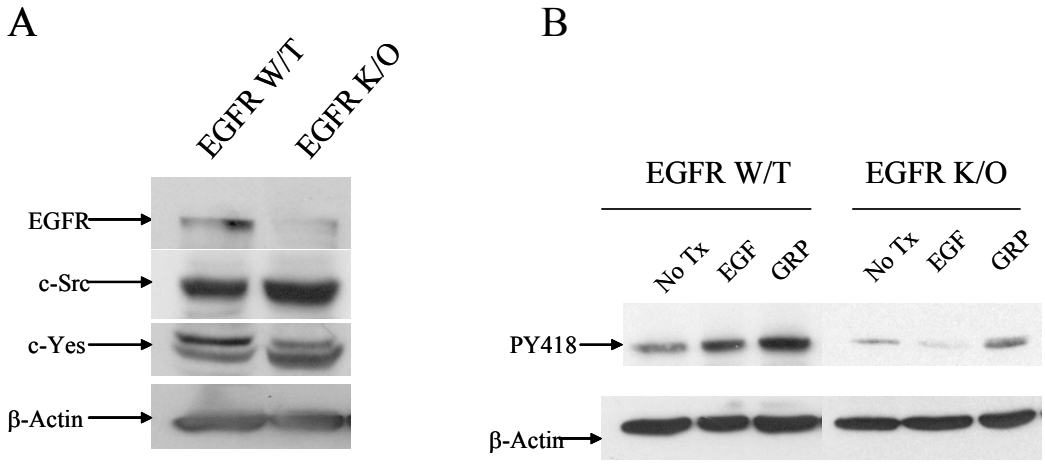


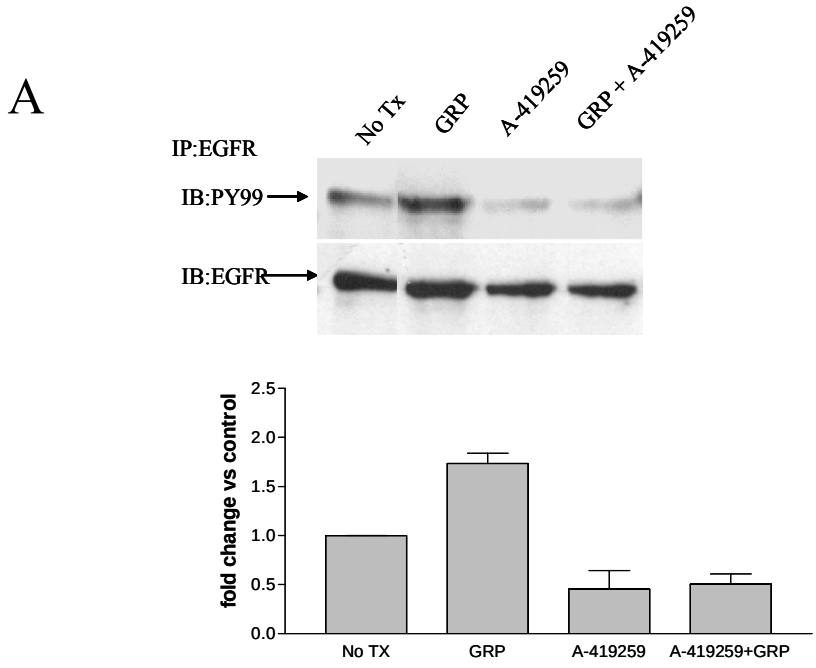
Figure 4: EGFR activity is required for maximum activation of Src family kinases by GRP.

(A) Cell lysates derived from EGFR knockout mice (K/O) or their wild-type littermates (W/T) were subject to western blotting for EGFR, c-Src and c-Yes. β-actin was used as a control for loading. (B) Cells derived from EGFR knockout mice (K/O) or their wild-type littermates

(W/T) were treated with recombinant EGF (10 ng/ml) or GRP (400 nM) for 10 min followed by immunoblotting for PY418 or β-actin (as a control for loading).

2.3.3. Src family kinases regulate EGFR activation by GRP in HNSCC cells

Src family kinases have been implicated in the crosstalk between EGFR and GPCRs (31, 39). However, the mechanism of how Src family kinases are involved in this process remains to be elucidated. To examine whether Src family kinases mediate EGFR activation by GRP in HNSCC, we treated HNSCC cells (1483) with each of three different Src inhibitors followed by GRP stimulation (Figure 5). The small molecule inhibitors A-419259, PP2 and PD0180970 have all previously been shown to inhibit Src-family kinases without inhibition of EGFR kinase activity in HNSCC cells (92, 95-97). As shown in Figure 5, GRP induced EGFR phosphorylation within 10 min, an effect that was nearly completely blocked by pretreatment with A-419259 (Figure 5A, p=0.014), PP2 (Figure 5B) or PD0180970 (Figure 5C). Similar results were obtained in another HNSCC cell line PCI-37a (data not shown). These results indicate that Src family kinases mediate EGFR activation by GRP in HNSCC cells.



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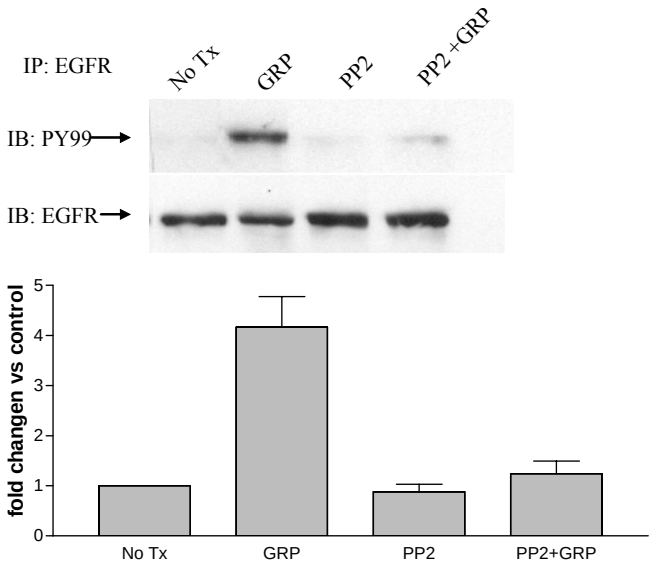
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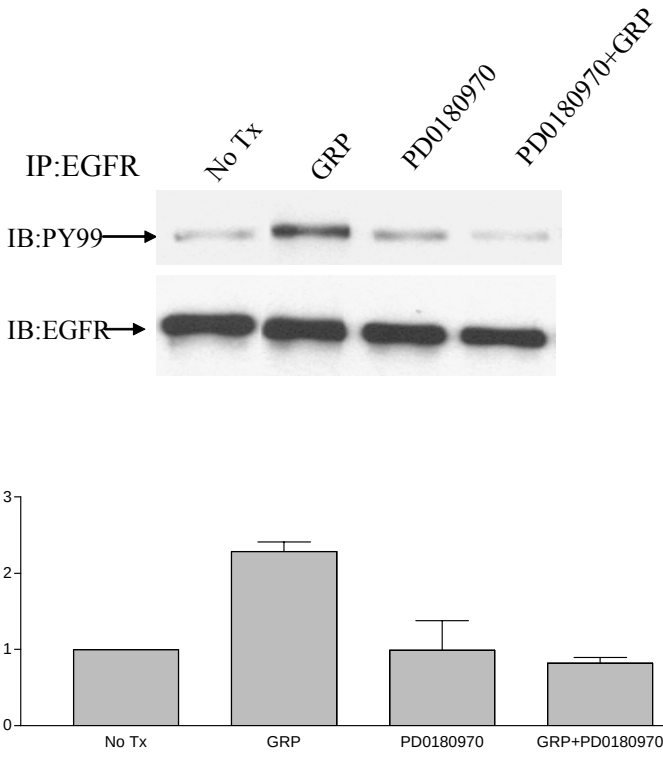
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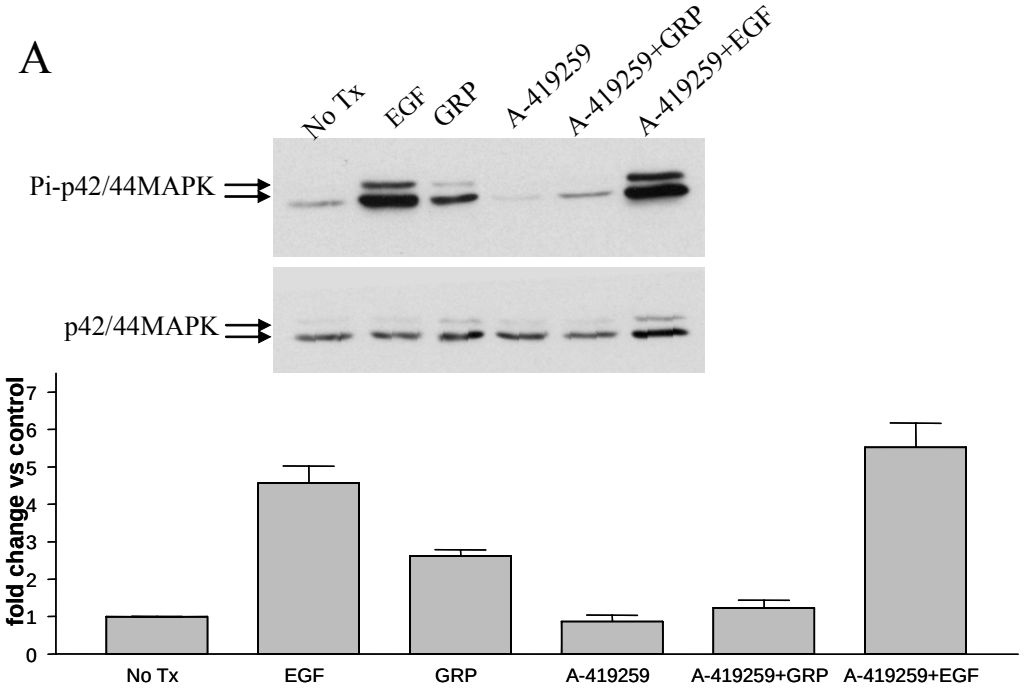
Figure 5: Src family kinases regulate EGFR activation by GRP.

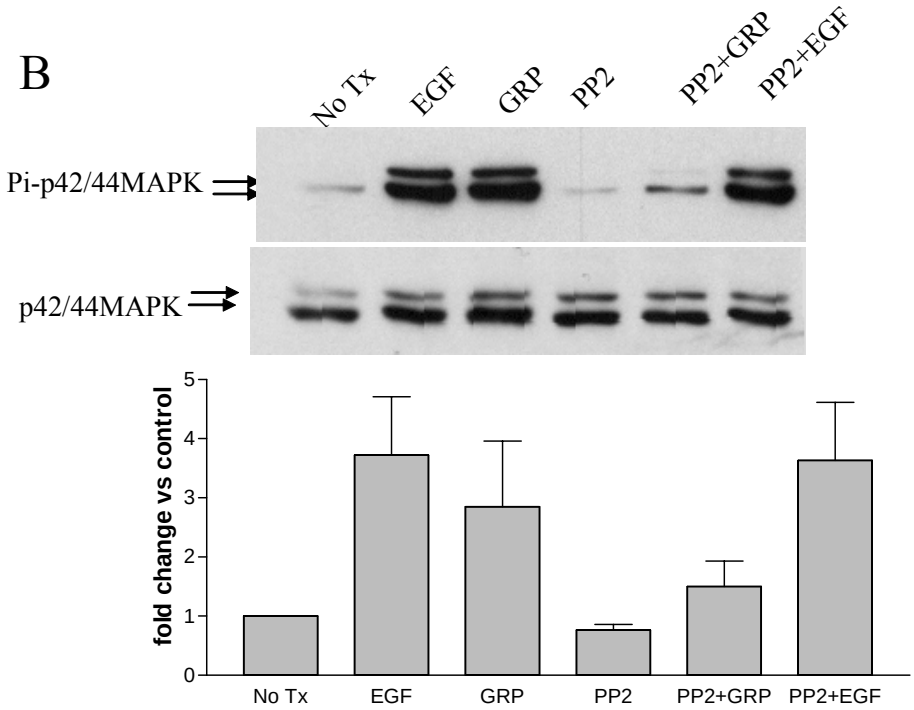
After 72 hours of serum starvation, representative HNSCC cells (1483) were pretreated with (A) A-419259 (100 nM), or (B) PP2 (5 μM), or (C) PD0180970 (500 nM) for 2 hours, followed by GRP (400 nM) treatment for 10 min. Cell lysates were collected and followed by EGFR phosphorylation determinations by immunoprecipitation with anti-EGFR antisera and immunoblotting with antiphosphotyrosine antibody (PY99). Total EGFR levels were determined by stripping the same membrane and probing for EGFR. In panel A, one tail Wilcoxon test was performed to test the significant differences. Cumulative results for each Src inhibitor are shown from 4 (A, p=0.014), 2 (B) or 3 (C) independent experiments.

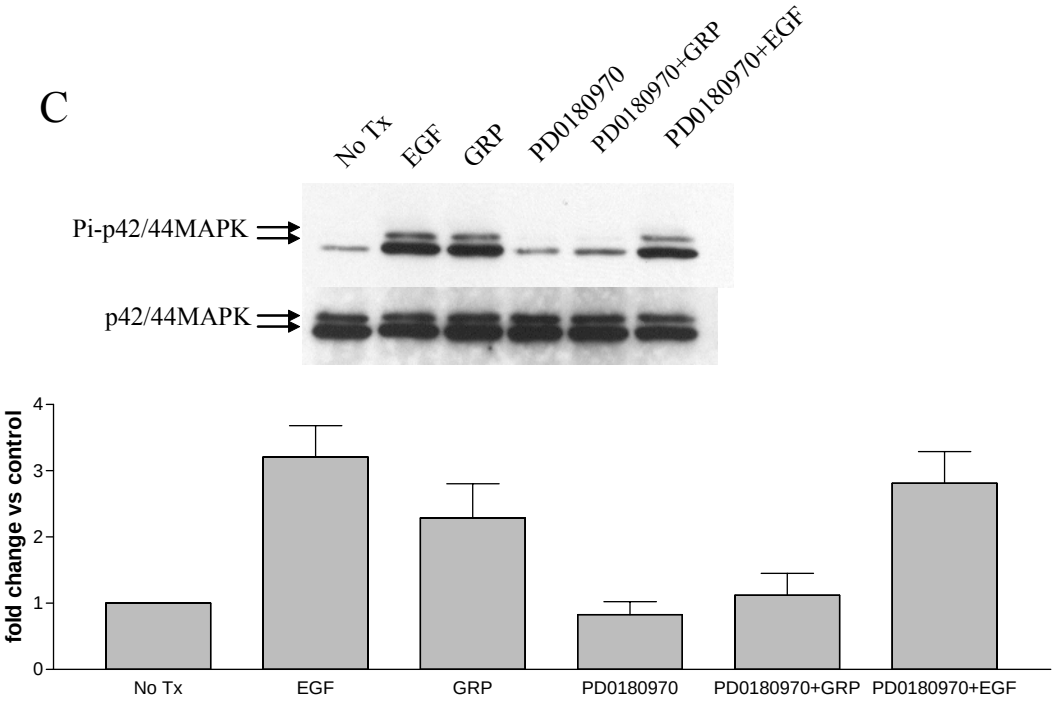
2.3.4. Src family kinases mediate GRP induced MAPK activation in HNSCC cells

We previously reported that MAPK activation by GRP occurs via EGFR phosphorylation in HNSCC (87). We also demonstrated that blockade of EGFR activity decreased MAPK activation by GRP (87). To investigate the role of Src family kinases in GRP-mediated MAPK activation, HNSCC cells (1483) were treated with each of the three different Src family kinase inhibitors described above. As shown in Figure 6, blockade of Src family kinases with A-419259 (A, p=0.0011), PP2 (B) or PD0180970 (C, p=0.014) inhibited MAPK activation by GRP. In contrast, activation of MAPK by EGF was not abrogated by Src family kinase blockade. Similar results were obtained in another HNSCC cell line PCI-37a (data not shown). These results indicate that Src family kinases mediate MAPK activation by GRP, but not by direct activation of EGFR by EGFR ligand. Further investigation using dominant-negative c-Src transfected HNSCC cells [previously shown to demonstrate reduced c-Src activation (92)] showed that GRP was able to stimulate MAPK activation in vector-transfected control cells but not in dominant-

negative c-Src-transfected cells (Figure 6D). MAPK activation by EGF was intact in dominant-negative c-Src-transfected cells, indicating that MAPK activation by GRP, but not EGF, requires c-Src activity.



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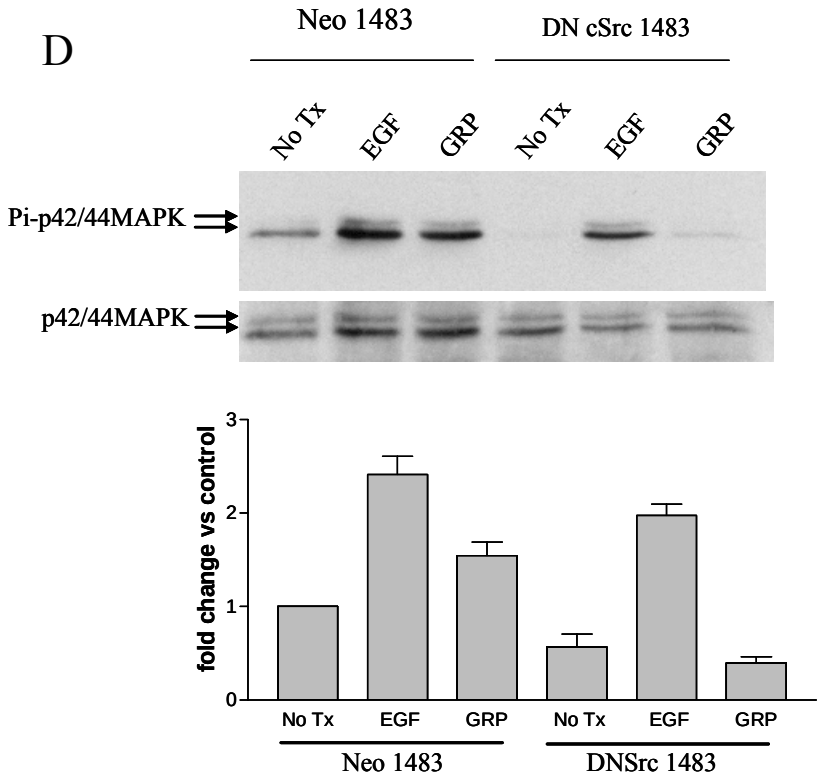
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DNSrc 1483

Figure 6: Src family kinases regulate MAPK activation by GRP.

HNSCC cells (1483) were pretreated (A) A-419259 (100 nM), or (B) PP2 (5 μM), or (C) PD0180970 (500 nM) for 2 hours, followed by GRP (400 nM) or EGF (10 ng/ml) treatment for 10 min. Cell lysates were followed by immunoblot analysis for phosphorylated MAPK and total MAPK. Cumulative results for each Src inhibitor are shown from 6 (Figure 6A, p=0.0011), 3 (Figure 6B) or 4 (Figure 6C, p=0.014) independent experiments. (D) Dominant-negative c-Src transfected HNSCC cells (1483) or vector-transfected control cells were serum starved for 72 hours followed by GRP (400 nM) or EGF (10 ng/ml) treatment for 10 min. Cell lysates were prepared followed by immunoblot analysis for phosphorylated MAPK and total MAPK. Cumulative results are shown from 2 independent experiments.

2.3.5. Amphiregulin, but not EGF, is cleaved by GRP stimulation in HNSCC cells

Previous reports have demonstrated that activation of EGFR by GPCRs can involve both intracellular and extracellular pathways (30, 31). We previously reported that TGF-α, but not HB-EGF, was implicated in the activation of EGFR by GRP in HNSCC (87) . In order to determine whether other EGFR ligands were involved in the activation of EGFR by GRP, ELISA assays were performed to examine Amphiregulin and EGF release. As shown in Figure 7A, GRP induced Amphiregulin secretion into HNSCC cell culture medium. Pretreatment of HNSCC cells with the MMP inhibitor Marimastat abrogated Amphiregulin release. In contrast, GRP treatment did not induce EGF release into HNSCC cell culture medium (Figure 7B,

p=0.343). These results indicate that in addition to TGF-α, Amphiregulin is cleaved by GRP stimulation in HNSCC cells. To confirm that Amphiregulin binding to EGFR mediated GRP-induce EGFR activation in HNSCC, HNSCC cells were treated with an Amphiregulin neutralizing antibody or an EGFR blocking antibody (C225) followed by GRP treatment. As shown in Figure 8, GRP-mediated EGFR phosphorylation and MAPK activation was abrogated by blockade of Amphiregulin or EGFR ligand binding.

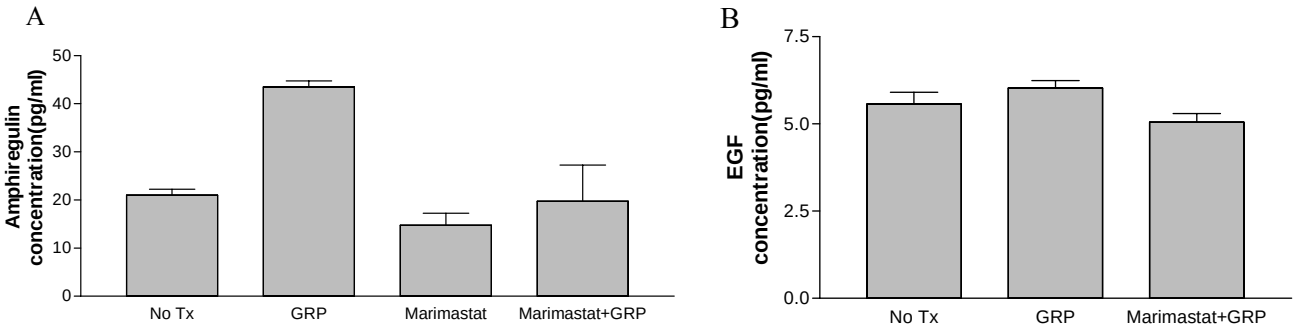


Figure 7: Amphiregulin, but not EGF, is cleaved by GRP stimulation.

Representative HNSCC cells (1483) were serum starved for 3 days followed by treatment with a metalloproteinase inhibitor (A) Marimastat (20 μM) for 2 hours. Cells were then stimulated with GRP (400 nM) for 10 min. Cell culture media were collected and cell debris was discarded using centrifugation at 1800 RPM for 10 min. An Amphiregulin ELISA was preformed on cell culture media according to the manufacturer's instructions. Cumulative results are shown from 2 independent experiments. (B) Representative HNSCC cells were serum starved for 3 days followed by Marimastat (20 μM) treatment for 2 hours. After that, cells were stimulated with GRP (400 nM) for 10 min. Supernatants were collected and cell debris was discarded using centrifugation at 1800 RPM for 10 min. An EGF ELISA was performed on cell

culture media according to the manufacturer's instructions. Cumulative results are shown from 4 independent experiments (p=0.343).

