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Role and Regulation of EphA2 in Pancreatic Cancer

Pavel A. Levin

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ROLE AND REGULATION OF EPHA2 IN PANCREATIC CANCER

by

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ROLE AND REGULATION OF EPHA2 IN PANCREATIC CANCER

A

DISSERTATION

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of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Pavel Aleksander Levin, B.S.

Houston, Texas

August, 2010

DEDICATION

This thesis is dedicated to all patients that have suffered and will suffer from pancreatic cancer with the hope that one day this work will help bring scientific and medical communities a little closer to improving the outcome of this terrible disease.

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I would like to thank my advisor Craig Logsdon for his constant support during my PhD. In the whole time that I did my PhD, he never questioned my ability to get to this point. He also never inhibited my creativity and never prevented me from following my ideas. In spite of running a busy lab, he was always available to discuss my research.

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ROLE AND REGULATION OF EPHA2 IN PANCREATIC CANCER

Publication No._____

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Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cancer cause of death in the US. Gemcitabine is the first-line therapy for this disease, but unfortunately it shows only very modest benefit. The focus of the current study was to investigate the role and regulation of EphA2, a receptor tyrosine kinase expressed in PDAC, to further understand this disease and identify new therapeutic targets.

The role of EphA2 was determined in PDAC by siRNA mediated silencing. In combination with gemcitabine, silencing of EphA2 caused a dramatic increase in apoptosis even in highly resistant cells *in vitro*. Furthermore, EphA2 silencing was found to be useful in 2 orthotopic models *in vivo*: 1) shRNA-pretreated Miapaca-2 cells, and 2) *in vivo* delivery of siRNA to established MPanc96 tumors. Silencing of EphA2 alone reduced tumor growth in Miapaca-2 cells. In MPanc96, only the combination treatment of gemcitabine plus siEphA2 significantly reduced tumor growth, as well as the number of lung and liver metastases. Taken together, these observations support EphA2 as a target for combination therapies for PDAC.

The regulation of EphA2 was further explored with a focus on the role of Ras. K-Ras activating mutations are the most important initiating event in PDAC. We demonstrated that Ras regulates EphA2 expression through activation of MEK2 and phosphorylation of ERK. Downstream of ERK, silencing of the transcription factor AP-1 subunit c-Jun or inhibition of the ERK effector RSK caused a decrease in EphA2 expression, supporting their roles in this process. Further examination of Ras/MEK/ERK pathway modulators revealed that PEA-15, a protein that sequesters ERK to the cytoplasm, inhibited expression of EphA2. A significant inverse correlation between EphA2 and PEA-15 levels was observed in mouse models of PDAC. In cells where an EGFR inhibitor reduced phospho-Erk, expression of EphA2 was also reduced, indicating that changes in EphA2 levels may allow monitoring the effectiveness of anti-Ras/MEK/ERK therapies.

In conclusion, EphA2 levels may be a good prognostic factor for anti-EGFR/anti-Ras therapies, and EphA2 itself is a relevant target for the development of new therapies.

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CHAPTER ONE: INTRODUCTION

INTRODUCTION TO THE PANCREAS

A brief overview of pancreas development, anatomy and function is provided below.

For more details please refer elsewhere (1-3).

Anatomy and development

Human digestive system is serviced by several glands: salivary glands, pancreas, and liver. Pancreas is an elongated organ that lies in the retroperitoneum, behind the stomach, between the duodenum on the right and spleen on the left. Adult pancreas is about 75 g to 100 g and 15 cm to 20 cm long (1). During foregut embryogenesis, pancreas arises from dorsal and ventral buds of endodermal cells. Bigger dorsal bud is connected to the developing duodenum through the duct of Santorini, while the smaller ventral bud arises from hepatic diverticulum and is connected to it by the duct of Wirsung. As development progresses, duodenum rotates and the ventral bud comes into proximity and fuses with the dorsal bud. Ventral bud becomes the uncinate process and the head of the pancreas, while the dorsal bud forms the body and the tail of the pancreas. The main pancreatic duct is formed by anastomosis of the duct of Santorini (proximal) and a distal portion of the duct of Wirsung (distal). The proximal portion of the duct of Wirsung forms an accessory pancreatic duct. In approximately 10% of people ducts fail to anastomose and remain separate (1, 2).

Function and histology

Pancreas has 2 distinct functions: exocrine and endocrine. Exocrine pancreas makes up 85% of the pancreas proper, while endocrine tissue is only 2%. The other 13% are made up of extracellular matrix, blood vessels, and major ducts.

Exocrine pancreas consists mostly of acinar cells (85%), which are arranged into spherical form, called acinus (40 acinar cells per acinus) (1, 4). Upon stimulation, acinar cells release digestive enzymes from the zymogen granules into the lumen of the acinus. Once in the lumen, digestive enzymes mix with the water and electrolyte secretions of the centroacinar cells. Centroacinar cells, as the name implies, are the cells that are located in the center of pancreatic acinus and are considered to be a part of pancreatic ductal system. From the acini, pancreatic juice travels through intercalated ducts, which join together to form very similar intralobular ducts. When intralobular ducts leave acinar lobules, they develop collagenous wall and become interlobular ducts. Several interlobular ducts form secondary pancreatic ducts that drain into the main pancreatic duct and later into the duodenum (1). On the way from acini to secondary pancreatic ducts, pancreatic juice receives secretions from duct cells (water, chloride and bicarbonate) to adjust the pH of pancreatic secretions and stabilize the proenzymes until they are activated in the duodenum (4). Overall, exocrine pancreas is responsible for producing on average from 500 ml to 800 ml a day of pancreatic juice, which contains amylases, proteases, and lipases that break down carbohydrates, protein, and fat, respectively (1).

Endocrine pancreas is made up of islets of endocrine cells within pancreatic parenchyma, called islets of Langerhan. Each islet consists of a mixture of 5 different cell

types: glucagon-secreting alpha cells, insulin-secreting beta cells, somatostatin-secreting delta cells, ghrelin-secreting epsilon cells and pancreatic polypeptide-producing cells (PP cells). These hormones are involved in regulation of blood glucose levels and general GI function (peristalsis, bile secretion, etc.) (1).

