Investigation of the mechanisms and therapeutic implications of crosstalk between G-protein-coupled receptors and the Epidermal Growth Factor Receptor in HNSCC

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Investigation of the mechanistic and therapeutic implications of crosstalk between G-protein-coupled receptors and Epidermal Growth Factor Receptor in HNSCC

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Head and neck squamous cell carcinoma (HNSCC) is characterized by the overexpression of the epidermal growth factor receptor. However, molecular targeting strategies against EGFR have not improved the 5-year survival rates of HNSCC patients. EGFR tyrosine kinase inhibitors displayed limited clinical responses in Phase II trials and the FDA-approved monoclonal antibody cetuximab (C225) did not prevent the occurrence of secondary tumors and distant metastases. G-protein-coupled receptor ligands; gastrin-releasing peptide (GRP), prostaglandin E2 (PGE2) and bradykinin (BK) have all been reported to activate EGFR in HNSCC via extracellular release of EGFR ligands TGF-α and AR. To improve the efficacy of EGFR inhibition in HNSCC, we investigated the efficacy of targeting common signaling intermediates involved in GPCR-EGFR crosstalk.

We previously reported that GRP mediated release of EGFR ligands via phosphoinositide-dependent kinase 1 (PDK1) – dependent phosphorylation of a disintegrin and metalloprotease 17 (ADAM17). We subsequently investigated whether PDK1 mediates EGFR activation downstream of PGE2, BK and LPA pathways and the efficacy of different PDK1 targeting strategies in HNSCC. PGE2, BK and LPA-mediated EGFR phosphorylation was abrogated in PDK1 siRNA-transfected HNSCC cells. PDK1 siRNA also decreased PGE2 and BK-mediated HNSCC growth *in vitro*. Expression of kinase-dead PDK1 (PDK1M) decreased PGE2 -mediated HNSCC growth. PDK1M HNSCC cells demonstrated reduced proliferation

iv

compared to control HNSCC cells. HNSCC cells displayed nanomolar sensitivity to the PDK1 inhibitor OSU-03012 compared to normal mucosal cells. Combined treatment with the EGFR TKIs erlotinib or AG1478, plus OSU-03012 enhanced anti-proliferative effects.

We have reported that PGE2 and BK mediated MAPK phosphorylation in the presence of EGFR inhibition, and combined GPCR and EGFR demonstrated additive to synergistic anti-tumor effects. To elucidate the EGFR-independent signaling mediated by GPCRs, we used a forward phase phosphoprotein array to identify potential molecular targets that can potentiate EGFR inhibition. We observed that p70S6K phosphorylation was induced in EGFR siRNA-transfected cells and sustained in cetuximab (C225)-treated cells following PGE2 or BK stimulation. Further investigation showed that p70S6K phosphorylation mediated by EGFR downmodulation was dependent on PDK1 and PKCδ expression. Combined targeting of EGFR with cetuximab and p70S6K with the mTOR inhibitor RAD001 decreased GPCR-mediated growth *in vitro* and *in vivo.*

The results from this study have indicated that targeting the GPCR signaling intermediates PDK1 and p70S6K in conjunction with EGFR may be beneficial therapeutic strategies for the subset of HNSCC patients that respond poorly to cetuximab treatment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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LIST OF ABBREVIATIONS

AR	Amphiregulin
BK	Bradykinin
COX-2	Cyclooxygenase-2
EGFR	Epidermal growth factor receptor
FCS	Fetal calf serum
FDA	Food and Drug Administration
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GRP	Gastrin-releasing peptide
GTP	Guanosine triphosphate
HNSCC	Head and neck squamous cell carcinoma
IRS1	Insulin receptor substrate 1
LPA	Lysophosphatidic Acid
MEF	Murine embryonic fibroblast
MMP	Matrix metalloprotease
mTOR	Mammalian target of rapamycin
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NoTx	No Treatment
NSCLC	Non small cell lung cancer
NTC siRNA	Non targeting control small interference RNA
OD	Optical density
PBS	Phosphate buffer saline
PDK1	Phosphoinositide-dependent kinase 1
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylimide gel electrophoresis
siRNA	Small interference RNA
TACE	TNF-α converting enzyme
TGF-α	Transforming growth factor alpha
TKI	Tyrosine kinase inhibitor
TMA	Tumor Micorarray

PREFACE

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1.0   INTRODUCTION

1.1   GENERAL INTRODUCTION

1.1.1   Introduction

Cancer cells harness multiple signaling pathways to proliferate, invade, and resist the cytotoxic effects of therapy, thereby contributing to tumor invasion and metastasis. Elucidation of these signaling pathways has enabled the identification of molecular targets that can be used for cancer therapy. Among the molecular targets discovered to date, growth factor receptors have been most amenable to the design of targeting strategies including monoclonal antibodies and kinase inhibitors. Growth factor receptors that are implicated in cancer development and/or progression include the epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGF-R), fibroblast growth factor receptor (FGF-R) and platelet-derived growth factor receptor (PDGFR).

EGFR is frequently overexpressed in epithelial tumors including those arising in the colon, lung, breast and head and neck where expression levels correlate with decreased five year survival rates [1]. These tumors also express high levels of EGFR ligands such as transforming growth factor α (TGF-α), amphiregulin, and heparin-binding EGF (HB-EGF), implicating autocrine regulatory pathways. Autocrine and paracrine activation of EGFR results in the

activation of the intracellular tyrosine kinase domain and the consequent recruitment of docking proteins that mediates downstream signaling. There are currently several EGFR inhibitors that are either FDA approved for cancer therapy or are under active clinical development. These inhibitors target the tyrosine kinase domain (e.g. erlotinib or gefitinib) or the extracellular ligand-binding domain (e.g. cetuximab or pamitumimab).

Despite the widespread overexpression of EGFR in these tumors and the correlation of EGFR levels with survival, targeting strategies that have demonstrated efficacy in preclinical models have proven effective in only a subset (10-15%) of cancer patients. One possible explanation for this low response rate is the interaction of EGFR with other cell surface receptors that mediate multiple signaling pathways. Several reports have indicated that interaction between IGF-1R and EGFR contributed to the increased proliferation and metastasis of pancreatic and breast cancer [2, 3]. EGFR was also reported to transactivate PDGFR in vascular smooth muscle cells (VSMC) and interact with Fas death receptor to affect cellular survival [4, 5]. GPCRs, the family of heptahelical receptors, have been shown to activate EGFR and play an integral role in cancer progression. GPCRs activate EGFR via two possible mechanisms: 1) increased EGFR ligand production; and 2) intracellular tyrosine kinase domain activation.

Studies on the GPCR-mediated activation of EGFR have implicated distinct signaling pathways, depending on the specific GPCR and cell type under investigation. Over 50% of the drugs that are currently developed target GPCRs due to the relative ease of inhibiting these receptors and their role in many diseases, in addition to cancer [6]. However, the heterogenous nature of tumors suggests that a single GPCR cannot be responsible for activating EGFR and contributing to cancer progression. The identification of heterotrimeric G-protein inhibitors [7] and common signaling intermediates among various GPCRs will allow for the

development of targeting strategies that block this process in general. In addition, reports indicating that GPCRs can mediate mitogenic signaling independent of EGFR activation suggest that combined targeting of EGFR and GPCRs may be an effective therapeutic strategy.

In this review, we will focus on tumors that are currently being treated with FDA approved EGFR inhibitors (Table 1), although GPCR-EGFR crosstalk has been reported in other malignancies. The similarities and differences in the signaling mechanisms among the specific GPCRs and tumor types will be highlighted.

1.1.2 Cancer

According to the National Cancer Institute (NCI), cancer is defined as multiple diseases that are characterized by the uncontrollable growth of deviant cells that may gain the ability to invade other tissues in the body[1]. There are many types of cancers, which are diagnosed according to the specific cell type and organ of origin. Cancer is the result of genetic abnormalities that can be induced by risk factors ranging from tobacco and alcohol abuse to viral infection and stress. Disruption of the DNA replication process and inheritance of specific mutations are also the result of the genetic abnormalities that cause cancer.

Advances in surgical and chemoradiation techniques, and drug development has led to a decrease in the diagnosis and mortality of cancer as reported in the 2009 version of NCI's Annual report. Although there is a decrease, the number of new cancer cases and related deaths in the United States for the year 2009 is 1,479,350 and 562,340 respectively[1].

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[1] http://www.cancer.gov/

1.1.3   Head and Neck Squamous Cell Carcinoma (HNSCC)

Approximately 90% of all head and neck cancers are of the squamous cell type. This type of cancer is located in the upper aerodigestive tract including the oral cavity, larynx, pharynx and nasal cavity [8]. HNSCC primarily metastasizes to the lymph nodes in the neck region and is usually the first diagnostic indicator of HNSCC [9]. HNSCC are primarily caused by tobacco and alcohol use [10]. A subset of HNSCC cases, specifically of the oropharynx, have also been attributed to HPV infection [11].

HNSCC is the 6th most common neoplasm worldwide accounting for approximately 50,000 deaths in the United States annually [12]. Current treatment includes surgery, chemotherapy and molecular targeted therapy. Although advances have been made in treatment techniques, the overall 5-year survival of HNSCC patients remains low. In 2006, the EGFR monoclonal antibody cetuximab (Erbitux) was FDA-approved for the treatment of HNSCC patients in combination with radiation therapy. However, HNSCC patients that develop recurrent metastatic HNSCC tumors display poor response to targeted therapy and poor survival rates [13].

1.2   EGFR IN CANCER

1.2.1   EGFR

EGFR or HER1 is a member of the ErbB family of cell surface tyrosine kinase receptors, which includes HER2/neu, HER3 and HER4. With the exception of HER2/neu, the other ErbB members are activated in a ligand-dependent manner. There are eight known ligands for EGFR

including EGF, TGF-α, amphiregulin, HB-EGF, betacellulin, epiregulin, epigen and crypto [14, 15]. The ligands for HER3 and HER4 are a group of proteins known as neuregulins [16]. Unlike HER2, HER3 contains an extracellular binding domain but has an inactive kinase domain. HER2 has a functional kinase domain, therefore HER2 and HER3 generate strong downstream signaling via heterodimerization with each other or with EGFR [17-19]. EGFR ligand binding induces homo- and/or hetero-dimerization of the ErbB receptors and phospho-tyrosine recruitment of proteins to docking sites on the intracellular portion of the receptor that mediate downstream signaling cascades [1, 20]. Heterodimers between EGFR and HER2 or HER3 result in more potent signaling cascades compared with EGFR homodimers, which include the MAPK, JNK and PI3K pathways. These signaling cascades have pleiotropic effects on cellular behavior.

EGFR activation in cancer cells results in increased DNA synthesis, proliferation, metastasis and angiogenesis [21]. Overexpression of EGFR in cancer has been correlated with poor prognosis in cancer patients [22]. There are several FDA approved EGFR inhibitors for specific cancer types and a large number of ongoing clinical trials using EGFR inhibitors in combination with other agents, including chemotherapy, radiation and/or molecular targeting strategies.

1.2.2   EGFR in HNSCC

Over 90% of HNSCC tumors overexpress EGFR and its ligand Transforming growth factor α (TGF-α) [23, 24]. The autocrine and paracrine secretion of TGF-α activates EGFR and its downstream mitogenic signaling pathways. The expression of EGFR is also correlated to advanced stage and poor differentiation of HNSCC tumors [23, 25]. In addition to overxpression in the tumors, EGFR expression is also observed in the normal adjacent regions. EGFR

overexpression in HNSCC is a result of transcriptional upregulation, which may be due to EGFR amplification and polymorphisms in intron 1 of the EGFR gene [26, 27]. EGFR expression in HNSCC is not only limited to the cell membrane but has been reported in the nucleus. Nuclear expression of EGFR has been linked to high recurrence rate and lower progression-free survival [28].

The overexpression of EGFR in HNSCC has made it an ideal therapeutic target in HNSCC. Preclinical targeting of EGFR in HNSCC displayed potent anti-tumor effects [29] . The EGFR monoclonal antibody Cetuximab displayed 100% patient responses in combination with radiation in HNSCC patients with no prior treatment [13]. The results from that clinical trial led to the FDA-approval of cetuximab to be used in combination with radiation for HNSCC patients. The EGFR targeting agent was the first FDA-approved agent for HNSCC in over 45 years.

1.3   G-PROTEIN COUPLED RECEPTORS (GPCRS) IN CANCER

1.3.1   GPCR Signaling

GPCRs are seven transmembrane receptors that mediate their signaling via a heterotrimeric G-protein complex. They comprise a large family of receptors that play critical roles in a wide variety of processes including sight, smell, cardiovascular health, and cancer progression [30]. GPCRs signal via a heterotrimeric small G-protein complex, Gαβγ. Agonist binding to GPCRs results in the exchange of GDP for GTP on the Gα subunit and its dissociation from the tightly bound Gβγ dimer [6, 31]. The Gα and Gβγ subunits mediate their own signaling cascades that are GPCR and cell type-specific. The Gα subunit is further divided into other

6

subtypes including G_i, G_q, G_s and $G_{12/13}$. The G_i and G_s subunits couple to the second messenger protein adenyl cyclase leading to inhibition and activation of adenyl cyclase and cAMP generation respectively. The Gq subunit activates phospholipase-beta (PLC-beta) and calcium signaling cascades while $G_{12/13}$ activates the guanine exchange factor, Rho. Recent studies on Gβγ have suggested that these subunits play a role in the activation of PLC, PI3K and adenyl cyclase [30]. Young *et al* identified the first oncogene that was a member of the GPCR family of receptors called *MAS* [32]. Following the identification of *MAS* in 1986, further investigation showed that overexpression of GPCRs and their respective ligands led to cancer phenotypes in breast and oral squamous cell carcinoma [33, 34].

1.3.2   GPCRs and HNSCC

G-protein coupled receptors have been reported to play significant roles in cancer progression [6]. Multiple GPCR agonists are expressed in the serum of cancer patients, where expression of these agonists is correlated with poor prognosis. Some of the GPCR agonists expressed in HNSCC include pro-inflammatory mediators PGE2 and bradykinin, lysophosphatidic acid (LPA), and thrombin. Bradykinin signals via two receptors B1R and B2R. It was previously shown that HNSCC tumors overexpress B2R compared to normal tissue [35]. In addition to other reports demonstrating increased COX-2 activity in HNSCC [36, 37], it was indicated that BK induces COX-2 transcriptional upregulation and subsequent extracellular release of another GPCR ligand PGE2 [35]. Reports have shown increased serum PGE2 levels in HNSCC patients [38]. In oral squamous cell carcinoma (OSCC), the PAR-1 receptors that are activated by thrombin are overepxressed [34]. Thrombin was also reported to activate EGFR in HNSCC cell lines [39]. LPA found in the serum has been shown to display high mitogenic

capabilities and is reported as a potential biomarker in gynecological cancers [40, 41]. Gschwind et al demonstrated that LPA induced EGFR phosphorylation in HNSCC cell lines [39].

1.4   GPCR-EGFR CROSSTALK IN CANCER

1.4.1   GPCR-EGFR Crosstalk

Daub e*t al* published the first report on the activation of EGFR by GPCRs in Rat-1 fibroblasts [42]. Stimulation of these cells with the GPCR ligands, lysophosphatidic acid (LPA), endothelin-1 (ET-1), and thrombin induced phosphorylation of EGFR and its downstream target Erk1/2. Following this discovery, the Ullrich group and others demonstrated that this transactivation phenomena occurred in different cell types including vascular smooth muscle cells (VSMC), keratinocytes, PC-12 cells and multiple cancer cell lines [39, 43, 44]. Activation of EGFR following GPCR stimulation is mediated by both ligand-dependent and ligand-independent mechanisms. Ligand-independent activation of EGFR was shown to occur via Src-dependent activation of the intracellular tyrosine kinase domain of EGFR [45, 46]. Another possible ligand-independent mechanism of GPCR activation of EGFR may be via inactivation of protein tyrosine phosphatase (PTP) by NADPH-mediated release of reactive oxygen species (ROS). Fisher et al reported the LPA-induced activation of the RTK c-Met via NADPH-mediated release of ROS [47]. The role of ROS in GPCR-mediated activation of EGFR in cancer is incompletely understood. Matrix metalloprotease (MMP) inhibitors abrogated GPCR-mediated EGFR activation in some cell systems leading to the development of the "triple membrane pass system" (TMPS) model where GPCRs mediated the cleavage of EGFR

proligands in a MMP-dependent manner [48, 49]. Further investigation demonstrated that the MMP involved in this process was from the ADAM (a disintegrin and metalloprotease) family of metalloproteases. ADAM family members 10, 12 and 17 have been reported to be responsible for the cleavage of TGF-α, AR and HB-EGF in a GPCR ligand and cell-type specific manner [39, 50, 51]. With respect to cancer, ADAM17 overexpression was observed and shown to mediate GPCR-induced ligand-dependent activation of EGFR in colon cancer [52, 53].

Table 1. GPCR Ligands reported to activate EGFR and tumorigenesis according to tumor type

Malignancy	GPCR Ligands	References
Colon Cancer	Thrombin PGE2 LPA ET-1	(41, 74) (35) (37, 75) (36)
NSCLC	GRP PGE2 IL-8	(46, 47) (48) (45)
HNSCC	LPA Thrombin GRP PGE2 BK	(24) (24) (22, 51) (54) (54)
Pancreatic cancer	CCK Vasopressin BK	(61) (59) (59)

1.4.2   The role of GPCR-EGFR Crosstalk in Cancer

1.4.2.1 Colon Cancer

In the United States, colon cancer is the second leading cause of cancer-related death. Crosstalk between GPCRs and EGFR play a critical role in the activation of the Wnt signaling pathway. In human colon cancer cell lines, the GPCR ligand prostaglandin E2 (PGE2)

transactivates both EGFR and c-Met-R and results in the increased nuclear accumulation of β-catenin and cellular invasion. Inhibition of EGFR abrogated PGE2- mediated invasion *in vitro* [54]. Endothelin-1 (ET-1) has been shown to promote tumorigenesis in colorectal cancer via the upregulated Endothelin A (ETA) receptor. In the HT29 colon cancer cell line, ET-1 stimulation induced increased proliferation and DNA replication. The ET-1 mediated effects were dependent on PI3K, protein kinase C (PKC) and EGFR. Inhibition of EGFR resulted in a significant decrease on ET-1 stimulated proliferation [55]. Lysophosphatidic Acid (LPA) was reported to activate EGFR and induce the expression of cyclooxygenase-2 (COX-2) in colon cancer cells [56]. LPA can interact with three GPCRs, LPA1, LPA2 and LPA3, although LPA2 is primarily overexpressed in colon cancer [57, 58]. The induction of COX-2 was shown to be dependent on the presence of EGFR and resulted in increased mitogenesis. This observation has therapeutic implications since COX-2 is responsible for the production of PGE2. Therefore, crosstalk between LPA and EGFR can further potentiate EGFR activation via PGE2. There is a report indicating that the pan-COX inhibitor sulindac decreased EGFR activation and expression in HT-29 cells [59].

Metastatic colon cancer has been correlated with the expression of the protease-activated receptors (PAR1). PAR1 is one of three PARs that are the cognate receptors for thrombin. Darmoul *et al* reported that thrombin-mediated colon cancer cell proliferation resulted via crosstalk between PAR1 and EGFR. The activation of EGFR was also shown to be dependent on Src and MMP-mediated release of the EGFR ligand TGF-α [60]. Antibody-based neutralization of TGF-α and tyrosine kinase inhibition of EGFR completely reverted the thrombin-induced increase in colon cancer cell proliferation *in vitro*. Unlike thrombin, interleukin-8 (IL-8) was shown to transactivate EGFR via the release of HB-EGF and promote Caco-2 proliferation and

migration [61]. Combined inhibition of EGFR, HB-EGF and MMPs completely blocked IL-8 mediated proliferation. Thus, there are multiple GPCRs that are overexpressed in colon cancer that promote tumor progression via the activation of EGFR where EGFR activation is dependent on MMP/ADAM-mediated cleavage of different EGFR proligands. Therefore, inhibition of MMP, Src and COX-2 may be effective therapeutic options for colon cancer patients.

The role of COX-2 and IL-8 in colon cancer emphasizes the link between cancer and inflammation. For example, COX-2 expression has been reported to play a significant role in ulcerative colitis-associated colon cancer [62]. The contribution of inflammatory mediators in bridging inflammatory disease to cancer is an expanding field of study. Crosstalk of GPCRs with EGFR in inflammatory diseases may reflect an early event in colon carcinogenesis that can be exploited for diagnostic purposes.

1.4.2.2 Non-small cell lung cancer

In comparison to other malignancies, lung cancer is among the most lethal [63]. Hiemstra et al showed that the cytokine interleukin-8 (IL-8) mediated the proliferation of NSCLC. Inhibition of EGFR with the tyrosine kinase inhibitor AG1478 and an EGFR blocking antibody decreased IL-8-mediated proliferation of A549 *in vitro*. The mechanism for IL-8-induced EGFR activation was shown to be MMP-dependent. The specific MMP or MMP family molecule responsible for EGFR activation is unknown, but IL-8 has been reported to activate the extracellular release of EGFR proligands [64]. Another G-protein coupled receptor that is aberrantly expressed in NSCLC is the gastrin-releasing peptide receptor (GRPR). Treatment of NSCLC cells with gastrin releasing peptide (GRP) was shown to activate EGFR and phosphorylation of Erk where Erk activation induced NSCLC proliferation [65, 66]. The

activation of EGFR by GRP was also shown to be sensitive to MMP inhibition and HB-EGF and TGF-α neutralization.

GPCRs that activate EGFR have also been described to activate proliferative signals independent of EGFR. PGE2 was reported to activate EGFR, however in the presence of EGFR inhibition, MAPK was still activated in NSCLC cell lines. The activation of MAPK was also reported to be resistant to Src and MMP-inhibition. The key finding in this report showed that PGE2-mediated activation of MAPK in NSCLC was dependent on PKC [67]. Combined inhibition of EGFR and COX-2 had a significant effect on decreasing proliferation.

1.4.2.3 Pancreatic cancer

Pancreatic cancer is one of the deadliest cancers of the digestive system. In 2004, approximately 31,000 new cases and deaths were recorded [68]. Pancreatic tumors overexpress EGFR and their respective ligands [20, 69, 70] which also correlates to chemotherapeutic resistance[71]. Pancreatic cell lines have also been shown to respond to multiple GPCR agonists including cholecystokinin (CCK), bradykinin, vasopressin and neurotensins [72, 73]. Piiper et al reported that CCK and gastrin activated EGFR in the AR42J pancreatic cell line [74]. CCK and gastrin-mediated activation of EGFR was also shown to be dependent on the Src family kinase, Yes. The co-immunoprecipitation of Yes to EGFR indicated that CCK and gastrin mediated a ligand-independent activation of EGFR and its downstream effector MAPK. However, CCK also activated MAPK by another pathway, which was PKC-dependent and EGFR-independent [74]. Another report showed that neurotensins also activated MAPK independent of EGFR via the PKC-dependent pathway [72]. PKC has been reported to activate MAPK following GPCR activation through the direct activation of Ras [75].

12

Src, PKC and Ras have been shown to play critical roles in GPCR-mediated activation of EGFR and MAPK in pancreatic cancer cell lines [72, 74]. In contrast to the colon, NSCLC and HNSCC, GPCR-EGFR crosstalk in pancreatic cancer has been reported to be primarily an intracellular process. Inhibition of these intracellular molecules in combination with erlotinib, which is FDA approved for pancreatic cancer [76], may be an efficacious therapeutic strategy.