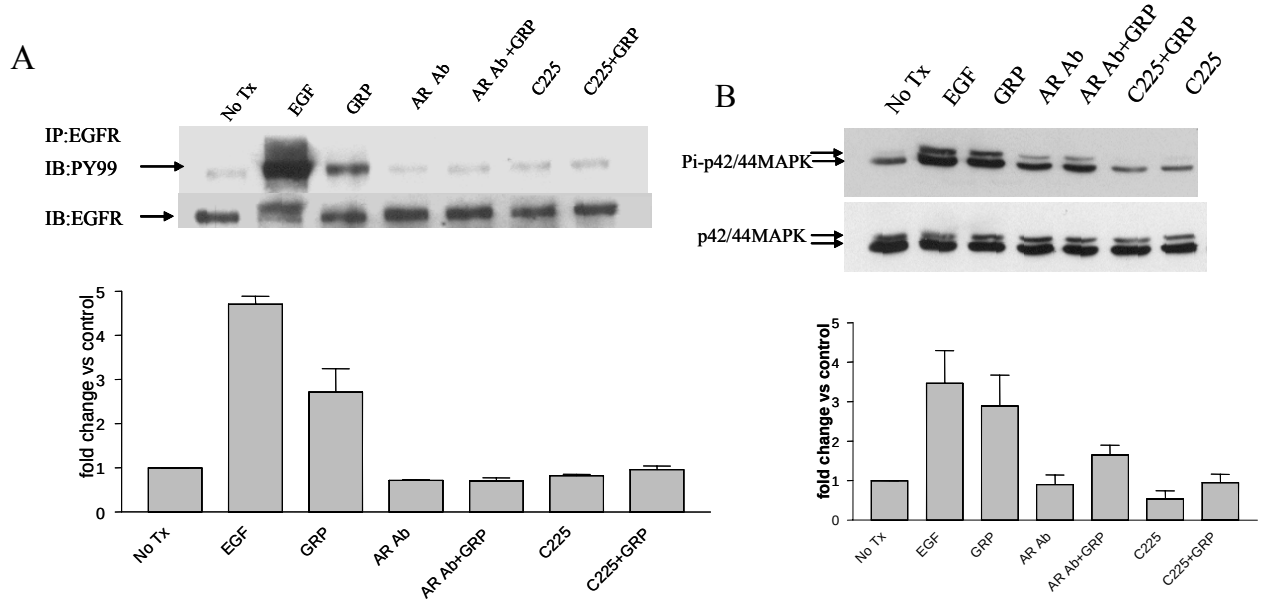
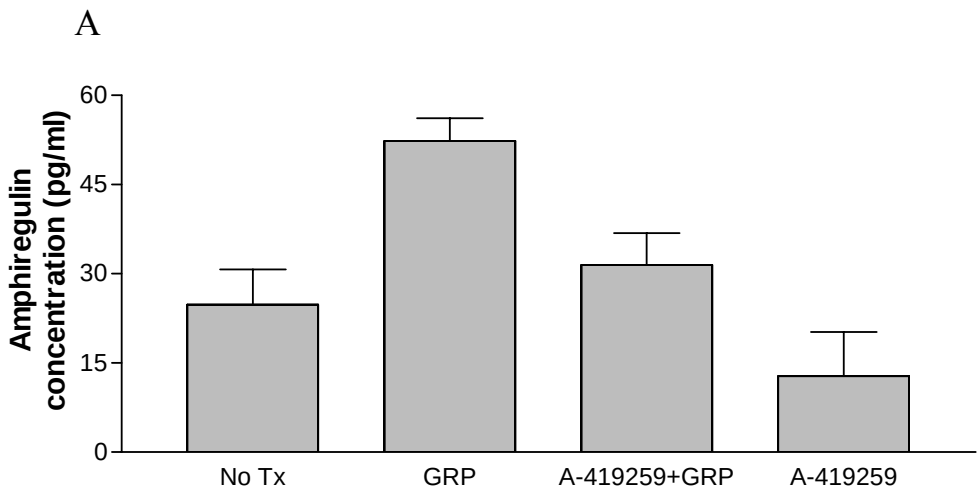


Figure 8: Amphiregulin release contributes to GRP-mediated EGFR and MAPK activation in HNSCC.

Representative HNSCC cells (1483) were serum starved for 72 hours and then treated with GRP (400 nM) for 10 min following 2 hours pretreatment with an AR antibody (15 μg /ml) or an EGFR blocking antibody C225 (6 μg/ml). EGF was used as a positive control. EGFR phosphorylation was determined by immunoprecipitation with anti-EGFR antisera followed by immunoblotting with antiphosphotyrosine antibody (PY99). Total MAPK as well as phosphorylated MAPK levels were determined by Western blotting. Cumulative results are shown from (Figure 8A) 2 or (Figure 8B) 4 independent experiments respectively.

2.3.6. Src family kinases mediate GRP induced EGFR ligand release into HNSCC cell culture medium

Our cumulative results suggest that Amphiregulin and TGF-α are the two specific EGFR pro-ligands cleaved following GRP treatment in HNSCC cells. In addition, Src family kinases mediate EGFR and MAPK activation by GRP. We therefore hypothesized that Src family kinases contribute to EGFR and MAPK activation by GRP through EGFR pro-ligand cleavage. In order to test this hypothesis, we treated HNSCC cells with the Src inhibitor A-419259 followed by GRP treatment and TGF-α or Amphiregulin determinations in cell culture medium. As shown in Figure 9, secretion of Amphiregulin or TGF-α following GRP treatment was abrogated by blockade of Src family kinases (Figure 9A, p=0.0143; Figure 9B, p=0.0011). These results indicate a novel role for Src family kinases in mediating EGFR proligand cleavage following treatment with a GPCR ligand.



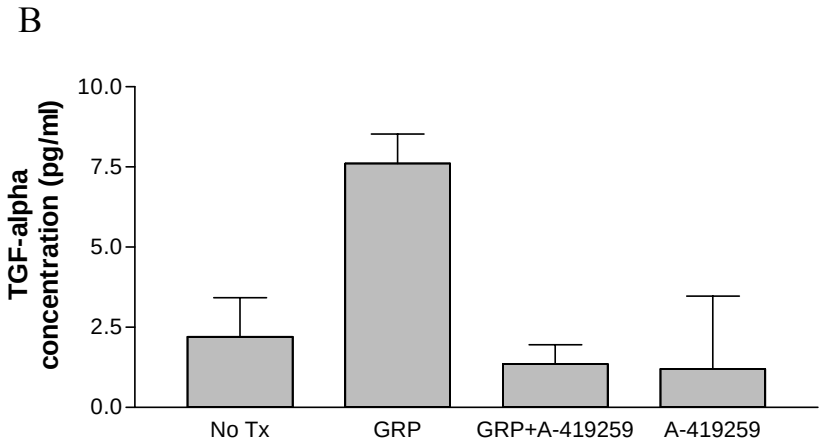


Figure 9: Src family kinases regulate GRP-induced TGF-α and amphiregulin release into cell line supernatants.

Representative HNSCC cells (1483) were serum starved for 3 days followed treatment with the Src-selective inhibitor A-419259 for 2 hours. Vector-transfected and dominant-negative c-Src-transfected HNSCC cells were plated and serum starved for 2 days followed by treatment with GRP (400 nM) for 10 min. Supernatants were collected and cell debris was discarded using centrifugation at 1800 RPM for 10 min. (A) Amphiregulin or (B) TGF-α ELISA assays were performed using supernatants according to the manufacturer's instructions. Cumulative results are shown from (A, p=0.014) 4 or (B, p=0.0011) 6 independent experiments.

2.3.7. HNSCC cell proliferation and invasion by GRP is dependent on c-Src activity

We previously reported that GRP induces HNSCC cell proliferation in a dose-dependent manner (76). Further investigation demonstrated that the GRP-mediated mitogenic effects in HNSCC cells occurred via a MEK/MAPK signaling pathway (87). In order to elucidate the role of c-Src activity on the proliferation of HNSCC cells by GRP, we treated dominant-negative c-Src-transfected HNSCC cells with GRP followed by cell count determinations using vital dye exclusion. As shown in Figure 10A, GRP induced proliferation of vector-transfected control

HNSCC cells (p=0.014), whereas no mitogenic effects were observed in dominant-negative c-Src-transfected cells. In order to confirm that cells expressing mutant c-Src were capable of proliferating, these cells were treated with 5% serum followed by cell count determinations (Figure 10A). These results suggested that c-Src activity is required for GRP-induced HNSCC cell proliferation.

Previous studies reported that the GPCR ligand LPA induced tumor cell migration and invasion (32, 98). We examined whether GRP treatment resulted in increased HNSCC cell invasion. In addition, we determined whether HNSCC cell invasion by GRP is mediated by c-Src activity. As shown in Figure 10B, GRP induced head and neck cancer cell invasion in vector-transfected control cells, an effect that was abrogated in dominant-negative c-Src transfected cells. In order to determine whether dominant-negative c-Src transfected cells could invade, these cells were treated with 5% serum followed by invasion determinations by Matrigel assay (99). These results suggest that c-Src activity is necessary for GRP-induced HNSCC cell invasion.

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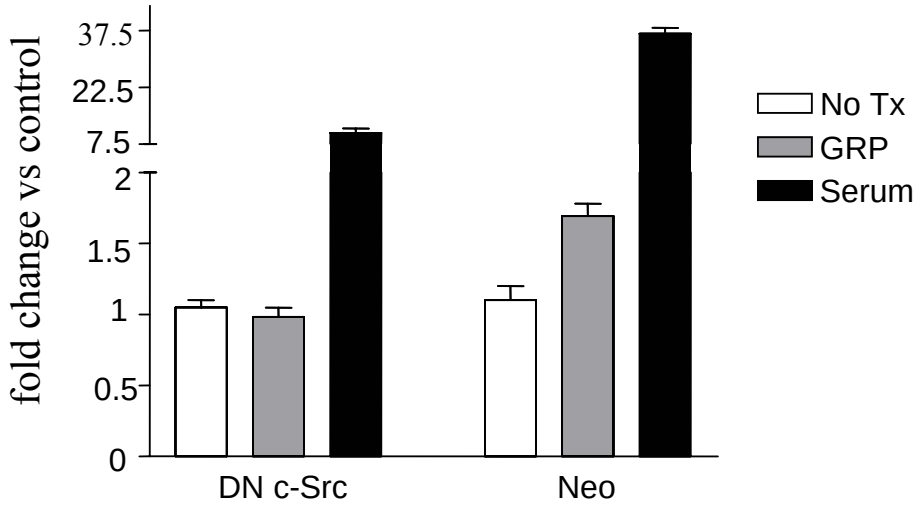


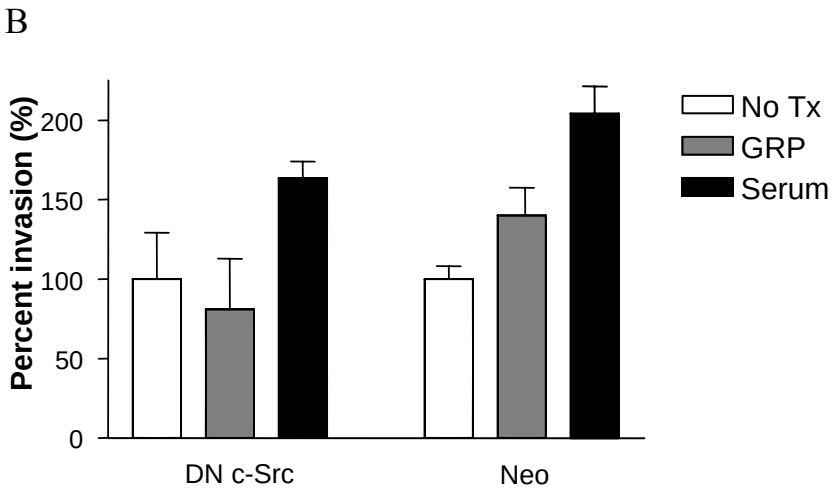


Figure 10: GRP-induced cell growth and invasion are dependent on c-Src activity.

(A) Dominant-negative c-Src transfected HNSCC cells (1483) or vector-transfected control cells were plated at a density of 2.5 x10^4 cells in a 24-well plate. After 3 days of serum starvation, GRP (400 nM) or 5% serum were added to the cells. After 24 hours, the percentage change in cell growth (compared to no treatment) was determined by cell counting via vital dye exclusion. Cumulative results are shown from 4 independent experiments ($p=0.014$). (B) Cells were plated at a density of 2×10^4 cells/well in DMEM with or without GRP (400 nM)/ 5% serum in the insert. The lower well contained 10% FBS with or without 400 nM GRP. After 48 h of treatment at 37°C in a 5% CO2 incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientifics, USA) according to the manufacturer's instructions. Invading cells in 4 representative fields were counted using light microscopy at 400X magnification. Cumulative results are shown from 2 independent experiments ($p<0.05$).

2.4. Discussion

Studies to date have shown that EGFR activation by GPCRs represents a paradigm of potential crosstalk between tyrosine kinase receptors and GPCRs (100). Although the biological significance of the initial studies performed in fibroblasts was undetermined, subsequent investigations in cancer cells have shown that activation of EGFR by GPCR ligands leads to downstream MAPK activation, tumor cell invasion and DNA synthesis (32, 101). We previously reported that HNSCC cell lines and tissues express increased levels of GRP and GRPR where the levels of GRPR in the primary tumor were correlated with patient survival (76). Further investigation demonstrated that blockade of GRP using the neutralizing antibody 2A11 inhibited HNSCC growth *in vitro* and *in vivo*, thus implicating an autocrine regulatory pathway involving this GPCR ligand and receptor in HNSCC (76). The importance of EGFR upregulation in HNSCC carcinogenesis has been well documented (5, 82, 83, 102). Further investigation demonstrated that treatment of HNSCC cells with GRP led to rapid phosphorylation of EGFR and MAPK activation with resultant cell proliferation (103). Others have reported that treatment of HNSCC cells with LPA, bradykinin, carbachol or thrombin induced rapid EGFR phosphorylation and MAPK activation with resulting cell invasion, thus suggesting that a variety of GPCR ligands may lead to EGFR transactivation and regulate behaviors critical to tumor progression (32, 101). These cumulative results implicate EGFR as a therapeutic target and support the potential significance of this growth factor receptor as an integration point for convergent signaling pathways in HNSCC cells. While a variety of potential intracellular and extracellular pathways have been described in the context of GPCR-mediated EGFR activation, studies to date suggest that the specific mechanisms are likely cell type-specific. The results of the present study underscore the importance of cleavage of the EGFR ligands, TGF-α and

Amphiregulin in this process in HNSCC cells and demonstrate a novel role for Src family kinases.

Src family kinases have been previously implicated in the crosstalk between EGFR and GPCRs (33, 89, 104). Activation of Src family kinases, including Fyn, c-Yes and c-Src, have been reported to be an early event following thrombin treatment in lung fibroblast CCL39 cells (105). A variety of GPCR ligands, including bombesin, bradykinin and vasopressin, can rapidly activate Src family kinases in Swiss 3T3 cells (106). In addition to fibroblasts, c-Src has been shown to be activated by Prostaglandin E2 (PGE-2) treatment in colon cancer cells in conjunction with EGFR activation (31). Since GRP is the "mammalian counterpart" of bombesin (103), our findings of increased phosphorylation of c-Src by GRP in HNSCC cells are consistent with these earlier studies. In all cell lines examined, activation of c-Src by GRP was more pronounced than the other Src family kinases, and was higher following treatment with this GPCR ligand compared with EGF. Interestingly, our preliminary experiments also suggest that GRP can modestly upregulate Lyn and Fyn activation. Furthermore, experiments in murine EGFR knockout cells demonstrated that EGFR was required for maximum induction of Src kinase activation by GRP. These results suggest that activation of Src family kinases by GRP is upstream of EGFR. Activation of Src family kinases has been implicated either upstream or downstream of EGFR phosphorylation, depending on the cellular context (39, 92, 104). In contrast, EGFR phosphorylation is required for EGFR ligand induced activation of Src family kinases (92).

Previous studies have demonstrated that Src family kinases can be activated by GPCRs (104, 105). However, the specific Src family kinase(s) involved in EGFR and MAPK activation by GPCRs in human cancer cells remain incompletely understood. To elucidate the role of

specific Src family kinases in EGFR phosphorylation and MAPK activation by GRP, we used several approaches including 3 different pharmacological inhibitors and generation of HNSCC cells expressing mutant c-Src. Compared with vector-transfected control cells, dominant-negative c-Src-transfected cells were found to specifically express lower protein levels of c-Src, but not Fyn, Lyn or c-Yes (92). Dominant-negative c-Src-transfected cells were also growth inhibited compared to vector-transfected controls (92). In the present study, GRP stimulated MAPK activation in vector-transfected control HNSCC cells but not in dominant-negative c-Src-transfected HNSCC cells, indicating that c-Src may play a critical role in MAPK activation by GRP. In contrast, we found that MAPK activation by EGF was not affected by decreased c-Src activity in HNSCC cells, suggesting that c-Src modulates activation of MAPK by GRP, but not EGF. In addition to MAPK activation, c-Src was found to also play a critical role in GRP-mediated HNSCC proliferation and invasion, indicating the importance of c-Src in GPCR-mediated EGFR activation and HNSCC progression.

EGFR ligand cleavage by the MMP and a distintegrin and metalloproteinase (ADAM) families of metalloproteinases from the plasma membrane has been shown to be involved in EGFR activation by GPCRs (33, 54, 107). However, the precise EGFR ligand(s) and cleavage enzymes that participate in this process appear to depend upon the specific different biological system being investigated (54). Evidence to date has indicated the involvement of several EGFR ligands including Amphiregulin, HB-EGF and TGF-α (30-33, 86, 103). However, there are no reports demonstrating that more than one EGFR ligand may be participating in EGFR activation by a single GPCR ligand in a specific tumor system. We previously reported that TGF-α, but not HB-EGF, is involved in EGFR activation by GRP in HNSCC (103). In the present study, we further demonstrate that Amphiregulin release into HNSCC cell line supernatants is also induced

by GRP. In fact, the concentration of Amphiregulin in the cell culture media following GRP treatment is 8-10 fold higher compared with TGF-α levels. While TGF-α has been extensively studied as an autocrine growth factor in HNSCC, there are few reports on the role of Amphiregulin in this tumor system. Our findings suggest that EGFR activation by GPCRs may involve cleavage of more than one EGFR proligand and its subsequent release from the plasma membrane.

Several studies have attempted to identify the specific cleavage enzyme involved in GPCR-mediated release of EGFR ligand (30, 33, 54). MMP-2 and MMP-9 have been shown to be involved in the release of HB-EGF and subsequent EGFR activation induced by Estradiol (E2) in breast cancer cells (33). Phorbol-12-Myristate-13-Acetate (TPA)-induced activation of ADAM9 was reported in HB-EGF shedding in Vero cells (50). A recent report demonstrated that TNF-alpha converting enzyme (TACE)/ADAM17 is involved in cleavage of proamphiregulin and downstream EGFR and MAPK activation in HNSCC cells (32). Zymography results demonstrated that MMP-2 and MMP-9 are not involved in the release of EGFR ligands induced by GRP in HNSCC cells (data not shown). Further studies are required to identify the precise cleavage enzyme involved in the GRP-induced EGFR ligand release.

The role of Src family kinases in mediating metalloproteinase activity and shedding of EGFR ligands has not previously been reported. We demonstrate here that several Src family kinases can be activated by GRP, with subsequent phosphorylation of EGFR and downstream MAPK activation. Emerging evidence suggests that Src family kinases may mediate metalloproteinase activity (33). Estradiol has been shown to induce MMP-2 and MMP-9 activity in MCF-7 cells, where the effect was attenuated by the Src family kinase inhibitor PP2 or a dominant negative c-Src construct (33). Although MMP-2 and MMP-9 do not appear to be

involved in GRP mediated EGFR ligand cleavage in HNSCC cells, TACE/ADAM17 has been shown to mediate cleavage of Amphiregulin by several GPCR ligands. A physical association between ADAM and Src family proteins has been previously reported (51, 53). In addition, it has been shown that Src family kinases induced ADAM-mediated release of L1 adhesion molecule from human tumor cells (108). These cumulative findings demonstrate a novel role for Src family kinases in mediating the release of EGFR ligands induced by GRP and contributing to HNSCC progression, possibly with the involvement of ADAM/MMP family members.

3. GRP INDUCED TACE PHOSPHORYLATION BY PDK1: A NOVEL MECHANISM FOR AMPHIREGULIN RELEASE AND EGFR ACTIVATION

3.1. Introduction

A variety of cancers are characterized by overexpression of EGFR leading to the development of therapeutic strategies that block this growth factor receptor. In a population of non-small cell lung cancer (NSCLC) patients with activating mutations in the EGFR, the responses to EGFR targeting therapy has been quite striking (26, 27). However, the frequency of response in patients without activating EGFR mutations in NSCLC and other cancers has been limited but not absent (23, 84, 85). The basis for these modest clinical responses in most patients, despite robust activity observed in preclinical models, is not completely understood. In addition to direct activation of EGFR by ligands, EGFR can be transactivated by GPCR in multiple cell types including fibroblasts, smooth muscle cells, neurons and tumor cells (29, 30, 39, 109). Upon EGFR activation, tumor cells demonstrate increased cell proliferation, invasion, decreased cell death and chemotherapy resistance (110). However, the molecular mechanism of GPCR ligand-induced EGFR activation appears to be both cell type and GPCR ligand specific. In Cos-7 cells, gonadotrophin-releasing hormone has been reported to activate EGFR through a cytoplasmic and EGFR ligand independent mechanism (111). Alternatively, extracellular EGFR ligand release (e.g. HB-EGF, TGF-α, amphiregulin) has been shown to be essential for EGFR activation by a variety of different GPCR ligands, including carbachol, lysophosphatidic acid (LPA), thrombin or bombesin in fibroblasts, colon, breast and head and neck cancer cells (30, 32, 86, 112).