PANCREATIC DUCTAL ADENOCARCINOMA

Epidemiology

Pancreatic ductal adenocarcinoma (PDAC) is a neoplasm of the cells of the exocrine pancreas. It is the deadliest cancer among all major malignancies. Although it is the 10th most common malignant tumor in both women and men, due to the high mortality associated with this malignancy, it is the 4th largest cancer killer in the US. According to the most recent statistics, it is estimated that almost 42,500 Americans were diagnosed with and 35,000 died from pancreatic cancer in 2009 (5).

PDAC is a disease of old age. According to the SEER statistics the median age of diagnosis is 72 years of age. The incidence is 30% higher in males than females (13.3 vs. 10.5 per 100,000 persons), and 30% higher in Blacks than in people of other races (15.5 vs. 11.7 per 100,000 persons) (6).

Pancreatic cancer has environmental and genetic risk factors. Environmental risk factors for pancreatic cancer include smoking (7), obesity, diet and lack of physical activity (8, 9). However, 5%-10% of pancreatic cancer patients have a known genetic syndrome or a positive family history. A first degree relative with pancreatic cancer increases individual's

risk at least 9 fold over the general population (10). Several known cancer-causing germline mutations are associated with an increased risk of pancreatic cancer, such as BRCA1 (11, 12) and BRCA2 (13), p16/CDKN2A (14), PRSS1 and STK11 (15, 16). The incidence of pancreatic cancer is also increased in individuals having ataxia-telangiectasia (17), familial adenomatous polyposis (18) and Lynch syndrome (19). Interestingly, the above syndromes and germline mutations account only for a small percentage of pancreatic cancer patients with a known familial susceptibility for pancreatic cancer (10). Therefore, further research of genes predisposing to development of pancreatic cancer is needed.

Other multifactorial risk factors include chronic inflammation of the pancreatic tissue, such as hereditary, or familial (20-22), or nonhereditary chronic pancreatitis (23). There is also a strong association between pancreatic cancer and diabetes (24-26). However, it is still unclear whether diabetes predisposes for the development of pancreatic cancer or is a direct result of pancreatic pathology caused by cancer development.

Diagnosis of pancreatic cancer

Pancreatic cancer patients do not exhibit any significant symptoms until late in the disease. Patient's history usually reveals recent weight loss, often accompanied by generalized gastrointestinal symptoms, such early satiety and diarrhea. The most common presenting symptom is a vague epigastric pain, which is present in 80% to 85% of patients with locally advanced tumors. As mentioned earlier, recent onset or exacerbation of diabetes in a patient over 50 years of age also should raise suspicion of a pancreatic tumor (26).

Physical findings of pancreatic cancer may include palpable abdominal mass, distended gallbladder, supraclavicular lymphadenopathy, and in a few cases migratory thrombophlebitis (Trousseau's syndrome) (27, 28). "Painless jaundice" is a rare presentation for pancreatic cancer. Patients that present with painless jaundice have an obstruction of the biliary tract and therefore come to medical attention earlier in the disease. These patients tend to have smaller tumors in the head of the pancreas that can be resected and result in a better prognosis. On the other hand, patients with tumors of pancreatic tail have fewer symptoms and therefore present at a later stage. These patients usually are not fit for surgery and have lower average survival (29).

Once pancreatic cancer is suspected, high resolution abdominal CT is used to confirm the diagnosis and to stage the disease. Tissue samples for pathology are often obtained by CT- or transabdominal/transesophageal ultrasound-guided biopsy. Surgical exploration (laparotomy) and pathologic confirmation of pancreatic cancer remain "gold standards" for the diagnosis and staging, but are not always required (30).

Management of pancreatic cancer

Once the diagnosis of pancreatic cancer is confirmed, the management depends on the extent of the disease. Pancreatic cancer can be divided into 3 groups: localized and resectable, locally advanced and unresectable, or metastatic (30).

Around 15%-20% of patients present with resectable pancreatic cancer. Resectable tumor does not have evidence of metastatic disease and does not invade superior mesenteric artery or celiac plexus. The treatment for a localized pancreatic cancer is surgical resection.

Most of the time the tumor is in the head of the pancreas, in which case surgical resection, called pancreaticoduodenectomy (Whipple procedure), is performed. Due to the developmental origin of the pancreas as an outgrowth of the GI tract, the blood supply to the pancreas is also shared by the stomach and duodenum. Therefore, the Whipple procedure entails quite an extensive resection of the head of the pancreas, duodenum, proximal part of jejunum, distal part of the stomach, gallbladder, cystic duct and the common bile duct, while attaching the pancreas and hepatic ducts to the jejunum. As would be expected, this major surgery is associated with high mortality (4%-16%) with high-volume centers showing significantly improved survival (31). When the tumor is in the tail of the pancreas, the surgical procedure is much simpler, involving resection of the pancreatic tail and spleen. Unfortunately, as mentioned above, tumor in the tail of the pancreas presents late with evidence of metastatic disease and often is not a subject for resection. Evidence showed that postoperative chemotherapy of gemcitabine improves patient survival (32). Overall 5-year survival for patients with resectable pancreatic cancer is 20%.

Patients with locally advanced and unresectable pancreatic cancer usually undergo palliative therapy to alleviate the symptoms. Procedures may include temporary stenting to eliminate obstruction of the bile duct. Gemcitabine is the first-line therapy for unresectable pancreatic cancer and has proven to be superior to an older drug 5-FU (33). However, only a small percentage of patients (15%-20%) have an objective response to gemcitabine therapy and most of them develop resistance very rapidly. The mechanism of action of gemcitabine and the mechanism of tumor resistance are described in more detail below. Other drugs are constantly being researched to achieve benefit over gemcitabine either by themselves or in

combination (34), with modest progress. Radiation has been used in combination with chemotherapy, but its benefit has not been proven (35).