1.4.3   GPCR-EGFR Crosstalk in HNSCC

Similar to NSCLC, GRPR is overexpressed in HNSCC compared to the normal mucosa [77]. Lui et al reported that GRP induced the activation of EGFR and its mitogenic surrogate MAPK in HNSCC cells [78]. Further investigation indicated that the activation of EGFR was dependent on Src, ADAM17 (TACE) and extracellular release of amphiregulin. A novel role for phosphoinositide-dependent kinase 1 (PDK1) was also discovered to induce phosphorylation of TACE following GRP stimulation [79, 80]. In addition, PDK1 downmodulation and EGFR inhibition significantly decreased HNSCC proliferation *in vitro*. Two other GPCR ligands, PGE2 and bradykinin (BK) have been shown to activate EGFR and promote HNSCC proliferation and invasion *in vitro* [81]. Activation of EGFR by PGE2 and bradykinin was reported to be dependent on Src, ADAM17 and TGF-α release. In addition to EGFR activation, PGE2 and BK were reported to activate MAPK in the presence of EGFR tyrosine kinase inhibition [81]. Combined inhibition of the PGE2 and BK pathways and EGFR resulted in significant decreases in HNSCC proliferation and invasion in vitro. Three other GPCR ligands, LPA, thrombin and carbachol were reported to activate EGFR in HNSCC cell lines, however their influence on a HNSCC phenotype were not investigated [39]. With respect to signaling mechanisms, LPA-mediated activation of EGFR was reported to be MMP-dependent.

13

Table 2. Preclinical studies of combined inhibition of GPCR and EGFR in different tumor types

Malignancy	Clinical Trials	Reference
Colon Cancer	Sulindac and EKI-569	(76)
NSCLC	CU201 and gefitinib sc58236 and erlotinib	(77) (48)
HNSCC	CU201 and gefitinib Sulindac and erlotinib PD176252 and erlotinib Celecoxib and gefitinib	(54) (54) (65) (78, 79)
Pancreatic Cancer	Celecoxib and erlotinib	(80)
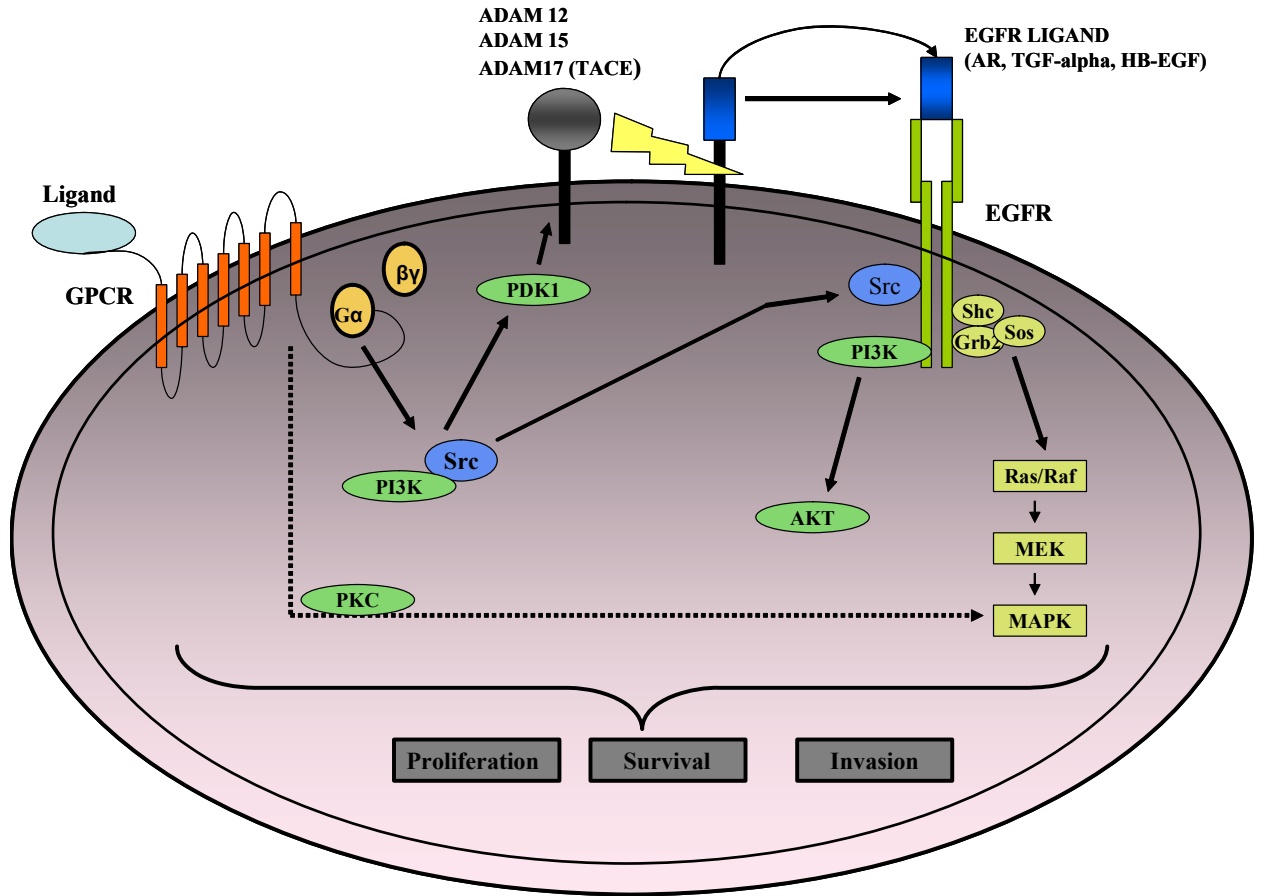
Figure 1. Model of GPCR-EGFR Crosstalk in Cancer.

Black unbroken arrows indicate GPCR-mediated activation of EGFR via both ligand-dependent and independent mechanisms. The broken Arrow indicates GPCR-mediated EGFR-independent activation of mitogenic signaling. Model illustrates key signaling intermediates involved in GPCR-EGFR crosstalk, which includes Src, PDK1 and ADAMs. GPCR, G-protein-coupled receptor; EGFR, epidermal growth factor receptor; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol-3 kinase; ADAM, a disintegrin and metalloprotease; AR, amphiregulin; TGF-α, transforming growth factor α; HB-EGF, heparin binding-epidermal growth factor.

15

1.5   SUMMARY AND HYPOTHESIS

1.5.1   Summary

Despite the widespread overexpression of EGFR in most epithelial malignancies, EGFR targeting alone has not resulted in dramatic clinical responses in the absence of EGFR activating mutations in selected NSCLC. Transactivation of EGFR by GPCRs may contribute to the continued growth of cancers in the setting of EGFR blockade. In addition, GPCRs have been shown to mediate mitogenic signaling pathways independently of EGFR [67, 72, 74, 81]. Both PGE2 and bradykinin have been shown to induce cancer cell proliferation in an EGFR-independent fashion in NSCLC and HNSCC. CCK and neurotensin were also shown to activate MAPK via a PKC-Ras interaction that is EGFR-independent. The EGFR-independent activation of ERK by GPCRs has been called multi-track signaling, where EGFR may effect a dual function by activating signaling components via its kinase domain or functioning as a scaffold to Ras/Raf/MEK complex which is necessary for Erk activation independent of its kinase function [82]. Preclinical studies have shown that combined inhibition of GPCR pathways and EGFR results in additive or synergistic growth inhibition in HNSCC and NSCLC [67, 81, 83]. Therapeutic strategies that inhibit both GPCR and EGFR pathways may prove effective as cancer treatment.

Preclinical studies inhibiting GPCR and EGFR pathways have been reported in colon, HNSCC, NSCLC and pancreatic cancer (Table 2). These studies have primarily utilized non-steroidal anti-inflammatory agents such as celecoxib or sulindac to inhibit the BK and PGE2 GPCR signaling modalities. Combined inhibition of COX-2 and EGFR has shown additive or synergistic effects in colon, NSCLC, HNSCC and pancreatic cancer models. Clinical trials to

evaluate the efficacy of COX inhibitors in combination with EGFR inhibitors are underway in several cancers. There were two clinical trials that combined the COX-2 inhibitor celecoxib with the EGFR inhibitors erlotinib and gefitinib in NSCLC [84, 85]. HNSCC and colon cancer patients are currently being enrolled on phase I/II studies using COX and EGFR inhibitors (www.clinicaltrials.gov).

In the absence of clinical inhibitors to other GPCRs such as thrombin, IL-8 and CCK, inhibition of proteins implicated in the transactivation mechanism may prove beneficial. For example, Src family kinases and TNF-α converting enzyme (TACE) have consistently been identified to mediate the activation of EGFR by a variety of GPCRs in several tumor systems. The Src family kinase inhibitor dasatanib (Sprycel) is FDA approved for the treatment of chronic myelogenous leukemia (CML) and selected leukemias and is under early stage investigation in solid tumors [86]. Because Src mediates both ligand-dependent and − independent GPCR-mediated activation of EGFR, dasatinib in combination with an EGFR inhibitor may have significant anti-tumor effects. TACE may be another key target in abrogating the effects of GPCR-EGFR crosstalk in tumors. The TACE inhibitor INCB3619 in combination with gefitinib decreased NSCLC proliferation both *in vitro* and *in vivo* [87]. Two other TACE inhibitors, TMI-2 and Ro-32-7315, have shown to be potent in the treatment of preclinical arthritis models [88, 89]. Both orally administered TACE inhibitors also lower associated toxicities compared to clinical grade broad range MMP inhibitors. The efficacy of TMI-2 and Ro-32-7315 are yet to be tested in a tumor model. Furthermore, TACE inhibition not only abrogates EGFR ligand production but HER3 ligand production also [87, 90, 91]. Therefore, targeting TACE may be an effective treatment strategy in tumors that are driven by GPCR-EGFR crosstalk and HER3 signaling [91]. However, it is unknown whether GPCRs can transactivate HER3 in a ligand-

dependent manner independently of EGFR. Gschwind et al reported that LPA induced the EGFR-dependent phosphorylation of HER2 in HNSCC, indicating the LPA induced EGFR homodimer and EGFR/HER2 heterodimer formation [39]. Future investigation of GPCR-mediated HER2/HER3 dimerization in tumor types driven by HER3 signaling is needed.

1.5.2   Hypothesis

Head and neck squamous cell carcinoma (HNSCC) is characterized by overexpression of the Epidermal Growth Factor Receptor (EGFR) where expression levels in the primary tumor correlate with survival [92]. Although EGFR-specific tyrosine kinase inhibitors (TKI) have shown promising preclinical results, low response rates (5%) have been observed when these agents were administered to HNSCC patients [93]. The modest response of HNSCC patients to EGFR TKI suggests that HNSCC progression may result from alternative routes of EGFR activation and/or EGFR-independent mechanisms. G-protein-coupled receptors (GPCRs) have been reported to activate EGFR and promote progression in many tumors including breast, colon, lung and HNSCC [67, 94-96]. We and others have reported that several GPCR ligands including prostaglandin E2 (PGE2), bradykinin (BK), gastrin releasing peptide (GRP), lysophosphatidic acid (LPA) and thrombin can activate EGFR signaling and contribute to HNSCC growth and invasion [39, 78, 96]. These cumulative findings suggest that targeting both GPCR and EGFR pathways may demonstrate therapeutic efficacy in this cancer. We have shown that combined inhibition of PGE2 and/or BK receptors with EGFR inhibition had additive or synergistic effects decreasing HNSCC survival *in vitro* and *in vivo* [96]. The heterogeneity and ubiquitous expression of GPCRs in HNSCC indicates that targeting a single upstream GPCR in combination with EGFR blockade may not effectively inhibit downstream signaling pathways.

However, multiple GPCRs signal through common intermediates that have been implicated in EGFR activation by GPCR including Src, PI3K, TACE and the EGFR proligands. There is an increasing availability of molecular targeting agents that are either approved for clinical use or in late pre-clinical development directed against these intermediates. **The central hypothesis of this thesis is that GPCRs mediate activation of EGFR and HNSCC progression via specific intermediates that may serve as effective therapeutic targets. Furthermore, I hypothesize that combined inhibition of the molecular targets downstream of GPCRs with EGFR blockade will have significant anti-tumor effects in HNSCC.**

In the following studies I used the GPCR ligands, PGE2, BK and LPA. In Chapter 3, I focused on BK because it was previously published that BK mediated the release of PGE2 and HNSCC tumors overexpressed the Bradykinin-2-receptor (B2R) compared to normal adjacent [35]. Those findings made the BK pathway more interesting to investigate.

2.0 TARGETING PDK1: A COMMON SIGNALING INTERMEDIATE IN GPCR-EGFR CROSSTALK IN HNSCC

2.1 INTRODUCTION

G-protein-coupled receptors are seven-transmembrane receptors that mediate various signaling pathways that contribute to growth, survival and cellular motility. The GPCR ligands gastrin-releasing peptide (GRP), Prostaglandin E2 (PGE2), bradykinin (BK) and lysophosphatidic acid (LPA) have all been shown to promote growth of Head and Neck Squamous cell carcinoma (HNSCC) [39, 80, 81]. The combined inhibition of GPCRs and EGFR has been further reported to result in improved anti-tumor effects in HNSCC [81, 83]. However, due to the heterogenous expression of GPCRs in HNSCC, multi-targeting of GPCRs and EGFR will be difficult to execute in a clinical setting. Identification and inhibition of a common signaling intermediate downstream of GPCR signaling pathways in HNSCC will be a more reasonable approach to treat HNSCC.

GRP was reported to mediate the release of EGFR ligands in a phosphoinositide-dependent kinase 1 (PDK1)-dependent manner [79]. Furthermore, downmodulation of PDK1 expression combined with erlotinib resulted in improved anti-proliferative and anti-invasive effects [79]. In addition to our report that PDK1 activates TACE, PDK1 is a serine/threonine kinase that has been demonstrated to activate multiple kinases from the AGC (Protein kinase A,

20

protein kinase <u>G</u>, protein kinase <u>C</u>) family of kinases such as p70S6K, PKB/Akt and p21-activated kinase (PAK) [97]. The pleiotropic capacity of PDK1 makes it a promising molecular and therapeutic target for HNSCC. However, our studies of PDK1 in HNSCC have been limited to its role in GRP-mediated crosstalk with the EGFR pathway. Therefore, we sought to further investigate the contribution of PDK1 in pathways mediated by other GPCR agonists detected in HNSCC such as prostaglandin E2 (PGE2) and bradykinin (BK).

In this study, we investigated the contribution of PDK1 in the activation of EGFR mediated by the GPCR ligands PGE2, BK and LPA. Furthermore, we investigated the contribution of PDK1 activity in GPCR-mediated growth and involvement in EGFR activation. We assessed the anti-tumor efficacy of the PDK1 inhibitor OSU-03012 as a monotherapy and in combination with EGFR inhibition. More importantly, this study will validate PDK1 as a therapeutic target than can enhance EGFR targeting modalities in heterogenous HNSCC tumors.

2.2   MATERIALS AND METHODS

2.2.1   Cell culture

PCI-37A, UM-22B, PCI-6B, UM-22A, and 1483 cells are of human origin. 1483 cells were derived from an oropharyngeal tumor, UM-22B and PCI-6B cell lines were derived from metastatic lymph nodes and PCI-37A was from a primary tumor in the epiglottis [98]. HET-1A cells were purchased from the American Type Culture Collection (Manassas, VA). The HET-1A cells are normal human esophageal mucosa cells immortalized by transfection with the SV40

large T antigen as described previously [99]. Cells were maintained in DMEM with 10% heat-inactivated FCS (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2.

2.2.2 Reagents

Epidermal growth factor (EGF) and Prostaglandin E2 (PGE2) were obtained from Calbiochem (San Diego, CA). Bradykinin was obtained from Bachem (Torrance, CA). Lysophosphatidic acid (LPA) was obtained from Sigma-Aldrich Corporation (St. Louis, MO). OSU-03012 was provided by Ching-Shih Chen [100]. C225 (Erbitux) was obtained from the University of Pittsburgh Cancer Institute pharmacy. Wild-type PDK1 and kinase-dead PDK1 (K110Q) cDNA plasmids were provided from Alexandra Newton (University of California San Diego).

2.2.3 Co-Immunoprecipitation and Immunoblotting

For immunoprecipitation, 300 µg of total protein were incubated overnight with 2µg of EGFR antibody (BD Transduction, San Jose, CA) and incubated overnight at 4ºC on a rotary shaker. Fourty microlitres of Protein G agarose beads (Upstate, Temecula, CA) were added to the lysates and allowed to incubate for 2 hours at 4ºC on a rotary shaker. The beads were collected by centrifugation at 4°C, 14,000 rpm for 1 minute. The beads were resuspended and washed three times with lysis buffer. The beads were resuspended in 30 µL of lysis buffer and 8 µl of 4x loading dye and boiled for 10 minutes at 95°C, followed by Western blot analysis. The immunoprecipitated proteins were then resolved on an 8% SDS-PAGE gel. After being transferred onto a nitrocellulose membrane, the membrane was blocked in 5% milk and blotted

with the antiphosphotyrosine antibody PY99 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 in 5% milk dissolved in TBST solution [0.6% dry milk powder, 0.9% NaCl, 0.5% Tween 20, and 50 mmol/L Tris (pH 7.4)]. After washing three times with TBST solution, the membrane was incubated with the secondary antibody (goat anti-rabbit/mouse IgG-horseradish peroxidase conjugate; Bio-Rad Laboratories) for 1 hour and washed three times for 10 minutes. The membrane was developed with Luminol Reagent (Santa Cruz Biotechnology) by autoradiography. Blots were stripped in Restore Western Blot Stripping buffer (Pierce, Rockford, IL) for 15 minutes at room temperature, blocked for 1 hour, and reprobed with EGFR antibody (Transduction Laboratories) at 1:500 dilution. Whole cell lysates were also resolved on 8% SDS-PAGE, transferred to nitrocellulose and probed for PDK1, p85 PI3K (Cell Signaling, Danvers, MA), and β-tubulin (Abcam, Cambridge, MA).

2.2.4   siRNA sequences

HNSCC cells were plated at a density of $2x10^5$ cells/ml in 10cm dishes and incubated overnight at 37C. 18 hours later, cells were transiently transfected with Non-Targeting Control siRNA (Dharmacon, LaFayette, CO) or PDK1 siRNA (Sense: 5'-CUGGCAACCUCCAGAGAAU-3' Antisense: 5' – AUUCUCUGGAGGUUGCCAG-3'). SiRNA was mixed with lipofectmaine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM media according to the manufacturer's instructions.

2.2.5 MTT and Trypan Blue dye exclusion growth assays

Cells were plated in a 24-well plate and incubated for 18 hours, followed by siRNA transfection and/or treatment with increasing concentrations of OSU-03012. At the end of the treatment, media was replaced with MTT solution and incubated for 20 minutes at 37°C. MTT (Sigma-Aldrich, St Louis, MO) solution was aspirated and replaced with DMSO (Sigma-Aldrich, St. Louis, MO). The optical density of the formazan product was determined using an ELISA-plate reader set at 570nM. The survival percentage was determined by the following formula: OD_{drug} / $OD_{vehicle}$ x 100. Trypan blue dye exclusion assay was performed by trypsinizing cells followed by cell counting in the presence of trypan blue solution.

2.2.6 Statistics

Statistical significance of biochemical assays was determined by Student's t-test. The group differences in in vitro proliferation, invasion and viability assays were tested with the exact Wilcoxon test. All p-values were determined using the StatXact Statistical Software Version 6.1. $P \leq 0.05$ was considered to be statistically significant.

2.3   RESULTS

2.3.1   PDK1 contributes to GPCR-mediated activation of EGFR

We previously reported that GRP mediated the activation of EGFR via phosphoinositide-dependent kinase 1(PDK1)-mediated TACE phosphorylation [79]. To determine whether PDK1 is a common signaling intermediate in the activation of EGFR by other GPCR ligands, we examined the effect of PDK1 knockdown on GPCR-induced phosphorylation of EGFR. Different HNSCC cell lines were treated with PGE2, BK and LPA followed by assessment of EGFR phosphorylation. In two different HNSCC cell lines transfected with PDK1 siRNA, PGE2 and BK-mediated phosphorylation of EGFR was abrogated (Figure 2A; $p<0.05$ and 2B). LPA-mediated phosphorylation of EGFR in HNSCC was also abrogated by PDK1 downmodulation (Figure 2C; $p<0.05$). Zhang *et al* further reported that GRP-mediated release of amphiregulin was also dependent on the upstream activator of PDK1, PI3-kinase [79]. We similarly demonstrated that PGE2-mediated phosphorylation of EGFR was abrogated in HNSCC cells transfected with siRNA targeting the p85 regulatory subunit of PI3-kinase (Figure 2D). These results suggest that the PI3K-PDK1 signaling axis contributes to GPCR-mediated activation of EGFR in HNSCC.