The mechanisms underlying GPCR-induced EGFR signaling involves both intracellular and extracellular pathways (54). EGFR activation by GPCR has been proposed to be mediated

by Src family kinases, PI-3 kinases and/or PKC signaling (54). Src family kinases have been reported to regulate GPCR ligand-induced EGFR phosphorylation in the colon cancer cell line Caco-2, gastric epithelial cells RGM1, Cos-7 cells, GT1-7 neuronal cells and head and neck cancer cells (31, 35, 89, 112). PI-3 kinase has also been implicated in linking GPCR to EGFR signaling (113-115). In addition to intracellular molecules involved in GPCR-induced EGFR activation, growing evidence suggests that transmembrane metalloproteinase mediate EGFR pro-ligand shedding in response to GPCR ligands (30, 45, 46, 116, 117). Cumulative results indicate that the metalloproteinase involved in EGFR pro-ligand cleavage is both cell type and GPCR ligand-specific. Matrix metalloproteinases (MMP-2 and 9) have been reported to mediate estrogen receptor (ER)/GPCR ligand induced EGFR ligand cleavage in breast cancer cells and gonadotropic cells respectively (33, 118), whereas a disintegrin and metalloproteinase-10 (ADAM10) was implicated in bombesin or LPA-induced HB-EGF cleavage and EGFR activation in Cos-7 cells (46). TACE/ADAM17 has been shown to mediate carbachol or LPA induced proamphiregulin release and downstream EGFR and MAPK activation in HNSCC cells (32). However, the precise intracellular signaling cascade coupling GPCR activation to metalloproteinase activation remains largely unknown. In particular, whether the intracellular Src and PI-3 kinases activation, extracellular metalloproteinases and EGFR family ligand release can be rationalized into a single signaling cascade remains largely unexplored.

We previously reported that Src family kinases regulate GRP-induced EGFR proligand cleavage, leading to downstream EGFR and MAPK activation in HNSCC (77, 112). Here we show for the first time that Src and PI-3 kinase associate with TACE following GRP treatment of HNSCC cells. This association is accompanied by phosphorylation of Src and TACE and translocation of both molecules to the cell membrane, an effect dependent on activation of the

PI-3 kinase pathway. Phosphorylation of TACE by GRP requires both Src family kinases and PI-3 kinases. Further investigation identified PDK1 kinase as the direct effector mediating GRP-induced TACE phosphorylation. Combined inhibition of PDK1 and EGFR dramatically enhanced anti-tumor effects. These results implicate PDK1 as a new therapeutic target in cancers where transactivation of EGFR by GPCR contributes to tumor progression.

3.2. Materials and Methods

3.2.1. Chemicals and reagents

Human gastrin-releasing peptide (GRP) was obtained from Sigma-Aldrich Corporation (St. Louis, MO). Human recombinant epidermal grown factor (EGF) was obtained from Oncogene Research Products (Boston, MA). Antibodies against epidermal growth factor receptor (monoclonal antibody) were obtained from the Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY). PD018970 was obtained from Pfizer Parke-Davis (Ann Arbor, MI); A-419259 was a kind gift from Abbott Bioresearch Center (Worcester, MA). Wortmannin, LY294002 and β-actin antibody were from EMD biosciences (San Diego, CA). Antibodies against p44/42 MAPK, phospho-p44/42 MAPK, p85, PDK1, Akt and phospho-Akt (Ser 473) were from Cell Signaling (Beverly, MA). c-Src and GST antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against the activation loop of Src (PY418) was purchased from Biosource international (Camarillo, CA). TACE antibody was from Chemicon International (Temecula, CA). Phospho-threonine and phosphor-serine antibody were from Zymed Biosciences (San Francisco, CA). The amphiregulin ELISA kits were purchased from R&D systems (Minneapolis, MN). PDK1 active enzyme was purchased from Upstate (Lake Placid, NY).

3.2.2. Cell culture

All HNSCC cell lines (PCI-37A ,PCI-15B) were of human origin (119-121). Cells were maintained in DMEM with 10 % heat inactivated fetal calf serum (Invitrogen, Carlsbad, CA) at 37°C with 5 % CO_2. HEK293 cells were maintained in DMEM with 10% heat inactivated fetal calf serum (Invitrogen).

3.2.3. Transfection of HNSCC cells with dominant-negative c-Src

PCI-37A and PCI-15B cells were transfected with a pUSEamp vector (Upstate Biotechnology, Inc. Lake Placid, NY) containing mutant Src [K296R/528F] cDNA using Lipofectamine (GIBCO Laboratories, Grand Island, NY) according to the manufacturer's recommendations. Stably transfected clones were selected for resistance to the neomycin analogue, G418 (800 μg/ml, Gibco BRL). Dominant-negative Src was generated by mutation of K296 to R, which rendered the kinase domain incapable of binding ATP (92).

3.2.4. Cell treatments

HNSCC cells were plated at a density of 6 x 10^5 cells/ml in 10 cm^2 plates. Twenty-four hours after plating, cells were serum-starved for 48 h in DMEM. During serum starvation the media was changed every 24 h. For the experiments with inhibitors cells were pretreated with either Src family kinase inhibitors A-419259 (100 nM), PD0180970 (500 nM) or PI-3 kinase inhibitors Wortmannin (250 nM) or LY294002 (20 μM) for 1 hour. After pretreatment where indicated, 400 nM GRP was added to the cells. At selected time point after GRP treatment, cells were washed three times with cold PBS, lysed with lysis buffer (10mM Tris HCl, pH 7.6, 50mM

$Na_4P_2O_7$, 50mM NaF, 1mM NaV_3O_4, 10 mM phenanthroline, 1% TritonX-100 and 1X protease inhibitor cocktail tablet that included a broad spectrum potent inhibitor of protein tyrosine phosphatases), scraped off the plate, and passed through a 26 and a half gauge needle 3-4 times. The lysate was then centrifuged at 4 °C, 14000 rpm for 20 mins. Supernatant was collected for protein quantitation using the Protein Assay Solution (BioRad Laboratories, Hercules, CA) and bovine serum albumin of known concentration as the standard.

3.2.5. RT-PCR

Total RNA were extracted from HNSCC cells (PCI-37A) cells by using RNA extraction kit (Qiagen, Germany). RNA was quantitated followed by RT-PCR with appropriate primer for 25 cycles. The primer sequences are as follows: ADAM10 (Forward primer: TCACATTACTTTTGCTCACGA. Reverse: TTCTACCATTCCATTTCCACA); TACE (forward primer: GCATTCTCAAGTCTCCACAAG. Reverse: CCTCATTCGGGGCACATTCTG); GAPDH (Forward: TGGAATTTGCCATGGGTG. Reverse: GTGAAGGTCGGAGTCAAC).

3.2.6. Immunoprecipitation

200 μg of total protein was incubated for 2 h at 4 °C with 3 μg of anti-TACE antibody (QED Biosciences), anti-EGFR antibody (Upstate technology) or 2μg anti-c-Src antibody (Santa Cruz Technology, Santa Cruz, CA) with gentle agitation. Forty microliters of Protein G agarose beads (Invitrogen, Carlsbad, CA) were added to the lysate and incubated overnight at 4 °C with gentle agitation. The beads were collected by centrifugation at 4 °C, 14000 rpm for 1 min, resuspended and washed with lysis buffer 3 times. The beads were resuspended in 20 μl of 2x

gel-loading buffer and boiled for 5 mins at 95 °C. the immunoprecipitated proteins were then resolved on an 8 % SDS-PAGE gel. After being transferred onto a Protran membrane, the membrane was blocked in 5% milk and blotted with the anti-phosphothreonine, anti-phosphoserine antibodies or the anti-phosphotyrosine antibody PY418 (Biosource International) in Blotto solution (0.6 % dry milk powder, 0.9 % NaCl, 0.5 % Tween 20 and 50 mM Tris, pH 7.4). The immunoblot was then developed with the Luminol Reagent (Santa Cruz Biotechnology, California). Blots were stripped in Restore Western Blot Stripping buffer (Pierce, Rockford, IL) for 15 mins at room temperature, blocked for 1 h and reprobed with TACE antibody (QED Biosciences, San Deigo, CA) , EGFR antibody (Transduction lab) or c-Src antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in blotto solution for 2 h.

3.2.7. Western blotting

15 μg of protein was resolved in an 8% SDS-PAGE gel and transferred onto a Protran membrane (Schleicher & Schuell Inc., Keene, NH) using a semi-dry transfer machine (BioRad Laboratories, Hercules, CA). After protein transfer, the membrane was blocked overnight with a blocking solution containing 5% non-fat dry milk, 0.2 % Tween 20 in 1x PBS. The membrane was incubated with the primary antibody (1:1000 phospho p44/42 MAPK or p44/42 MAPK) for 2 hrs and then washed with the Blotto solution (0.6 % dry milk powder, 0.9 % NaCl, 0.5 % Tween 20 and 50 mM Tris, pH 7.4) 3 times for 10 mins. The membrane was then incubated with the secondary antibody (Goat anti-rabbit IgG-HRP conjugate, Bio-Rad Laboratories, Hercules CA) for 1 h and washed 3 times for 10 mins. The membrane was quickly rinsed with a rinsing solution and the blot was developed with Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

3.2.8. siRNA transfection

Transfection of 21 nucleotide siRNA duplexes for targeting endogenous genes was carried out by using Lipofecatamine 2000 (Invitrogen)**.** Cells (PCI-37A and PCI-15B) were plated at a density of 1x 10^5 cells/ml in 10 cm^2 plates. Sixteen hours after plating, cells were transfected with TACE siRNA(32), p85α siRNA (M-003020-02, Dharmacon Research, Lafayette, CO), c-Src siRNA (M-003175-03, Dharmacon Research, Lafayette, CO), ADAM10 siRNA (Target sequence: AAAGACAUUAUGAAGGAUUAU) or PDK1 siRNA (Target sequence: AACUGGCAACCUCCAGAGAAU). 4 hours after transfection, cells were serum starved for 2 days followed by treatment with GRP (400 nM).

3.2.9. Matrigel invasion assay

Cells were plated in duplicate at a density of 2 x 10^4 cells/well in DMEM. The lower well contained 10% fetal bovine serum with DMSO, PD176252, Erlotinib or PD176252+ Erlotinib. After 48 hours of treatment at 37°C in a 5% CO_2 incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientifics, Hampton, NH) according to the manufacturer's instructions. Invading cells in four representative fields were counted using light microscopy at 400x magnification.

3.2.10. *In vitro* apoptosis assay

After treating HNSCC cells with PD176252, Erlotinib or PD176252+ Erlotinib, cells were detached by trypsinization, counted and pelleted (1000 r.p.m. for 5 min). Cell pellets were

washed once with PBS (pH 7.4) and resuspended in 100 µl Annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 5 µl of Annexin V-Cy3 (BioVision Research Products, 2455-D Old Middlefield Way, Mountain View, CA) was added per tube and allowed to incubate at room temperature for 15 min in the dark. Then the stained cell suspension was dropped on the slides and covered with coverslips. The membrane of apoptosis cells are stained a bright orange color when analyzed with fluorescence microscope. The ratio (percentage) of apoptotic to total cells (apoptotic plus nonapoptotic cells) was calculated for each high-power field. For each treatment, 5–10 high-power fields of view were quantitated on each section.

3.2.11. TUNEL assay

HNSCC cells were plated on coverslips in 24 well plates. After treatment with PD176252, Erlotinib or PD176252+Erlotinib, cells were fixed with formalin followed by TdT mediated dUTP nick-end labeling (TUNEL) assay according to the manufacturer's instruction. This assay is designed to specifically detect apoptotic cell DNA by catalytically incorporating fluorescein-12-dUTP at the 3'-OH DNA ends using the terminal deoxynucleotidyl transferase (TdT) enzyme.

3.2.12. Cell cytotoxicity analysis

HNSCC cells were plated at 4×10^4 cells/well in the 24 well plate. After treating cells with PD176252, Erlotinib or PD176252+ Erlotinib for 72 hours, MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to determine the cytotoxic effects of drug treatment. Percentage of cell killing was determined by comparing drug treatment to vehicle. The equation to calculate the percentage of killing is $(OD_{vehicle}-OD_{drug})/ OD_{vehicle}$ X100%.

3.2.13. Colony formation assay

60mm culture plates were covered with a layer of 0.5% agar in medium supplemented with 20% FBS in combination with PD176252, Erlotinib or PD176252+Erlotinib. Cell suspensions (500 cells per well) were prepared in 0.3% agar and poured into 60mm culture plates. The plates were incubated at 37°C in a humid atmosphere of 5% CO_2 for 2 weeks until colonies appeared. The colonies were stained with 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide (1-2 mg/mL) and counted.

3.2.14. ELISA assays

HNSCC cells were plated at a density of 6 x 10^4 cells/ml in 10 cm^2 plates. Twenty-four hours after plating, cells were transfected with TACE siRNA as indicated. After overnight incubation, cells were serum-starved for 48 h in serum-free DMEM. During serum starvation the media was changed every 24 h. After treating cells with GRP, supernatants were collected and cell subjected to centrifugation at 1300 rpm for 10 min. Pellets were discarded and the supernatant was tested for levels of amphiregulin as per the manufacturer's instructions.

3.2.15. Cloning of the human TACE cytoplasmic domain (TACEc) and its expression as GST-TACEc fusion protein

Human TACE wild type cDNA was provided by Dr. Huizhui Fan. For amplification of the cDNA fragment encoding the cytoplasmic domain of TACE, total TACE wild type cDNA was subjected to PCR by using the following specific primer (Forward: 5'CCCGGATCCCATTGTGTGGATAAG 3' and Reverse:

5'CCCGAATTCTTAGCACTCTGTTTCTTTGCTG3'). The PCR products were cut with BamHI and ECoRI enzymes (Roche Diagnostics) before in-frame ligation with T4 DNA ligase (New England Biolabs, Beverly, MA) into the *Bam*HI-*Eco*RI-restricted multiple cloning site of the pGEX-2T expression vector (Amersham Pharmacia Biotech, Freiburg, Germany). Then, the recombinant expression vector was transformed with DH5α competent cells. PCR-tested colonies were used to inoculate 50 ml LB medium containing 75 µg/ml ampicillin. After overnight culture, the starter culture was diluted with 500 ml LB medium containing 75 µg/ml ampicillin until the culture reached an OD (A_{600}) of 1.0. Then, isopropyl-1-thio-ß-D-galactopyranoside (IPTG, 0.1mM) was added to induce protein expression for 4 more hours at 37 °C. GST-TACEc was solubilized in 50 mM Tris-HCl (pH 8) containing 0.02% sodium azide. Then, GST-TACEc was eluted with glutathoine and subject to dialysis against 20 mM Tris-HCl (pH=7.5).

3.2.16. *In vitro* kinase assay

PCI-15B or PCI-37A cells were serum starved for two days followed by cell lysates accumulation. Cell lysates were subject to TACE immunoprecipitation followed by three washes with lysis buffer and two washes with the kinase buffer (50 mM Tris-HCl, pH7.5, 0.1mM EGTA, 0.1mM EDTA, 1% 2-mercaptoethanol). Cell pellets or recombinant GST proteins were incubated with 100 ng activated PDK1 (Upstate) and 10 µci [γ-^{32}P]-ATP in 20 µl kinase buffer for 10 min at 30°C followed by boiling with gel loading buffer. Proteins were resolved in 8% or 12% SDS-PAGE gel followed by transferring to the nitrocellulose membranes. TACE phosphorylation was detected by autoradiography.

3.2.17. Statistics

The group differences were tested with the exact Wilcoxon test or paired t test. For exact Wilcoxon test, the p values from multiple comparisons within the same experiment were adjusted with the Bonferroni procedure.

3.3. Results

3.3.1. Combined inhibition of GRPR and EGFR enhances antitumor effects

We previously reported that inhibition of GRP decreased HNSCC proliferation through blocking EGFR and MAPK signaling(77, 112). Blockade of EGFR has shown some promise in clinical trials in HNSCC, especially when combined with irradiation(22, 122). To determine whether targeting GRPR and EGFR pathways in combination would enhance the therapeutic effects compared with each treatment alone, we used the EGFR tyrosine kinase inhibitor Erlotinib (Tarceva, OSI-774) and the GRPR antagonist PD176252. As shown in Figures 11A and 11B, the IC50 for Erlotinib ranged from 12-13 μM, while the IC50 for PD176252 was approximately 8 μM for both cell lines tested.

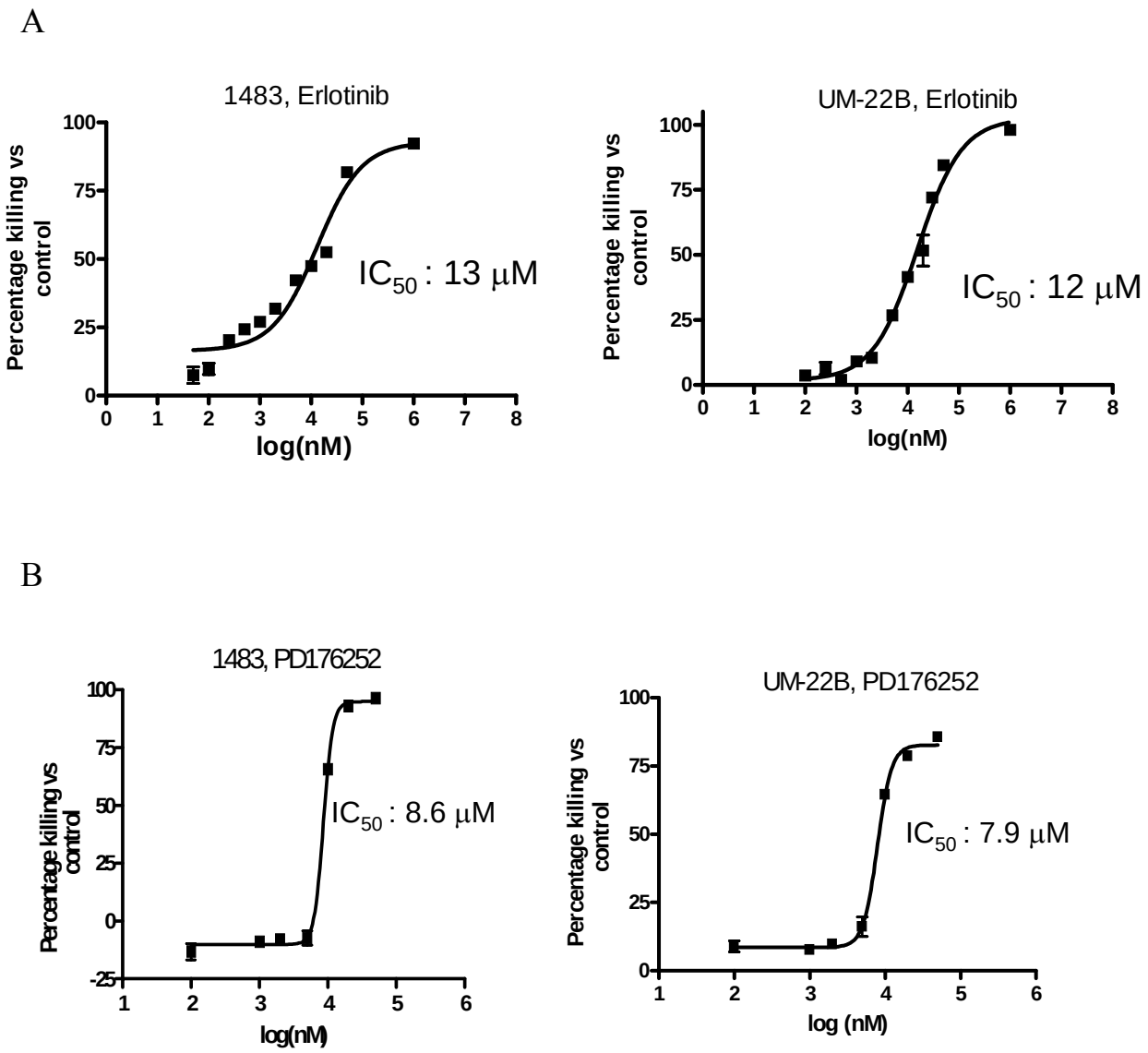
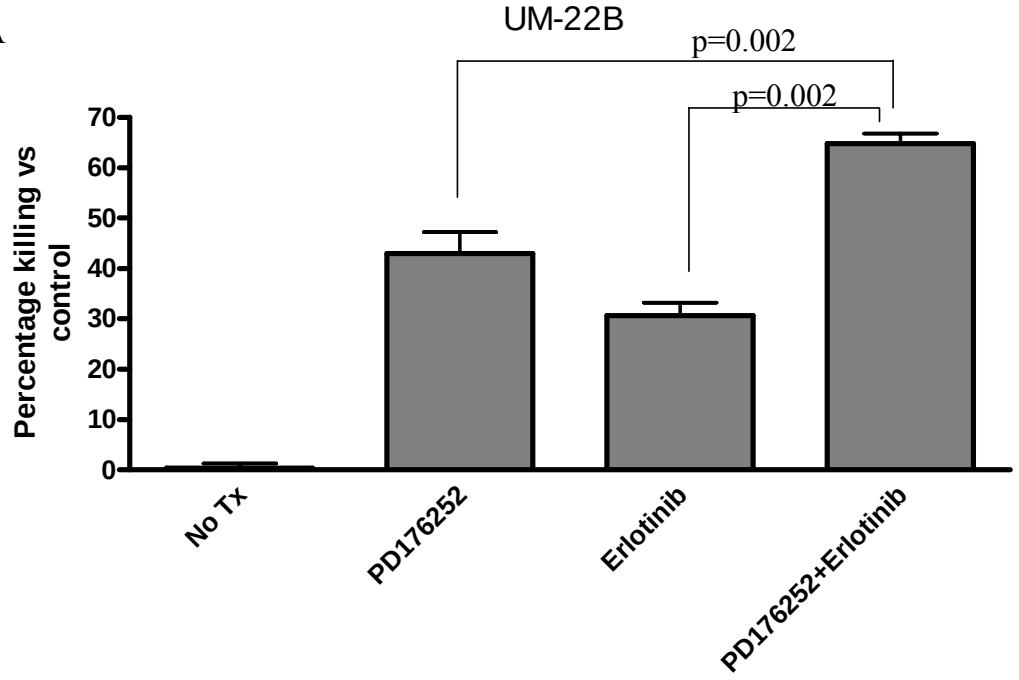


Figure 11: IC50 determination of Erlotinib and PD176252.

HNSCC cells (1483, UM-22B) were treated with (A) Erlotinib or (B) PD176252 followed by MTT assay 3 days later. The data were analyzed using Prism (GraphPad Software) to determine the IC_{50} dose of both drugs. The experiment was repeated at least 3 times with similar results.

In order to determine whether combined therapy targeting GRPR and EGFR resulted in enhanced inhibition of proliferation or invasion, half of the IC50 dose for each drug was used to treat cells followed by MTT or Matrigel invasion assay. As shown in Figures 12A and 12B, combined inhibition of both GRPR and EGFR led to enhanced growth inhibition compared with either treatment alone (p=0.002).