Pancreatic cancer patients that have evidence of metastatic disease have very poor prognoses. The response rate to chemotherapy is very low. Therefore, potential benefit of chemotherapy is usually carefully weighed against possible toxicity, before the decision is made on disease management.

Recently, a different therapeutic algorithm has been proposed for patients with borderline resectable pancreatic cancer (36). Borderline resectable patients do not have evidence of metastatic disease, based on CT. However, the local disease may not satisfy the strict definition of resectable tumor, due to limited vascular involvement, and may potentially be surgically excised with a high chance of positive margin. The management of these patients varies from center to center, but it has been proposed to treat these patients with chemotherapy before surgical resection. After these patients go through chemotherapy, the tumor mass is reevaluated and the decision is made whether it is amenable to surgical resection.

Gemcitabine

The first-line therapy for unresectable pancreatic cancer is a deoxycytidine analog, gemcitabine. Its mechanism of action has been well characterized (37). Gemcitabine is a prodrug and has to be phosphorylated by deoxycytidine kinase (rate-limiting step) into gemcitabine monophosphate in order to be activated. It is phosphorylated further by other enzymes into diphosphate and triphosphate forms. The triphosphate forms incorporate into

DNA and block DNA synthesis. Gemcitabine is different from other nucleotide analogs, because it has a “self-potentiating” effect (37). For example, dCTP decreases effect of gemcitabine, because dCTP: 1) competes with gemcitabine triphosphate for incorporation into DNA, 2) inhibits deoxycytidine kinase, and 3) is required for the activity of gemcitabine inactivating enzyme, dCMP deaminase. However, gemcitabine diphosphate inhibits ribonucleotide reductase activity and therefore decreases synthesis of dCTP.

As mentioned above, the vast majority of pancreatic cancers are resistant to gemcitabine at the initiation of therapy or become resistant soon after. The mechanism of resistance in the clinic is not clearly understood, but is likely to be multifactorial (38). Pancreatic cancer is hypovascular with extensive stroma separating cancer cells from blood vessels (39). Evidence suggests that low vascularity of pancreatic cancer may be responsible for the diminished delivery of drugs to cancer cells and therefore higher drug resistance (39). Moreover, many pancreatic cancer cell lines have a high endogenous resistance to gemcitabine, even when grown in cell culture or as a xenograft with reduced stroma (40). Many cell alterations have been described that can increase gemcitabine resistance. For example, low expression levels of membrane transporter hENT1, which is responsible for gemcitabine uptake into the cancer cells, have been correlated to decreased patient survival after gemcitabine therapy (41, 42). Similarly, some resistant cell lines have reduced activity of deoxycytidine kinase and therefore have diminished ability to convert gemcitabine into its active form (43). Ribonucleotide reductase, an enzyme involved in dNTP metabolism is also associated with gemcitabine resistant of pancreatic cancer xenografts (44). Other resistance mechanisms are more generalized and have to do with regulation of cell survival and cell death. For example, antiapoptotic protein Bcl-XL is overexpressed in 92% of pancreatic

cancer samples (45) and proapoptotic gene BNIP3 is undetectable in 59% and down-regulated in 90% of samples (46). Proteins that have been implicated in gemcitabine resistance also include NFκB, Src, Fak, XIAP, and others (38, 47, 48). Therefore, a treatment that can decrease expression or activity of antiapoptotic proteins while increasing that of proapoptotic, may prove to be very useful as a combination therapy with gemcitabine.

Molecular pathology of pancreatic cancer

For the last several decades, pancreatic cancer has been puzzling researchers and physicians by defeating their best attempts at treating this disease. However, there have been many advances in understanding the mechanisms of disease development and progression. Since pancreatic cancer cells form duct-like structures, it was long believed that pancreatic cancer originates from duct cells. This dogma has been recently challenged and it was established that acinar cells also possess the ability to transform and create a disease histologically identical and molecularly similar to PDAC (49, 50). In contrast, expression of K-Ras, an oncogene believed to drive pancreatic cancer, in mature duct cells has not caused cell transformation or development of PDAC (51). Currently, it is believed that in the process of oncogenic transformation, duct, or more likely acinar cells, form preneoplastic lesions, called pancreatic intraepithelial neoplasia 1, 2, and 3 (PanIN 1, 2, and 3). As transformation progresses, cells eventually break through the basement membrane and develop into PDAC (52).

Pancreatic cancer is a highly heterogeneous disease and has a complex molecular pathology. Jones and colleagues reported on average as many as 63 mutations relevant to tumor progression per tumor sample (53). In spite of the heterogeneity of an established pancreatic cancer, there are a few molecular alterations that are present in almost all pancreatic cancer samples. These alterations include K-Ras (75%-100%), p16INK4A (27%-98%), p53 (40%-75%) and SMAD (50%-55%) and their occurrences seems to parallel the disease progression (30, 54).

K-Ras mutations are present in almost all pancreatic cancer patients and are believed to play a central role in pancreatic cancer development and progression. Due to its importance, K-Ras is described in more detail below.

In addition to K-Ras, protein expression or function of tumor suppressors p16 INK4A, p21 CIP1/WAF1 and p53 is altered in a majority of pancreatic cancer cells. These tumor suppressors normally regulate checkpoints of the cell cycle and prevent uncontrolled cell division (55). The evidence suggests that in the presence of functional tumor suppressors, Ras activity may not be sufficient to cause pancreatic cancer and instead leads to cell senescence (50). In order for the cells to pass through G1-S checkpoint and continue proliferation cyclin D1 is upregulated. It binds to cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and phosphorylates retinoblastoma protein (Rb). In non-proliferating cells, Rb stays bound to transcription factor E2F preventing its activity. However, when Rb is phosphorylated it dissociates from E2F, thereby relieving its inhibition. E2F regulates expression of numerous proteins involved in DNA synthesis and metabolism, and E2F activity ensures transition into the S phase. Protein p16 INK4A prevents Rb phosphorylation and therefore cell cycle progression by binding and inactivating CDK4 and CDK6. Rb is

also phosphorylated by cyclin E-CDK2 complex late in G1 phase, which in turn is inhibited by p21 CIP1/WAF1. Tumor suppressor p53 prevents cell cycle progression in response to cell injury and DNA damage. It is a transcription factor for many intracellular proteins, including most importantly p21 CIP1/WAF1. Mutation in p53 decreases p21 expression levels and relieves the inhibition on cyclin E-CDK2 complex, allowing phosphorylation of Rb and continuation of cell cycle. Mechanisms of tumor suppression are described in more detail elsewhere (55).