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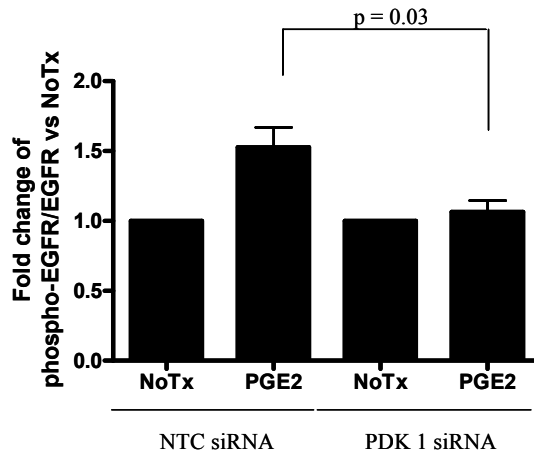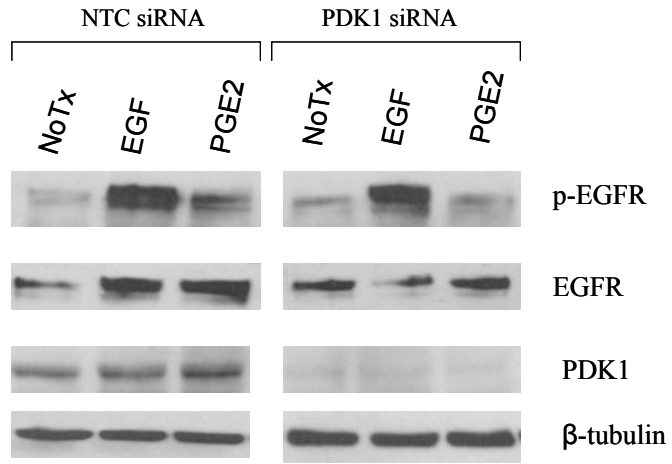
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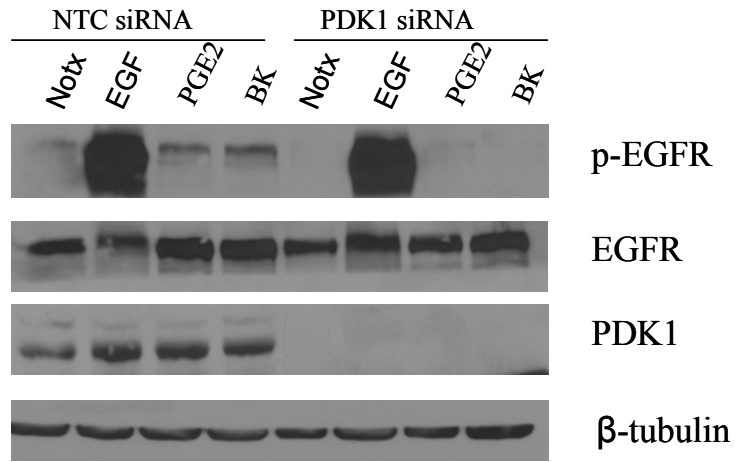
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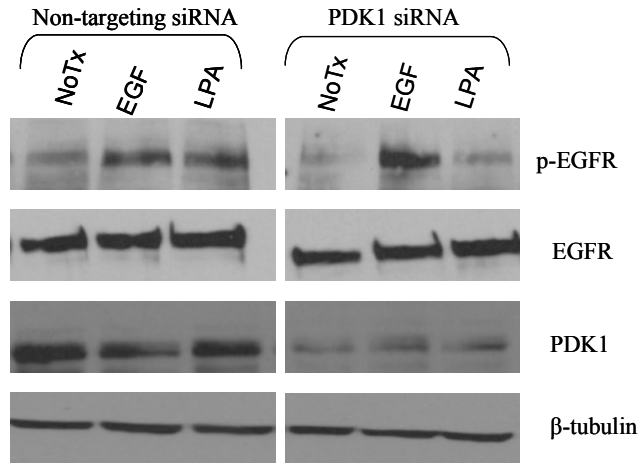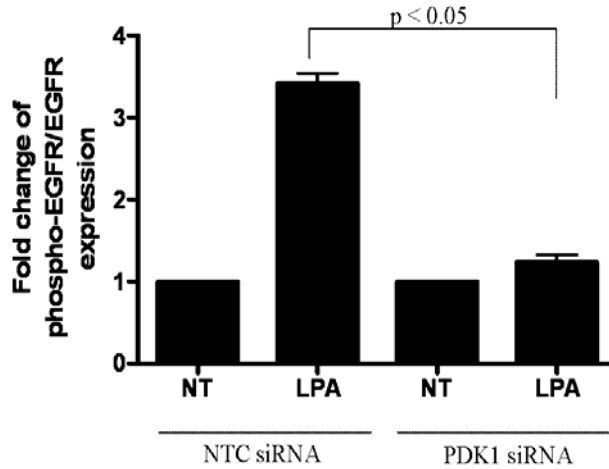
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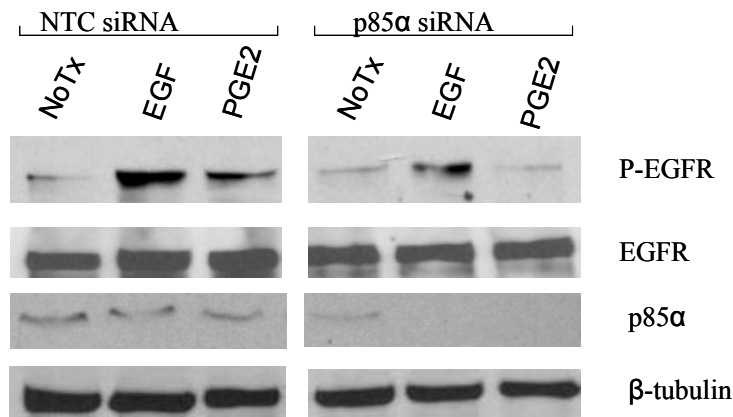
Figure 2. PDK1 contributes to PGE2 and BK-mediated EGFR phosphorylation

(A) PCI-37A, (B) UM-22A and (C) PCI-6B cells were transfected with non-targeting control (NTC) or PDK1 siRNA followed by 72hr serum starvation and then stimulation with 10 ng/ml EGF, 10 nM PGE2, 10nM BK or 10μM LPA for 5 minutes. Cell lysates were collected and immunoprecipitated with EGFR and immunoblotted with an anti-phosphotyrosine antibody (upper panel) and EGFR (lower panel) respectively. PDK1 levels were determined by western blotting with an anti-PDK1 antibody. The experiment was repeated 3 times with similar results ($p < 0.05$ for (A) and (C)). (D) PCI-37A cells were transfected with non-targeting control or p85 siRNA followed by 72hr

serum starvation and then stimulation with 10 ng/ml EGF or 10 nM PGE2 for 5 minutes. Cell lysates were collected and immunoprecipitated with EGFR and immunoblotted with an anti-phosphotyrosine (upper panel) and EGFR (lower panel) respectively. p85α levels were determined by western blotting with an anti-p85 antibody. The experiment was repeated 3 times with similar results.

2.3.2   PDK1 contributes to GPCR-mediated proliferation

We have previously reported that PDK1 siRNA decreases HNSCC survival[79] . However, the effect of PDK1 downmodulation on GPCR-mediated HNSCC proliferation has not been reported. HNSCC cells were transfected with control and PDK1 siRNA for 48 hours before 24-hour stimulation with PGE2 or BK. In Figure 3A we showed that PGE2 and BK induced an approximate 20% increase in growth of control siRNA-transfected cells after 24 hours. In PDK1 siRNA transfected cells, PGE2 and BK-mediated a 5-10% increase in growth (p=0.002). Next, we transiently transfected HNSCC cells with the kinase-dead PDK1 (PDK1M) construct and stimulated with PGE2 for 48 hours. Vector control-transfected cells displayed a 40% increase in growth after 48 hours. However, the PDK1M-transfected cells displayed a modest 10% increase in growth with PGE2 stimulation (Figure 3B; p=0.05). These results suggest that in addition to EGFR activation, PDK1 is an important mediator of GPCR-induced HNSCC growth.
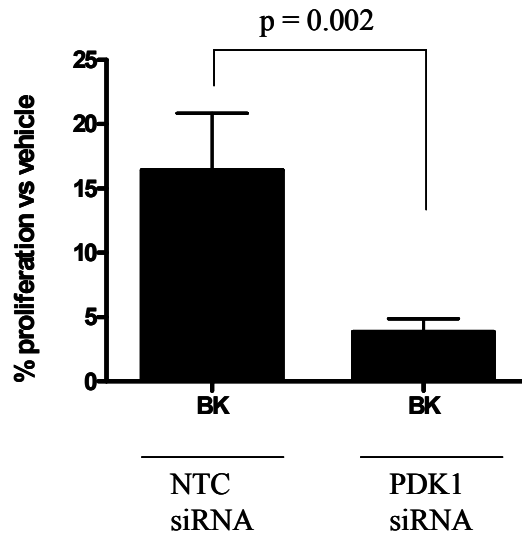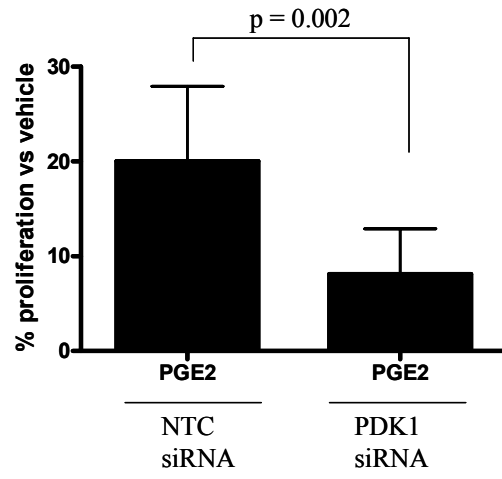
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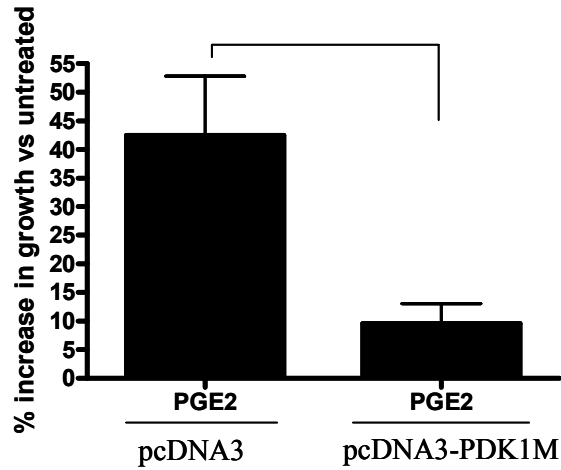
Figure 3. PDK1 contributes to GPCR-mediated growth.

UM-22A cells were transiently transfected with (A) PDK1 or non-targeting control siRNA or (B) pcDNA3 or

pcDNA3-PDK1M (K110Q), serum starved for 48 hrs, followed by stimulation with 10nM PGE2 or 10nM BK or

vehicle for 24(A) or 48 hours (B). Percentage increase in proliferation was determined by trypan blue dye exclusion

assay. Results were graphed using GraphPad Prism Software. Experiment was done twice in triplicate with similar

results (p=0.002 for A, p=0.05 for B).
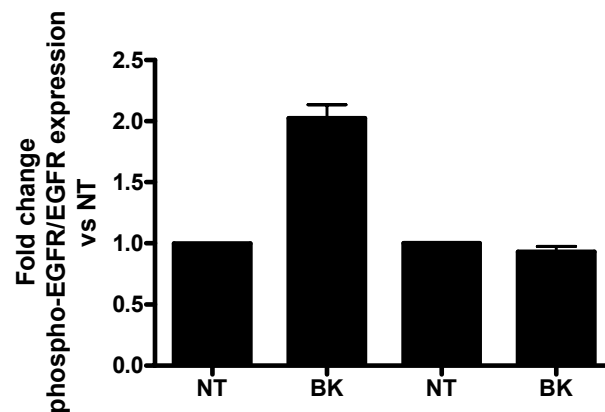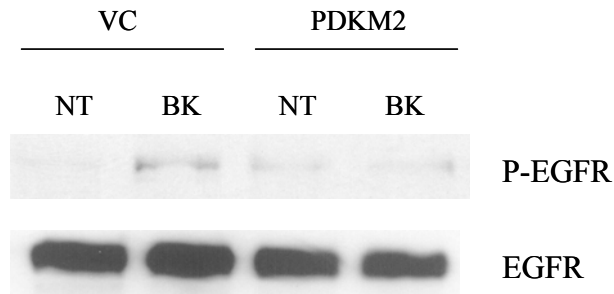
Figure 4. Kinase-dead PDK1 abrogates BK-mediated EGFR phosphorylation

1483 VC and PDKM2 cells were plated and serum-starved for 72 hours. Cells were treated with 10nM BK for 10 minutes. Lysates collected were immunoprecipitated with anti-EGFR antibody and probed with an anti-phosphotyrosine antibody. Figure is representative of 2 independent experiments.

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2.3.3   HNSCC cells demonstrate sensitivity to pharmacological PDK1 inhibition

Establishing that PDK1 and its kinase activity contributed to GPCR-mediated signaling and HNSCC growth, we wanted to assess the efficacy of pharmacological inhibition of PDK1 activity in HNSCC using the PDK1 inhibitor OSU-03012. Firstly, we determined the effect of

OSU-03012 on GPCR-mediated EGFR phosphorylation. In Figure 4, we observed that PGE2-mediated EGFR phosphorylation was abrogated in the presence of the kinase-dead PDK1 mutant. In the presence of OSU-03012, PGE2-mediated EGFR phosphorylation was abrogated (data not shown). Next, we investigated the effect of OSU-03012 on HNSCC proliferation and we observed that HNSCC cell lines displayed a range of IC_{50} values from 74nM to 700nM (Figure 5A). In addition to HNSCC cell lines, we assessed the sensitivity of the normal mucosal epithelial cell line Het-1A. The IC_{50} value of OSU-03012 for Het-1A cells was 6μM, which was approximately 9-fold higher than the IC_{50} for UM-22B cells. To assess the ability of OSU-03012 to inhibit its target, we determined the inhibitory effect of OSU-03012 on Akt phosphorylation, which is a readout for PDK1 activity. Various reports showed that OSU-03012 inhibited Akt phosphorylation while others have shown that OSU-03012 had no effect on Akt phosphorylaion [101-103]. We showed that OSU-03012 abrogated phospho-Akt (S473) levels at 1μM in both cell lines (Figure 5B). To determine whether the decreased proliferation was due to increased apoptosis we used PARP cleavage as the apoptotic readout. PARP cleavage was first observed at 300nM and 1μM concentrations in PCI-37A and UM-22B cells respectively (Figure 6A). Using the similar range of OSU-03012 concentrations in Figure 6A, Het-1A cells displayed no cleaved PARP (Figure 6B). In addition to PARP, we looked at the effect of OSU-03012 on survivin expression. Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins that is overexpressed in HNSCC, which is involved in mediating cell proliferation and inhibiting apoptosis [104, 105]. OSU-03012 decreased survivin expression at 100nm and 1μM concentrations in PCI-37A and UM-22B respectively (Figure 6C). These results indicate that the PDK1 inhibitor OSU-03012 effectively decreases HNSCC growth, mitogenic signaling and promotes apoptosis.

2.3.4 Anti-proliferative effect of OSU-03012 is partially dependent on PDK1

In light of recent reports indicating that OSU-03012 mediated various PDK1-independent effects in different cancer models [102, 103, 106], we chose to look at the contribution of PDK1 to the increased sensitivity of HNSCC to OSU-03012. PDK1 siRNA transfected cells displayed an approximate 2-fold higher IC_{50} for OSU-03012 compared to control transfected cells (Figure 7A and 7B). Kinase-dead PDK1-expressing HNSCC cells also displayed a 2-fold higher IC_{50} value to OSU-03012 compared to vector transfected cells (data not shown). Therefore, OSU-03012 effects were partially dependent on PDK1 expression and activity. PDK1 was reported to mediate cellular proliferation via cyclinD1 control, furthermore cyclin D1 expression has been shown to confer HNSCC resistance to gefitinib [107, 108]. Therefore, we compared the inhibitory effect of OSU-03012 on cyclin D1 expression between PCI-37A and UM-22B cells, the most and least sensitive HNSCC cell lines to OSU-03012. In Figure 7C, we observed that OSU-03012 mediated downmodulation of cyclin D1 at 100nM and 1μM in PCI-37A and UM-22B cells respectively. The results from this section indicate that HNSCC cells sensitivity to OSU-03012 is partially dependent on PDK1 expression and activity. Furthermore, OSU-03012-mediated inhibition of the PDK1 proliferative mediator cyclin D1 is possibly associated with PDK1 inhibitory activity of OSU-03012.

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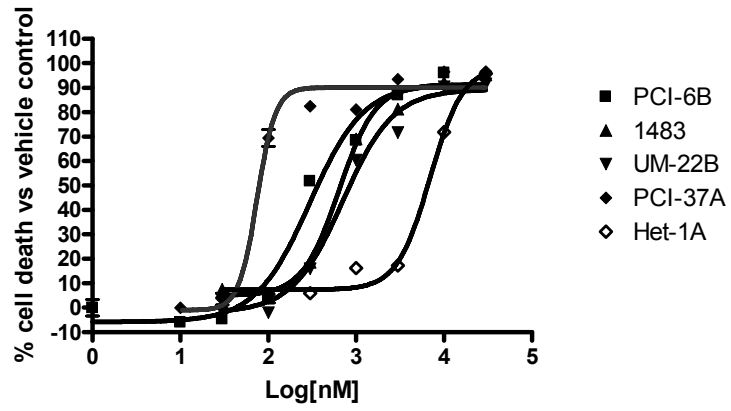
Cell Line	IC$_{50}$ (nM)
PCI-37A	74
PCI-6B	297
1483	310
UM-22B	708
Het-1A	6000

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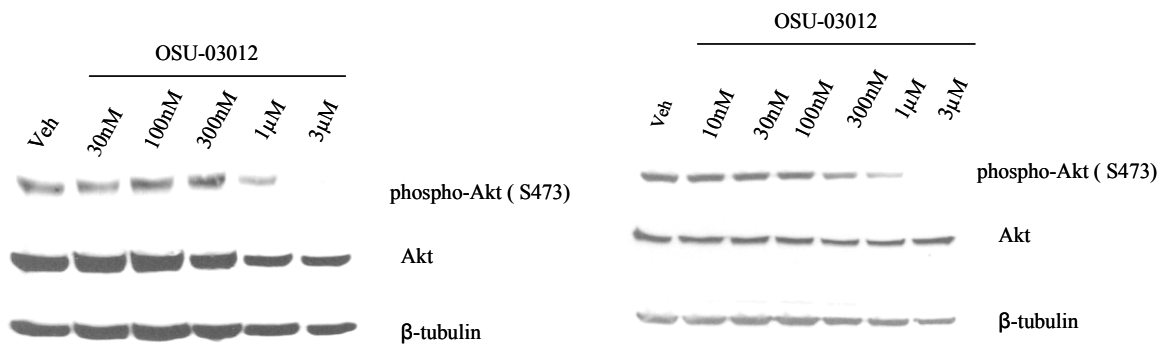
Figure 5. OSU-03012 inhibits HNSCC proliferation and Akt phosphorylation

(A) HNSCC and a normal mucosal epithelial cell line Het-1A cells were treated with increasing concentrations of OSU-03012. After 72 hours, MTT assay was performed and the IC_{50} values were determined using Prism (GraphPad) Software. The experiment was repeated 3 times with similar results. (B) PCI-37A and UM-22B cells were treated with increasing concentrations of OSU-03012 for 72 hours. Lysates were assessed by immunoblotting for phospho-Akt , total Akt and β-tubulin. Representative figure from 3 independent experiments is shown.

A

PCI-37A (IC_{50} =74nM)   UM-22B (IC_{50} = 704nM)

B

OSU-03012

C

OSU-03012

DMSO | 10nM | 30nM | 100nM | 300nM

survivin

β-tubulin

PCI-37A (IC$_{50}$ =74nM)

OSU-03012

DMSO | 10nM | 30nM | 100nM | 300nM | 1uM

survivin

β-tubulin

UM-22B (IC$_{50}$ =708nM)
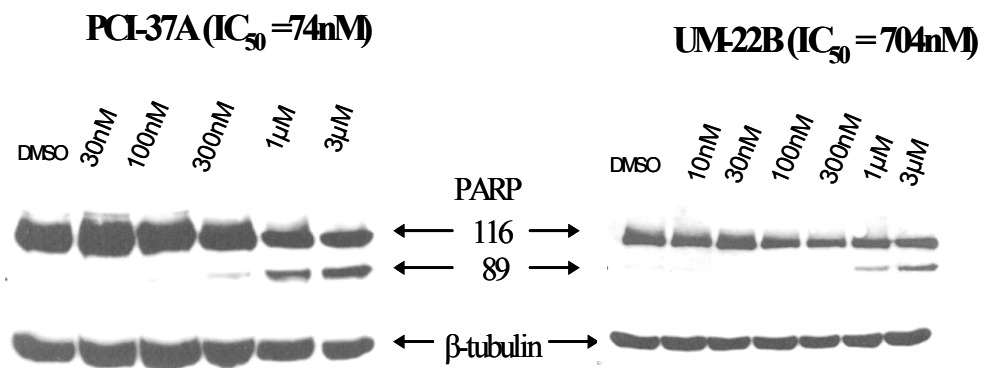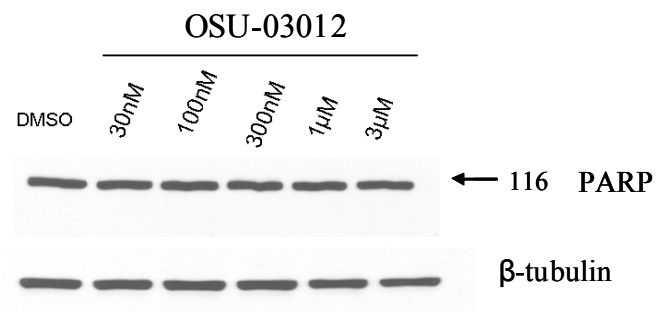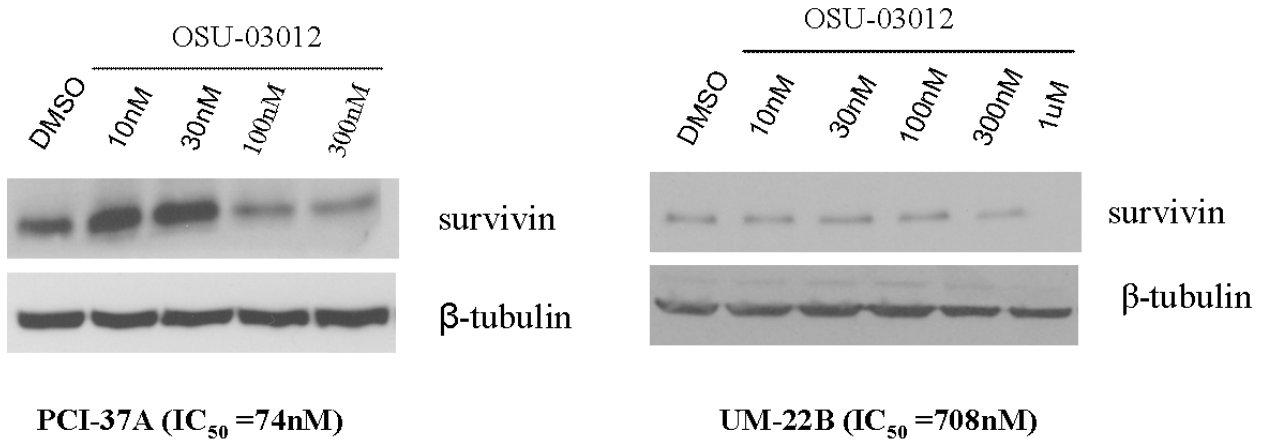
Figure 6. OSU-03012 induces pro-apoptotic signaling

(A) PCI-37A, UM-22B and (B) HET-1A cells were treated with increasing concentrations of OSU-03012 and analyzed by immunoblotting for cleaved PARP (116 and 89Kd bands). Representative figure from three independent experiments is shown. (C) HNSCC cells were treated with OSU-03012 and assessed for survivin expression by immunoblotting. Figure is a representation of three independent experiments.