A



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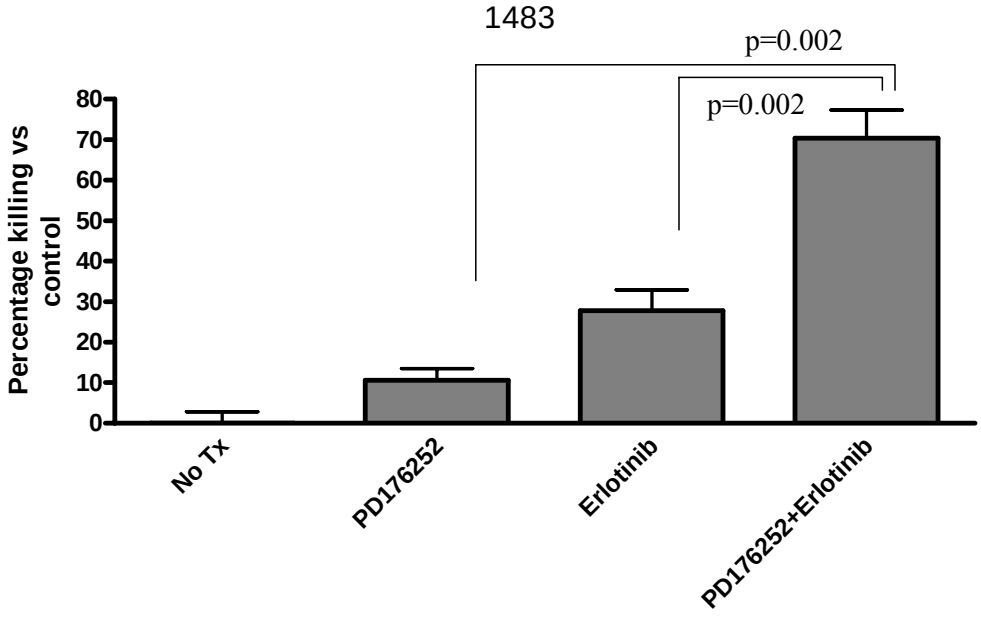
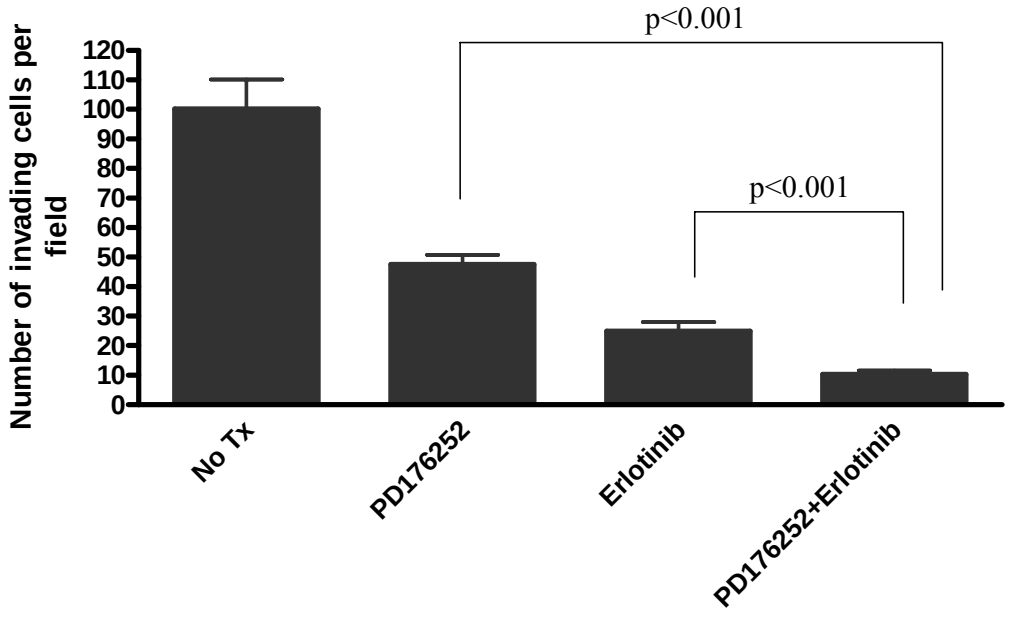


Figure 12: Combined targeting of GRPR and EGFR enhances HNSCC cell toxicicity.

UM-22B (A) or 1483 (B) cells were plated on 24 well plates followed by treatment with PD176252 (4 μM), Erlotinib (6 μM) or PD17625 (4 μM) + Erlotinib(6 μM) in triplicate. MTT assay was performed 3 days later. The percentage of cell killing was calculated according to the equation as indicated previously. Experiments were repeated 6 times. p values were determined by comparing combined treatment to PD176252 or Erlotinib treatment alone (p=0.002).

Cell invasion and colony formation was also significantly reduced by combination therapy compared to single treatment (Figure 13 A and 13B, p<0.001).

A



B

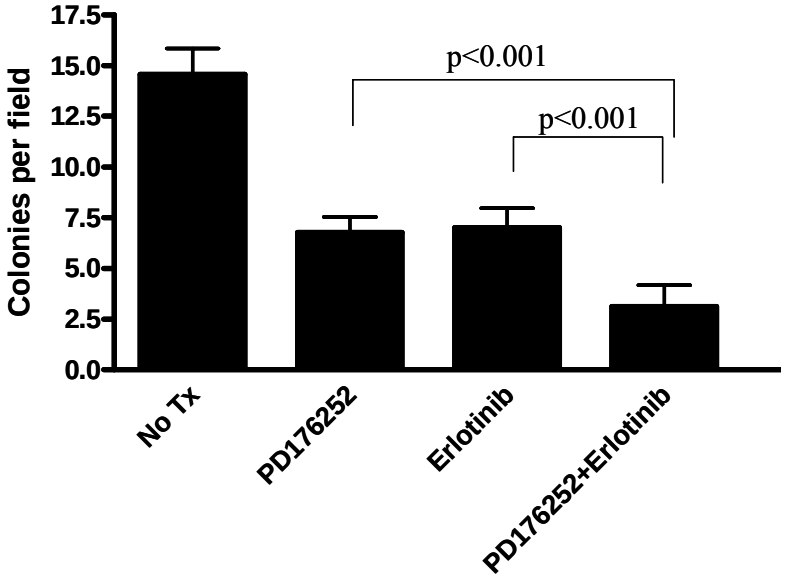


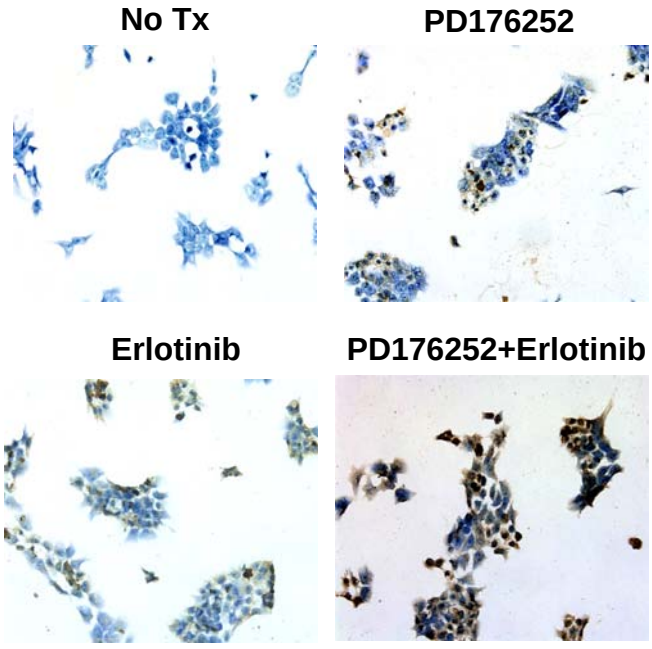
Figure 13: Combined inhibition of GRPR and EGFR decreases HNSCC cell invasion and colony formation.

(A)PCI-37A cells were plated in Matrigel invasion chamber in triplicates followed by treatment with PD176252 (4 μM), Erlotinib (6 μM) or a combination of PD176252 and Erlotinib for 48 hours. Invading cells in 4 representative fields were counted using light microscopy at x400 magnification. Mean ± SE was calculated from two independent experiments. p values were determined by comparing combined treatment to PD176252 or Erlotinib treatment alone ($p<0.001$). (B) 37A cells were plated on 60mm culture plates that were covered with a layer of 0.5% agar in medium supplemented with 20% FBS in combination with PD176252 (4 μM), Erlotinib (6 μM) or a combination of PD176252 and Erlotinib. Cell suspensions (500 cells per well) were prepared in 0.3% agar and poured into 60mm culture plates. The plates were incubated at 37°C in a humid atmosphere of 5% CO2 for 2 weeks until colonies appeared. The

colonies were stained with 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazolium bromide (1-2 mg/mL). 10 different fields were counted by the light microscopy for each treatment ($p<0.001$).

To study whether enhanced cell growth inhibition resulted from increased apoptosis, by using TUNEL (Figure 14A) or annexin V assay (Figure 14B), the apoptotic cell population increased significantly with combined targeting of GRPR and EGFR (Figure 14B, $p=0.008$).

A



B

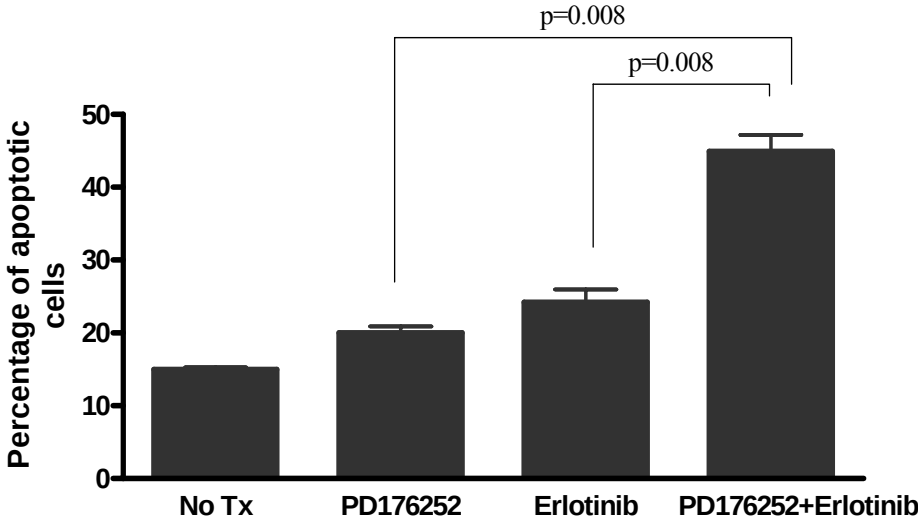


Figure 14: Combined inhibition of GRPR and EGFR increases HNSCC cell apoptosis. 1483 cells were treated with PD176252 (4 μM), Erlotinib (6 μM) or a combination of PD176252 and Erlotinib for 24 hours followed by TUNEL (A) or Annexin-V assay (B) . p values were determined by comparing PD176252+Erlotinib treatment to single drug treatment (B, p=0.008).

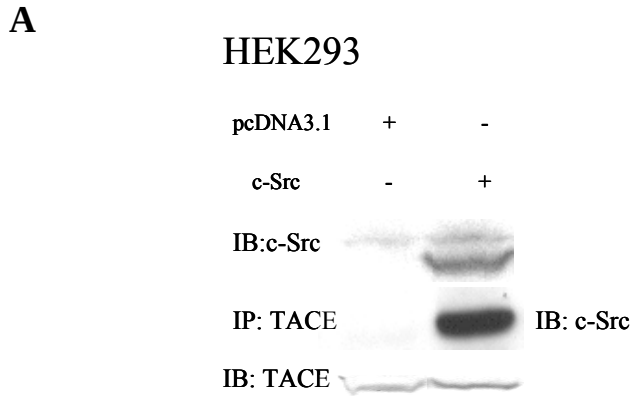
3.3.2. GRP induces the association between TACE and c-Src

The enhanced antitumor effects of combined GRPR and EGFR targeting suggest that the crosstalk between these signaling pathways presents a potential target for therapy. We have previously shown that Src family kinases contribute to GRP-induced EGFR and MAPK activation by facilitating the release of tethered EGFR ligands in HNSCC (112). However, the signaling pathways and the proteases involved in Src-mediated ligand cleavage have not been fully elucidated. EGFR ligand cleavage in response to activation of GPCR can be mediated by members of the metalloproteinase family (30, 45, 116). Indeed, TACE has been shown to contribute to LPA-induced EGFR activation in HNSCC (32, 45). We therefore examined whether Src family kinases contribute to EGFR ligand cleavage by physical association with TACE.

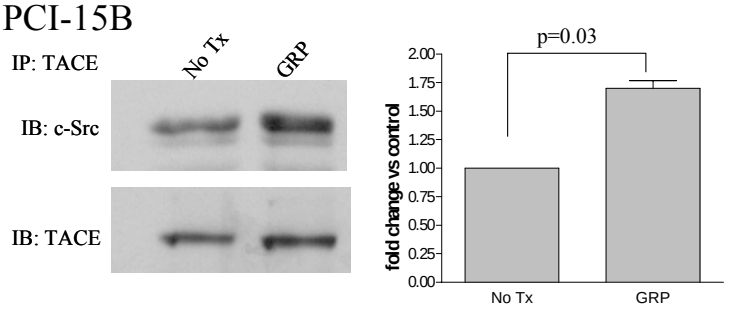
In order to test whether TACE and c-Src associate either constitutively or following GPCR activation, we transfected HEK293 cells with a wild type c-Src expression plasmid followed by co-immunoprecipitation. As shown in Figure 15A, TACE and c-Src association increases upon c-Src transfection. To investigate the effect of GRP on the association between

endogenous TACE and c-Src in a biologically relevant model, HNSCC cells (PCI-37A and PCI-15B) were treated with GRP followed by determinations of TACE and c-Src association. In both HNSCC cell lines, GRP increases the association between TACE and c-Src (Figure 15B, p=0.03 and Figure 15C, p=0.004).

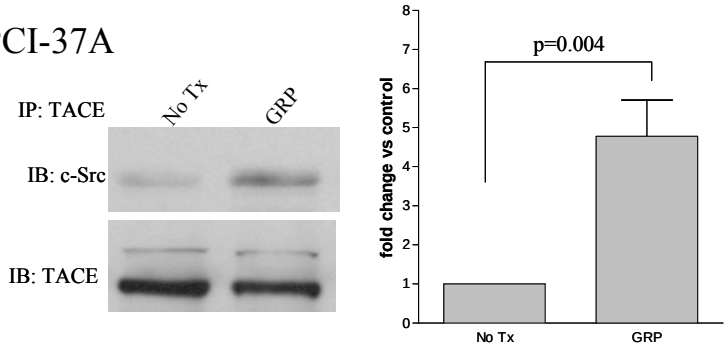
12-O-tetradecanoylphorbol-13-acetate (TPA) has been reported to induce the redistribution of TACE from the perinuclear region to the cell membrane, where it colocalizes with Rac (123). As a result of relocalization, TACE contributes to CD44 cleavage. We next examined whether GRP could induce TACE and c-Src association by intracellular translocation of these proteins using confocal microscopy. In the absence of GRP, TACE and Src are present mainly in the cytoplasm and exhibited a punctuate staining pattern. Based on the merged signals demonstrating overlap, there may be a degree of interaction of TACE and Src in resting cells. Short-term treatment with GRP induces a coordinate redistribution of both Src and TACE to the cell periphery (Figure 15D). These results suggest that GRP induces association of TACE and c-Src as well as translocation to the cell membrane where TACE can cleave EGFR proligands.



B


PCI-15B
IP: TACE
No Tx
GRP
IB: c-Src
IB: TACE
p=0.03
fold change vs control
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
No Tx
GRP

C


PCI-37A
IP: TACE
No Tx
GRP
IB: c-Src
IB: TACE
p=0.004
fold change vs control
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7
6
5
4
3
2
1
0
No Tx
GRP

D

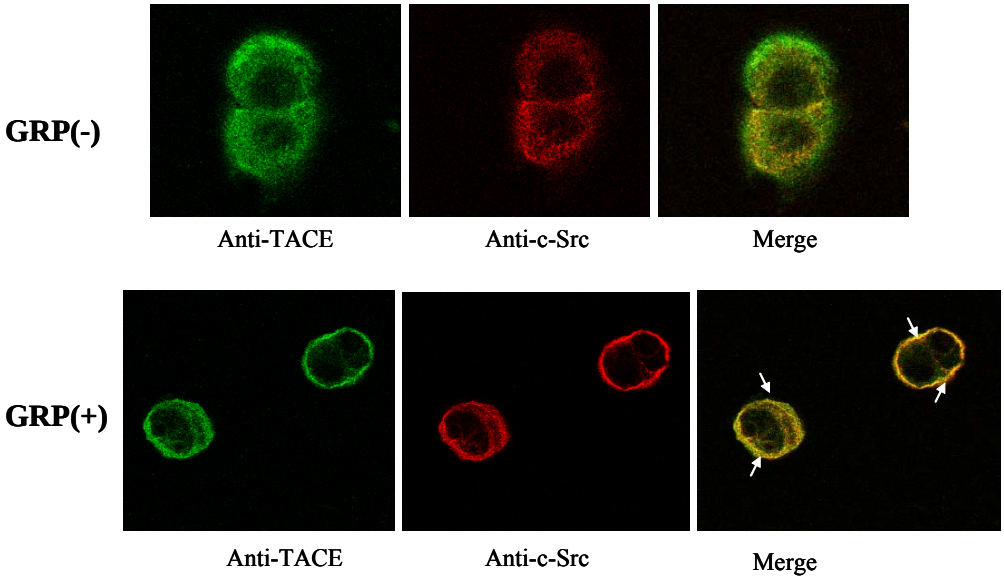

GRP(-)
Anti-TACE
Anti-c-Src
Merge
GRP(+)
Anti-TACE
Anti-c-Src
Merge

Figure 15: GRP induces TACE and c-Src association.

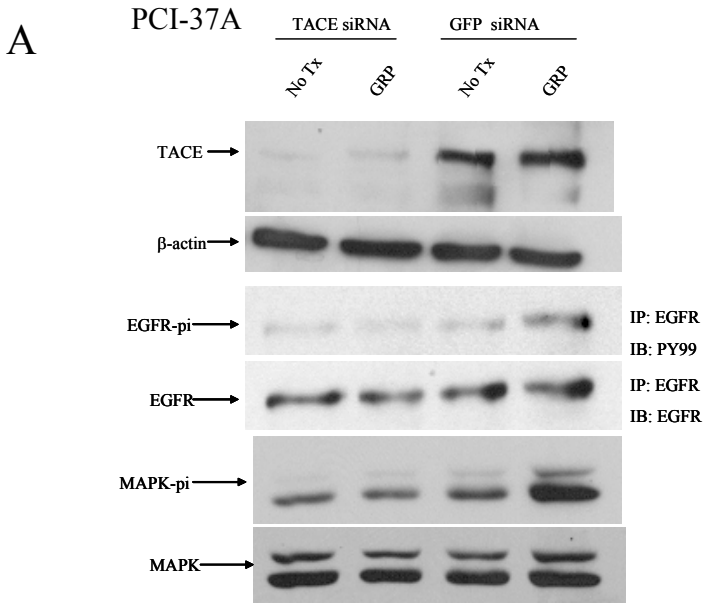
(A) Lysates from HEK293 cells transfected with c-Src or pcDNA3.1 (empty vector) were used to immunoblot for c-Src (*upper panel*) or immunoprecipitated with TACE followed by immunoblotting for c-Src (*middle panel*). The membrane was stripped to immunoblot for TACE (*lower panel*). (B, C) Representative HNSCC cells (PCI-37A or PCI-15B) were treated with GRP (400 nM) for 10 min followed by immunoprecipitation with TACE and immunoblotting for c-Src. The membrane was stripped to immunoblot for TACE to ensure equivalent loading. Cumulative results are shown from 3 independent (Figure 15B, p=0.03) or 5 independent experiments (Figure 15C, p=0.004). (D) PCI-15B cells were plated on coverslips followed by serum starvation for 2 days. Upon incubation with GRP (400 nM) for 5 min, cells were double stained with anti-TACE (green) and anti-c-Src (red) antibody. Arrowheads indicate the redistribution of TACE and c-Src to the cell membrane upon GRP stimulation.

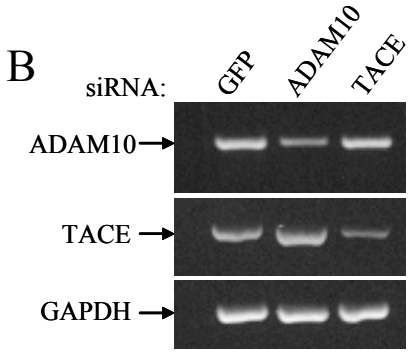
3.3.3. TACE/ADAM17 is the major metalloproteinase involved in GRP-induced EGFR proligand cleavage

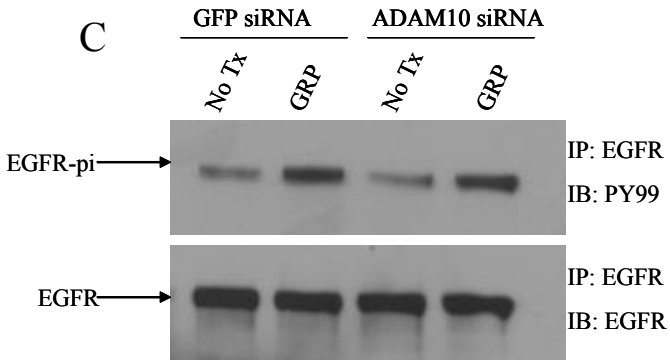
TACE/ADAM17 has been implicated in thrombin-induced cleavage of proamphiregulin with subsequent EGFR and MAPK activation in HNSCC cells (32). To determine the role of TACE in GRP-induced EGFR and MAPK activation, HNSCC cells (PCI-37A and PCI-15B) were transfected with TACE siRNA or negative control GFP duplex followed by GRP treatment. As shown in Figure 16A, GRP fails to induce EGFR and MAPK activation following suppression of TACE expression suppression in both cell lines. However, in the GFP siRNA duplex-transfected cells, GRP retains the ability to induce EGFR and MAPK activation. Similar results were observed in PCI-15B cells (data not shown).

In addition to TACE, we also investigated several other MMPs, including MMP-2, MMP-9 and ADAM-10. MMP-2 and MMP-9 have been shown to be involved in the release of HB-EGF and subsequent EGFR activation induced by Estradiol (E2) in breast cancer cells (33). Zymography demonstrated that GRP did not induce MMP-2 and MMP-9 activity HNSCC cells, suggesting that MMP-2 and MMP-9 are not involved in the release of EGFR ligands induced by GRP (data not shown). In addition, silencing of ADAM-10 expression by siRNA does not affect GRP-induced EGFR phosphorylation in HNSCC cells (Figures 16B and C). These results suggest that TACE is the major metalloproteinase involved in GRP-induced EGFR transactivation.