Smad4 is deleted in a half of all pancreatic cancer patients (30). This protein is responsible for growth suppressive effect of TGF- β . However, upon deletion of Smad4, TGF- β is not able to suppress cell cycle progression and instead leads to epithelial-to-mesenchymal transition (EMT) of cancer cells. EMT promoting effect of TGF- β in addition to its immunosuppressive action causes increased cancer cell proliferation and invasion (56).

Oncogenic K-Ras and downstream signaling

K-Ras4A and K-Ras4B (later referred to as K-Ras) belong to a family of small Ras GTPases that also include H- and N-Ras (57). Ras proteins cycle through active GTP-bound and inactive GDP-bound states. Under basal conditions in a normal cell, Ras proteins are mostly inactive GDP-bound. However, upon growth factor stimulation, receptor (ie. EGFR) gets activated and autophosphorylated. Activated receptor recruits an adaptor protein that binds guanine nucleotide exchange factors (GEF). GEF is a family of enzymes that bind inactive GDP-bound Ras and promote GDP to GTP switch. GTP-bound Ras interacts and activates a number of downstream proteins eventually increasing cell proliferation and

promoting cell survival. GTP-bound active Ras encounters one of the members of GTPase-activating protein (GAP) family, which promote hydrolysis of bound GTP into GDP, thereby inactivating the protein. Ras proteins possess the ability to hydrolyze GTP, but without the presence of GAP catalysts they are very inefficient.

K-Ras, H-Ras and N-Ras are very closely related, having 85% of amino acid identity (57). However, recent evidence demonstrates that these proteins may perform different functions. Gene knockout studies revealed that while H-Ras and N-Ras are dispensable for normal development, deletion of K-Ras is embryonic lethal (58). Although the domains involved in GTP and effector binding are identical between the isoforms, the hypervariable region (HVR) is <15% similar between different Ras proteins and is responsible for membrane binding. Not surprisingly, studies of Ras intracellular localization also point to the fact that 3 isoforms tend to localize to different intracellular compartments (59).

Ras mutations are very common and present in 20% of human cancers. These mutations occur mostly in codon 12, but also in codons 13 and 61 of the gene (57). They compromise GAP-mediated GTPase activity of Ras protein, causing accumulation of constitutively active GTP-bound Ras. Among Ras mutations, changes in the K-Ras, N-Ras and H-Ras account for 85%, 15% and <1%, respectively (57). Pancreatic cancer stands out among other cancers, however, because activating mutations in the K-Ras gene are present in as high as 75%-100% of all patients (30). Moreover, sequence analysis showed K-Ras mutations occur very early and therefore may be the central initiating event in the cancer progression (60). In support of this theory, our group has previously demonstrated that in transgenic mice, high levels of mutant K-Ras alone or endogenous low level of mutant K-Ras in combination with p53 deletion in pancreatic acini is enough to induce spontaneous

development of pancreatic cancer (50). Similarly, silencing of K-Ras in pancreatic cancer cells decreases their capacity to form tumors (61). Therefore, understanding of K-Ras function and downstream signaling is crucial for the future progress in pancreatic cancer research.

The list of Ras effectors is still growing. However, 5 different downstream signaling targets of K-Ras were recently discovered to be important for its oncogenic properties: Raf serine/threonine kinases, phosphoinositol 3-kinases (namely p110 subunits), Tiam1 (GEF specific for Rac small GTPase), RalGEF and PLC ϵ (62). Among these effectors, Raf was the first one identified and currently the most studied. The importance of Raf in Ras-mediated oncogenesis is underlined by the following facts: 1) B-Raf mutations have been identified in a number of human cancers, 2) mutant B-Raf is capable of transforming rodent fibroblasts, and 3) activating mutations in Ras and Raf occur in nonoverlapping pattern, suggesting that they employ similar mechanism of tumorigenesis (63).

Traditionally Raf-MAPK pathway is described simplistically with Ras activating Raf, which in turn activates MEK1 and MEK2. ERK is a serine/threonine kinase, which is also called mitogen-activated protein kinase (MAPKs) and it is the only reported effector of MEK. MEK phosphorylates ERK1 and ERK2 and they phosphorylate a growing list of downstream targets. It is currently estimated that 160 proteins are regulated by phospho-Erk, which include nuclear (eg, Fos, Fra-1, Ets1), cytoplasmic (eg, RSK, cPLA2), membrane and cytoskeletal targets (64). However, the biology of this pathway is much more complicated than the simple signal progression through second messengers and involves many regulatory inputs (63). In addition to Ras and MEK, Raf interacts with a number of proteins, such as 14-3-3, HSP90, and others. More importantly, the nature of Raf interaction with MEK, and

MEK interaction with ERK depends on the adaptor proteins involved, such as KSR, Sef, MP1, and IQGAP1 (65). These adaptor proteins may regulate the extent of downstream activation and the intracellular localization of the phosphorylated effectors. MEK1 and MEK2 originated from a common ancestor and are structurally very similar proteins. However, there is a growing body of evidence suggesting that they perform slightly different functions, possibly through influencing ERK localization (66-68). Unlike MEK, ERK doesn't have a nuclear export sequence and therefore is free to translocate between nuclear and cytoplasmic domains. ERK localization is an important regulatory point in MAPK pathway. It is influenced by ERK dimerization and various adaptor proteins (69). It is believed that oncogenic properties of ERK come from its nuclear effectors. However, this theory is constantly being challenged and some new evidence suggests that while nuclear phospho-ERK regulates cell proliferation, cytoplasmic phospho-ERK may increase cell survival (68).