A

37A NTC
37A PDK1

B

C

OSU-03012

DMSO | 10nM | 30nM | 100nM | 300nM

Cyclin D1

β-tubulin

PCI-37A (IC$_{50}$ =74nM)

OSU-03012

DMSO | 10nM | 30nM | 100nM | 300nM | 1uM

Cyclin D1

β-tubulin

UM-22B (IC$_{50}$ =708nM)
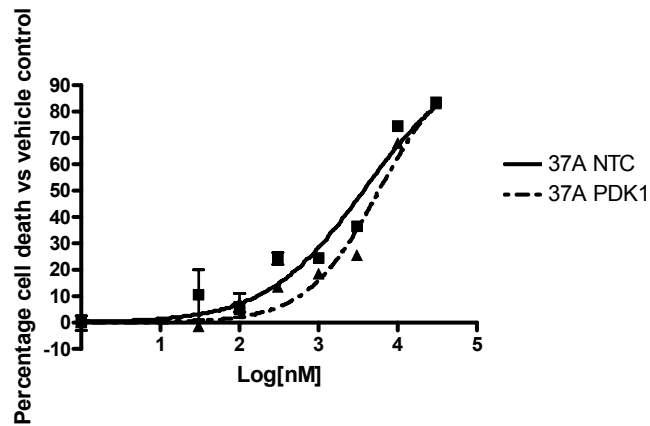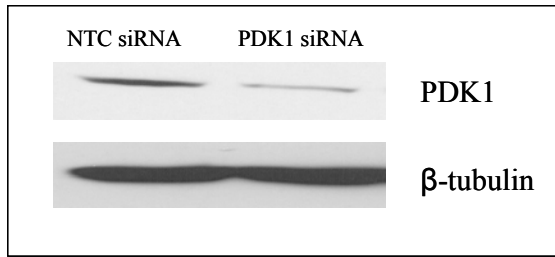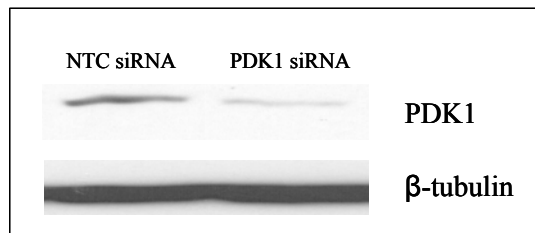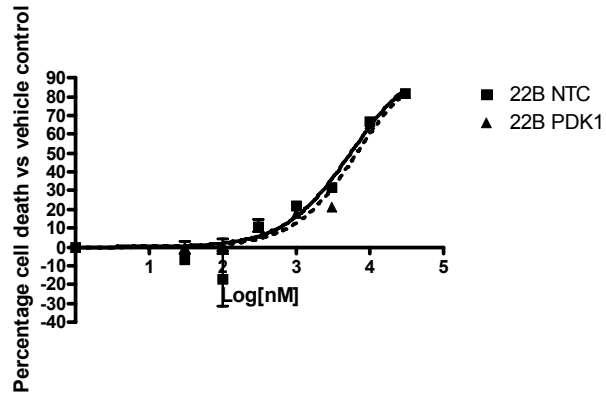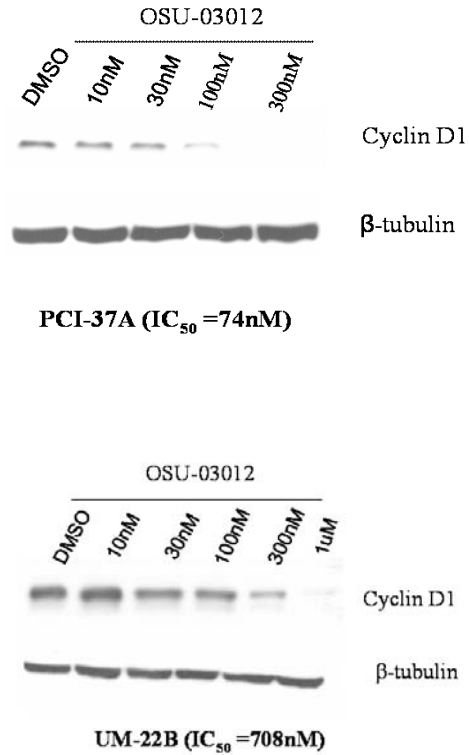
Figure 7. PDK1 expression contributes to HNSCC sensitivity to OSU03012

A) PCI-37A and (B) UM-22B cells were seeded in 10 cm plates and transfected with NTC or PDK1 siRNA for 48 hours. Cells were trypsinized and seeded into 24-well plates. The remaining cells were treated with lysis buffer to generate lysate for SDS-PAGE analysis. 24-well plates were treated with various concentrations of OSU-03012 18 ours later. After 24 hours, MTT assay was performed and the EC$_{50}$ values were determined using Graph Pad Prism. (C) PCI-37A and UM-22B cells were treated with increasing concentrations of OSU-03012 for 48 hours. Lysates collected were resolved by SDS-PAGE and membranes were probed for cyclin D1 and β-tubulin. Representative figure from three independent experiments is shown.

2.3.5 Combined treatment with OSU-03012 and EGFR TKIs displays enhanced anti-proliferative effects

Combination of molecular inhibitors is being currently tested in multiple clinical trials due to enhanced anti-tumor effects and decreased toxicities compared to monotherapy. We previously reported that RNA silencing of PDK1 enhanced the anti-proliferative and anti-invasive capacity of the EGFR TKI erlotinib [79]. Therefore, we determined whether OSU03012 treatment and PDK1M expression increased HNSCC sensitivity to EGFR inhibition. In Figure 8A, we showed that combined treatment of sub-IC_{50} concentrations of OSU03012 and the EGFR TKI AG1478 (Tyrphostin) resulted in an approximate 50% decrease in cell growth compared to either agent alone ($p = 0.003$). Similar results were observed with the EGFR TKI erlotinib (Figure 8B; $p=0.003$). However, treatment of 1483 PDK1M HNSCC cells with EGFR TKIs did not result in enhanced cytotoxicity compared to 1483VC cells *in vitro* (data not shown). These results indicate that in addition to PDK1 downmodulation, OSU-03012 can improve the anti-tumor effects of EGFR inhibition of HNSCC, and may be a valid therapeutic strategy pending results from *in vivo* studies in the future.

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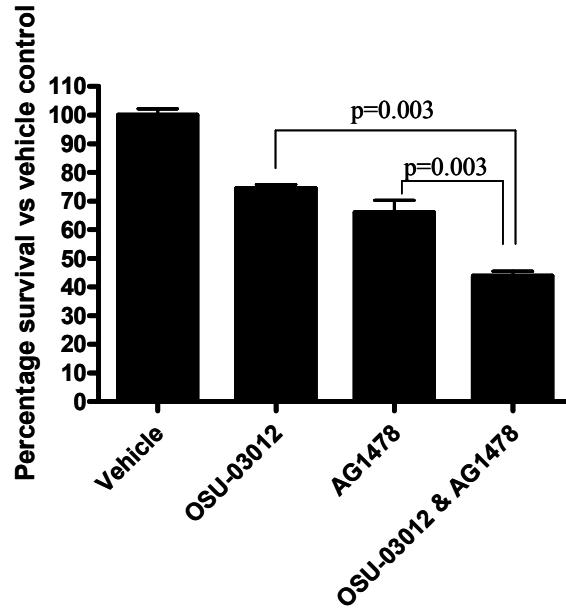
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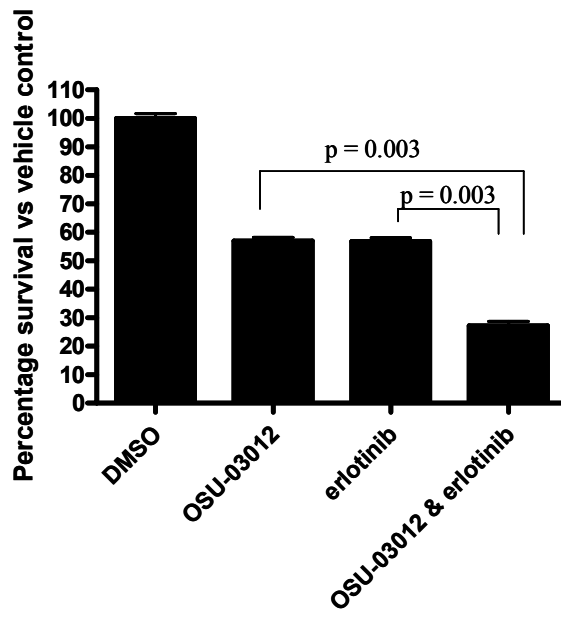
Figure 8. OSU-03012 additively improves EGFR inhibition in HNSCC

UM-22B cells were treated with OSU-03012 (200nM) , (A)AG1478(6µM), (B) erlotinib (7µM) or a combination of OSU-03012 plus (A)AG1478 or (B) erlotinib. MTT assay was performed after 72 hours and the data analysis was done using GraphPad Prism Software. Experiments were done in 6 replicates thrice (p=0.003).

2.4   DISCUSSION

GPCR-EGFR crosstalk has been demonstrated to mediate tumorigenesis in different cancer models via the extracellular release of EGFR ligands [39, 41, 42, 45, 53, 64, 109-111]. Although the combined targeting of GPCRs and EGFR display additive and synergistic growth suppression effects, it is unfeasible to target multiple GPCRs and EGFR in a clinical setting due to the heterogenous nature of tumors. Identification of a common "druggable" signaling intermediate involved in GPCR-EGFR crosstalk may pave a more rational path to overcome GPCR and EGFR-mediated tumorigenesis. A previous report from our lab showed that PDK1 mediated EGFR ligand release in response to GRP stimulation [79]. PDK1 downmodulation also enhanced EGFR inhibition of proliferation and invasion. In this study, we show that in addition to GRP, PDK1 is a common signaling mediator of GPCR-mediated EGFR phosphorylation in HNSCC (Figure 9). Furthermore, we showed that targeting PDK1 expression and activity abrogated GPCR-mediated growth and enhanced EGFR inhibition also.

Multiple reports have indicated that GPCR ligands mediate release of EGFR ligands in a Src and MMP-dependent manner [39, 80-82, 112, 113]. We first reported the involvement of the PI3K-PDK1 signaling complex in the release of EGFR ligands in HNSCC mediated by the

42

bombesin, GRP. However, it was unknown whether PI3K and PDK1 were common signaling mechanisms involved in GPCR-EGFR crosstalk in HNSCC. We observed that both PI3K and PDK1 were involved in PGE2, BK and LPA-mediated phosphorylation of EGFR. Reports from our lab also indicated that PGE2 and BK-mediated release of TGF-α was abrogated with TACE siRNA [81]. In conjunction with findings from this study, PDK1-mediated TACE phosphorylation is critical to PGE2, BK and LPA–mediated EGFR phosphorylation. The interaction between PDK1 and TACE in response to GPCR stimulation may also be critical to other types of cancers such as breast cancer. In addition to EGF ligands [114], TACE has been shown to mediate release of heregulin that activates HER3 [115]. Targeting the release of these ligands via PDK1 inhibition may have therapeutic benefits over a broader range of tumor types.

PDK1 is a pleiotropic kinase that mediates proliferative, invasive and survival signaling pathways via activation of multiple substrates [97]. We previously reported that PDK1 downmodulation decreased HNSCC growth [79], however, there were no reports on the contribution of PDK1 in specific GPCR-mediated growth in HNSCC or any other tumor model. With both PDK1 siRNA and a kinase-dead PDK1 construct, GPCR-mediated growth was inhibited. We previously reported that blockade of EGFR significantly abrogated GPCR-mediated growth and invasion [116]. Our findings in Figure 3A further corroborate our findings that GPCRs mediate HNSCC growth via EGFR ligand release and consequent EGFR activation [81]. Although, we have shown that PDK1 mediates GPCR stimulated growth via EGFR activation, PDK1 may contribute to GPCR-mediated growth in the presence of EGFR blockade. We and others have reported that PGE2 and BK-mediate mitogenic signaling in the presence of EGFR inhibitors [67, 116]. In NSCLC and an ovarian cancer cell line, PGE2 and BK mediated EGFR-independent signaling via PI3K and PKC [67, 117, 118]. PI3K is upstream of PDK1 and

PKC is also a PDK1 substrate [97, 119]. Therefore, GPCR-mediated activation of PDK1 may also have phenotypic implications independent of EGFR ligand release. This hypothesis is currently being investigated in our laboratory.

Celecoxib was demonstrated to inhibit Akt activity via inhibition of PDK1 [120, 121]. The celecoxib derivative OSU-03012 was reported to inhibit PDK1 activity in different cell models [122]. However, in some cancer models, OSU-03012 demonstrated cytotoxic effects via PDK1-independent pathways [102, 103, 123, 124]. OSU-03012 was reported to activate CDKs and mediate oral cancer apoptosis in a p21-dependent manner [106, 125]. However, it was unclear whether OSU-03012-mediated cytotoxicity in oral cancer was via PDK1 inhibition. We show that the most OSU-03012-sensitive HNSCC cell line displayed a 2-fold resistant phenotype in the absence of PDK1. The least OSU-03012 sensitive cell line UM-22B displayed a minimal increase in resistance in the absence of PDK1. We showed that Akt phosphorylation was abrogated with 1uM of OSU-03012 in both 37A and 22B cells which displayed $IC_{50}s$ of 74 and 700nM respectively. These observations together indicate that OSU-03012 induced cell death via PDK1-dependent and independent mechanisms. However, it is still possible that OSU-03012 is mediating its anti-tumor effects via inhibition of the PDK1 effectors instead of the PDK1 kinase itself. One report showed that OSU-03012 had a greater affinity for the PDK1 substrate, p21-activated kinase 1 (PAK1) than for PDK1 itself. Structurally, it was further illustrated that OSU-03012 was bound to the ATP binding pocket of PAK1[126]. Therefore, the enhanced sensitivity of HNSCC cells to OSU-03012 may be due to its ability to inhibit other AGC kinases that function differently between the various HNSCC cell lines.

In HNSCC and other cancer models, the combined targeting of GPCRs and EGFR has demonstrated improved anti-tumor effects. Approximately 50% of new drugs developed target

GPCRs, hence emphasizing their importance to disease progression. The autocrine/paracrine release of TGF-α was reported to correlate with poor HNSCC patient prognosis. Therefore, inhibition of EGFR ligand release by both GPCR and EGFR-mediated activity is a potent treatment strategy. In addition to abrogating PDK1-mediated activation of proliferative and motility effectors such as p70S6k and PAK1, inhibition of TACE-mediated release of TGF-α mediated by PGE2 and BK will enhance the inhibitory efficacy of EGFR inhibition and its consequent autocrine release of EGF ligands. We previously reported that PDK1 siRNA improved the anti-proliferative and anti-invasive effects of the erlotinib. In this present study we further indicated that the PDK1 inhibitor OSU-03012 enhanced EGFR TKI inhibition. These results are similar to the findings observed in NSCLC. OSU-03012 was demonstrated to enhance the anti-tumor efficacy of erlotinib [124]. Furthermore, combined inhibition of GRPR and EGFR in HNSCC also additively decreased the phosphorylation of the PDK1 substrate, p70S6K, indicating that GPCRs may mediate p70S6K phosphorylation independently of EGFR. In closing, targeting PDK1 by inhibition of kinase activity or expression is a viable therapeutic option for HNSCC patients treated with EGFR inhibitors. Further preclinical studies including *in vivo* experiments are currently under way to further justify this treatment strategy in HNSCC.
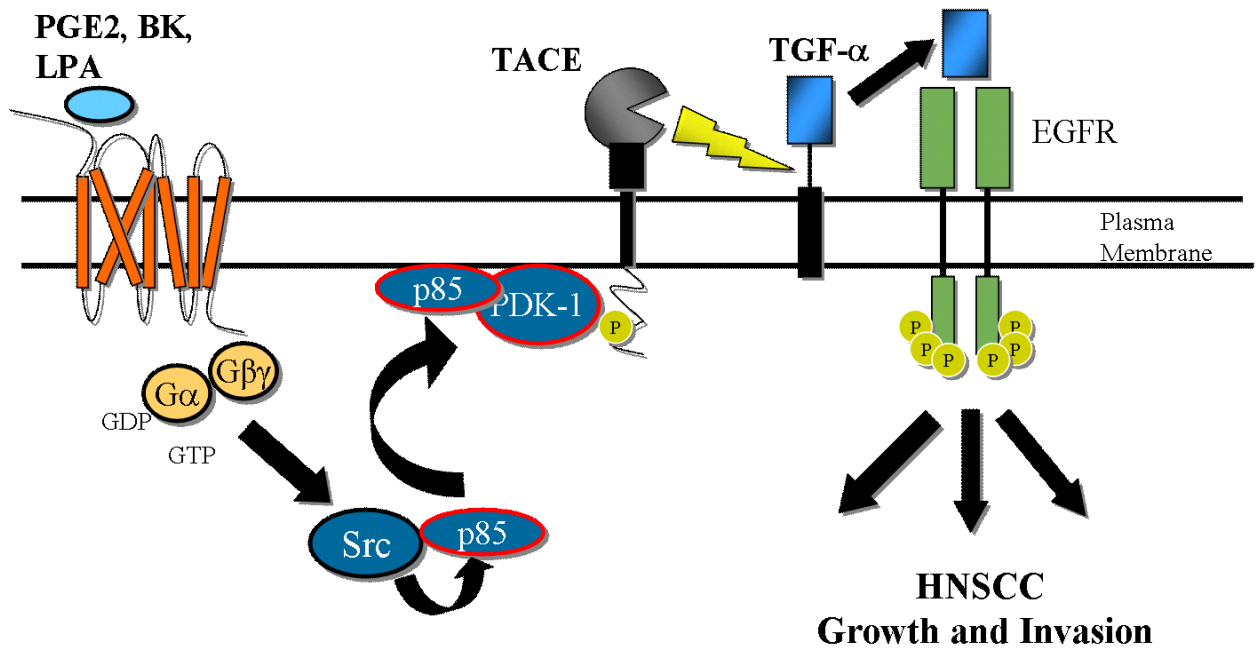
Figure 9. Model for PGE2, BK and LPA-mediated crosstalk in HNSCC.

PGE2, BK and LPA stimulation induces EGFR phosphorylation via TACE-mediated EGFR ligand release. Inhibition of PDK1 and the p85 regulatory subunit of Class 1A PI3K abrogate GPCR-mediated EGFR phosphorylation in different HNSCC cell lines. PDK1 phosphorylates the cytoplasmic domain of TACE which mediates TGF-α release in response to GPCR stimulation.

3.0   INDUCTION OF P70S6K SIGNALING BY EGFR INHIBITION: IMPLICATIONS FOR MECHANISMS OF RESISTANCE TO EGFR BLOCKADE AND THERAPEUTIC COTARGETING STRATEGIES

3.1   INTRODUCTION

Head and Neck Squamous Cell carcinoma (HNSCC) results to 13,000 deaths annually in the United States and is characterized by the overexpression of the tyrosine kinase receptor, Epidermal Growth Factor Receptor (EGFR)[23]. EGFR overexpression in HNSCC has been correlated to poor patient outcome, regardless of primary therapy [29]. The addition of the EGFR monoclonal antibody cetuximab (C225, ™Erbitux) to radiation therapy improved survival leading to the FDA approval of this agent for HNSCC in 2006 [13]. However, more than 50% of patients with primary HNSCC tumors develop advanced secondary tumors that are highly metastatic [127]. Treatment of the patients diagnosed with advanced metastatic HNSCC with C225 resulted in a more limited clinical response (11%). The resistance of these secondary tumors to EGFR –targeting may be the result of activation of alternative signaling pathways that contribute to HNSCC progression.

G-protein-coupled receptors (GPCRs) are seven transmembrane receptors that mediate cell growth, motility and differentiation via stimulation with cognate agonists [6, 31]. HNSCC

tumors express elevated levels of the GPCR, bradykinin receptor 2 (B2R) which stimulates the upregulation of cyclooxygenase 2 (COX-2) and its downstream effector PGE2, which is another GPCR ligand that has been observed in HNSCC [35, 128]. We and others have shown that GPCR ligands PGE2, BK, GRP and lysophosphatidic acid (LPA) mediate HNSCC proliferation and invasion via the autocrine release of EGFR ligands and the consequent activation of EGFR [39, 77, 81]. Furthermore, the combined inhibition of GPCRs and EGFR displayed additive effects on decreasing HNSCC growth, invasion and survival, indicating that targeting GPCRs is a potential HNSCC treatment [81, 83]. However, due to the heterogenous nature of HNSCC, it is therapeutically impractical to inhibit multiple GPCRs in conjunction EGFR. Identification of a common signaling intermediate downstream of the GPCRs may elucidate a new therapeutic target for HNSCC patients, which can augment clinical responses when combined with an EGFR inhibitor.

The present study was carried out to identify a "druggable" target that contributes to GPCR-mediated HNSCC progression when EGFR expression is downmodulated. We used a high-throughput antibody microarray to identify proteins that were activated by GPCRs in an EGFR-independent manner. We investigated the pharmacological inhibition of the molecular target identified by the microarray as monotherapy and in combination with EGFR inhibitors under using preclinical HNSCC models *in vitro* and *in vivo* conditions.

3.2   MATERIALS AND METHODS

3.2.1   Cell Lines

All the HNSCC cell lines (PCI-37A, 1483, UM-22B) were of human origin. 1483 cells were derived from an oropharyngeal tumor, UM-22B cell line was derived from metastatic lymph nodes and PCI-37A and UM-22A were from a primary tumor in the epiglottis [129]. Cells were maintained in DMEM with 10% heat-inactivated FCS (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2.

3.2.2   Reagents

Epidermal growth factor (EGF), Prostaglandin E2 (PGE2), and Rottlerrin were obtained from Calbiochem (San Diego, CA). Bradykinin was obtained from Bachem (Torrance, CA). Lysophosphatidic acid (LPA) was obtained from Sigma-Aldrich Corporation (St. Louis, MO). RAD001 was provided by Novartis (Basel, Switzerland) and C225 (Erbitux) was obtained from the University of Pittsburgh Cancer Institute pharmacy.

3.2.3   Phospho-protein Antibody Array

PCI-37A cells were seeded in four 10cm culture dishes; 1) NTC siRNA and No treatment, 2) NTC siRNA and PGE2, 3) EGFR siRNA and No Treatment, 4) EGFR siRNA and BK. Cells were transiently transfected with non-targeting control (NTC) siRNA and EGFR siRNA, serum-starved for 72 hours and treated with vehicle or 10nM PGE2. Cells were lysed

with Extraction buffer provided as described according to manufacturer's instructions. Protein samples were biotinylated using Biotin reagent dissolved in N, N-Dimethylformamide. Ten microlitres of protein sample was mixed with 40μl of Labeling Buffer followed by addition of Biotin/DMF reagent at a 1:7 ratio. Biotin-labeled protein samples were conjugated to the cancer/apoptosis phospho-antibody microarray. The Cancer/Apoptosis phospho-antibody microarray was purchased from FullMoon Biosystems (Sunnyvale, CA; http://www.fullmoonbiosystems.com/Products/AntibodyArrays/PAC155.htm). Antibody microarray slides were incubated with blocking solution provided on a rotating shaker at room temperature. Slides were then rinsed thrice in water and allowed to dry. Protein coupling mix was added over the array slide and incubated at 4°C overnight. Slides were washed twice for 10 minutes each with 1X Wash Solution. Cy3-streptavidin solution was then added to the slides for 60 minutes at room temperature with shaking. Slides were scanned using the GenePix 4300 Array Scanner. The PGE2-treated intensities from either NTC or EGFR siRNA-transfected groups were normalized to the Vehicle treated intensities of either NTC or EGFR siRNA-transfected groups respectively. Next, the fold change in intensities from six replicates between PGE2-treated NTC siRNA and PGE2-treated EGFR siRNA cells was calculated.

3.2.4 Immunoblotting

Cells were lysed with lysis buffer and quantitated as described previously. Lysates were resolved by 8% or 10% SDS-PAGE. After being transferred onto a nitrocellulose membrane, the membrane was blocked in 5% milk and blotted with various primary antibodies in 5% milk dissolved in TBST solution [0.6% dry milk powder, 0.9% NaCl, 0.5% Tween 20, and 50 mmol/L

Tris (pH 7.4)]. After washing three times with TBST solution, the membrane was incubated with the secondary antibody (goat antirabbit/mouse IgG-horseradish peroxidase conjugate; Bio-Rad Laboratories) for 1 hour and washed three times for 10 minutes. Membranes were developed with Luminol Reagent (Santa Cruz Biotechnology) by autoradiography. Blots were stripped in Restore Western Blot Stripping buffer (Pierce, Rockford, IL) for 25 minutes at room temperature, blocked for 1 hour, and reprobed with primary antibodies.