While HB-EGF is cleaved in response to GPCR stimulation in a cell- and ligand-dependent manner (116), our prior studies in HNSCC demonstrated that amphiregulin and TGF-α, but not HB-EGF or EGF, are released following treatment with GRP (77). To determine the role of TACE in GRP-mediated EGFR ligand release, we performed an amphiregulin ELISA assay following GRP stimulation in cell supernatants. As shown in Figure 16D, suppression of TACE expression with specific siRNA abrogates GRP-induced amphiregulin release into the conditioned medium ($p=0.0011$).


A
PCI-37A
TACE siRNA
GFP siRNA
No Tx
GRP
No Tx
GRP
TACE
β-actin
EGFR-pi
IP: EGFR
IB: PY99
EGFR
IP: EGFR
IB: EGFR
MAPK-pi
MAPK


B
siRNA:
GFP
ADAM10
TACE
ADAM10
TACE
GAPDH


C
GFP siRNA
ADAM10 siRNA
No Tx
GRP
No Tx
GRP
EGFR-pi
IP: EGFR
IB: PY99
EGFR
IP: EGFR
IB: EGFR

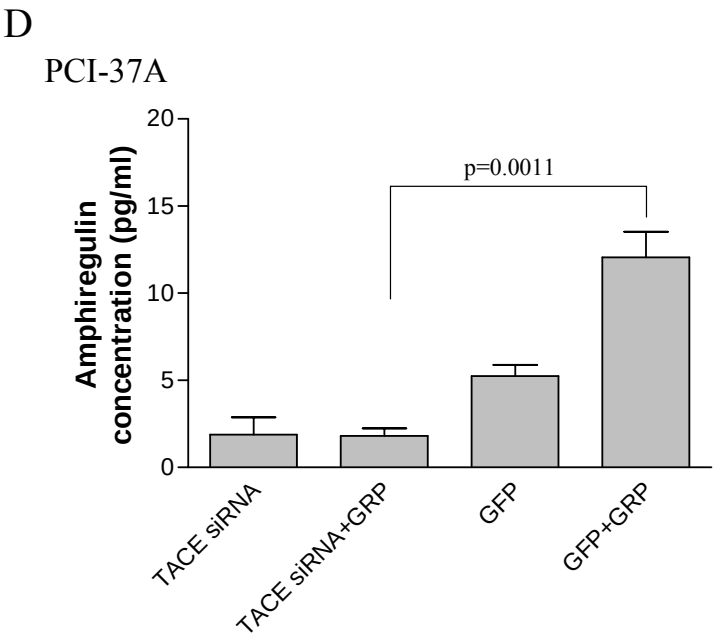

D
PCI-37A
Amphiregulin concentration (pg/ml)
20
15
10
5
0
p=0.0011
TACE siRNA
TACE siRNA+GRP
GFP
GFP+GRP

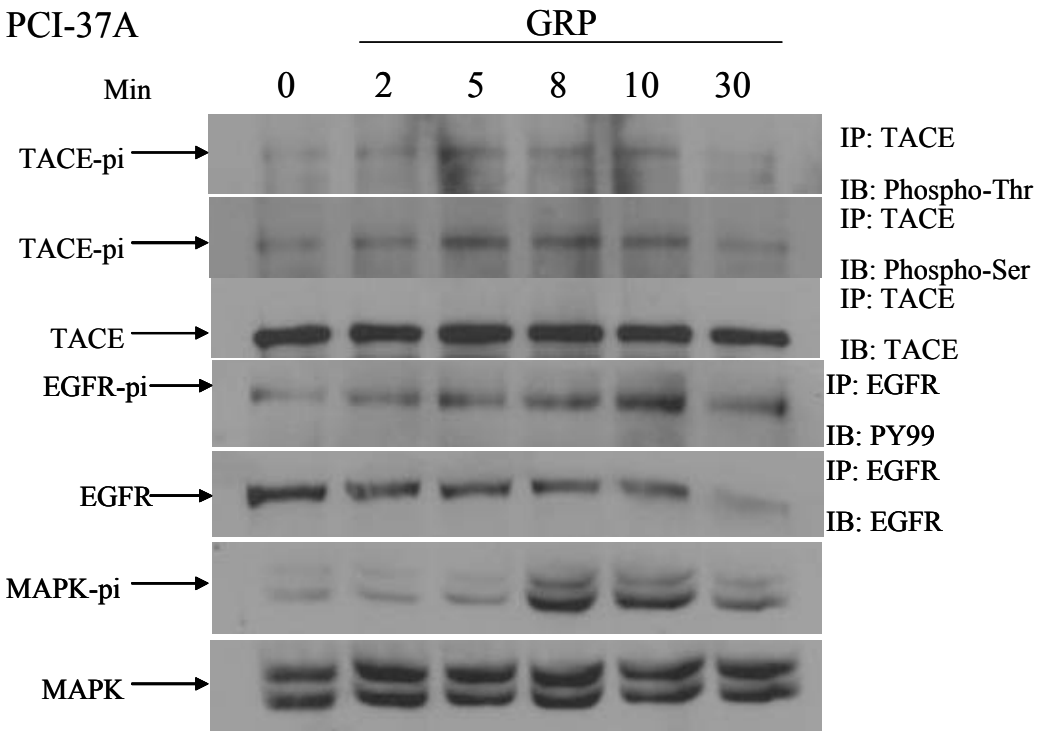
Figure 16: TACE mediates GRP-induced EGFR activation.

(A) Representative HNSCC cells (PCI-37A) were plated on 6 well plates followed by TACE siRNA transfection or transfection with negative control GFP duplex. After serum starvation for two days, cells were treated with GRP (400 nM) for 10 mins followed by immunoblotting for TACE and β-actin. Cell lysates were also subjected to immunoprecipitation for EGFR followed by phospho-tyrosine (PY99) immunoblotting. In addition, cell lysates were collected followed by immunoblot analysis for phosphorylated MAPK and total MAPK. (B, C) HNSCC cells (PCI-37A) cells were transfected with TACE, ADAM10 or GFP siRNA followed by RNA extraction and RT-PCR for ADAM10, TACE and housekeeping gene GAPGH. Cell lysates were accumulated followed by immunoprecipitation for EGFR and immunoblotting with PY99. (D) PCI-37A cells transfected with TACE siRNA or GFP duplex were serum starved for 2 days followed by GRP treatment for 10 min. Supernatants were collected and cell debris was discarded using centrifugation at 1800 RPM for 10 min. An amphiregulin ELISA was preformed on cell culture media according to the manufacturer's instructions. (TACE siRNA+GRP) treatment was compared to (GFP siRNA+GRP) treatment. Cumulative results are shown from 6 independent experiments and ($p=0.0011$).

3.3.4. GRP induces TACE, EGFR and MAPK phosphorylation

TPA, a well known shedding activator, has been reported to induce TACE phosphorylation on threonine residues (124, 125). EGF also induces TACE serine phosphorylation (126). In order to elucidate the mechanisms by which GRP leads to TACE relocalization and subsequent amphiregulin release, we investigated TACE serine and threonine

phosphorylation following GRP treatment in HNSCC cells. GRP stimulates TACE phosphorylation as early as 2 min after addition in PCI-37A cells (Figure 17A, p=0.04). In PCI-15B cells, TACE phosphorylation reaches maximal levels by 5 min (data not shown). GRP-induced EGFR and MAPK phosphorylation are first detectable at 5 min and peak at 10min in PCI-37A cells (Figure 17A), compatible with TACE acting upstream of EGFR and MAPK phosphorylation. While phosphorylation was readily detected at both serine and threonine residues following immunoprecipitation and western blotting with phospho-Ser and phospho-Thr antibodies, we could not detect TACE phosphorylation on tyrosine residues by immunoprecipitation of TACE and western blotting with anti-phosphotyrosine antibodies (data not shown).



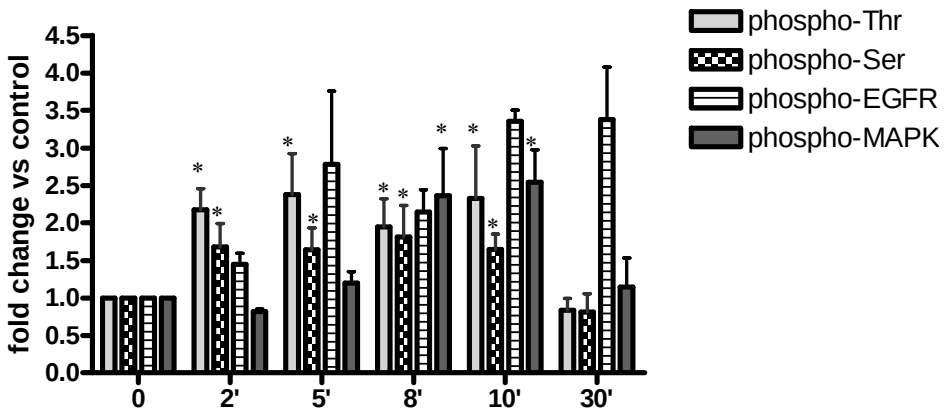


Figure 17: Src family kinases mediate GRP induced TACE phosphorylation in HNSCC.

PCI-37A cells were serum starved for 2 days followed by GRP (400 nM) treatment for different time points as indicated. After treatment, cell lysates were collected followed by immunoprecipitation with TACE and immunoblotting using phospho-specific threonine or serine or total TACE antibodies. Also, Cell lysates were subject to immunoblotting for phospho-MAPK and total MAPK or EGFR immunoprecipitation followed by immunoblotting for PY99. All of the time points were compared to no treatment group. Cumulative results are shown from 4 independent experiments for TACE phospho-serine, phospho-threonine and phosphor-MAPK. Cumulative results are shown from 2 independent experiments for phosphor-EGFR. Significant differences between no treatment and treatment were indicated by asterisks ($p<0.05$) determined by Wilxocon test and adjusted with the Bonferroni procedure.

3.3.5. Src family kinases are required for GRP induced TACE phosphorylation

The mechanism underlying GRP-induced TACE phosphorylation is unknown. Since c-Src translocates to the plasma membrane after GRP treatment, where c-Src associates with TACE, we hypothesized that GRP-induced Src family kinase activation could contribute to TACE

phosphorylation. However, as Src is strictly a tyrosine kinase the effect would be indirect requiring the activation of an intermediary kinase. Two different Src family kinase inhibitors, A-419259 (100 nM) and PD0180970 (500 nM), were used to test this hypothesis. As shown in Figure 18, GRP-induced TACE serine and threonine phosphorylation are abrogated upon blockade of Src family kinases in PCI-37A cells (p=0.028). Similar results were observed in PCI-15B cells (data not shown).

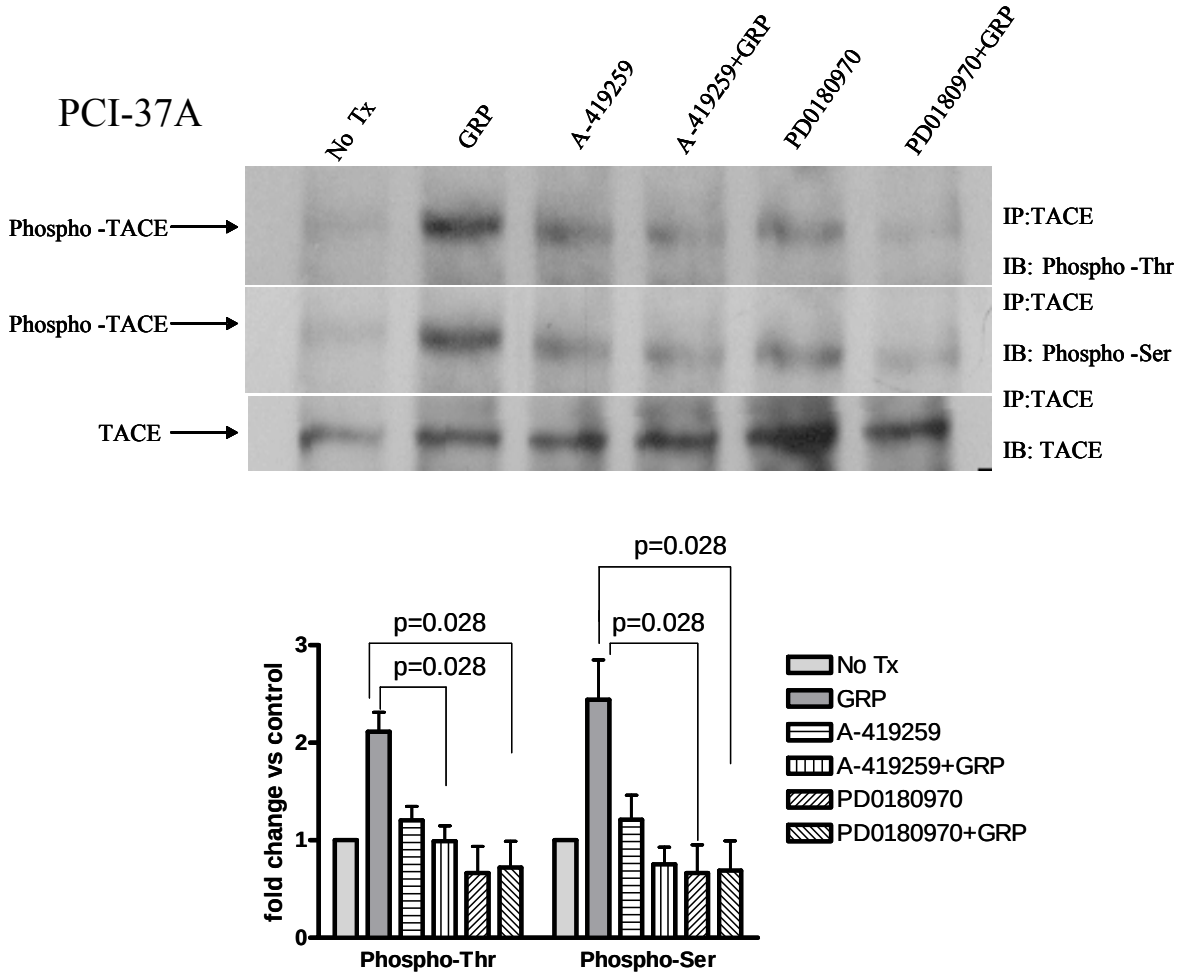


Figure 18: Src kinases mediate GRP induced TACE phosphorylation.

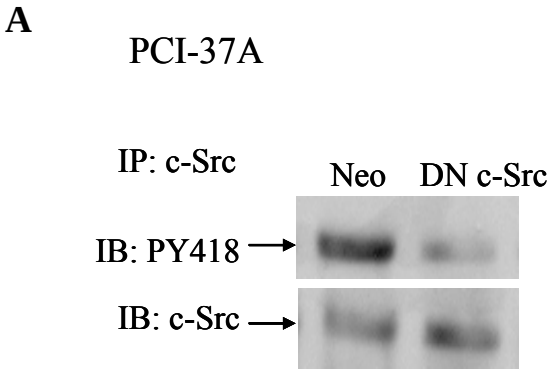
PCI-37A cells were plated in 10 cm plates followed by serum starvation for 2 days. After serum starvation, a Src kinase inhibitor [A-419259 (100 nM) or PD0180970 (500 nM)] was used

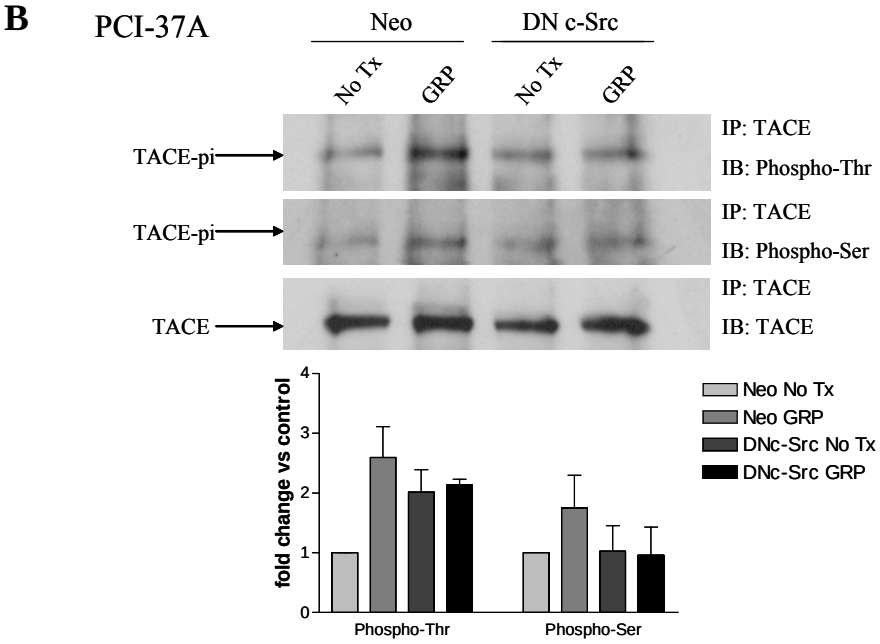
to pretreat cells followed by GRP (400 nM) treatment for 10 min. Cell lysates were collected followed by TACE immunoprecipitation and immunoblotting for phospho-threonine or phospho-serine. The same membrane was stripped followed by immunoblotting for TACE. (A419259+GRP) or (PD0180970+GRP) treatment was compared to GRP treatment alone. Cumulative results are shown from 4 independent experiments ($p=0.028$). TACE phosphorylation levels were normalized to TACE total expression levels. Fold changes were compared to no treatment group.

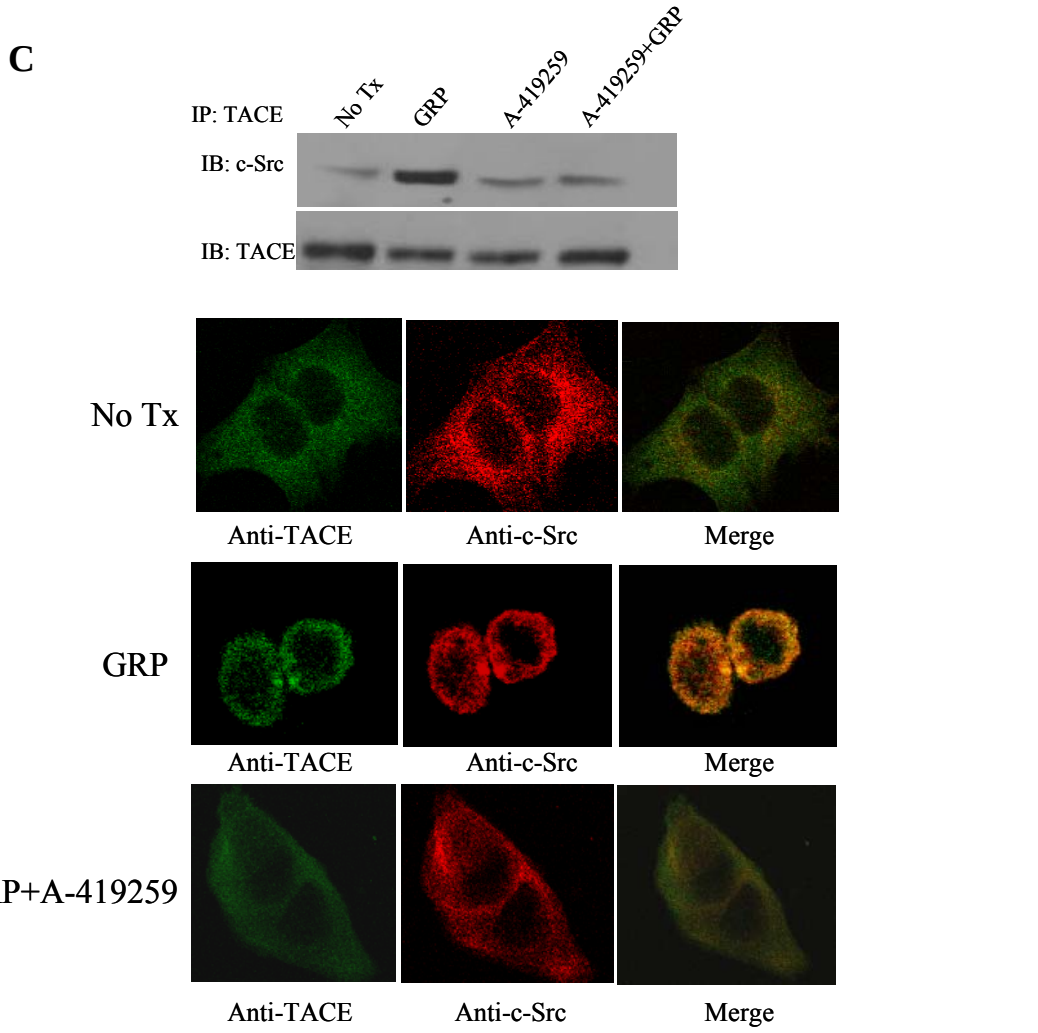
In addition to pharmacological inhibitors, we also generated HNSCC cells stably transfected with dominant-negative c-Src (DN c-Src). To characterize stably transfected cells, c-Src was immunoprecipitated from cell lysates followed by immunoblotting with an antibody that recognizes the activation loop of Src family kinases. As shown in Figure 19A, the level of activated c-Src is downregulated in DN c-Src compared to empty vector (Neo) transfected cells. GRP is able to induce TACE phosphorylation in Neo transfected cells, but fails to induce phosphorylation in DN c-Src transfected cells (Figure 19B), compatible with an obligatory role for c-Src in TACE phosphorylation. Further investigation shows that c-Src activity is important for GRP induced TACE translocation. Upon treating HNSCC cells with A-419259, the association and translocation of TACE and c-Src induced by GRP was abrogated (Figure 19C).

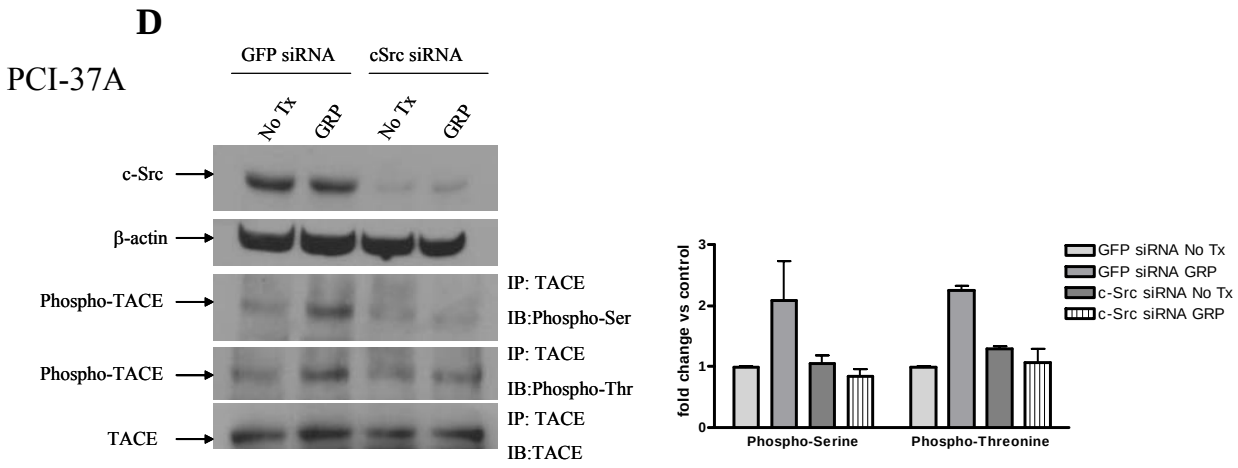
To further confirm the role of c-Src on GRP-induced TACE phosphorylation, HNSCC (PCI-37A) cells were transfected with c-Src siRNA followed by GRP treatment. As shown in Figure 19E, upon knockdown of c-Src expression, GRP failed to induce TACE phosphorylation. In addition, GRP fails to induce amphiregulin release into the supernatants in the presence of c-

Src siRNA (Figure 19F, p=0.0011). These results suggest that Src family kinases mediate GRP-induced TACE phosphorylation, thereby contributing to EGFR transactivation.