Considering the importance of Ras-Raf-MAPK pathway in pancreatic cancer, it is not surprising that the pharmaceutical manipulation of this pathway is a constant subject of research. Different chemical MEK inhibitors are under scrutiny in the clinic. As was mentioned earlier, EGFR is a known stimulator of Ras-MAPK pathway (70). Several antibodies, and chemical inhibitors that block this receptor have been approved for use in different types of cancer (70). Since the EGF receptor is acting upstream from Ras protein, mutational activation of Ras is believed to negate the importance of EGFR as a potential target in pancreatic cancer. In support of that theory, Phase III clinical studies in colon cancer showed that anti-EGFR antibody is only effective against tumors that have wild-type K-Ras and the antibody failed to achieve significant improvement in patients with K-Ras

mutation (71, 72). However, a study in pancreatic cancer demonstrated that by blocking EGFR activity with a chemical inhibitor, erlotinib, a physician may increase patient survival (73). Although the effect of erlotinib in pancreatic cancer was very mild, it is the only therapy that has been shown to improve gemcitabine treatment. It is also possible that there is a subpopulation of pancreatic cancer patients that may be very responsive to anti-EGFR therapy, but the survival benefit for that population is diluted out by nonresponsive patients.

There are other ways that Ras-MAPK pathway can be manipulated. For example, a novel adaptor protein phosphoprotein enriched in astrocytes 15 (PEA-15) has been found to bind phospho-Erk without blocking its kinetic activity (74). PEA-15 possesses a nuclear export sequence and therefore, it keeps phospho-ERK anchored in the cytoplasm upon binding. High expression of this protein decreases tumorigenic potential of breast cancer cells, and in breast cancer tissue samples high PEA-15 was associated with low tumor grade (75). Moreover, high PEA-15 expression in ovarian cancer causes cell autophagy and is correlated with better patient survival (76). It would be interesting to know the role PEA-15 plays in pancreatic cancer and whether there are ways to induce PEA-15 expression to block nuclear function of Erk downstream of oncogenic K-Ras.

EPHA2 RECEPTOR

Structure of Eph receptors and ephrin ligands

Eph receptors make up the largest family of tyrosine kinase receptors, consisting of 14 members. The family is divided into 2 classes: 9 EphA and 5 EphB receptors, based on the structure of their extracellular domain (77). Eph receptors bind to their ligands, Eph

receptor interacting proteins (ephrins). Normally, ephrins are membrane-bound and interact with an Eph receptor upon cell-to-cell contact. However, recent evidence suggests that the ligands can be cleaved and released into the interstitial space, although the physiological function of cleaved ligands is not clear (78, 79). Ephrins are also divided on 2 structural types – ephrin-As and ephrin-Bs, each of which has several different ligands (eg, ephrin A1, A2, etc.) (77). In general, ephrin-A ligands bind to EphAs and ephrin-B ligands bind to EphBs, with the exception of EphA4 binding ephrin-B2/3 (80) and EphB2 binding ephrin-A5 (81).

Eph receptors share a similar structure. Like other receptor tyrosine kinases, they have the extracellular, transmembrane and cytoplasmic portions. The Eph receptor extracellular portion starts at the N-terminus and consists of ephrin-binding globular domain, followed by a cysteine-rich region, and 2 fibronectin-type III repeats. Intracellular portion of the receptor consists of a juxtamembrane region with 2 autophosphorylation tyrosine sites, a highly conserved tyrosine kinase domain, sterile alpha motif (SAM) and a PSD-95 postsynaptic density protein, Discs large, Zona occludens tight junction protein (PDZ) bindings motif. As was mentioned above, extracellular portion, namely globular domain, is what determines ephrin binding specificity of the receptor. This structural interaction of receptor to ligand is well characterized and is reviewed in detail elsewhere (82).

Ephrin structure is very different between the subclasses. While both ephrin-As and ephrin-Bs are membrane anchored proteins, ephrin-Bs posses a transmembrane region, and a cytoplasmic portion containing a phosphorylation site and PDZ binding domain. Ephrin-As do not have a transmembrane region and instead are linked to the membrane by a short

glycosylphosphatidylinositol (GPI) anchor. Interestingly, unlike conventional ligands, both ephrin-As and Bs are capable of “reverse” signaling upon binding to Eph receptors and therefore can also be regarded as receptors (83).

Eph receptor role in physiology and cancer

In normal physiology, the role of Eph receptors was the most characterized in developing nervous system. Eph receptors and ephrins demonstrate complementary patterns of expression and form gradients that allow neurons to be targeted to a specific location in the brain, based on the level and type of Eph/ephrins protein expression. Eph receptor expression in CNS persists into adulthood, but is limited areas of the brain undergoing tissue repair or involved in neuronal plasticity, such as hippocampus. Outside of nervous system, Eph-ephrin interaction has been shown to play an important role in immune system (thymocyte differentiation), glucose homeostasis (insulin secretion by β cells), bone development and maintenance (osteoblast differentiation) and intestinal homeostasis (epithelial cell differentiation and migration) (83).

Eph receptors are strongly linked to cancer development. The name for this family of receptors comes from the erythropoietin producing hepatocellular carcinoma cell line, from which the first receptor EphA1 was identified. It was discovered as a result of a screening for oncogenic tyrosine kinase receptors with a similarity to virus v-fps in 1987 (84). The same group that identified the receptor also found that it was highly expressed in tumor tissues (84) and was capable of transforming NIH3T3 upon overexpression (85).

Incidentally, the first Eph ligand, ephrin-A1 was also identified and isolated from cancer cell lines (86).

EphA2 receptor expression in cancer

EphA2 is one of the most studied members of Eph receptor family, due to its prominent role in cancer. High EphA2 receptor expression both on protein and mRNA levels was found cancers of breast (87), ovary (88, 89), cervix (90), prostate (91, 92), kidney (93), bladder (94), lung (95), esophagus (96), stomach (97), colon (98), and pancreas (99). EphA2 is also highly expressed in melanomas (100) and glioblastomas (101-103). High EphA2 expression in tumor tissues has also been significantly associated with higher tumor grade and stage (88, 89, 93-95, 99, 102). It was also found to be predictive of shorter patient survival (88, 90, 93, 95, 96). In addition to being present on tumor cells, both EphA2 and the ligand, ephrins-A1, have been found in the tumor microenvironment, mostly on endothelial cells (104, 105).