3.2.5   siRNA Transfection

Silencing RNA oligonucleotides targeting EGFR, p70S6K, PDK1, PKCδ was obtained from Dharmacon (Lafayette, CO). PDK1 siRNA was designed to the target the following sequence: 5'-CUGGCAACCUCCAGAGAA-3'. EGFR siRNA was designed to target the following sequence: 5' – CUCUGGAGGAAAAGAAA-3'. P70S6K siRNA was designed to target the following sequence: 5'- CCAAGGUCAUGUGAAACUA-3'. PKCδ siRNA was designed to target the following sequence: 5'- GAUGAAGGAGGCGCUCAG-3'. 2 x 10^6 cells were seeded in 10cm plates and allowed to incubate overnight at 37ºC. Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

3.2.6 Growth Assays

HNSCC cells were seeded and incubated overnight at 37C. Cells were treated with inhibitors or transfected with siRNA for different time points. Cells were trypsinized and stained with trypan blue solution before being transferred to a hemocytometer and viable cells were counted. Growth-inhibitory effects were also determined using the fluorimetric resazurin-based Cell Titer Glo Assay (Promega, WI) according to manufacturer's instructions. Cells were read using a Victor3 multilabel counter at $560_{ex}/590_{em}$ wavelengths.

3.2.7 Matrigel Invasion Assay

In vitro invasion assays were performed in the growth-factor reduced Matrigel-coated Transwell chambers (BD Biosciences, San Jose, CA). 1483 cells were plated in a 6-well plate. Twenty-four hours later, 4 wells were treated with vehicle, C225, RAD001, and C225 and RAD001 in serum-free media for 48 hours. Cells were trypsinized, counted and plated in serum-free media into the Transwell chambers. The lower well contained 10% serum-containing media and cells were allowed to invade for 24 hours at 37°C and 5% CO_2. The cells on the insert were removed by gently wiping with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema3 Solution (Fisher Scientifics, Hampton, NH). Invading cells in 5 representative fields were counted at 400X magnification using light microscopy.

3.2.8 Xenograft Studies

Athymic nude mice were injected subcutaneously with 2×10^6 1483 or 22B cells in the right flank. Tumor-bearing mice were randomized into four groups; Vehicle (saline), C225, RAD001, or C225 plus RAD001. C225 was administered by IP injection at a dose of 0.8mg/mouse twice weekly while RAD001 was administered 5 days a week at a dose of 5mg/kg over a four-week period. RAD001 was provided as a 2% microemulsion, which was dissolved in fresh 5% glucose daily before administration. For UM-22B xenografts, mice were treated with 5mg/kg placebo or RAD001 daily for 12 days.

3.2.9 Statistics

Statistical significance of biochemical assays was determined by Student's t-test. The group differences in *in vitro* proliferation, invasion and viability assays were tested with the exact Wilcoxon test. All p-values were determined using the StatXact Statistical Software Version 6.1. $P \leq 0.05$ was considered to be statistically significant. The differences between treatment groups in xenograft experiments were tested with the exact Wilcoxon Mann Whitney 1-sided test.

3.3   RESULTS

3.3.1   Expression of phosphorylated p70S6K is increased by GPCR stimulation of HNSCC cells in the absence of EGFR

GPCR ligands including PGE2, BK, GRP and LPA have each been shown to activate EGFR and promote proliferation of many types of cancer cells including HNSCC [39, 77, 81]. However, EGFR inhibition strategies have demonstrated limited clinical efficacy to date implicating persistent signaling through oncogenic signaling pathways in the setting of EGFR blockade. To begin to identify which proteins are phosphorylated by GPCRs in the absence of EGFR, we performed a phospho-protein antibody array using lysates from HNSCC cells that were transfected with control and EGFR siRNA, and then stimulated with (or without) the GPCR ligand PGE2. The array was performed using cells where EGFR siRNA was shown to abrogate EGFR expression (Figure 10A). From the phospho-protein array, six phosphorylated proteins, out of a total 155 examined, were induced greater than 2-fold in EGFR siRNA transfected HNSCC cells stimulated with PGE2 for 10 minutes (Table 3). Levels of phosphorylated p70S6K were induced to the greatest degree (5.6-fold). Immunoblotting of the same lysates used in the phospho-protein array was performed to validate these findings. As shown in Figure 10B, p70S6K phosphorylation was induced approximately 3-fold. To extend these observations to other HNSCC models and GPCR ligands, we examined the effect of EGFR downmodulation on GPCR-mediated p70S6K phosphorylation and found that bradykinin (BK) induced a 4-fold induction of p70S6K phosphorylation in UM-22B cells following EGFR siRNA transfection (Figure 10C; $p < 0.05$) cells and a 2.5-fold induction of phospho- p70S6K in EGFR siRNA-transfected 1483 (Figure 10D; $p < 0.05$) cells treated with BK. In addition to PGE2 and BK, we

also found that LPA induced p70S6K phosphorylation in UM-22B EGFR siRNA-transfected cells compared to the control siRNA-transfected cells (data not shown).

Table 3. Fold-increase in levels of phosphorylated proteins induced by PGE2 in EGFR siRNA-transfected cells compared to PGE2-stimulated control siRNA-transfected cells

Phospho-protein	Fold induction of PGE2-stimulated EGFR siRNA cells vs PGE2 stimulated NTC siRNA cells
Phospho-p70S6K	5.6
Phospho-PKCδ	3.6
Phospho-PKCθ	3.1
Phospho-IkBα	2.9
phospho-IRS1	2.3
Phospho-MAPK	2.2

To test the effects of EGFR loss in a clean genetic system, we looked at the expression of total and phosphorylated p70S6K in murine embryonic fibroblasts (MEFs) derived from EGFR WT and EGFR KO mice. As shown in Figure 10E, EGFR KO MEFs expressed a 3-fold higher level of phosphorylated p70S6K. These results indicate that EGFR downregulation induces phosphorylation of p70S6K, which is further augmented by multiple GPCR ligands. Therefore,

p70S6K may be a critical signaling molecule to target under conditions where EGFR expression

is downregulated in HNSCC.

A

B

C

UM-22B

NTC siRNA   EGFR siRNA

NT   BK   NT   BK

phospho-p70S6K (T389)

p70S6K

EGFR

B-tubulin

D

1483

p <0.05

Fold change in
phospho-p70S6K/p70S6K

NoTx — BK — NoTx — BK

NTC
siRNA

EGFR
siRNA

E

WT — KO

Phospho-p70S6K

p70S6K

EGFR

Fold change of
phospho-p70S6K/p70S6K

EGFR WT — EGFR KO

59
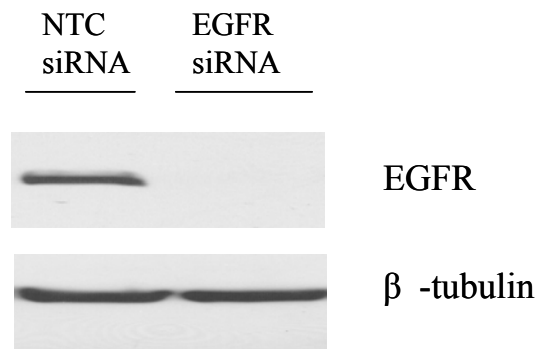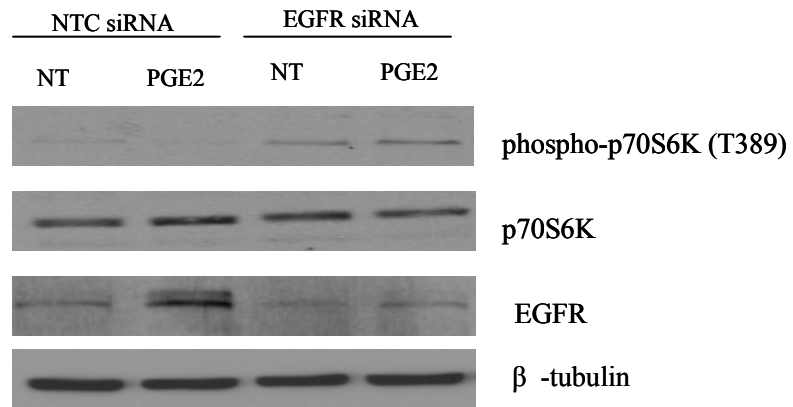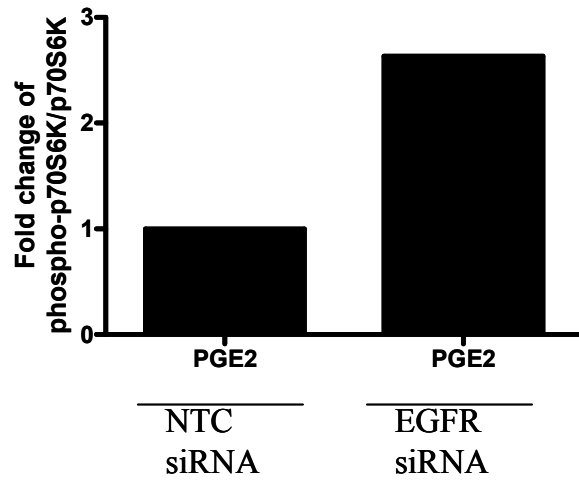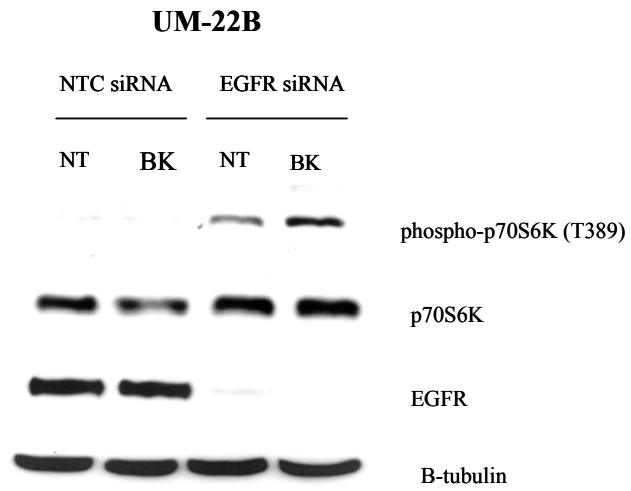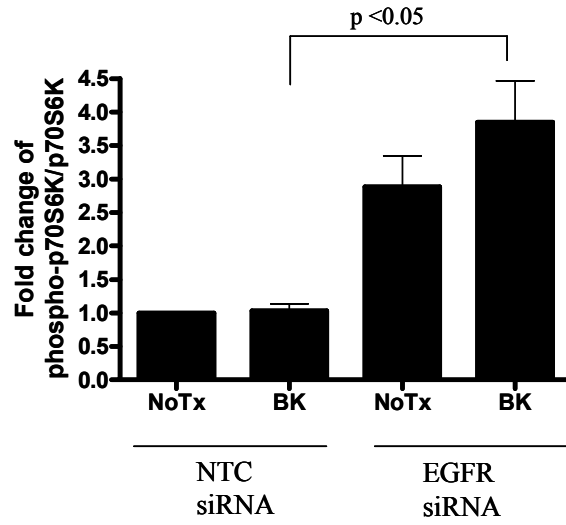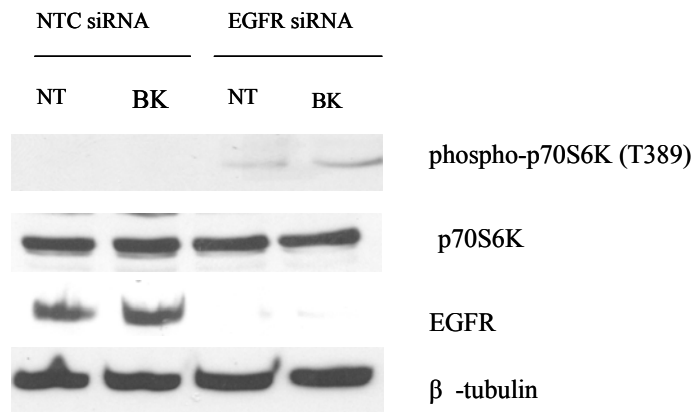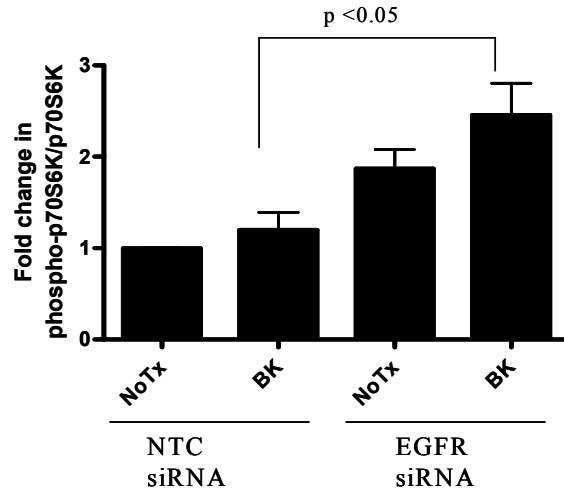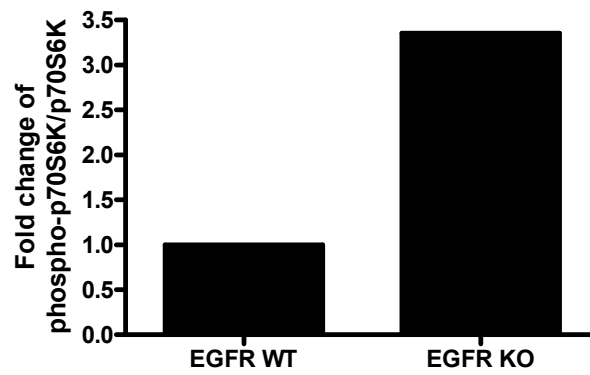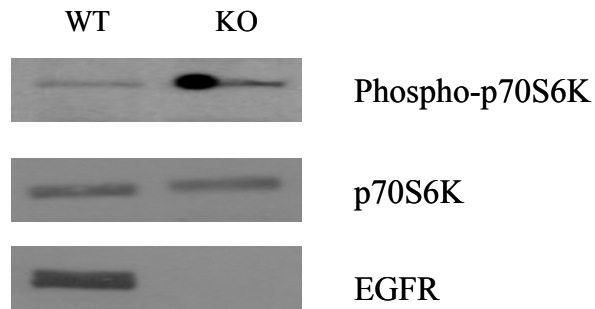
Figure 10. Decreased EGFR induces upregulation of p70S6K phosphorylation

(A) PCI-37A cells were transfected with NTC or EGFR siRNA for 72 hours. Cell lysates were examined for EGFR and β-tubulin. The experiment was repeated twice with similar results. (B) HNSCC cell lysates (PCI-37A) used for phospho-protein antibody array were assessed for expression of phosphorylated p70S6K, total p70S6K and total EGFR. (C) UM-22B and (D) 1483 cells were transiently transfected with EGFR siRNA, serum-starved for 72 hours and stimulated with either vehicle or BK (10 nM) for 10 minutes. Lysates were collected and resolved by SDS-PAGE. Denistometry represents the results of 3 independent experiments (p<0.05). (E) MEFS derived from EGFR WT or EGFR KO mice were plated in serum-containing media for 72 hours. Lysates were collected and resolved by SDS-PAGE. Denistometry represents the results of 2 independent experiments.

3.3.2 Inhibition of EGFR ligand binding does not affect BK-mediated p70S6K phosphorylation

EGFR targeting strategies that are FDA-approved or in clinical development include primarily monoclonal antibodies and small molecule tyrosine kinase inhibitors (TKI). To begin to determine the effects of inhibition of EGFR ligand binding or tyrosine phosphorylation on p70S6K phosphorylation mediated by GPCR ligands, we investigated the effects of the EGFR TKI erlotinib, and the EGFR monoclonal antibody cetuximab (C225), the latter of which is FDA-approved for the treatment of HNSCC. Treatment of HNSCC cells with 3μM of erlotinib, followed by BK or EGF stimulation resulted in the abrogation of p70S6K phosphorylation (Figure 11A). In contrast, while treatment of HNSCC cells with C225 decreased EGF-mediated

p70S6K phosphorylation, BK-mediated p70S6K phosphorylation was moderately increased in the presence of cetuximab (Figure 11B; p<0.05). To extend these *in vitro* observations to an *in vivo* HNSCC model, cetuximab-treated xenografts were assessed for expression of total and phosphorylated p70S6K. As shown in Figure 11C, treatment of HNSCC tumor-bearing mice with cetuximab led to sustained (not reduced) expression levels of phosphorylated p70S6K. Two out of the seven cetuximab treated tumors displayed lowered phosphorylated p70S6K. These observations indicate that cetuximab does not decrease p70S6K signaling, which may be critical to promoting tumor survival in the setting of blockade of EGFR ligand binding.

A

B

C

Vehicle   C225

Phospho-p70S6K

p70S6K

EGFR

β -tubulin

(Top graph — y-axis: Mean fold induction of phospho-p70S6K/p70S6K vs Vehicle)

Vehicle   C225

(Bottom graph — y-axis: Ratio of EGFR/b-tubulin vs vehicle)

p<0.05

Vehicle   C225
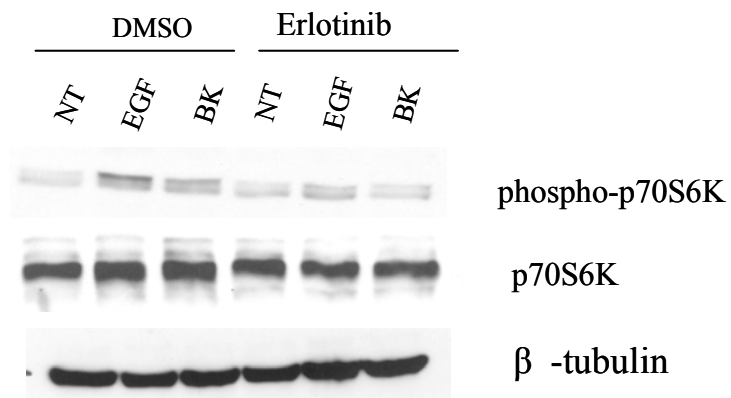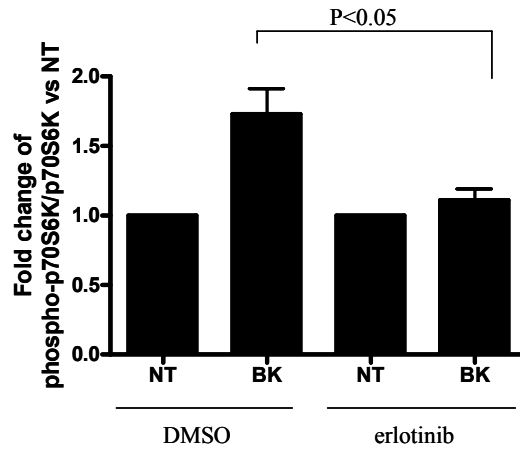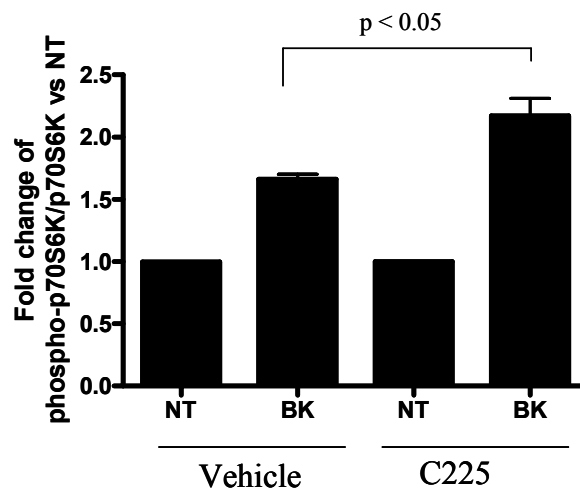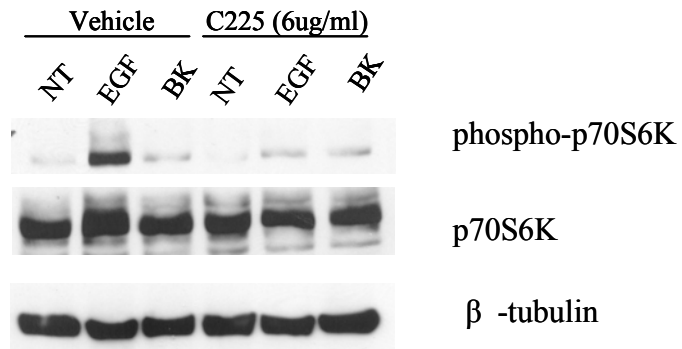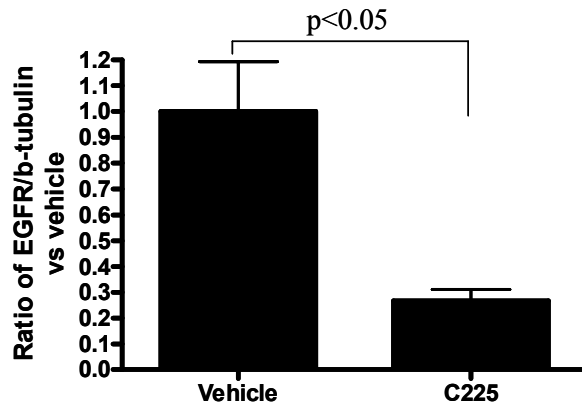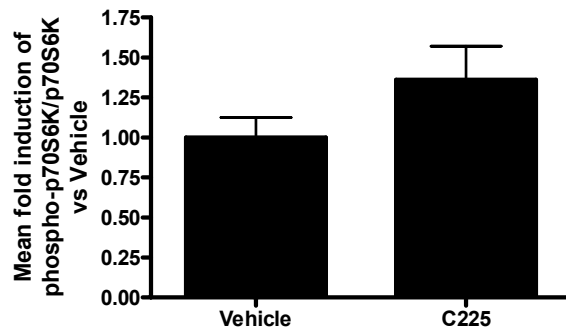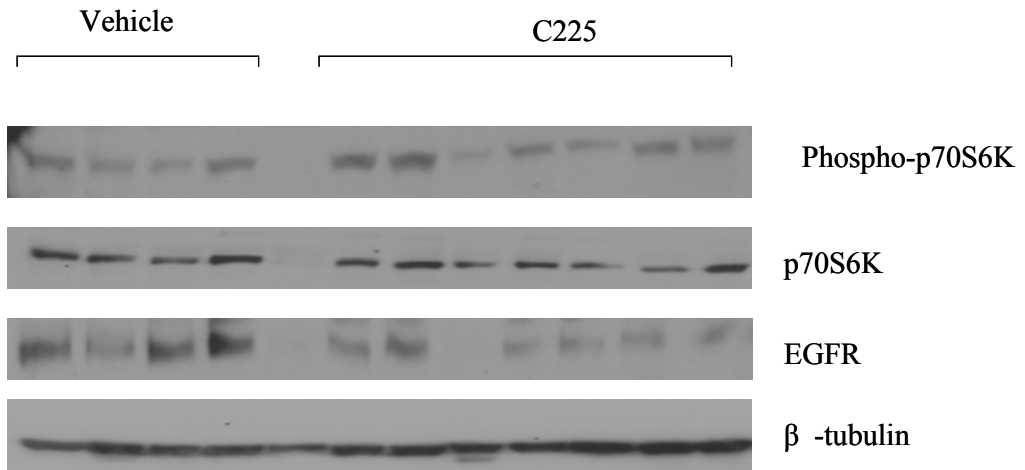
Figure 11. P70S6K phosphorylation is abrogated by erlotinib but is sustained with cetuximab treatment.

1483 cells were serum-starved for 72 hours, pre-incubated with (A) erlotinib (3μM) or (B) C225 (6μg/ml) for 2 hours and stimulated with EGF (10 ng/ml) or BK (10 nM) for 10 minutes. Lysates were collected and resolved by SDS-PAGE. Densitometry represents the values from three independent experiments (p<0.05). (C) HNSCC (1483) xenograft lysates from vehicle and C225-treated mice were analyzed for phospho-p70S6K by immunoblotting. Densitometric analysis of phosphorylated p70S6K and EGFR expression was calculated and graphed.