C
IP: TACE
No Tx
GRP
A-419259
A-419259+GRP
IB: c-Src
IB: TACE
No Tx
Anti-TACE
Anti-c-Src
Merge
GRP
Anti-TACE
Anti-c-Src
Merge
GRP+A-419259
Anti-TACE
Anti-c-Src
Merge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GFP siRNA
cSrc siRNA
No Tx
GRP
No Tx
GRP
c-Src
β-actin
Phospho-TACE
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IB:Phospho-Ser
Phospho-TACE
IP: TACE
IB:Phospho-Thr
TACE
IP: TACE
IB:TACE
fold change vs control
Phospho-Serine
Phospho-Threonine
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GFP siRNA GRP
c-Src siRNA No Tx
c-Src siRNA GRP

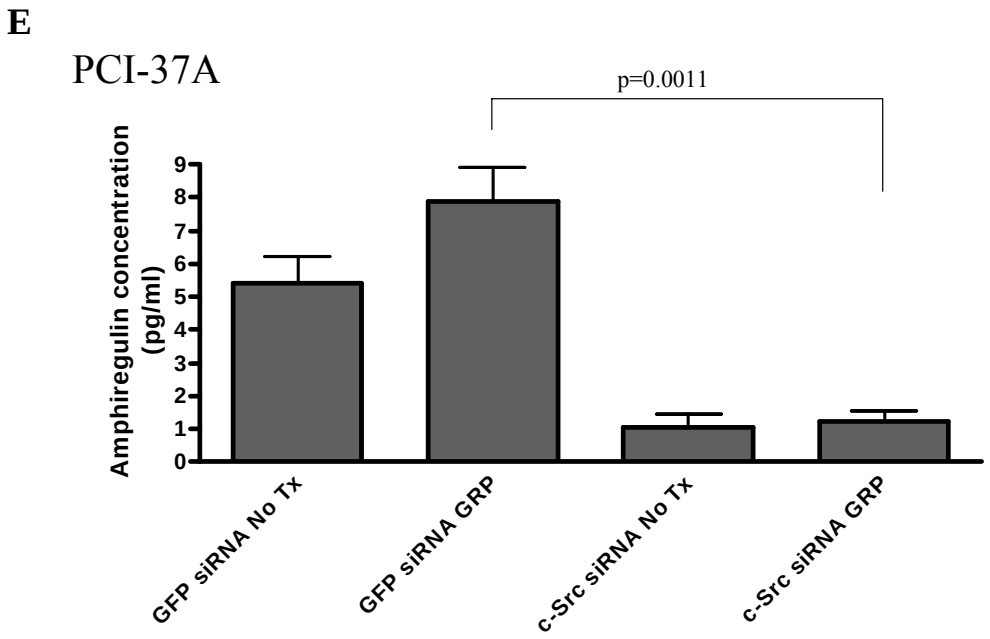


Figure 19: Src kinases mediate GRP induced TACE translocation and phosphorylation.

(A) PCI-37A cells were transfected with dominant negative c-Src (DN c-Src) or empty vector (Neo) followed by selection with G418 (600 μg/ml). Stable cell clones were isolated and expanded. Cells were subject to immunoprecipitation with c-Src followed by immunoblotting with PY418. The same membrane was stripped followed by immunoblotting with c-Src antibody. (B) DN c-Src or Neo transfected PCI-37A cells were serum starved for 2 days followed by GRP treatment for 10 min. Cell lysates were used to detect phosphorylated TACE levels or followed by immunoprecipitation by TACE and immunoblotting with c-Src antibody. Cumulative results are shown from 2 independent experiments. (C) PCI-15B cells were plated on 10 cm plates or coverslips followed by serum starvation for two days and GRP treatment for 5 min in the presence or absence of A-419259 (100 nM) two hour pretreatment. Cell lysates were harvested and subject to TACE immunoprecipitation followed by immunoblotting with c-Src.

Cells on coverslips were double stained with anti-TACE (green) and anti-c-Src (red) antibody. Merged fluorescence was shown as yellow. (D) PCI-37A cells were transfected with c-Src siRNA followed by serum starvation for 2 days and treatment with GRP (400 nM) for 10 min. Cell lysates were subject to western blotting for c-Src expression and TACE immunoprecipitation. Cumulative results are shown from 3 independent experiments. (E) An amphiregulin ELISA was preformed on cell culture media according to the manufacturer's instructions. c-Src siRNA+ GRP treatment was compared to GFP siRNA+ GRP treatment. Cumulative results are shown from 6 independent experiments ($p=0.0011$).

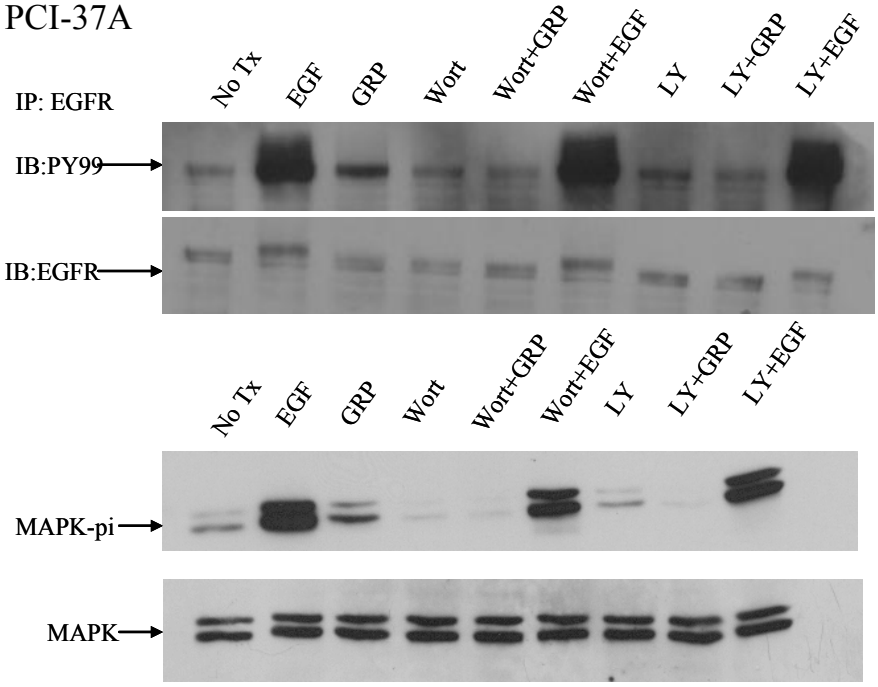
3.3.6. PI-3 kinase acts as an intermediate molecule in GRP induced EGFR and MAPK phosphorylation

Since Src family kinases are tyrosine kinases and phosphorylate substrates on tyrosine residues and TACE is phosphorylated on serine/threonine residues, a serine/threonine kinase must act as an intermediary between Src and TACE phosphorylation. The PI-3 kinase pathway contains multiple serine/threonine kinases and PI-3 kinase has been reported to be required for GPCR ligand-induced MAPK activation via both EGFR independent and dependent pathways (39, 42, 43, 127). To determine the role of PI-3 kinase in GRP-induced EGFR and MAPK activation, HNSCC cells were incubated with the PI-3 kinase inhibitor Wortmannin (250 nM) or LY294002 (20 μM) followed by GRP treatment. As shown in Figure 20A, GRP-induced EGFR and MAPK phosphorylation are blocked by both inhibitors in PCI-37A cells, indicating that PI-3 kinase mediates GRP-induced EGFR signaling. In contrast, neither Wortmannin nor LY294002 inhibits EGF-induced MAPK phosphorylation demonstrating specificity and further that the

effects of Wortmannin and LY294002 and thus PI-3 kinase are upstream of EGFR activation. To confirm that PI-3 kinase acts upstream of GRP-induced EGFR signaling, amphiregulin release was assessed by ELISA. Upon PI-3 kinase blockade by Wortmannin or LY294002, GRP-induced amphiregulin release is abrogated (Figure 20B, p=0.028), indicating that PI-3 kinase mediated GRP-induced EGFR signaling by contributing to EGFR ligand release.

Src family kinases have been reported to activate PI-3 kinase by interaction between Src SH2 domains and the p85 regulatory subunit of PI-3 kinase with subsequent phosphorylation of Y688 and release of the inhibitory of activity of p85 on the p110 catalytic subunit (128, 129). Further Src family kinases can inhibit PTEN activity also leading to increased signaling through the PI-3 kinase pathway. To examine whether the PI-3 kinase pathway was involved in GRP-induced EGFR signaling by acting downstream of Src family kinases, HNSCC cells (PCI-37A) were treated with the Src family kinase inhibitor A-419259 (100 nM) followed by GRP treatment. Using phospho-Akt as a surrogate marker for PI-3 kinase pathway activation, we found that GRP-induced PI-3 kinase activity is suppressed by Src family kinase blockade (Figure 20C). However, EGF-induced Akt phosphorylation is not affected by Src inhibition. Thus, direct activation of the PI-3 kinase/Akt pathway by the EGFR does not require c-Src. In contrast, activation of the PI-3 kinase pathway induced by GRP proceeds via a c-Src dependent mechanism (Figure 20C). Similar results were observed in PCI-15B cells (data not shown).

A


PCI-37A
No Tx
EGF
GRP
Wort
Wort+GRP
Wort+EGF
LY
LY+GRP
LY+EGF
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IB:PY99
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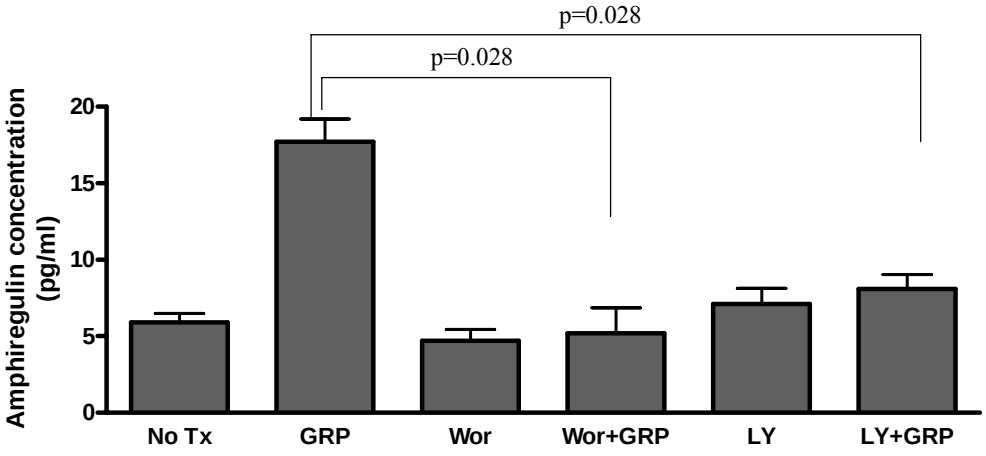
MAPK

B


p=0.028
p=0.028
Amphiregulin concentration (pg/ml)
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15
10
5
0
No Tx
GRP
Wor
Wor+GRP
LY
LY+GRP

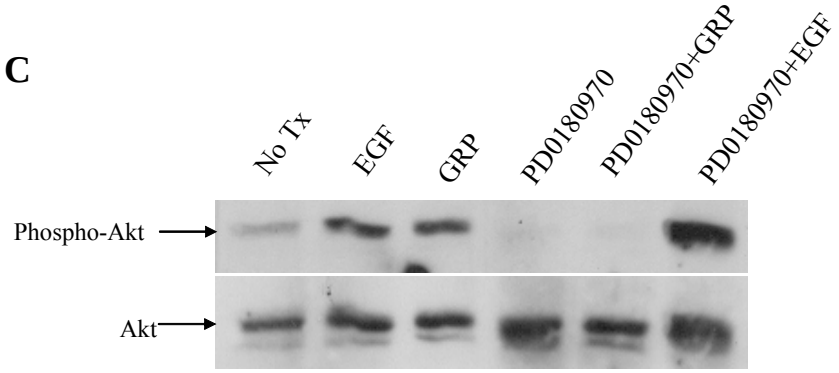


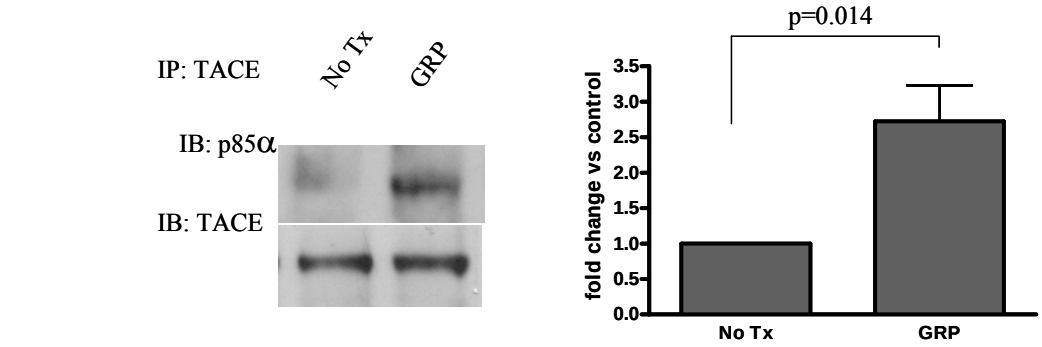
Figure 20: PI-3 kinase mediates GRP induced EGFR and MAPK phosphorylation.

(A, B) PCI-37A cells were plated followed by 2 days of serum starvation. The PI-3 kinase inhibitors Wortmannin (Wort, 250 nM) or LY294002 (LY, 20 μM) were used to pretreat cells for 1 hr followed by GRP (400 nM) or EGF (10 ng/ml) treatment for 10 min. Cell lysates were collected followed by immunoblotting for phospho-MAPK and total MAPK. EGFR phosphorylation levels were also examined by immunoprecipitation with EGFR followed by immunoblotting with phospho-tyrosine antibody (PY99). Cell supernatants were accumulated followed by an ELISA assay for amphiregulin. Cumulative results are shown from 4 independent experiments ($p=0.028$). (C) PCI-37A cells were serum starved for 2 days. After pretreatment with the Src family kinase inhibitor PD0180970 (500 nM) for 2 hours, cells were treated with GRP or EGF for 10 min. Cell lysates were subject to immunoblotting with phospho-Akt (Ser473) and total Akt.

3.3.7. PI-3 kinase activity is required for GRP induced TACE phosphorylation

ADAM12 can directly associate with the SH3 domains of the p85α subunit of PI-3 kinase in C2C12 fibroblast cells (52). To determine whether GRP could induce interaction of TACE with the p85α subunit of PI-3 kinase, HNSCC cells were treated with GRP followed by immunoprecipitation of TACE and western blotting with p85α. As shown in Figure 21A, GRP increases the association between TACE and p85α in HNSCC cells ($p=0.014$). Since PI-3 kinase mediates GRP-induced EGFR and MAPK activation, we next examined whether PI-3 kinase activity is required for GRP-induced TACE phosphorylation. As shown in Figures 21B, blockade of PI-3 kinase by either of two PI-3 kinase inhibitors, Wortmannin (250 nM) or LY294002 (20 μM), eliminates GRP-induced TACE phosphorylation in HNSCC cells ($p=0.008$). In addition to PI-3 kinase inhibitors, we used p85α siRNA to determine the effect of p85 knockdown on GRP-induced TACE phosphorylation (Figure 21C). GRP fails to induce TACE phosphorylation in p85α siRNA-transfected cells; while GRP retains the ability to stimulate TACE phosphorylation in control siRNA-transfected HNSCC cells (Figure 21C). Furthermore, p85α siRNA abrogates GRP-induced amphiregulin release ($p=0.014$) (Figure 21D). Similar results were observed in PCI-15B cells (data not shown). Thus, PI-3 kinase is required for GRP-induced TACE phosphorylation and EGFR ligand release in HNSCC cells.

A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No Tx
GRP
IB: p85α
IB: TACE
p=0.014
fold change vs control
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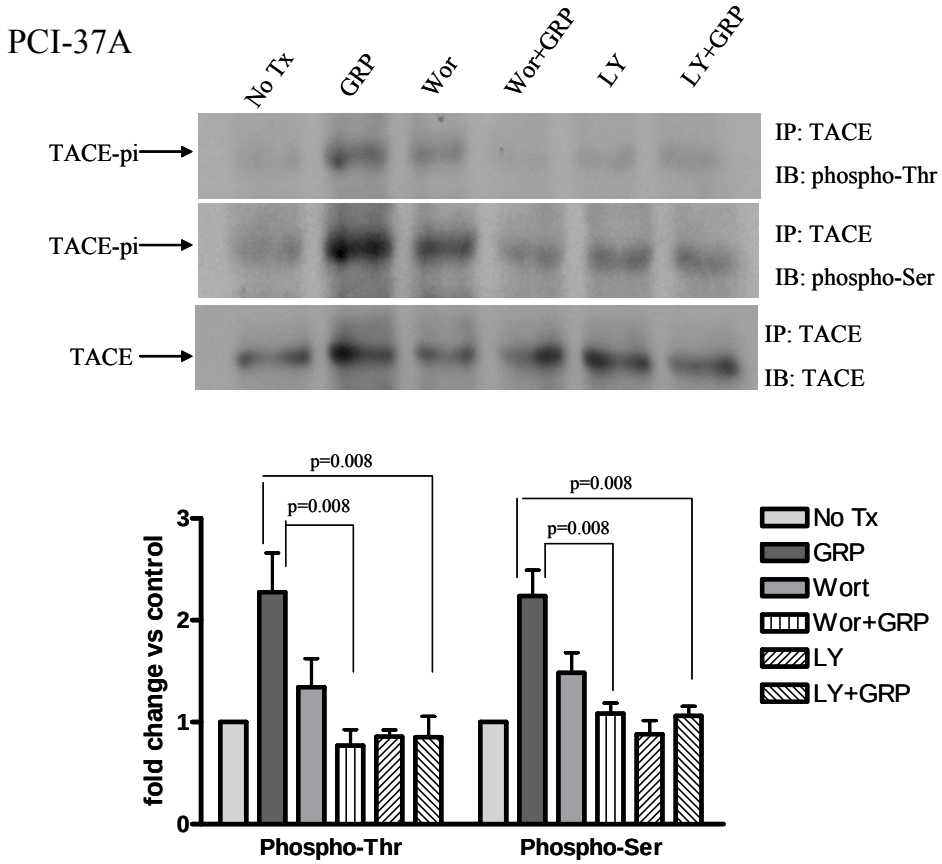
3.0
2.5
2.0
1.5
1.0
0.5
0.0
No Tx
GRP

B


PCI-37A
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GRP
Wor
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IB: phospho-Ser
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p=0.008
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p=0.008
p=0.008
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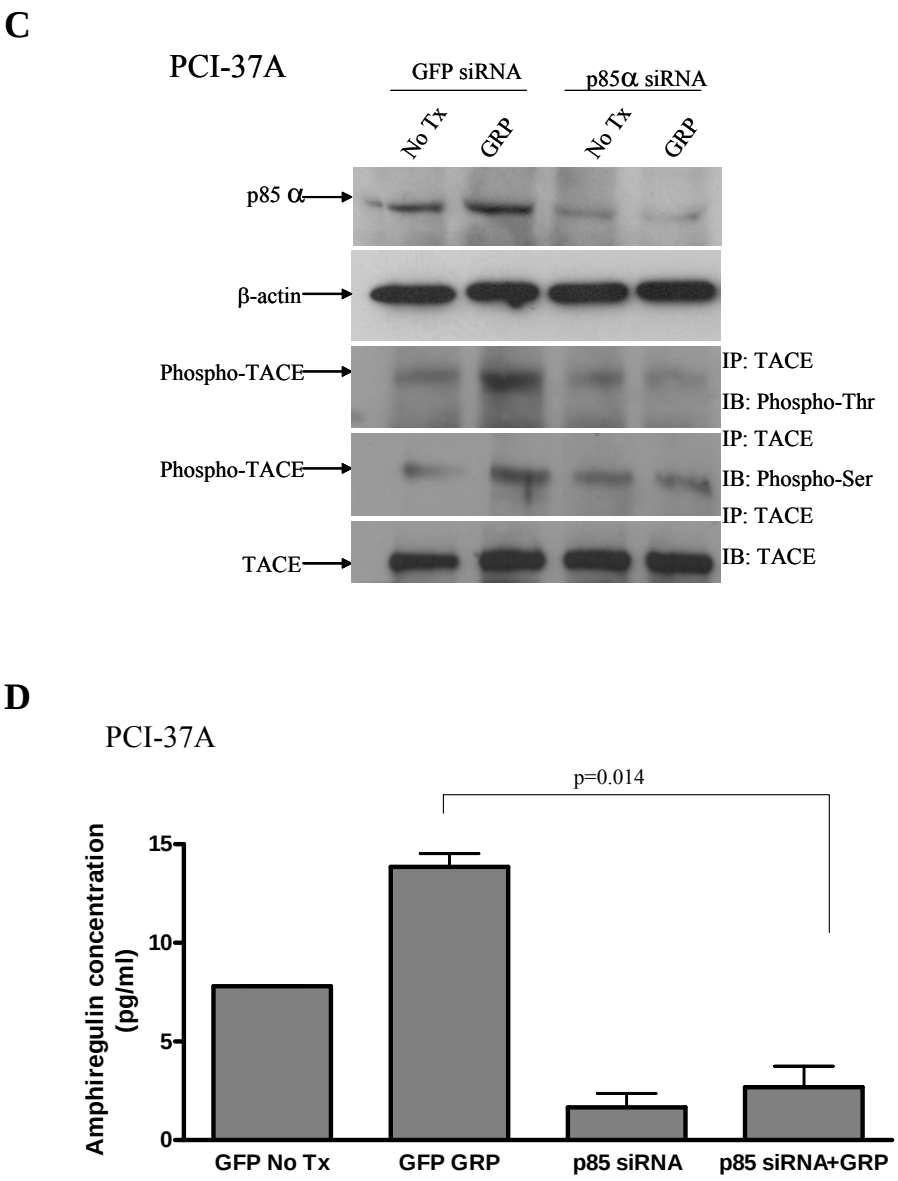


Figure 21: PI-3 kinase is required for GRP induced TACE phosphorylation in HNSCC cells.