Regulation of EphA2 receptor expression

In spite of wide overexpression of EphA2 receptor in tumor tissues, the regulation of EphA2 expression is not clear. The EphA2 gene is located on chromosome 1p36 and is transcribed and translated into a 130 kDa, 976-amino acid transmembrane glycoprotein. In breast and lung cancer cells, EphA2 expression appears to be regulated, at least in part, by the activity of Ras-MAPK pathway and may be induced with exogenous EGF (106, 107).

However, the exact mechanism of this regulation and the transcription factors involved remain unknown. Surprisingly, several studies conclusively demonstrated that EphA2 can be induced in response to p53, p63, or p73 activation (108) and EphA2 promoter has a binding site for tumor suppressor p53 (109). This perplexity in receptor induction by both tumor suppressor p53 and an oncogene K-Ras is not well understood, but is reflected in EphA2 function. EphA2 has been reported to promote both cell apoptosis (108-110) and cell survival (87, 111, 112), as will be described in detail below. Enhancer region of EphA2 gene is also regulated by homeobox transcription factors, namely HoxA1 and HoxB1. In neural development, they have been shown to restrict EphA2 expression to rhombomere 4 (113).

EphA2 receptor signaling and function in cancer

Although wide overexpression of EphA2 implies its involvement in cancer biology, it may arguably be a bystander effect, and in itself does not determine receptor's significance in cancer progression. This question was addressed by Zelinski and colleagues, who demonstrated that ectopic overexpression of EphA2 receptor in nontransformed breast epithelial cells confers tumorigenic phenotype, namely the ability to grow in soft agar and form tumors in vivo (87). Reduction of EphA2 receptor expression either by siRNA or through antibody/ligand-mediated degradation, on the other hand, leads to decrease in tumor growth and metastasis (114-118).

The effect of EphA2 on cancer cell biology is determined by more than just mere level of receptor's expression. The complexity stems from the fact that the receptor is capable of signaling in the absence of the ligands or receptor phosphorylation (114).

Moreover, in the presence of the ligand, EphA2 receptor functions differently depending on the clustering ratio of ligand to receptor (119). Accumulating evidence suggests that unphosphorylated receptor drives tumorigenesis, and receptor phosphorylation is detrimental to cancer progression and results in rapid receptor internalization and degradation. Consistent with that theory, examination of EphA2 phosphorylation status in non-transformed and cancer breast epithelial cells revealed that the ratio of unphosphorylated to phosphorylated receptor is higher in neoplasia (87, 120).

It appears that there are several mechanisms that cancer cells employ to prevent receptor phosphorylation and therefore increase receptor stability and oncogenic function. Macrae et al., demonstrated that in breast cancer cells maintained in culture, ephrin expression is inversely related to EphA2 (107). Further, they discovered that the MAPK pathway, the pathway that increases EphA2 expression, is also involved in downregulation of ephrin-A1. Whether ephrin-A1-EphA2 inverse relationship persists in vivo is unclear, since our data suggest that ephrins are also upregulated in cancer tissue (unpublished). Several reports revealed that EphA2 receptor are often localized to cytoplasm or membrane ruffles, and therefore may not be available for interaction with ephrins (87, 120, 121). Interestingly, E-cadherin restores membrane localization of EphA2, thereby increasing EphA2 phosphorylation (120). Lastly, EphA2 phosphorylation can be reversed by tyrosine phosphatases, such as LMP-PTP (122). Overexpression of LMP-PTP is present in multiple cancers and by itself can lead to oncogenic cell transformation (122).

Unphosphorylated EphA2 receptor associates with and possibly stabilizes phosphorylated focal adhesion kinase (FAK) in prostate and pancreatic cancers (118, 123). Consequently, high EphA2 expression also translates into high level of FAK

phosphorylation, which in turn leads to secretion of matrix metalloproteinases, such as MMP2 (118). Changes in FAK phosphorylation and extracellular levels of MMP2 are directly responsible for cell mobilization and invasion into surrounding tissues, respectively (124, 125). Not surprisingly, EphA2 expression has been correlated with tumor metastasis (91, 126-128).

In addition to FAK, EphA2 has been shown to regulate MAPK pathway activity, although the literature is contradictory. Miao and colleagues demonstrated that activation of EphA2 receptor with ephrin-A1 causes decrease in ERK phosphorylation with 5 minutes and persists for at least 2 hrs (129). Dephosphorylation of ERK by EphA2-ephrin-A1 binding was also confirmed by Macrae et al (107). However, in attempt to replicate findings by Miao et al., Pratt and colleagues achieved increase in ERK phosphorylation in response to EphA2 stimulation with ephrins-A1 in the same PC-3 cell line (130). More research is needed to characterize the effect of EphA2 on MAPK pathway.

There are 2 reports linking EphA receptors to the Jak/STAT pathway. Lai and colleagues have demonstrated that upon activation, EphA4 receptor binds and phosphorylates Jak2, causing activation of STAT3 (131). The same year, a different study showed that Eph receptor family is the only RTK family that has a Thr in the (p+1) loop, which allows these receptors to constitutively activate STAT3. Amino acid substitution of Thr to Met removes this ability from Eph receptor. On the other hand, substitution of Met to Thr in (p+1) loops of other RTKs, such as RET and MET, allows them to constitutively activate STAT3 (132). Although this study needs to be confirmed, it gives another evidence linking EphA2 receptor to a pathway that plays a major role in multiple cancers (133), including pancreatic (134).

The numerous aforementioned signaling pathways directly regulated by EphA2 receptor, are joined by a plethora of intracellular changes indirectly regulated by EphA2 through binding to and activating of other receptor tyrosine kinases. Eph receptors are known to bind in cis- and activate or modulate activity of a variety of transmembrane proteins, from RTKs to channels and pores (135). EphA2 has been recently found to interact with members of EGFR family, such as EGFR1 and ErbB2 (106, 136). EphA2 binds to phosphorylated EGFR1, and silencing of EphA2 decreases EGF-induced cell migration (106).