3.3.3 Targeting p70S6K inhibits cell proliferation, an effect that is enhanced in the setting of EGFR downregulation or inhibition

Since p70S6K phosphorylation was induced by both downmodulation of EGFR expression and inhibition of EGFR ligand binding, we next examined the phenotypic effect of targeting p70S6K in HNSCC. siRNA completely abrogated the protein expression of p70S6K in both UM-22B and 1483 cell lines 72 hours post-transfection in conjunction with significant growth inhibition compared to control siRNA-treated cells (p=0.002; Figure 12A and B). To test the hypothesis that p70S6K contributes to proliferation in a clean genetic system, we treated MEFs from EGFR KO and WT mice with p70S6K siRNA. As shown in Figure 12C, knockdown of p70S6K significantly abrogated proliferation in EGFR KO MEFs but did not affect the growth of EGFR WT MEFs, suggesting that blockade of p70S6K selectively inhibits proliferation in the absence of EGFR (P=0.002; Figure 12C). To determine the effect of cetuximab-mediated p70S6K phosphorylation on HNSCC growth, we treated control and p70S6K siRNA-transfected HNSCC cells with cetuximab followed by growth determinations. Compared to cetuximab or

p70S6K siRNA alone, we observed that the combination of cetuximab plus p70S6K siRNA

abrogated HNSCC growth in both HNSCC cell lines tested (1483 and UM-22B) (p=0.05; Figure

12D).

A

B

C

D
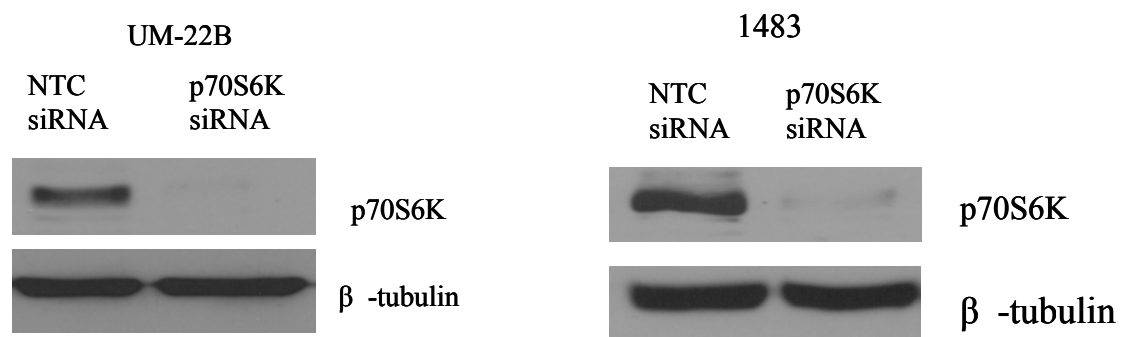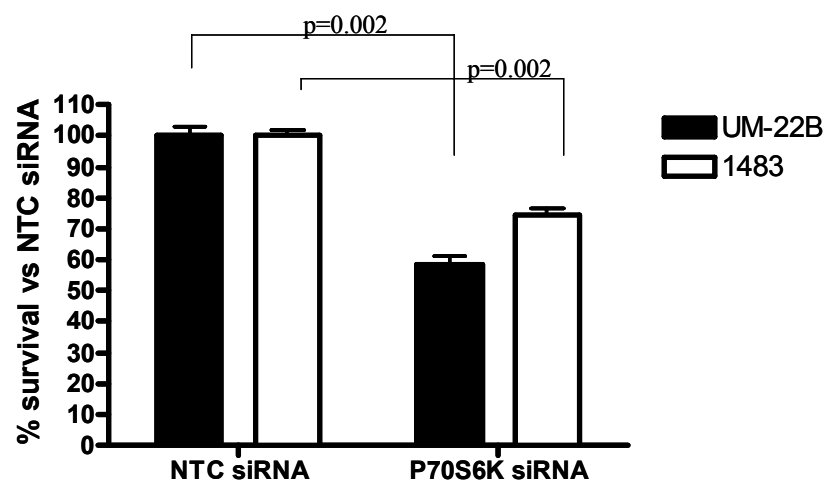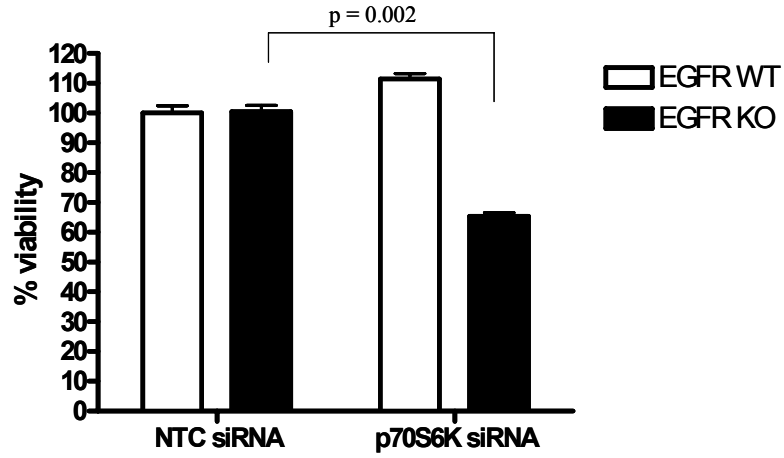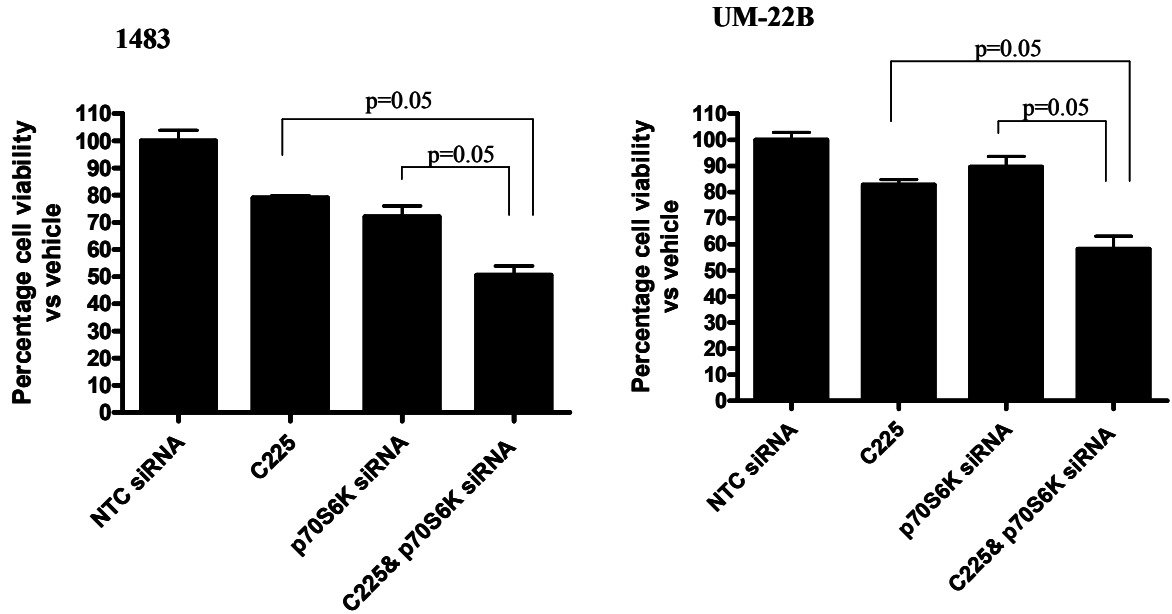
1483

UM-22B

Figure 12. p70S6K downmodulation decreases HNSCC cell proliferation in the presence of EGFR downmodulating agents.

(A) UM-22B and 1483 cells were transiently transfected with p70S6K siRNA for 72 hours. Lysates were analyzed for p70S6K expression. β-tubulin was used as a loading control. (B) UM-22B and 1483 cells were seeded in 24-well

plates and transiently transfected with control (NTC) or p70S6K siRNA for 72 hours. MTT assay was performed and percentage survival was calculated. Percentage survival was graphed using GraphPad Prism Software. The experiment was performed twice with six replicates each (p=0.002). (C) EGFR WT and EGFR KO MEFs were transfected with NTC or p70S6K siRNA for 72 hours. Cell-Titer Glo solution was added to each well and OD values were obtained. Percentage viability was calculated and graphed using GraphPad Prism Software. The experiment was performed twice with six replicates for each experiment (p=0.002). (D) 1483 and UM-22B cells were transfected with NTC or p70S6K siRNA. 24 hours later, siRNA-transfected cells were trypsinized, counted and seeded in 96-well plates. 24hours later, cells were treated with either saline or 6 µg/ml C225 for 24 hours. Cell-Titer Glo assay was performed according to manufacturer's instructions. Percentage viability was calculated and graphed using GraphPad Prism Software. The experiment was repeated twice in triplicate wells for each experiment (p =0.05).

3.3.4   RAD001 inhibits p70S6K phosphorylation and HNSCC tumor growth

P70S6K is a serine/threonine kinase that is activated downstream of the mammalian target of rapamycin (mTOR). The mTOR inhibitor RAD001 (everilomus) was previously shown to completely abrogate p70S6K phosphorylation in ovarian cancer cells at a concentration of 10 nM [130]. We observed that 10 nM of RAD001 completely abolished p70S6K phosphorylation in UM-22B after 48 hours of treatment (Figure 13A). For 1483 cells complete abrogation of p70S6K phosphorylation was observed at 100 nM (Figure 13A). Next, we investigated the effect of RAD001 on HNSCC proliferation in 3 HNSCC cell lines where IC_{50}'s ranged from 10nM to 14µM (data not shown).   To test the effects of mTOR inhibition on HNSCC tumor growth, xenograft-bearing mice were treated with RAD001 (5 mg/kg 5 times a week) in conjunction with tumor volume determinations. As shown in Figure 13B, RAD001 significantly abrogated HNSCC tumor growth *in vivo* (p=0.001). These results suggest that p70S6K is a feasible

therapeutic target in HNSCC where the mTOR inhibitor RAD001 effectively inhibits p70S6K *in vitro* and HNSCC growth *in vivo*.

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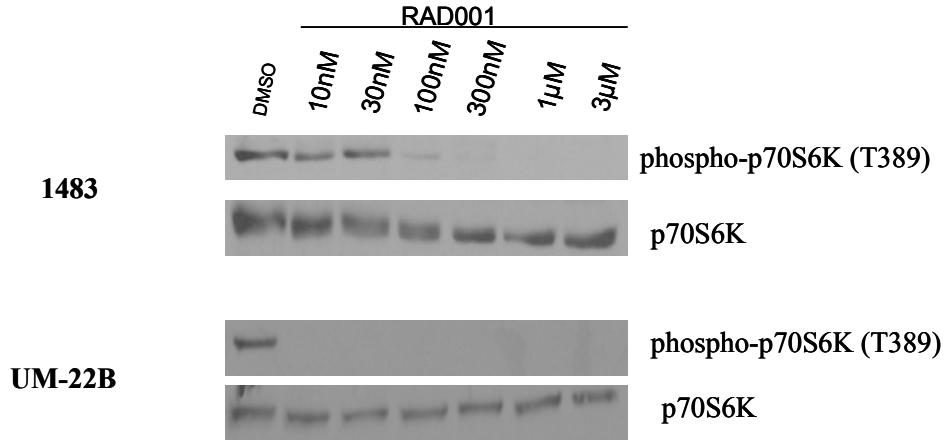
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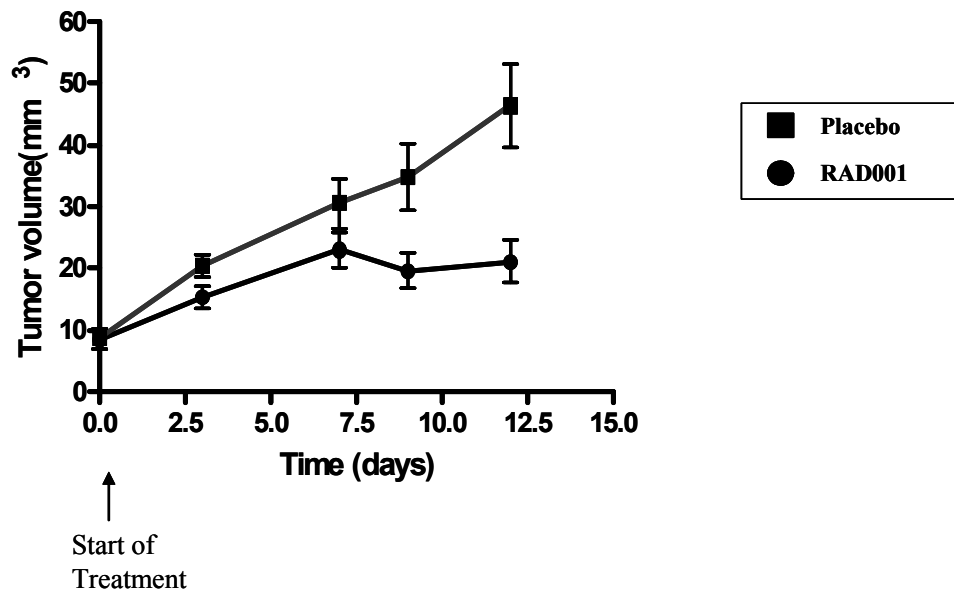
Figure 13. RAD001 abrogates p70S6K phosphorylation and inhibits HNSCC growth.

(A) 1483 and UM-22B cells were seeded and treated with various concentrations of RAD001 for 72 hours. Lysates were collected and resolved by SDS-PAGE. The experiment was repeated 3 times with similar results. (B) UM-22B cells were inoculated into the right flank of athymic nude mice. After the formation of tumor nodules (7 days), tumors were measured and mice were randomized and treated with placebo or RAD001 (5 mg/kg) daily. Tumors were measured 3 times weekly and the tumor volumes were calculated. RAD001 abrogated tumor growth (p=0.001).

3.3.5 Cetuximab combined wtih RAD001 enhances HNSCC growth inhibition *in vitro* and *in vivo*

To begin to determine whether RAD001 can enhance the effects of EGFR inhibition, we determined the effect of cetuximab and RAD001 on HNSCC growth. Although C225 decreased phosphorylated Akt, phosphorlyated p70S6K remain unchanged (Figure 14A). RAD001 treatment abrogated p70S6K phosphorylation when used alone and in combination with C225. Next, we sought to assess the combined effect of C225 and RAD001 on GPCR-mediated growth. As shown in Figure 14B, we observed that BK promoted a 1.6 or 3-fold increase in HNSCC cell growth after 24 hours in UM-22B and 1483 cells, respectively. Preincubation with cetuximab or RAD001 alone had modest effects on BK-mediated growth in both HNSCC cell lines. However, combined treatment with cetuximab plus RAD001 significantly inhibited BK-mediated cell proliferation in both models (p=0.05). In addition to proliferation, we investigated the combined effect of C225 and RAD001 on HNSCC invasion. C225 and RAD001 alone decreased 1483 invasion by 50%. However, combination of C225 and RAD001 abrogated 1483 invasion by 75% (Figure 14C). To determine the effects of combined inhibition of EGFR and mTOR/p70S6K *in vivo*, we inoculated athymic nude mice with HNSCC cells (1483) and divided mice into four treatment groups; vehicle, 0.8 mg C225 twice weekly, 5 mg/kg RAD001 fives days/week, or

combined treatment with C225 and RAD001. After 28 days we observed that the combination of cetuximab plus RAD001 significantly decreased tumor growth compared to cetuximab alone (Figure 14D; p=0.026). Tumor lysates from the mice were assessed for expression of EGFR and p70S6K by immunoblotting (Figure 14E). As shown in Figure 14E, cetuximab decreased EGFR expression in HNSCC xenografts in conjunction with sustained expression of phosphorylated p70S6K while combined treatment with cetuximab and RAD001 abrogated expression of both EGFR and phosphorylated p70S6K.

A

Phospho-p70S6K (T389)

p70S6K

β -tubulin

B

C

71
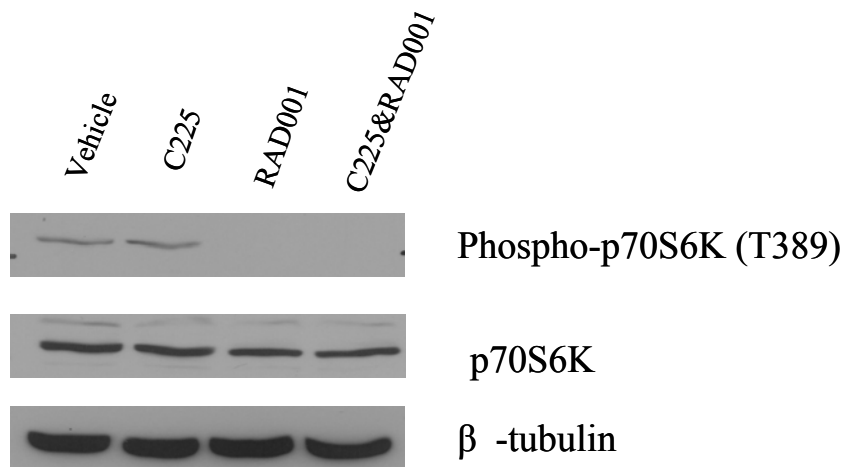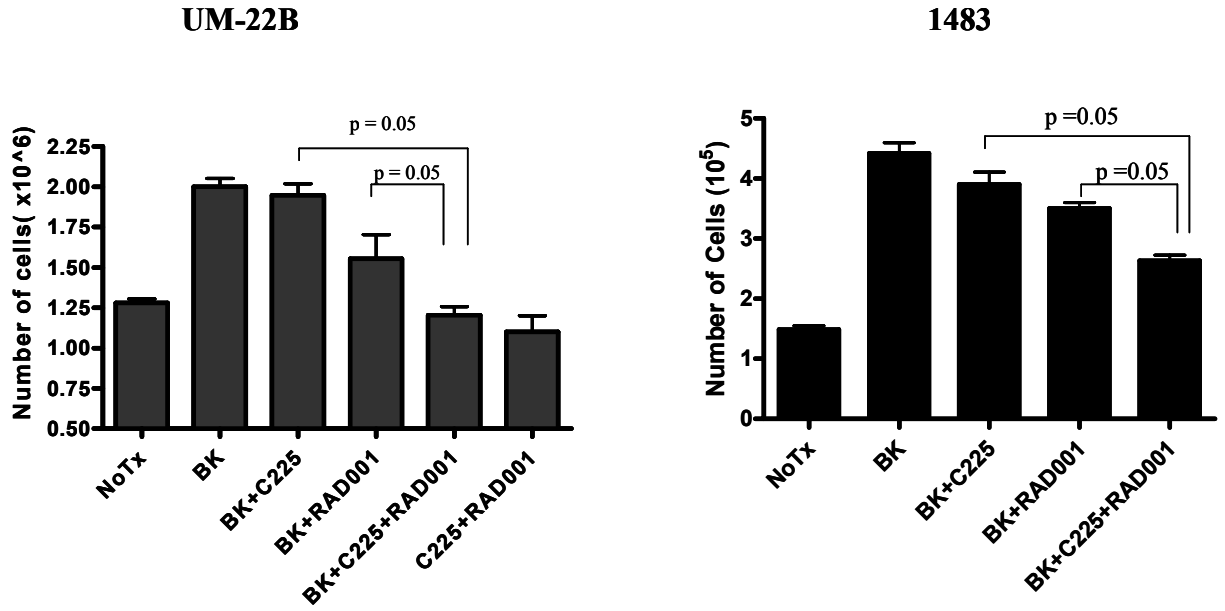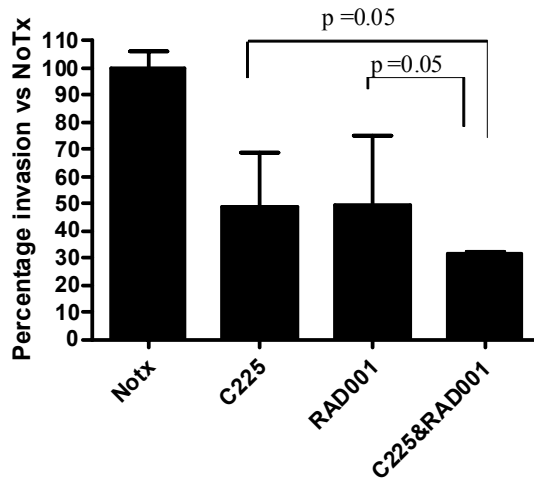
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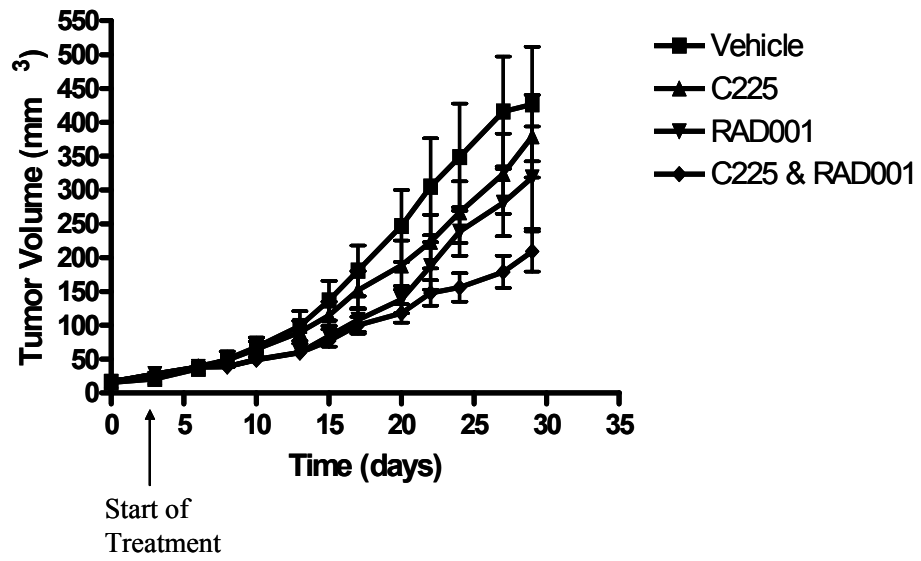
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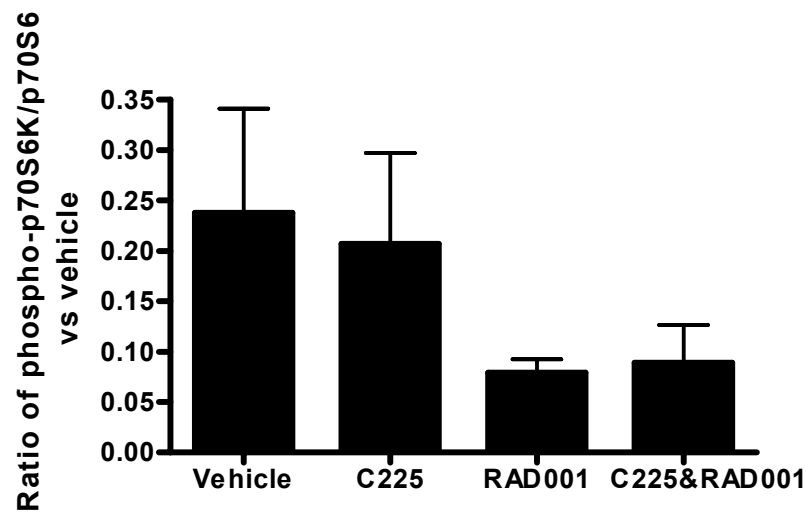
Figure 14. Cetuximab in combination with RAD001 abrogates HNSCC growth and invasion in vitro and in vivo.