(A) PCI-37A cells were treated with GRP (400 nM) for 10 min followed by immunoprecipitation with TACE and immunoblotting for p85α. The membrane was stripped to immunoblot for TACE to ensure equivalent loading. GRP treatment was compared to no treatment. Cumulative results are shown from 4 independent experiments (p=0.014). (B) PCI-

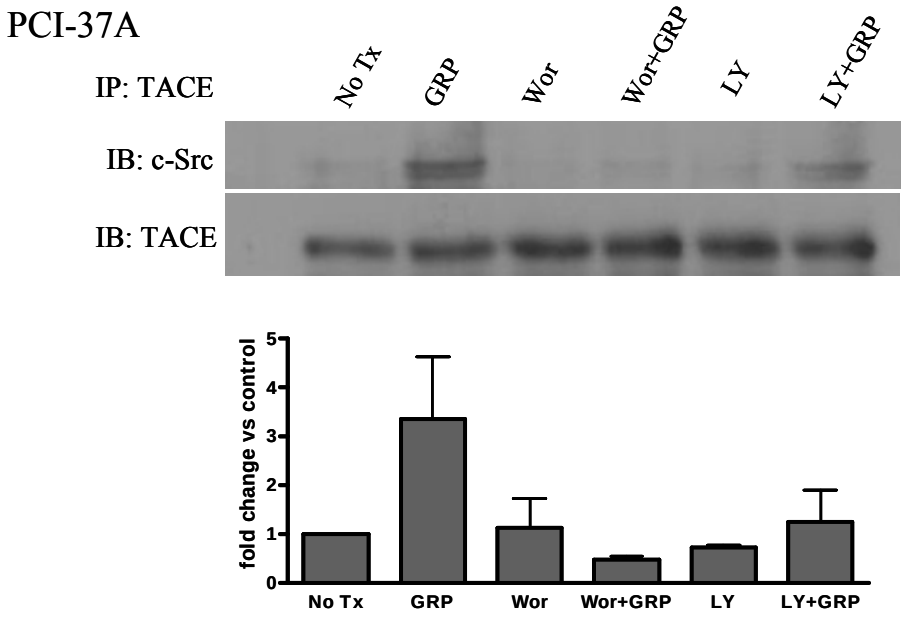
37A cells were plated followed by 2 days of serum starvation. PI-3 kinase inhibitors [Wortmannin (Wort, 250 nM) or LY294002 (LY, 20 μM)] were used to pretreat cells for 1 hr followed by GRP treatment for 10 min. Cell lysates were collected followed by immunoprecipitation with TACE followed by immunoblotting for phospho-threonine or phospho-serine. The same membrane was stripped followed by immunoblotting for TACE. Wor+GRP or LY+GRP treatment was compared to GRP treatment alone. Cumulative results are shown from 5 independent experiments (p=0.004). (C) PCI-37A cells were transfected with p85α siRNA followed by serum starvation for 2 days and treatment with GRP (400 nM) for 10 min. Cell lysates were subject to western blotting for p85α expression and TACE immunoprecipitation. Supernatants were collected and cell debris was discarded using centrifugation at 1800 RPM for 10 min. (D) An amphiregulin ELISA was preformed on cell culture media according to the manufacturer's instructions. p85 siRNA+ GRP treatment was compared to GFP siRNA+ GRP treatment. Cumulative results are shown from 4 independent experiments (p=0.014).

3.3.8. PI-3 kinase activity is required for GRP induced TACE and c-Src phosphorylation

Since GRP induced PI-3 kinase activity in a Src kinase dependent fashion, leading to downstream TACE phosphorylation and amphiregulin cleavage, we hypothesized that PI-3 kinase mediates GRP-induced TACE and c-Src association. In order to test this hypothesis, HNSCC cells (PCI-37A) were treated with Wortmannin or LY294002 or transfected with p85α siRNA or GFP siRNA followed by assessment of TACE and c-Src interaction by co-

immunoprecipitation. As shown in Figures 22A&B, GRP induced TACE and c-Src association is blocked upon PI-3 kinase blockade by either pharmacological inhibitors (Wortmannin or LY) or p85α siRNA. These results suggest that PI-3 kinase plays a pivotal role in mediating GRP-induced TACE and c-Src association in HNSCC cells.

A



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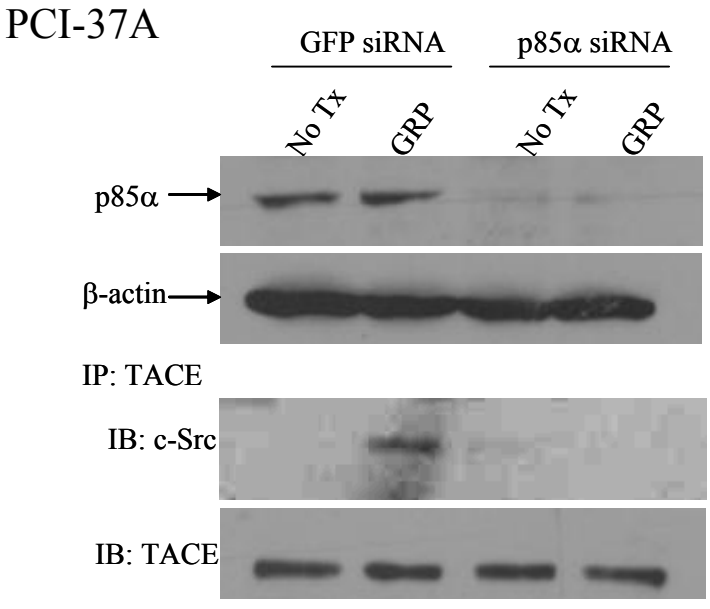


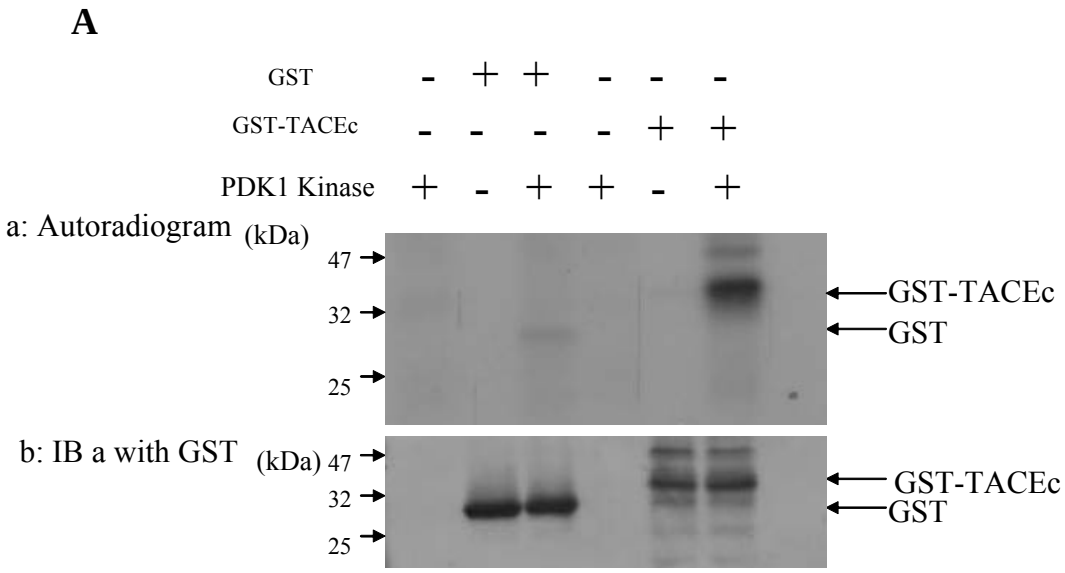
Figure 22: PI-3 kinase mediates GRP-induced TACE and c-Src association in HNSCC cells.

(A) PCI-37A cells were pretreated with Wortmannin (Wor, 250 nM), LY294002 (20 μM) followed by serum starvation for 2 days and treatment with GRP (400 nM) for 10 min. Cell lysates were used to perform co-immunoprecipitation between TACE and c-Src. The same membrane was stripped followed by immunoblotting with anti-TACE antibody as the loading control. (B) PCI-37A cells were transfected with p85α or GFP siRNA followed by serum starvation for 2 days and GRP (400 nM) for 10 min. Cell lysates were subject to co-immunoprecipitation between TACE and c-Src. The same membrane was stripped followed by immunoblotting with anti-TACE antibody as the loading control.

3.3.9. PDK1 is the effector responsible for GRP induced TACE phosphorylation

While PI-3 kinase does have serine/threonine protein kinase activity, the only identified PI-3 kinase protein substrate is PI-3 kinase itself. Thus, we reasoned that an alternative intermediary kinase must directly phosphorylate TACE in response to GRP stimulation. There are a number of possible candidates as the 3 phosphorylated phosphatidylinostides produced by PI-3 kinase activate, both directly and indirectly, a broad kinase cascade including all protein kinase C (PKC) isoforms, phosphoinositide-dependent kinase 1 (PDK1), PDK2, Akt and others. In order to identify the kinase that phosphorylates TACE, we purified a GST fusion protein linked to the cytoplasmic domain of TACE (GST-TACEc). As shown in Figure 23A, PDK1 kinase phosphorylates TACE on the cytoplasmic domain *in vitro*, implicating PDK1 kinase as the effector mediating GRP-induced TACE phosphorylation. In addition to PDK1 kinase, we also

examined other kinases including PKC. The PKC active enzyme (mixture of α,β and γ isoforms) failed to induce GST-TACEc phosphorylation (data not shown). To confirm that PDK1 could increase full length TACE phosphorylation, HNSCC (PCI-15B and 37A) cells were serum starved followed by an *in vitro* kinase assay with recombinant PDK1 enzyme. In both cell lines, PDK1 increasesTACE full length phosphorylation (Figures 23B and 23C). To investigate the requirement of PDK1 in GRP-induced TACE phosphorylation, PDK1 siRNA was used to knockdown endogenous PDK1 expression (Figure 23D). Upon PDK1 knockdown, GRP fails to induce TACE phosphorylation which is accompanied by abrogation of amphiregulin release compared to GFP siRNA transfected cells (Figure 23E, p=0.014). These results demonstrate that PDK1 can act as an effector mediating GRP-induced TACE phosphorylation.



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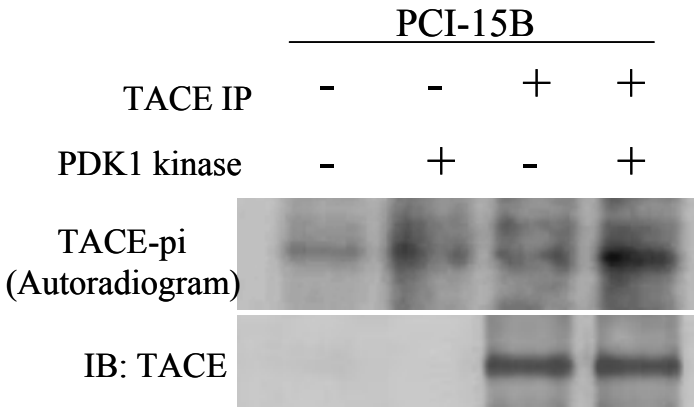
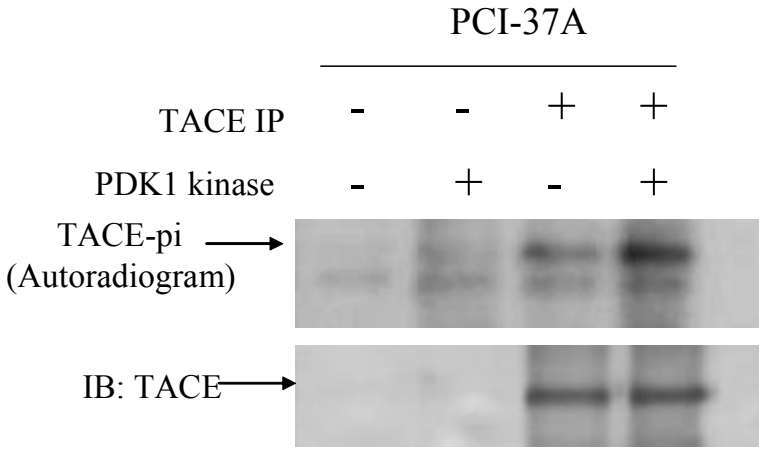

PCI-15B
TACE IP
- - + +
PDK1 kinase
- + - +
TACE-pi
(Autoradiogram)
IB: TACE

C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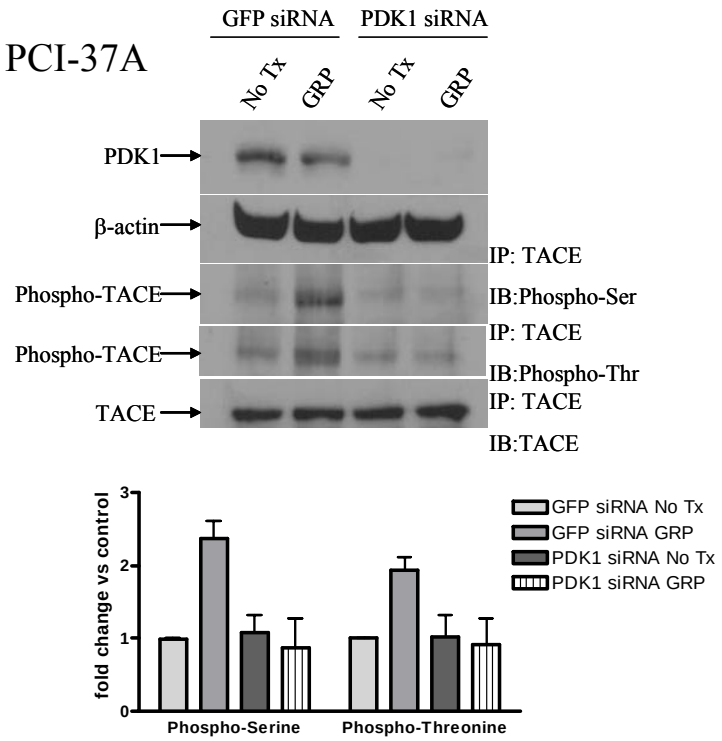
TACE IP
- - + +
PDK1 kinase
- + - +
TACE-pi
(Autoradiogram)
IB: TACE

D


PCI-37A
GFP siRNA
PDK1 siRNA
No Tx
GRP
No Tx
GRP
PDK1
β-actin
Phospho-TACE
Phospho-TACE
TACE
IP: TACE
IB:Phospho-Ser
IP: TACE
IB:Phospho-Thr
IP: TACE
IB:TACE
fold change vs control
GFP siRNA No Tx
GFP siRNA GRP
PDK1 siRNA No Tx
PDK1 siRNA GRP
Phospho-Serine
Phospho-Threonine

E

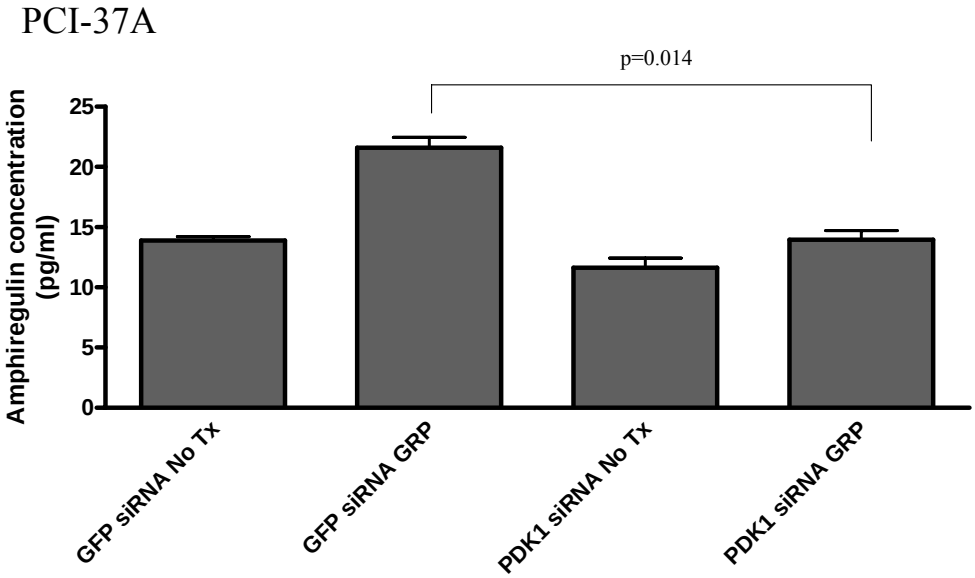

PCI-37A
p=0.014
Amphiregulin concentration (pg/ml)
GFP siRNA No Tx
GFP siRNA GRP
PDK1 siRNA No Tx
PDK1 siRNA GRP

Figure 23: PDK1 kinase phosphorylates TACE upon GRP treatment in HNSCC.

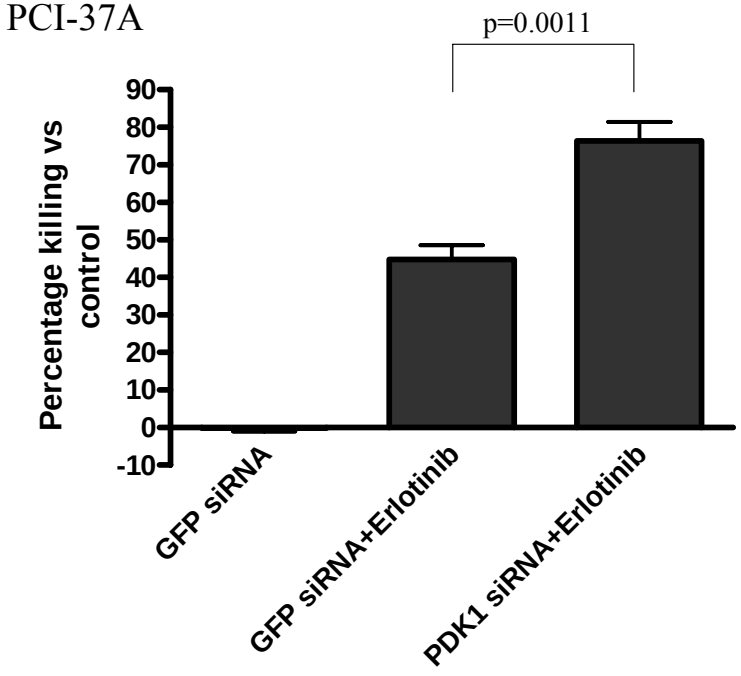
(A) Active PDK1 kinase was incubated with recombinant GST or GST-TACEc followed by *in vitro* kinase assay. Autoradiography was performed. The same membrane was subject to immunoblotting with anti-GST antibody to ensure equal loading. (B, C) HNSCC cells (PCI-15B and 37A) were serum starved for 2 days followed by TACE immunoprecipitation. PDK1 enzyme was used to incubate with the cell pellets followed by *in vitro* kinase assay. Autoradiography was performed. The same membrane was used to probe for total TACE. (D) HNSCC cells (PCI-37A) were transfected with PDK1 siRNA or negative control (GFP) siRNA followed by immunoblotting with PDK1 antibody or immunoprecipitation with TACE followed by immunoblotting with phospho-threonine or serine antibody. Results were shown from 3 independent experiments. (E) An amphiregulin ELISA was performed by using the supernatants accumulated. PDK1 siRNA+ GRP treatment was compared to GFP siRNA+ GRP treatment. Cumulative results are shown from 4 independent experiments ($p=0.014$).

3.3.10. Targeting of PDK1 enhances the anti-tumor effects of EGFR inhibition

EGFR inhibitors, including erlotinib, have resulted in limited clinical responses in cancer patients whose tumors do not contain activating EGFR mutations(23). Since GRP stimulates HNSCC growth through transactivation of EGFR, we hypothesized that the anti-tumor effects of an EGFR inhibitor could be enhanced by simultaneous targeting of PDK1. As shown in Figure 24A, erlotinib treatment (1 μM) resulted in approximately 50% growth inhibition at 24 hours. The cytotoxic effects of erlotinib were increased significantly when combined with PDK1

siRNA (Figure 24B, p=0.0011). In addition, PDK1 siRNA significantly reduced HNSCC cell invasion ability when combined with erlotinib (p<0.001). These results indicate that the anti-tumor effects of EGFR inhibitors can be enhanced by PDK1 blockade.

A



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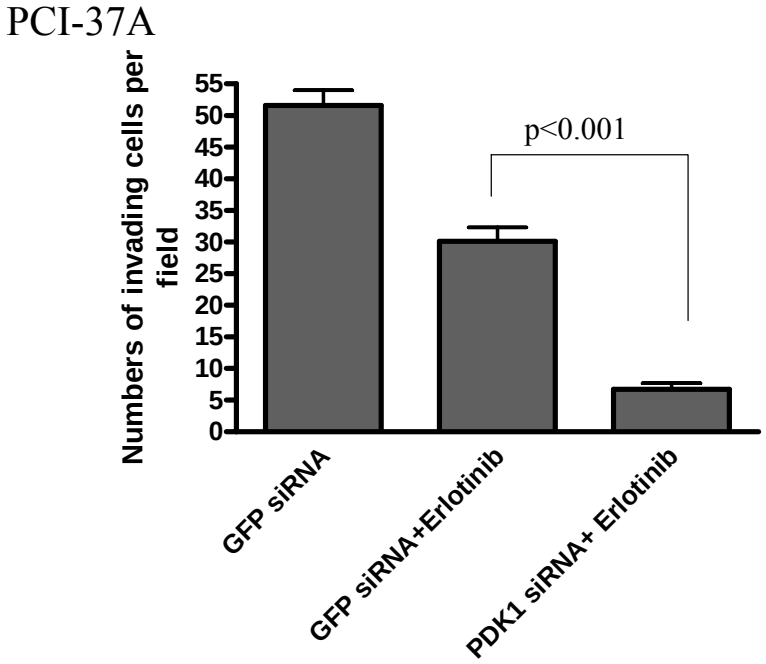

Figure 24: Enhanced anti-tumor effects by combined targeting of PDK1 and EGFR.

HNSCC (PCI-37A) cells were plated on 24 well plates followed by transfection with PDK1 siRNA or GFP duplex diRNA. 24 hours after transfection, erlotinib (1 μM) was added to the well. (A) MTT assay was performed after 24 hour Erlotinib treatment. Results from 6 independent experiments (p=0.0011). (B) Cells were plated in Matrigel invasion chamber in duplicates followed by treatment with erlotinib (1 μM) for 24 hours. Invading cells in 10 representative fields were counted using light microscopy at x400 magnification.