EphA2 targeting

In view of widespread EphA2 overexpression in cancers and EphA2 activation of multiple known oncogenic pathways, it is not surprising that EphA2 targeting is a subject of constant research and improvement.

There have been several studies targeting EphA2 receptor using either ligand-mediated receptor downregulation or RNA interference (RNAi). Posttranslational downregulation of Eph receptors using mimetic antibody/ligand-mediated internalization and degradation has been attempted and was successful at reducing tumor growth in several studies (101, 116, 118, 137). However, there are several flaws to posttranslation EphA2 targeting: 1) due to receptor-ligand promiscuity, this downregulation may not be specific to any one member of Eph receptor family, 2) treatment of cells with ligands or mimetic antibodies/peptides increases “forward” signaling by the receptor, making the outcome difficult to interpret, and 3) ligand clustering and receptor oligomerization may produce

different signaling output depending on receptor/ligand ratio and thereby confound the outcome (119). The above flaws may explain contradicting findings regarding MAPK pathway activity after stimulation of PC-3 cells with ephrin-A1 (129, 130).

EphA2 receptors were also successfully targeted using RNAi (106, 115, 117, 138-140). RNAi-mediated posttranscriptional gene silencing exploits a natural mechanism of gene expression regulation that has been originally discovered in *C. elegans*, but is also present in mammalian cells. This mechanism has been well characterized (141). Briefly, double stranded RNA is cleaved into small fragments by an endoribonuclease Dicer. These fragments, small interfering RNA (siRNA), are 21 base pairs long. They assemble into the RNA-induced silencing complex (RISC). One of the strands of RNA gets degraded, while the other one, the guide strand, binds the target mRNA forming siRISC-mRNA complex. Upon binding, an endoribonuclease AGO2, which is part of the activated siRISC complex, cleaves the target mRNA.

RNAi offers several important advantages over posttranslational means of EphA2 downregulation: 1) using RNAi technology ensures receptor downregulation through inhibition of receptor synthesis and therefore completely prevents receptor function and 2) using siRNA allows specific downregulation of any member of Eph receptor family, without directly affecting other members. However, there are also disadvantages of siRNA usage. Naked siRNA has a very low half-life in the body, because of the high rate of degradation and renal clearance (142). Naked siRNA also demonstrates low organ uptake and systemic toxicity due to high interferon response (143). Recent advances have improved systemic siRNA delivery, including delivery to solid tumor, by using chemical modification of siRNA backbone or packaging siRNA into protective nanoparticles (143, 144).

EphA2 in pancreatic cancer

EphA2 is highly expressed in 92% pancreatic cancer samples. High levels of expression of EphA2 are detectable early, from preneoplastic lesions, and expression increases as the disease progresses (99). Few groups have examined the function of EphA2 in pancreatic cancer settings. The first study to examine EphA2 targeting used soluble EphA2-Fc chimeras to trap EphA2 ligands in a subcutaneous and orthotopic models of pancreatic cancer (145). Observed decrease in tumor growth was attributed to inhibition of angiogenesis, by blocking EphA2 receptors on endothelial cells. However, blocking of ephrin-A1 binding to endogenous EphA2 receptors on pancreatic cancer cells prevents basal ligand-induced receptor degradation, thereby increasing EphA2 stability and pancreatic cancer cell survival, diminishing the intended cytotoxic effect of the treatment. Moreover, trapping the EphA2 ligands, such as ephrin-A1, may decrease ephrin-A1-EphA4 interaction. EphA4 is also highly expressed in pancreatic cancer (data not shown) and has a higher affinity for ephrin-A1 (146). In addition, EphA2-Fc may stimulate ephrin-A1 reverse signaling. These additional variables make it difficult to attribute the outcome of the experiment to EphA2 receptor alone.

Duxbury and colleagues have used an ephrin-A1 chimera conjugated to the Fc portion of the antibody to induce ligand-mediated EphA2 receptor degradation (118). Upon EphA2 downregulation, they observed a decrease in pancreatic cancer invasiveness and reduction in cell resistance to anoikis in vitro (118). Anoikis, or cell death due to detachment from substrate, is considered a hallmark of a metastatic cancer. Further examination of

EphA2 silencing in a subcutaneous pancreatic cancer model by systemic delivery of naked siRNA yielded decreased tumor growth and metastasis (126). Proper native environment is essential in studying tumor behavior, especially because Eph receptors are physiologically responsible for setting tissue boundaries and are heavily regulated by cell-to-cell contact. It is not clear from the study of Duxbury et al. whether systemic siRNA delivery to a hypovascular orthotopic pancreatic tumor would be sufficient to silence the EphA2 receptor, and if silenced, whether orthotopic tumor growth would be inhibited.

The only study examining downregulation of EphA2 in an orthotopic pancreatic cancer was performed recently by Ansuini et al. using a single cell line, MiaPaca-2 (147). This group used 2 monoclonal antibodies, one to stimulate the EphA2 receptor and induce ligand-mediated receptor degradation (similar to Ephrin-A1-Fc) and the other to block the EphA2 receptor without inducing its phosphorylation (147). Both antibodies achieved similar decrease in tumor growth and metastasis, although the 2 antibodies produced seemingly opposite effects on EphA2 receptor phosphorylation and expression. Authors attribute the effects of treatment to either inhibition of pancreatic cancer cell growth directly or inhibition of angiogenesis. Similar to the studies above, using mimetic antibody increases EphA2 “forward” signaling prior to receptor degradation. Since EphA2 functions regardless of ligand binding or phosphorylation, blocking antibody against EphA2 does not block its oncogenic signaling, but rather prevents its interaction with ephrins and may decrease ephrin-mediated signaling.