(A) 1483 cells were treated with C225 (6μg/ml), RAD001 (50nM) or a combination of both C225 and RAD001 at the same concentrations for 72 hours. Lysates were analyzed for phospho-p70S6K, total p70S6K, phospho-Akt, total Akt and β-tubulin. (B) 1483 or UM-22B cells were serum-starved for 48 hours, followed by treatment with different combinations of BK (10 nM), C225 (6 μg/ml), RAD001 (100 nM), alone or in combination. Cell growth was determined by trypan blue dye exclusion. The experiment was performed twice in triplicate with similar results ($p=0.05$). (C) 1483 cells were seeded in 6-well plates and treated with C225 (6 ug/ml), RAD001 (100 nM), or a combination of C225 and RAD001 at the same concentrations in serum-free media for 48 hours. Cells were trypsinized and replated in the inserts of the invasion chambers in duplicate. 48 hours later, invaded cells were counted from 6 representative fields using light microscopy. Percentage invasion was calculated and graphed ($p=0.05$) (D) 1483 cells were inoculated in the right flank of athymic nude mice. After the formation of tumor nodules (7 days), mice were randomized into four treatment groups to insure comparable starting tumor volumes across treatment groups (10 mice per group); 1) vehicle (400 μl saline and 5 mg/kg placebo), 2) C225 (0.8 mg twice weekly), 3) RAD001 (5 mg/kg 5 days/week), and 4) C225 and RAD001 at the same doses. Tumors were measured 3 times weekly and tumor volumes were calculated (vehicle vs C225&RAD001; $p=0.0217$, C225 vs C225&RAD001; $p=0.03$). (E) Lysates from tumor xenografts were analyzed by immunoblotting for phospho-p70S6K, total p70S6K, total EGFR and β-tubulin (loading control). Densitometric analysis of phospho-p70S6K/p70S6K from immunoblot of tumor lysates

3.3.6 P70S6K phosphorylation induced by EGFR inhibition is dependent on novel PKCs.

From the phospho-protein array, we observed that two PKC isozymes from the novel subfamily of PKCs, PKCδ and PKCθ were increased 3-fold in PGE2-treated cells transfected with EGFR siRNA (Table 1). Evidence to date suggests that PKCδ can activate p70S6K directly

or via activation of mTOR, an upstream activator of p70S6K [131, 132]. First, we analyzed the EGFR WT and KO MEFS for phosphorylated levels of PKCδ/θ. In Figure 15A, we observed that EGFR KO MEFS expression of phosphorylated PKCδ/θ was approximately five-fold greater than in EGFR WT MEFS. To determine the contribution of PKCδ and PKCθ to the increased p70S6K phosphorylation levels observed in HNSCC cells exposed to EGFR siRNA or cetuximab, we treated HNSCC cells transfected with EGFR siRNA with the selective PKC inhibitor Rottlerin. Rottlerin is a highly selective inhibitor of PKCδ and PKCθ. As shown in Figure 15B, Rottlerin abrogated EGFR siRNA-induced p70S6K phosphorylation, similar to what was observed with the mTOR inhibitor RAD001. Since Rottlerin targets both PKCδ and PKCθ, we wanted to determine which PKC isozyme, PKCδ or PKCθ, was critical for p70S6K phosphorylation. PKCδ has been reported to contribute to p70S6K and ERK activation [133]. Using siRNA designed against PKCδ, we found that PKCδ contributes to the p70S6K phosphorylation observed in EGFR siRNA treated cells (Figure 15C). These results indicate that the novel PKC, PKCδ plays a role in EGFR siRNA-mediated p70S6K phosphorylation.

A

B

C
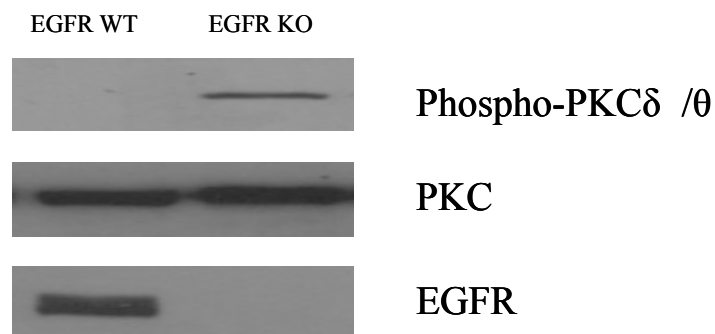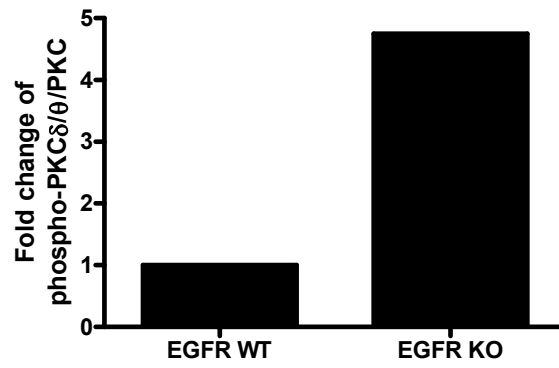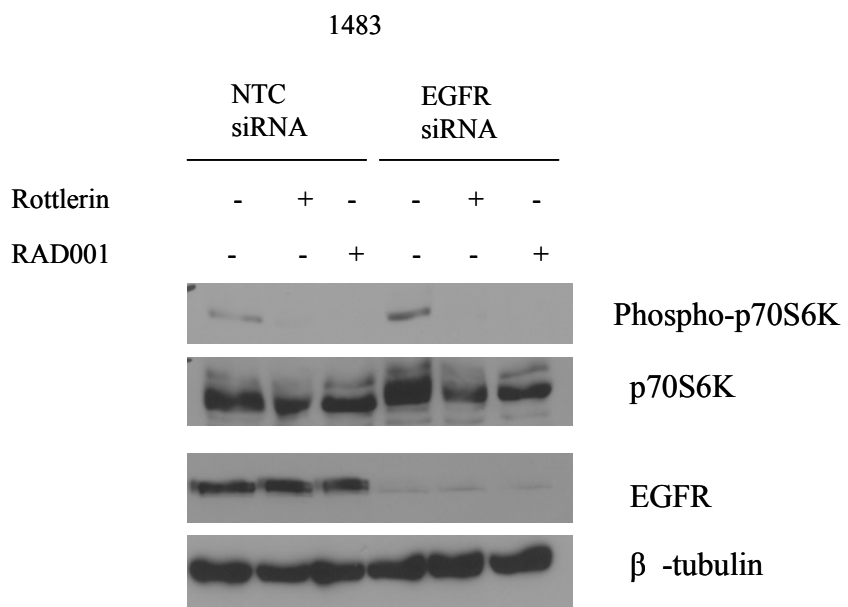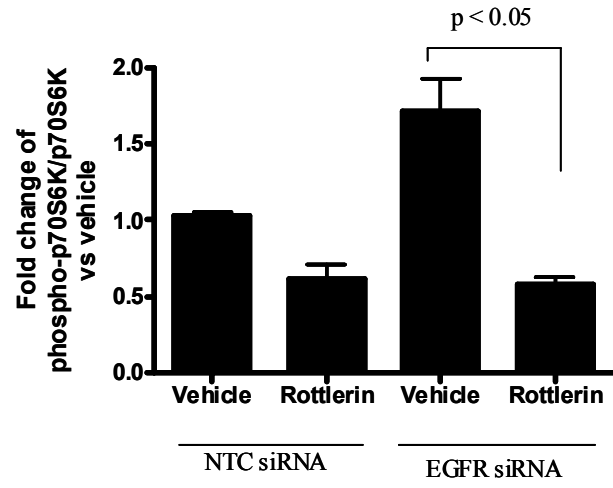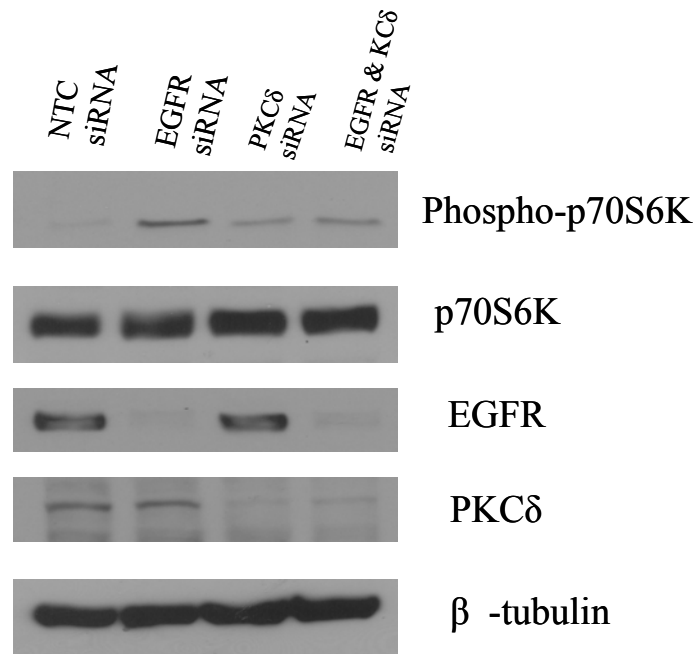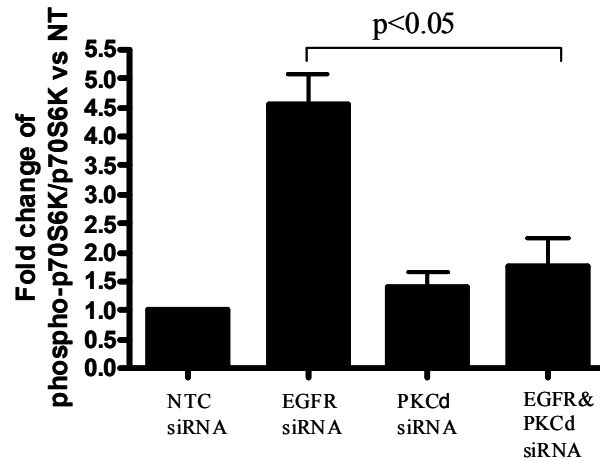
Figure 15. PKCδ inhibition abrogated p70S6K phosphorylation mediated by EGFR downmodulation

(A) EGFR MEFS derived from EGFR WT and EGFR KO mice were plated in serum-containing media for 72 hours. Lysates were collected and resolved by SDS-PAGE for PKCδ and PKC expression. Experiment was repeated twice with similar results (B) 1483 cells were transiently transfected with EGFR siRNA, 24 hours post-transfection, cells were treated with either DMSO, Rottlerin (3μM) or RAD001 (50nM) for 48 hours. Lyastes were collected and resolved by SDS-PAGE. The experiment was repeated 3 times with similar results ($p<0.05$). (C) 1483 cells were transiently transfected with NTC, EGFR, PKCδ, or both EGFR and PKCδ siRNA for 72 hours. Lysates were collected and resolved by SDS-PAGE. Experiment was repeated thrice with similar results

3.3.7 PDK1 contributes to EGFR siRNA and C225-mediated p70S6K phosphorylation

The PI3K/Akt pathway has been reported to phosphorylate and activate different PKC isoforms including PKCδ [134] .However, we observed that both EGFR siRNA and C225 abrogated Akt phosphorylation (data not shown), therefore mTOR and p70S6K phosphorylation was mediated by an Akt-independent mechanism. PDK1 is a central serine/threonine kinase that phosphorylates multiple members of the AGC kinase family including Akt, PKC, PAK1 and p70S6K [97]. Given our finding that PKCδ contributes to p70S6K phosphorylation induced by GPCR ligands, we investigated the possible role of PDK1 in mediating p70S6K phosphorylation in EGFR siRNA-treated cells. As shown in Figure 16A, we observed that PDK1 siRNA abrogated PDK1 protein expression 72 hours post-transfection. EGFR knockdown resulted in phosphorylation of p70S6K, which was inhibited in cells transfected with PDK1 siRNA. Next, we looked at the contribution of PDK1 in C225 and BK-treated cells. We observed that BK-mediated p70S6K phosphorylation in the presence of C225 was decreased in PDK1 downmodulated cells (Figure 16B). These cumulative results suggest a new model where GPCR stimulation induces oncogenic pathways in the setting of EGFR blockade using the clinical agent cetuximab. Cetuximab treatment induces activation of p70S6K via a pathway that involves PDK1, selected PKC isoforms, and mTOR.

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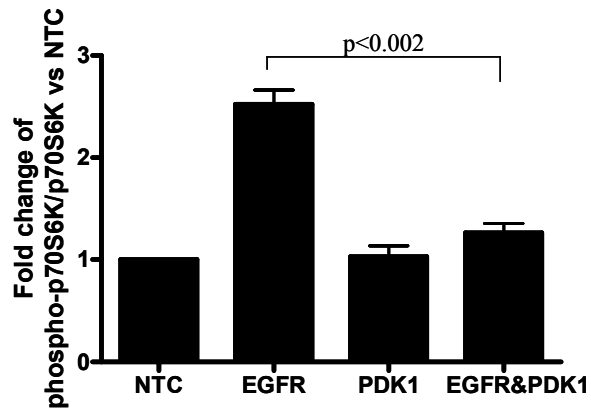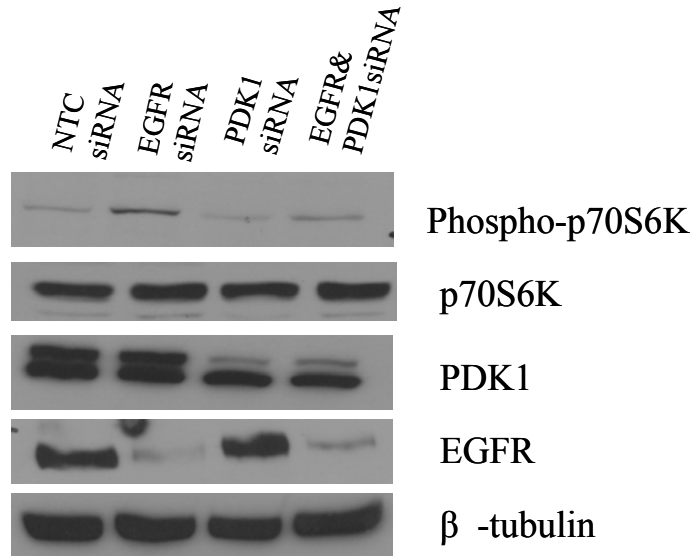
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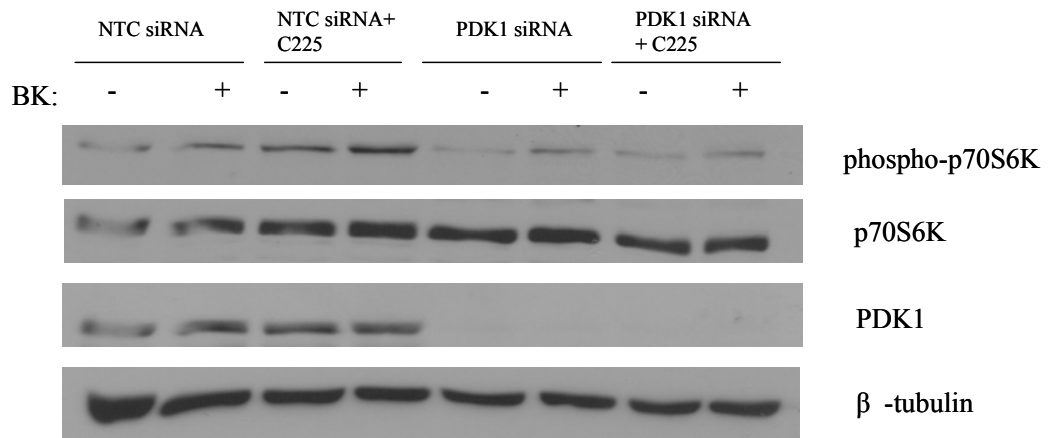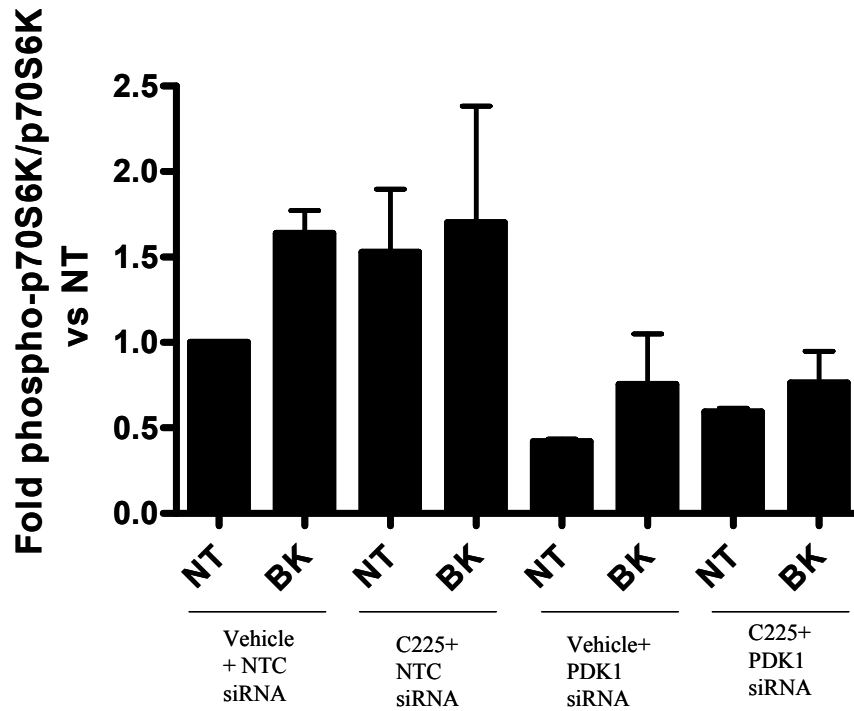
Figure 16. PDK1 contributes to EGFR siRNA and C225-mediated p70S6K phosphorylation

(A) 1483 cells were transiently transfected with NTC, EGFR, PDK1 or both EGFR and PDK1 siRNA for 72 hours.

Lysates were resolved and analyzed for phospho-p70S6K. Experiment was repeated twice with similar results. (B)

HNSCC cells were transiently transfected with control or PDK1 siRNA and serum-starved for 72 hours. Cells were

either preincubated with saline or 6ug/ml C225 for 2 hours followed by stimulation with 10nM BK for 10 minutes.

Lysates were collected and resolved by SDS-PAGE. Experiment was performed thrice with similar results
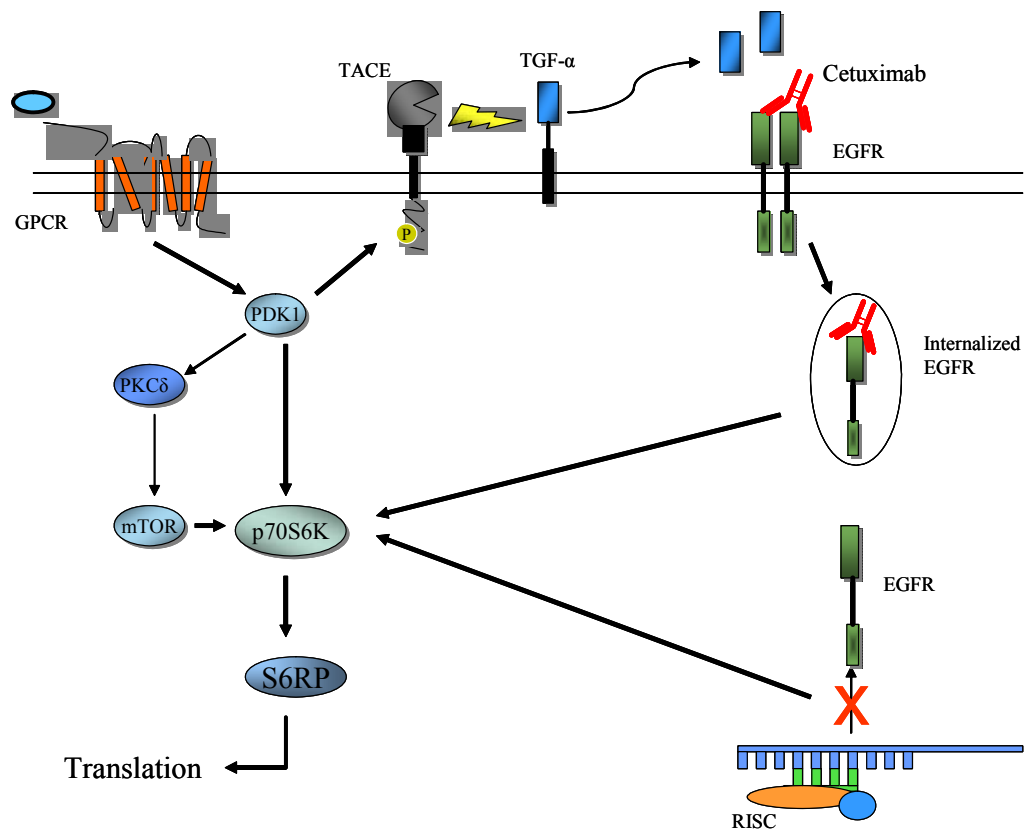
Figure 17. Model of BK-mediated p70S6K phosphorylation in presence of EGFR downmodulating agents

Downmodulation of EGFR by EGFR siRNA induces p70S6K phosphorylation. Further augmentation of p70S6K phosphorylation was observed with GPCR ligand stimulation. Cetuximab (C225) treatment does not abrogate p70S6K phosphorylation in the presence and absence of GPCR stimulation. Phosphorylation of p70S6K in the presence of EGFR siRNA and C225 is dependent on the novel PKC, PKCδ, and the serine/threonine kinase PDK1. p70S6K and PKCδ activity is inhibited by RAD001 and Rottlerin respectively.

3.4   DISCUSSION

EGFR is an established therapeutic target for HNSCC based on studies performed using preclinical models [23, 29]. To date, HNSCC patients have demonstrated limited clinical response to EGFR tyrosine kinase inhibitors [135, 136]. The monoclonal EGFR antibody cetuximab (C225/Erbitux) was FDA-approved for the treatment of primary HNSCC in combination with radiation [13]. A more recent phase III trial demonstrated prolonged survival using cetuximab in combination with chemotherapy in recurrent or metastatic HNSCC [127]. Despite widespread EGFR expression, cetuximab is only effective in a subset of HNSCC patients. The reasons for the limited response to EGFR blockade in HNSCC are incompletely understood. G-protein-coupled receptors (GPCRs) have been shown to promote the aggressive phenotype of different cancer models including HNSCC via both EGFR-dependent and EGFR-independent mechanisms [6, 33, 60, 78, 111, 116, 137]. Combined inhibition of GPCRs and EGFR resulted in enhanced anti-tumor effects indicating that GPCRs contribute to tumorigenic signaling in the setting of EGFR blockade [83, 116]. The results of the present study indicate that EGFR downmodulation by cetuximab (or siRNA) in the presence and absence of GPCR ligands induces oncogenic signaling via activation of a PDK1/PKC/mTOR pathway resulting in increased phosphorylation of p70S6K and tumor cell survival (Figure 17).