3.4. Discussion

The integration of EGFR and GPCR signaling pathways has been shown to contribute to carcinogenesis in a variety of cancer models (30, 33, 86, 116, 130). The precise mechanism of EGFR activation by GPCR has been incompletely understood. The results of the present study suggest that following GPCR activation, Src kinase is activated leading to downstream induction of PI-3 kinase. Following PI-3 kinase and downstream PDK1 activation, TACE is phosphorylated on threonine and serine residues and translocated to the cell membrane thereby mediating EGFR proligand (e.g. amphiregulin) cleavage and subsequent EGFR and MAPK phosphorylation (Figure 25). Thus, autocrine or paracrine GRP activates a novel cascade with sequential activation of c-Src, PI-3 kinase/PDK1, TACE, amphiregulin, EGFR and MAPK with subsequent downstream signaling and functional outcomes including proliferation, survival and invasion.

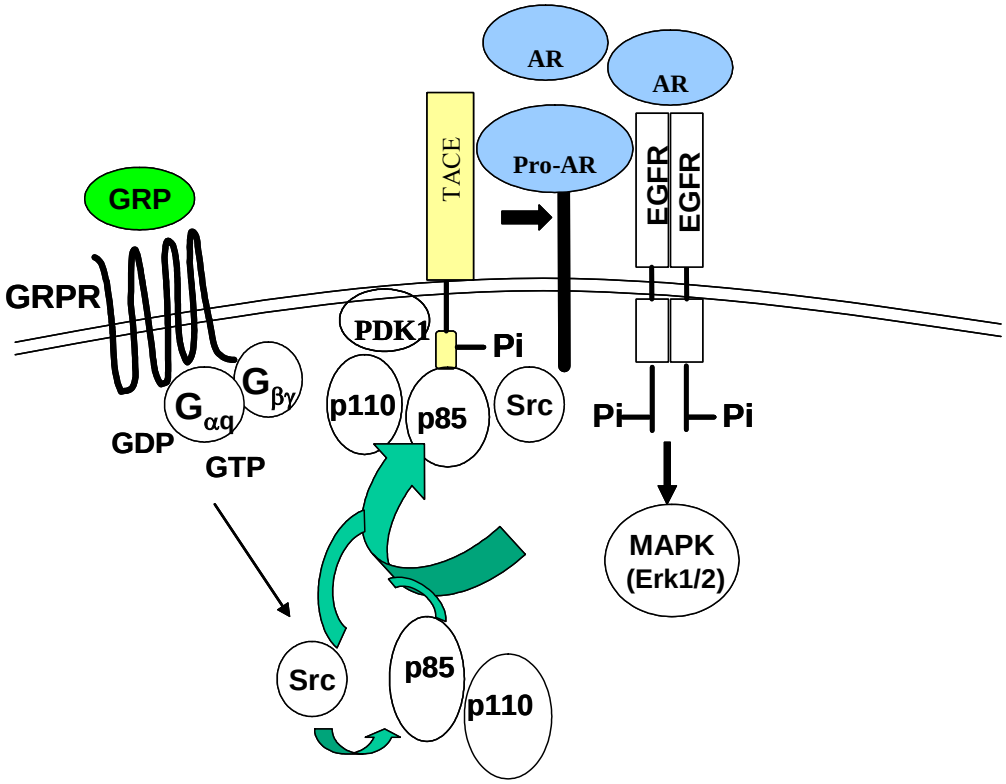

Figure 25: Proposed mechanism of GRP-induced EGFR signaling.

Upon GRP binding to GRPR, Src family kinases are activated. As a result of Src activation, downstream PI-3 kinase and PDK1 are activated, which contributes to TACE phosphorylation and EGFR proligand cleavage (e.g., amphiregulin). Consequently, mature EGFR ligand binds and activates EGFR, leading to downstream MAPK phosphorylation.

Following GRP treatment, c-Src, p85 and TACE form a complex which translocates to the plasma membrane. This is likely a consequence of phosphorylation of TACE and association of TACE with c-Src and p85. Both c-Src and TACE are located in punctuate foci in the cytosol

compatible with the association of c-Src and TACE with intracellular membranes through myristylation of the Src N-terminal domain and TACE transmembrane domain respectively. Following activation by GRP, both c-Src and TACE translocate to the cell membrane where they co-associate. However, the possibility that c-Src and TACE associate and then rapidly translocate to the membrane remains a possibility. GRP induces immediate phosphorylation of TACE on serine and threonine residues without concurrent detectable tyrosine phosphorylation. Although c-Src and PI-3 kinase are both required for TACE phosphorylation and translocation, they are unlikely to be the direct mediators as c-Src is exclusively a tyrosine kinase and PI-3 kinase has not been demonstrated to phosphorylate any exogenous proteins. PDK1 kinase, a downstream molecule of PI-3 kinase that is activated by 3-phosphorylated phosphatidylinositides and produced upon activation of PI-3 kinase, can phosphorylate TACE *in vitro*. Downregulation of PDK1 blocks TACE phosphorylation compatible with PDK1 being the effector mediating GRP-induced TACE phosphorylation. This represents a previously unknown function of PDK1 and implicates this kinase as potential therapeutic target for cancer treatment.

Transactivation of EGFR signaling pathways by GPCR ligands has been reported to contribute to carcinogenesis in several tumor cell types, including colon, gastric, prostate, breast and head and neck cancers (29, 31, 32, 86). The mechanisms underlying EGFR activation by GPCR ligands include both intracellular and extracellular pathways (54). In general, ectodomain shedding of EGFR ligands mediated by metalloproteinase activity appears to be essential for EGFR activation, leading to downstream MAPK activation, contributing to increased invasion and anti-apoptosis in tumor cells (45, 54). ADAM family members have a variety of functions, playing roles in fertilization, angiogenesis, neurogenesis and transmembrane molecule shedding (47, 131). Cumulative evidence suggests that ADAM family members mediate EGFR pro-ligand

cleavage (32, 45, 131). In this study, we demonstrate that TACE/ADAM17 is the primary protease involved in GRP-induced amphiregulin release and subsequent EGFR activation. This finding is consistent with previous reports implicating TACE/ADAM17 in the cleavage of proamphiregulin and downstream EGFR and MAPK activation in lung caner cells and HNSCC (32, 130). It is noteworthy that different ADAM family members mediate the release of specific EGFR ligands. Thus, release of TGF-α, amphiregulin, HB-EGF and epiregulin, was observed in ADAM17$^{-/-}$ compared to wild-type murine fibroblasts in the absence or in the presence of TPA (47), while cleavage of betacellulin and EGF release was mediated by ADAM10 (47). In addition, in response to specific GPCR ligands, tumor cells may use different mechanism to induce cell surface proteolysis. In human glioma cells, Ca^{2+} influx has been reported to activate ADAM 10 and lead to CD44 ectodomain cleavage. PMA stimulation activates ADAM17 through PKC, which also induces CD44 proteolysis suggesting a potential redundancy among proteolytic mechanisms (123).

The mechanisms underlying TACE phosphorylation in the context of GPCR ligand stimulation of EGFR have not been previously identified. We reported that Src family kinases contribute to GRP-induced EGFR phosphorylation in HNSCC (112). In the present study, we demonstrate by coimmunoprecipitation and confocal microscopy in HNSCC cells that TACE associates with c-Src at the cell membrane after incubation with GRP. The mechanism by which GRP induces TACE association with c-Src is unknown but could involve the proline-rich sequence in the cytoplasmic domain of TACE and the SH3 domain of c-Src. Alternatively, as p85 binds TACE and also binds c-Src, p85 could be an intermediary between TACE and c-Src. In addition to TACE, other ADAM family members (including ADAMs 10, 12, 15 and 17) contain proline-rich sequences in their cytoplasmic domain, which may interact with signaling

molecules (45, 132, 133). Several observations suggest that the interaction between TACE and c-Src is of physiological significance. First, the transactivation of EGFR and the activation of downstream MAPK in response to GRP require active c-Src. Second, stimulation of HNSCC cells with GRP resulted in increased levels of TACE and c-Src association. Third, TACE translocated with c-Src to the plasma membrane where cleavage of amphiregulin by TACE occurs. Finally, GRP stimulation of HNSCC cells induced TACE phosphorylation in a c-Src dependent fashion. Since phosphorylation plays a critical role in intracellular signaling, c-Src-mediated TACE phosphorylation may be a mechanism that underlies TACE activation in response to GRP stimulation. However, as c-Src is a tyrosine kinase and TACE is phosphorylated on serine/threonine residues, it is likely that TACE is phosphorylated as a consequence of a Src-dependent kinase. Consistent with this notion, we show that various reagents that block TACE phosphorylation strongly inhibited the transactivation of EGFR by GRP, most likely due to abrogation of proamphiregulin cleavage by TACE. In addition, the phosphorylation of TACE may result in its translocation to its targets or the formation of a functional complex of p85, Src and TACE on the membrane. The precise role of TACE phosphorylation in the cleavage of proamphiregulin and EGFR transactivation will require further investigation including the identification of amino acids in TACE that are phosphorylated in response to GRP, and functional analyses of an unphosphorylatable TACE mutant.

In addition to phosphorylation, translocation of TACE to the plasma membrane likely plays an important role in TACE function by placing it in the proximity of its target, proamphiregulin. Further studies will be needed to determine whether phosphorylation of TACE directly mediates the translocation or whether TACE first translocates to the membrane where it

is phosphorylated as a consequence of association with c-Src, p85, PDK1 or other molecules in the activation nidus.

We show that GRP-induced phosphorylation of TACE occurs on serine and threonine, but not on tyrosine residues. Therefore, we reasoned that additional signaling molecules are required in the regulation of TACE phosphorylation. In addition to c-Src, the p85 subunit of PI-3 kinase also associates with TACE. GRP stimulation activates the PI-3 kinase signaling pathway, as indicated by increased levels of the phosphorylated form of Akt, which is phosphorylated as a consequence of activation and/or recruitment of PDK1 and PDK2 by 3 phosphorylated phosphatidylinositols by PI-3 kinase. Moreover, inhibition of PI-3 kinase activity by pharmacological approaches or siRNA to the p85 regulatory subunit reverses the ability of GRP to induce TACE phosphorylation, EGFR ligand release and the activation of EGFR and MAPK, suggesting that similar to c-Src, PI-3 kinase activity is also required for the GRP-induced EGFR transactivation and consequent cell proliferation. PI-3 kinase has been reported to be activated by Src SH3 domain binding with its p85 subunit (128). Since inhibition of Src activity prevents GRP-induced Akt phosphorylation, c-Src is likely upstream of the PI-3 kinase pathway. Subsequent results show that abrogation of PI-3 kinase by p85α siRNA eliminates GRP-induced TACE and c-Src association and translocation. Although p85α mediated GRP-induced TACE and c-Src association and translocation, thereby contributing to EGFR ligand release and downstream EGFR and MAPK phosphorylation, it remains to be determined whether PI-3 kinase/p85α plays a similar relevant role for other cell types.

Although c-Src and PI-3 kinase are both required for TACE phosphorylation and translocation, they are unlikely to be the direct mediators as c-Src is exclusively a tyrosine kinase and PI-3 kinase has not been demonstrated to phosphorylate any exogenous proteins. The

products of PI-3 kinase activation (PIP2 and PIP3) bind with PDK1 pleckstrin homology (PH) domain and are necessary for PDK1 docking at the plasma membrane (134). PDK1 has been reported to facilitate the activation of several AGC protein kinases including PKA, PKG and PKC (135-137). Here we show that in addition to AGC protein kinases, PDK1 can also phosphorylate TACE. PDK1 is mainly cytoplasmic with some localization on the plasma membrane under basal condition (138, 139). Unlike other kinases, PDK1 exists as a constitutively active kinase even in the absence of exogenous stimulation. Furthermore, phosphorylation of PDK1 appears to be resistant to agonist stimulation of PI-3 kinase (136, 137, 140). Consistent with these previous findings, upon GRP treatment, we did not observe increased PDK1 phosphorylation by *in vitro* kinase assay. Given that PDK1 does not contain any SH3 domain, we propose the following model of PDK1 induced TACE phosphorylation: (1) GRP induces c-Src activation and downstream PI-3 kinase activation, giving rise to PIP2 and PIP3 production; (2) these lipid molecules elicit the translocation of PDK1 and TACE from the cytoplasm to the plasma membrane; (3) TACE undergoes a conformational change which can serve as a substrate for PDK1, through recognition of multiple PXXP motifs on the TACE cytoplasmic domain by PDK1. This previously unknown function of PDK1 identifies this kinase as potential therapeutic target for cancer treatment.

The products of PI-3 kinase activation (PIP2 and PIP3) bind with PDK1 pleckstrin homology (PH) domain and are necessary for PDK1 docking at the plasma membrane (134). PDK1 has been reported to facilitate the activation of several AGC protein kinases including PKA, PKG and PKC (135-137). Of particular interest, PDK1 specific docking with these substrates appeared to be required for efficient phosphorylation, which was localized to the hydrophobic Phe-Xaa-Xaa-Phe (PXXP) domain on the substrates (141, 142). Since TACE

contains several PXXP domains on the cytoplasmic domain, it is likely that upon PI-3 kinase activation by GRP, PDK1 translocates to the membrane, where it recognizes TACE cytoplasmic PXXP domain and regulates TACE phosphorylation. Thus, we have identified in HNSCC a GPCR-initiated signaling cascade involving sequential activation of c-Src, PI-3 kinase, PDK1 and TACE, leading to proamphiregulin cleavage and the subsequent activation of EGFR and MAPK.

Identification of these intermediate signaling molecules in GRPR-EGFR crosstalk can potentially benefit cancer therapy. In this paper, combined targeting of GRPR and EGFR enhanced anti-tumor effects. However, due to the limited availability of GRPR antagonists, alternative targeting strategy is needed to be combined with EGFR inhibitors. Here, we show that PDK1 targeting with siRNA dramatically enhanced cytotoxicity of the EGFR tyrosine kinase inhibitor Erlotinib. In addition, HNSCC invasion ability was further decreased by combining PDK1 siRNA and Erlotinib together. Combined EGFR targeting with additional targeting strategies including Src or PI-3 kinase may improve the efficiency and outcome of cancer therapy.

4. SUMMARY AND DISCUSSIONS

This thesis mainly focuses on elucidating the mechanism of GRPR and EGFR crosstalk in head and neck cancer. Since EGFR monoclonal antibodies or EGFR tyrosine kinase inhibitors have been reported to have limited anti-tumor effects when these agents have been administered to cancer patients without EGFR activating mutations (84, 85), enhanced understanding of the mechanism of crosstalk may lead to improved treatment approaches which could be combined with EGFR .

4.1. Src family kinases act as the key intracellular molecules mediating GRP induced EGFR signaling

Despite the fact that Src is one of most studied protooncogenes, the role of Src family kinases in cancer remains largely unknown. Src has been reported to mediate tumor cell adhesion, motility and invasion. However, the role of Src mediated cancer cell proliferation remains controversial. Src activity is important to maintain fibroblast and precancerous cell division and proliferation. In contrast, overexpression of c-Src was reported to have no effect on colon cancer cell proliferation(143, 144).

Intracellular pathways involving Src family kinases have also been implicated in GPCR-EGFR transactivation (93). Interaction between Src kinases and EGFR is well documented (145). We have accumulated evidence demonstrating that Src family kinases are activated by EGFR ligand (TGF-α or EGF) in HNSCC cells where they mediate growth pathways. All 9 HNSCC cell lines examined were found to express phosphorylated c-Src, Lyn, c-Yes and Fyn in response to EGFR ligand treatment (92).

In this thesis, I showed that Src kinase mediates GRP induced HNSCC cell proliferation and invasion. GRP induced Src kinase activity reached maximum levels in the presence of EGFR, indicating that Src kinase could act both upstream and downstream of EGFR. More importantly, Src was shown to play a pivotal role as the key intracellular intermediate mediating GRPR induced EGFR signaling. Upon Src activation by GRP, Src could contribute to downstream PI-3 kinase, PDK1 activation, which is followed by TACE phosphorylation and EGFR ligand release. This novel finding of Src-mediated GRP-induced EGFR ligand release opens a new research perspective. Src can serve as a potentially therapeutic target to achieve anti-tumor effects by inhibiting cancer cell growth and invasion. Currently, there are several c-Src inhibitors in preclinical or phase I clinical trials, such as SU6656 and SKI606 (146, 147). In addition to monotherapy, when combined with EGFR inhibitor gefitinib, the Src inhibitor A-419259 enhanced the effect of gefitinib on cytotoxicity by 30%. Also, cell invasion ability was further decreased by Src inhibitor treatment (data not shown).

4.2. Identification of TACE/ADAM17 as the major ADAM family member responsible for GRP induced EGFR signaling

Previous research showed that TACE/ADAM17 mediated GPCR ligand-induced EGFR phosphorylation in HNSCC. However, the mechanism underlying the GPCR ligand-induced TACE activation remains to be elucidated. The two most important domains of TACE are the metalloproteinase domain and the cytoplasmic domain. The metalloproteinase domain mainly mediates transmembrane molecule cleavage upon its activation, while the cytoplasmic domain is chiefly responsible for interacting with intracellular molecules and mediating TACE activity. Since the cytoplasmic domain of TACE contains multiple PXXP domains and proline rich

sequences, it's not surprising that Src can interact with TACE and mediate its activity. Here I show that upon GRP induced Src activation, TACE undergoes a Src kinase dependent translocation from the cytoplasm to the periphery part of cells, where TACE is phosphorylated directly by PDK1 kinase followed by EGFR ligand cleavage. The remaining question is whether TACE phosphorylation is correlated with its activity. We have some preliminary data showing that when blocking Src kinase, TACE activity is dramatically reduced, indicating that TACE phosphorylation is important for its activity (data not shown). Further research will be needed to characterize the PDK induced TACE phosphorylation sites on the cytoplasmic domain *in vitro* and *in vivo.* Once confirmed, TACE mutants will be used to test whether TACE phosphorylation is important for its activity.

Broad spectrum inhibitors for MMP and ADAM family members used in clinic trials to date have not shown good therapeutic potential due to serious side effects such as tendonitis or fibroplasias (148). The MMP inhibitor Marimastat was used to treat pancreatic cancer patients in phase III clinical trials but there was no survival advantage compared to placebo treated patients (149). A TACE inhibitor developed by Dupont/Bristol Myers was discontinued after phase II clinical trials, possibly due to toxic side effects (150). The potential drawback for TACE inhibition strategy is that TACE mediates the cleavage of many transmembrane molecules and nonselective inhibition could cause malfunction of normal cells. The mechanism by which TACE mediates normal cell function remains to be elucidated. Inhibitors that specifically block TACE function in cancer cells but not in normal cells would be ideal therapeutic reagents but may not be feasible.

4.3. PDK1 as a novel therapeutic target

Elucidation of the critical mediators of GPCR/EGFR crosstalk has important clinical implications. Here we show that PDK1 directly mediated TACE phosphorylation induced by GRP. PDK1, as a kinase at the hub of many signaling pathways, has been reported to bring key signaling molecules into proximity, activate cell signaling through translocation and induce receptor signaling complex nuclear localization for downstream molecule activation such as NF-κB (151). Inhibition of PDK1 and Akt with a broad-spectrum kinase inhibitor, staurosporine, promotes apoptosis in a variety of cancer cells (152). However, because of nonselectivity, this compound produces very toxic effects (153). Another potent PDK1 inhibitor, 7-hydroxystaurosporine (UCN-01), has been reported to inhibit tumor cell growth and promote apoptosis, which is supported by promising results in phase I clinical trails (154, 155). Unfortunately, this drug is not specific for PDK1, either. Design of specific PDK1 inhibitors would be desirable. PDK1 antisense oligonucleotide treatment has shown to reduce glioblastoma cell proliferation and survival (156). Since PDK1 has only one isoform, compared with three isoforms of Akt, the design of a specific inhibitor for PDK1 is feasible and may provide more efficacious therapy for cancer patients.

Results of the present study suggest that the combination of PDK1 targeting with EGFR blockade may enhance the therapeutic effects of EGFR inhibitors. Amphiregulin secretion has been reported to inhibit gefitinib induced cacner cell apoptosis, which is responsible for the resistance of lung cancer cells to gefitinib, one of the EGFR tyrosine kinase inhibitors (157). Since PDK1 activity contributes to EGFR ligand release, inhibition of PDK1 may enhance the anti-tumor effects by increasing the sensitivity of tumor cells to EGFR inhibitor treatment. In addition to EGFR pathways, PDK1 targeting may block EGFR independent pathways that might

contribute to cancer cell mitogenic signaling and invasion ability. By reverse phase protein microarray (RPPA), p70S6K was identified to be the protein that acts downstream of PDK1 but indepdently on EGFR. Targeting PDK1 kinase may provide an efficient and novel strategy to inhibit tumor cell growth, survival and invasion.

4.4. Combined targeting of GRPR and EGFR in head and neck cancer

The observation that elevated levels of growth factor receptors are associated with adverse cancer outcome, has led to the development of approaches which specifically interrupt these autocrine pathways. The Grandis lab targeted EGFR *in vitro* and *in vivo* using several strategies and found selective growth inhibition and anti-tumor efficacy (56, 57). Based on the promising results of phase I/II studies, phase III clinical trials (using monoclonal antibodies or EGFR-specific tyrosine kinase inhibitors) are presently testing the efficacy of EGFR targeting strategies in patients with head and neck cancer. However, the response rates of HNSCC patients treated with EGFR inhibitors alone remain below 20%. The research in this thesis investigated EGFR activation by GRPR signaling pathways. In addition, GRP has been suggested to promote expression of proangiogenic factor expression and induce angiognesis in endothelial and cancer cells (158, 159). GRP/GRPR may serve as a therapeutic target in GRPR-expressing malignancies. GRP blocker 77427 treatment inhibited tumor growth in vitro and in vivo (159). Studies have also demonstrated anti-tumor efficacy using GRPR-specific inhibitors RC-3940II and RC3095 in preclinical animal models (160, 161). A phase I clinical trial in lung cancer patients using a monoclonal antibody 2A11 against GRP demonstrated no evidence of toxicity (162). Anti-tumor activity has been observed with this anti-GRP Ab in patients with small cell lung cancer (163). 2A11 treatment in a phase II trial in relapsed small-cell lung cancer showed

partial response in patients(164). This thesis provides evidence of enhanced anti-tumor effects of GRPR and EGFR targeting strategies in head and neck cancer. Our results suggest that combined inhibition of GPCR and EGFR pathways in a variety of cancer types may potentially improve cancer therapy.

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6. APPENDIX

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Figure 1. The diversity of the EGFR signaling network

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