Cancer cells have been shown to selectively activate alternative signaling pathways in the setting of single pathway inhibition [138, 139]. Stommel *et al* reported that in glioblastoma cell lines, xenografts and primary tumors, various receptor tyrosine kinases are simultaneously activated resulting in the sustained activation of signaling pathways in the face of RTK monotherapy [140]. Blockade of specific pathways have been shown to initiate feedback mechanisms that trigger pro-survival signaling cascades in cancer. For example, inhibition of the

PI3K/Akt pathway stimulates the MAPK/ERK signaling cascade in different cancer models [141, 142]. Here, we show that EGFR downmodulation led to increased p70S6K phosphorylation, which was further augmented with GPCR stimulation. This indicates that p70S6K signaling is enhanced by GPCR ligands present in the tumor microenvironment and may represent a compensatory signaling pathway initiated by EGFR targeting agents. While studies to date have not identified p70S6K as a possible feedback mechanism in response to EGFR downmodulation, several reports demonstrate that inhibition of EGFR results in activation of the insulin growth factor receptor pathway, which signals via the PI3K/Akt/p70S6K pathway [2, 138, 139]. Furthermore, IGF1R downmodulation has been shown to augment EGFR signaling [143]. Therefore, it is possible that EGFR downmodulation in HNSCC leads to increased p70S6K phosphorylation via increased insulin signaling. The results from our phospho-antibody array indicated that Ser312 IRS-1 phosphorylation was also increased in EGFR siRNA cells (Table 1). P70S6K activation induces Ser312 phosphorylation and inhibits IGF1R-mediated signaling by a negative feedback mechanism [132, 144-146]. The possible role of the insulin-signaling pathway in mediating p70S6K phosphorylation remains to be elucidated in cancers treated with EGFR inhibitors.

The serine/threonine kinase p70S6K phosphorylates the S6 ribosomal subunit to mediate translation of proteins that contribute to cell survival [147]. P70S6K is a multi-phosphorylation site protein kinase that is activated by the PKB/Akt substrate, mTOR. Interestingly, we observed that EGFR siRNA or cetuximab increased p70S6K phosphorylation whereas the EGFR TKI erlotinib decreased p70S6K phosphorylation. A recent report showed that cancer cells were able to survive EGFR TKI inhibition via sustained glucose transport and metabolism [148]. Therefore, the increased p70S6K phosphorylation observed with EGFR downmodulation may be

a compensatory signaling pathway used to overcome the decreased glucose transport in HNSCC cells.

The induction of p70S6K phosphorylation by EGFR downmodulation was augmented by treatment with GPCR ligands. GPCRs have been shown to augment EGF-mediated p70S6K signaling in non-transformed cells. We previously reported that combined inhibition of EGFR and the GPCR gastrin-releasing peptide receptor resulted in additive abrogation of phospho-p70S6K indicating the possible involvement of p70S6K in an EGFR-independent manner in HNSCC [83]. In NSCLC and ovarian cancer cell lines, EGFR-independent signaling pathways were shown to be dependent PKCs [67, 118]. However, no investigation into the specific isoforms of PKCs involved was undertaken. The results of our forward phase array in EGFR siRNA-treated HNSCC cells (Table 3), as well as findings using EGFR KO MEFs, indicated augmentation of phosphorylated PKCδ/θ. The precise mechanism of GPCR-mediated activation of PKC has not been elucidated. PKCδ and PKCθ are members of the novel subfamily of PKCs. PKCδ has been shown to be either pro or antimitogenic in different model systems [149-152] , while PKCθ has been implicated in the survival of T cells by activating the transcription factors NFAT and AP-1, leading to IL-2 production [153, 154]. While total PKCδ expression has been reported in HNSCC, phosphorylated PKCδ was not detected in a panel of cell lines tested [155]. We found that inhibition or downmodulation of PKCδ abrogated p70S6K phosphorylation in the presence of EGFR siRNA. These findings are consistent with previous reports indicating that PKCδ mediates both p70S6K and 4EBP-1 phosphorylation via direct activation of mTOR [131, 156]. However, these prior studies have not shown PKCδ-mediated p70S6K phosphorylation in

an EGFR-independent setting. These cumulative observations indicate that PKCδ activity in HNSCC may be induced by EGFR downregulation and/or blockade.

PKCδ is reported to participate in the PKB/Akt pathway [134]. Furthermore, PKCδ is a direct substrate of the critical serine threonine kinase PDK1 [119, 157]}. We previously reported that PDK1 mediates GPCR-induced EGFR ligand cleavage where its downmodulation enhances EGFR inhibition in HNSCC [79]. In the present study, PDK1 downmodulation abrogated p70S6K phosphorylation in presence of EGFR targeting agents. PDK1 also directly phosphorylates the T229 site on p70S6K leading to complete activation of p70S6K [158]. One possible mechanism of p70S6K phosphorylation may involve PDK1-mediated activation of PKCδ, which leads to mTOR activation and phosphorylation of p70S6K. Therefore, the PDK1 signaling pathway not only promotes EGFR ligand release but can also induce pro-survival signaling in the absence of EGFR by activation of p70S6K.

Increasing evidence suggests that blockade of a single signaling pathway may induce activation of alternative pathways by feedback mechanisms and pathway cross-talk in cancer cells. EGFR is the only FDA-approved molecular target in HNSCC and patients are generally treated with cetuximab alone or in combination with chemotherapy or external beam radiation, leading to anti-tumor responses in only a small subset of cases. The results of the present study suggest that EGFR downmodulate by cetuximab induces p70S6K phosphorylation, which mediates HNSCC survival in the presence of EGFR targeting agents. We further demonstrate that combined administration of cetuxmiab and the mTOR inhibitor RAD001 abrogated p70S6K phosphorylation and HNSCC *in vitro* and tumor growth *in vivo* growth. Phosphorylation of

p70S6K by EGFR downregulation was dependent on PDK1 and PKCδ. To date, there are no specific PDK1 inhibitors approved for clinical use. Potential inhibitors of PDK1 such as UCN-01 and OSU03012 have also been shown to inhibit other kinases with similar affinities to PDK1 [101, 159, 160]. The PKCδ inhibitor, KAI9803 [161], recently demonstrated safe and prevention of injury associated with cardiac arrest in Phase I clinical trial. However, isoform-specific PKC inhibitors have not yet being widely tested. The effects of mTOR inhibitors have been shown to be promising in preclinical HNSCC models [162-164]. Therefore, p70S6K represents a promising therapeutic target, particularly in combination with EGFR blockade using cetuximab in HNSCC, as EGFR downmodulation appears to induce survival pathways that can be blocked by inhibition of p70S6K.

4.0   SUMMARY AND DISCUSSION

The poor efficacy of EGFR monotherapy in HNSCC patients has led us to investigate the mechanisms contributing to sustained HNSCC tumorigenesis. In this thesis we focused on investigating the therapeutic efficacy of targeting signaling intermediates involved in GPCR-mediated tumorigenesis in both EGFR-dependent and –independent backgrounds. Identification and targeting of these common signaling intermediates together with EGFR may have promising clinical benefits in HNSCC therapy.

4.1   PDK1 IS A COMMON SIGNALING INTERMEDIATE IN GPCR-EGFR CROSSTALK IN HNSCC

PDK1 is a pivotal serine/threonine kinase that activates multiple substrates that contribute to cell motility, differentiation and growth[97, 108]. PDK1 has been demonstrated to activate kinases including PKC, p70S6K, PAK1 and Akt. Previous studies in our laboratory showed that the GPCR ligand, GRP induced amphiregulin (AR) and TGF-α release via src and TACE-dependent mechanisms[165]. Additionally, we demonstrated that GRP stimulated PDK1-mediated phosphorylation of TACE, which led to EGFR ligand release. We also reported that two pro-inflammatory GPCR ligands, PGE2 and BK also mediate EGFR activation via TGF-α ligand release in a TACE-dependent manner [116]. However, the possible role of PDK1 in PGE2

and BK-mediated EGFR activation was unknown. In this study, we demonstrated that PDK1 contributed to both PGE2 and BK-mediated phosphorylation of EGFR. Studies undertaken by Gschwind *et al* illustrated that LPA-mediated EGFR phosphorylation in HNSCC cells. We observed that LPA-mediated EGFR phosphorylation was also dependent on PDK1.

These results indicate that similar to Src and TACE, PDK1 is a common signaling intermediate in GPCR-mediated EGFR activation. The autocrine pathway between TGF-α and EGFR contributes to HNSCC growth indicating the important role of EGFR activation plays in HNSCC phenotype. In addition to EGFR activation, this study showed that GPCR-mediated growth was partially dependent on PDK1 as shown in Chapter 2. HNSCC tumors have increased expression levels of GRPR, B2R and PGE2 [35, 166]. Reports from our laboratory have shown that combined inhibition of those GPCR pathways and EGFR led to improved anti-tumor effects [REF]. However, targeting one GPCR does not inhibit the signaling mediated by another, considering the heterogeneity and high serum levels of GPCR ligands in HNSCC tumors. Furthermore, it is clinically irrational to target multiple GPCRs and EGFR for therapeutic purposes. Identification of PDK1 as a common signaling intermediate in HNSCC provides a much more feasible therapeutic avenue for treating HNSCC patients in the future. HNSCC patient subsets that demonstrate high GPCR and GPCR ligand expression may benefit from PDK1 targeting therapy. Furthermore, the improved anti-tumor efficacy of PDK1 knockdown and EGFR inhibition is testament to the benefits of targeting PDK1 and EGFR in HNSCC [79].

4.2   OSU-03012 IS AN EFFECTIVE THERAPEUTIC AGENT FOR HNSCC

Along with PDK1, PI3K, src and TACE are common signaling intermediates in GPCR-EGFR crosstalk in HNSCC. However, PDK1 is a more feasible and promising molecular target in HNSCC and cancer in general. In addition to having only one isoform, PDK1 hypomorphic mice are viable indicating that targeting PDK1 in cancer will not have a lethal effect on normal cells [167]. PDK1 also activates various serine/threonine kinases such as PKC, Akt and PAK1 that promote cellular proliferation, invasion and motility [97, 108, 158]. C-Src is one of 4 members of the Src family of kinases (SFKs) activated by TGF-α in HNSCC [168]. Use of non-selective src kinase inhibitors have been used to show the role of Src in GPCR-mediated activation of EGFR [80, 81]. However, the specific SFK involved in GPCR-EGFR crosstalk remains to be elucidated. PI3K has remained a prominent therapeutic target among all cancer models [169]. Unlike PDK1, PI3K has many isoforms for which selective inhibitors are being designed to inhibit [170].

With all the above advantages, there is still a paucity of specific PDK1 inhibitors available. The cyclin-dependent kinase (CDK) inhibitor, UCN-01 was reported to inhibit PDK1 with an IC_{50} of 33nM range and inhibit tumorigenesis in preclinical models [159, 171]. Furthermore, HNSCC also displayed promising responses to UCN-01 treatment from *in vitro* and *in vivo* studies [172]. Phase I clinical trials also demonstrated promising responses [173]. However, UCN-01 also inhibited other kinases with nM affinity in addition to PDK1 [159]. Another report discussed different classes of inhibitors that demonstrated inhibition of PDK1 in addition to other kinases [174].

From a screen of celecoxib derivatives, Chen *et al* identified a candidate PDK1 inhibitor OSU-03012, which inhibited PDK1 kinase activity and PC3 proliferation with IC_{50s} of 3μM and

2 μM respectively [122]. Our studies demonstrate that OSU-03012 inhibits phospho-Akt. However, we further observed that HNSCC cell lines displayed sensitivity to OSU-03012 with IC_{50s} in the nM range. This was in contrast to other tumor models such as breast cancer, glioblastoma, chronic myelogenous leukemia (CML) and rhabdomyosarcoma that demonstrated IC_{50s} in the μM range[101, 175-177] . Multiple reports of OSU-03012 indicated various PDK1-independent biochemical and phenotypic effects in different cancer models also. More importantly, we showed that normal epithelial cells had an IC_{50} which was 10 fold higher than the IC_{50} observed in HNSCC cell lines. We observed that PDK1 expression contributed to the sensitivity of HNSCC cell lines to OSU-03012. Furthermore, OSU-03012 inhibited GPCR-mediated growth and enhanced the anti-proliferative effects of EGFR tyrosine kinase inhibition.

Although OSU-03012 induced cytotoxic effects via pleiotropic inhibition of molecules involved in proliferation, cell cycle and apoptosis and enhanced EGFR inhibition, it remains a non-specific PDK1 inhibitor. However, it is worth noting that OSU-03012 demonstrated a greater affinity for a PDK1 substrate PAK1. Therefore, based on the promiscuous role of PDK1, OSU-03012 may demonstrate greater affinity for the ATP binding pockets of PDK1 substrates compared to PDK1, which may explain its cytotoxic efficacy in HNSCC compared to other cancer models. OSU-03012 was handed over to Arno Therapeutics for clinical development. The FDA approved the testing of the modified OSU03012 compound, now called AR-12, for a Phase I clinical trial in adults with solid tumors or lymphoma for which no standard therapy is available[2]

Another group of PDK1 inhibitors have been reported to inhibit PDK1 with greater specificity than UCN-01 and OSU03012. BX-320, BX-795 and BX-912 displayed anti-tumor

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[2] http://www.arnothera.com/pr090511.html

effects with IC_{50}s in the nM range [160]. However, there are no current reports on the clinical development of these compounds.

4.3 P70S6K IS ACTIVATED IN THE PRESENCE OF EGFR DOWNMODULATING AGENTS.

In 2006, Cetuximab was FDA-approved for the treatment of HNSCC patients in combination with radiation. The promising responses of patients to cetuximab were in contrast to patients with recurrent/metastatic HNSCC [13, 178]. The patients with recurrent/metastatic HNSCC displayed an 11% response to cetuximab. Therefore, more aggressive HNSCC tumors may express alternate signaling patterns that contribute to survival in the presence of cetuximab. Cetuximab is a monoclonal antibody that mediates it action by inhibiting the ligand-mediated activation of EGFR. Cetuximab bound to the ligand-binding domain of EGFR subsequently results in EGFR internalization and degradation [179]. In our studies, we observed that EGFR siRNA and cetuximab augmented p70S6K phosphorylation. Furthermore, we showed that GPCR-mediated p70S6K was sustained in the presence of cetuximab. This is the first report linking EGFR downmodulation to sustained or increased p70S6K phosphorylation.

P70S6K activity is critical to cell biology by promoting cell growth, motility, survival and inhibiting apoptosis. The increased expression of p70S6K phosphorylation in EGFR siRNA-transfected cells may be indicative of a feedback mechanism that promotes HNSCC survival. The initiation of feedback mechanisms further indicate that some tumors do not display

oncogene "addiction", where they can activate alternative membrane receptors and their respective intracellular signaling cascades in the presence of monotherapeutic agents [141].

P70S6K phosphorylates the S6 ribosmal subunit to promote translation of proteins involved in growth and survival [158, 180]. Inhibition of p70S6K by rapamycin and its analogs, have displayed enhanced anti-angiogenic effects via inhibition of VEGF release [181-184]. Therefore, the induction of p70S6K activity may not only provide a growth and invasive advantage but promote angiogenesis which further nourishes the tumor to survive in the presence of cetuximab. Further studies are warranted to verify the link between EGFR downmodulation, p70S6K phosphorylation and VEGF release.

4.4   TARGETING P70S6K AND EGFR IS A FEASIBLE THERAPEUTIC STRATEGY FOR HNSCC

P70S6K has been described as a therapeutic target in HNSCC and combined inhibition of EGFR and p70S6K demonstrated additive anti-tumor effects *in vitro* and *in vivo* [164]. In addition, our study showed that combination of the FDA-approved cetuximab with the mTOR inhibitor RAD001 displayed significant anti-tumor efficacy compared to C225 treatment alone. This study is the first report showing the preclinical efficacy of the "rapalog", RAD001 (everolimus), in HNSCC. Primarily used as an immunosuppressant for organ transplants,

RAD001 was FDA-approved on March 30 2009 for treatment of kidney cancer[3]. There are currently 3 clinical trials recruiting HNSCC subjects to assess the efficacy of RAD001 in combination with other therapeutic agents[4].

Although rapamycin and its analogs demonstrate anti-tumor effects both *in vitro* and *in vivo*, they only inhibit the mTORC1 complex and not the mTORC2 complex[185-187]. However, one report has shown that RAD001 decreased both mTORC1 and mTORC2 in Acute myelogenous leukemia (AML)[188]. mTORC2 phosphorylates Ser473 of Akt, and treatment with RAD001 has shown to increase Akt phosphorylation despite reports that indicate that prolonged rapamycin treatment disrupts mTORC2 in prostate cancer cells[189]. The increased activation of Akt may promote prolonged survival in cells treated with mTOR inhibitors. However, inhibition of Akt phosphorylation has been reported to be biochemical readout for cetuximab activity, therefore strengthening the rational for combining cetuximab and RAD001 for HNSCC therapy. There are currently dual PI3K/mTOR inhibitors being developed that target both mTORC1 and mTORC2 pathways. Preliminary results in our lab show that one such compound NVP-BEZ235 displays potent anti-proliferative and anti-invasive effects alone and in combination with EGFR inhibitors in HNSCC. Therefore, targeting p70S6K by using mTOR inhibitors may increase the clinical response of HNSCC patients to cetuximab.

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[3] http://www.novartis.com/newsroom/media-releases/en/2009/1301801.shtml

[4] http://www.clinicaltrials.gov/

4.5   FUTURE DIRECTIONS

The National Cancer Institute defines translational research as "the transformation of scientific discoveries in laboratory, clinical, population studies into clinical applications to reduce cancer incidence, mortality and morbidity"[5]. To fulfill the requirements for translational research, we need to validate the finding that the sustained p70S6K phosphorylation observed in vitro and in vivo with clinical samples. Analysis of pre- and post-treatment HNSCC patient tumor microarrays for phosphorylated p70S6K is currently being done in the laboratory. The tumor microarray (TMA) possesses tumor cores from pre-treatment biopsies and biopsies from patients treated with cetuximab alone or cetuximab and radiation. The data from the TMA will indicate whether p70S6K phosphorylation is a marker for low clinical response to cetuximab and beneficial response to mTOR inhibitors such as RAD001.

Unlike other tumor types such as breast and colon cancer, oncogenic mutations are uncommon in HNSCC. The presence of mutations can serve as clinical biomarkers that can predict the response of patients to specific targeting therapies. The only common mutation in HNSCC is the inactivating or deletion mutation of the tumor suppressor p53 [190, 191]. Approximately 50-60% of HNSCC patients display mutations of p53 [192, 193]. However, treatment strategies that can produce a clinical response in these patients have not been discovered. A recent report using an inducible oral-specific SCC mouse model showed that induction of the K-*ras* activating mutation and deletion of p53 resulted in SCC growth in the tongue [194]. However, in the presence of both mutations, mTOR inhibition by rapamycin significantly reduced SCC growth. Studies in colorectal cancer showed that *KRAS* mutations did

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[5] http://www.cancer.gov/trwg/TRWG-definition-and-TR-continuum

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not correlate with response to cetuximab [195]. Furthermore, *KRAS* mutations are observed in 3-4% of HNSCC patients [196]. However, two p53 homologs have been reported to be upregulated or downregulated in HNSCC, p63 and p73 respectively [197, 198]. The presence of the p63 was shown to prevent p73-mediated apoptosis in HNSCC.

In a recent study using breast and lung cancer models, p73 was induced in the presence of rapamycin indicating that mTOR pathway regulates p73 [199]. Furthermore, p73 was reported to regulate expression of autophagy-related genes such as ATG5, ATG7 and UVRAG [200]. Zhang *et al* showed that EGFR downmodulation by siRNA induced autophagic cell death in breast cancer cells by downmodulating the expression of the glucose transporter SGLT1 [148]. Therefore, we hypothesize that induction and sustained levels of p70S6K phosphorylation from EGFR siRNA and cetuximab may be protecting HNSCC cells from p73-mediated apoptosis and autophagic cell death (Figure 18). We intend to look at whether treatment with RAD001 or downmodulation of p70S6K by siRNA induces p73 expression in the presence of cetuximab.
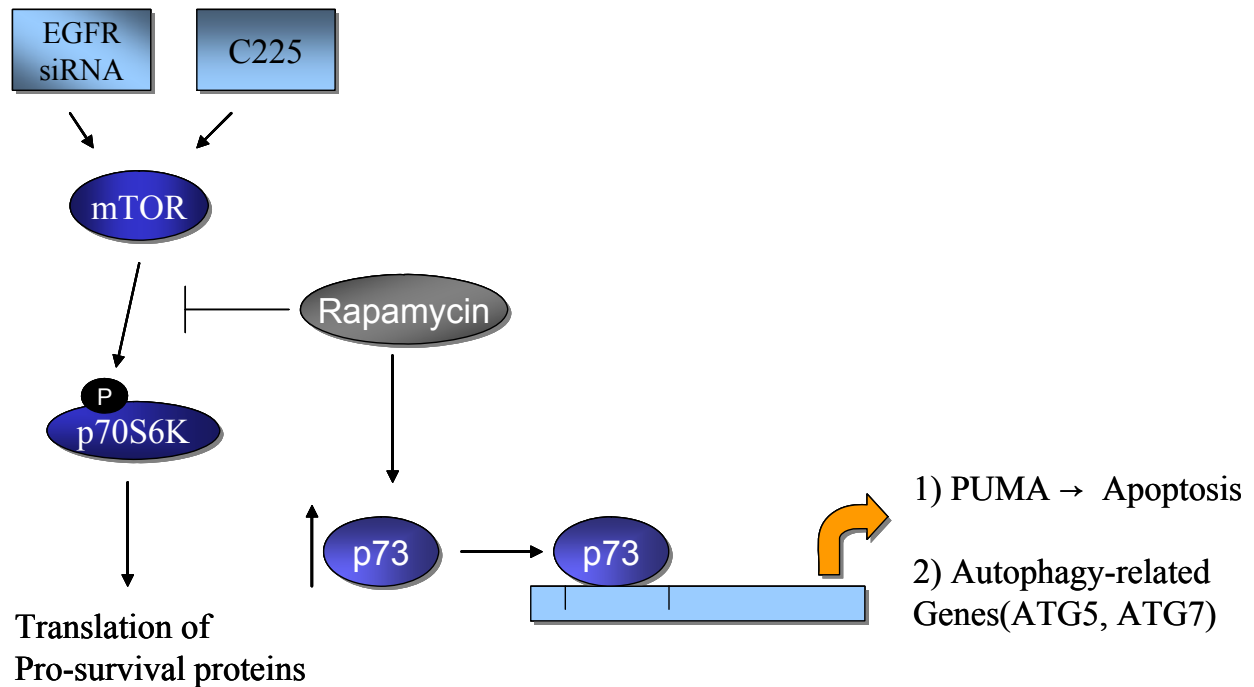
Figure 18. mTOR-p70S6K signaling axis may prevent p73 induction in the presence of EGFR downmodulating agents.

Expression of p73 is reported to be proapoptotic and proautophagic. mTOR inhibition with rapamycin induces expression of p73 in breast cancer. EGFR siRNA treatment in breast cancer resulted in autophagic cell death. This model indicates that p70S6K phosphorylation in the presence of EGFR siRNA or cetuximab may possibly prevent p73 induction and its consequent phenotypic effects.

These results will indicate whether activation of the mTOR-p70S6K pathway prevents p73-mediated phenotypic effects in the presence of EGFR siRNA and cetuximab. In addition to investigating p73 induction, we will determine p63 expression levels in HNSCC and assess whether p63 expression correlates with sensitivity to p70S6K targeting.

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