EXPERIMENTAL RATIONALE AND HYPOTHESIS

As was mentioned above, pancreatic cancer is a rapid and very deadly disease. Currently, no therapies are available that improve patient survival beyond the median of 6 months. Gemcitabine is considered the first-line therapy for unresectable pancreatic cancer, but other therapies are constantly being investigated in combination with gemcitabine to improve patient outcome. It is clear that new rational therapies are highly needed to improve patient prognosis.

Based on the available evidence, the hypothesis for this dissertation was that **EphA2 is regulated by oncogenic K-Ras and may be an important therapeutic target for the treatment of pancreatic cancer**. This dissertation addresses the role and regulation of EphA2 in pancreatic cancer. In order to accomplish this, the first aim focused on the inhibition of receptor expression in gemcitabine-resistant pancreatic cancer cells. This aim examined the effect of EphA2 silencing in combination with gemcitabine on cell proliferation and death in vitro. This aim also explored tumor formation, growth and metastasis of gemcitabine-resistant pancreatic cancer cells grown orthotopically after silencing of EphA2 alone or in combination with gemcitabine.

The second aim of this dissertation explores regulation of EphA2 in pancreatic cancer, focusing primarily on Ras-MAPK signaling. In addition, this aim addresses modulation of the Ras-MAPK pathway activity in the presence of constitutively active K-Ras by blocking EGFR activity and utilizing an adaptor protein PEA-15.

**CHAPTER TWO: EPHA2 RECEPTOR SILENCING INCREASES
PANCREATIC CANCER CELL APOPTOSIS IN VITRO AND
SENSITIZES GEMCITABINE RESISTANT ORTHOTOPIC
PANCREATIC TUMORS IN VIVO.**

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the US. According to the most recent estimates, 42,500 people were diagnosed with and 35,000 died from pancreatic cancer in the US in 2009 (5). Median survival for patients with pancreatic cancer is 6 months, while 5-year survival is only 5%. Despite many attempts, there has been little by way of significant improvement in the treatment or prognosis of patients with pancreatic cancer and the only potentially curative treatment remains surgery. Unfortunately, pancreatic cancer is highly invasive and only 15%-20% of patients are resectable at the time of diagnosis (148). Among those few patients, the 5-year survival after resection is only 15%-20% (148). Gemcitabine is the first-line pharmacological treatment for patients with advanced pancreatic cancer and was found to be superior to the well-tested older drug 5-FU (33). Other drugs, including targeted therapeutics, are being investigated in combination with gemcitabine to improve survival further, however, the benefit to date has been modest (73). Therefore, further research of pancreatic cancer biology and especially mechanisms of invasion and metastasis is crucial for the improvement of patients' treatment and prognosis.

Our laboratory previously observed that a member of Eph receptor family, EphA2 was specifically expressed in pancreatic tumor tissues (149). EphA2 receptors are also highly expressed in breast, colon, prostate, gastric, lung adeno- and squamous carcinomas, and in aggressive melanomas (82). Ectopic expression of EphA2 in non-transformed breast epithelial cells confers a malignant phenotype and results in tumor formation in vivo (87). Silencing of the EphA2 receptor using EphA2 siRNA- or antibody-mediated degradation in ovarian carcinoma resulted in decreased tumor size, decreased number of metastases and

increased mouse survival (115, 116). Therefore, this receptor may play an important role in the biology of PDAC.

The erythropoietin-producing hepatocellular carcinoma (Eph) receptor family is the largest family of receptor tyrosine kinases. Eph receptors are divided into two subclasses, EphAs and EphBs, based on similarities of their extracellular domains (77). This division into 2 classes also corresponds to the Eph receptor binding preferences to their membrane-bound ligands, ephrin-As and ephrin-Bs, respectively. Normally, Eph receptors are involved in determining tissue patterning. In development, they regulate somite formation and distribution, while in the nervous system they are responsible for a proper topographic map formation and axonal guidance (150). Consequently, altered expression or function of these molecules is likely to influence cell-cell interactions. In cancer this may lead to altered tumor-stroma interactions and misguided malignant cell invasion into a healthy tissue, followed by a formation of metastasis.

In pancreatic cancer, analysis of human pancreatic tissue samples supported the specific expression of this receptor and illustrated increasing EphA2 expression from normal pancreas to PanINs to carcinoma (99). High levels of EphA2 were further shown to be associated with increased pancreatic cancer cell invasiveness (118). Duxbury and colleagues have demonstrated that EphA2 silencing using siRNA in vitro leads to increased susceptibility of PDAC cells to anoikis. Using naked siRNA, Duxbury et al also performed in vivo silencing of EphA2 in a subcutaneous model of pancreatic cancer. They observed a decrease in tumor growth/metastasis and an increase in cell apoptosis (126). However, the subcutaneous pancreatic cancer model does not recapitulate the appropriate tumor microenvironment for PDAC. Eph receptors are involved in cell-to-cell contact mediated

regulation; therefore it is likely that interactions with native pancreatic stroma are important. Furthermore, the potential interactions between targeting of EphA2 and gemcitabine were not evaluated.

In this study, we examined the effect of EphA2 silencing alone and in combination with gemcitabine on pancreatic cancer cells in culture. Further, we explored silencing of EphA2 with and without gemcitabine treatment on progression and metastasis of gemcitabine-resistant PDAC cells using orthotopic models and 2 different RNAi approaches. The results were encouraging, as EphA2 silencing in combination with gemcitabine caused a dramatic reduction in tumor growth even in these normally gemcitabine resistant models.

MATERIALS & METHODS

Cell Culture and Treatment

MPanc96 cells (151) were provided by Dr. Timothy J. Eberlein (St Louis, MO). The cells were cultured in Dulbecco Modified Eagle Media, supplemented with 10% fetal bovine serum and antibiotics. Cells were kept at 37°C in a humidified atmosphere of 5% CO₂. For in vivo studies, luciferase expressing MPanc96 cells were developed as previously described (152). Luciferase expression in these cells was confirmed in (0-10) x 10⁵ cells per well in a 24-well plate by measuring the light emission after adding luciferin (150 µg/ml) using the IVIS system (Xenogen Corp, Alameda, CA), and emitted light was directly proportional to cell number.

Reverse Transcription and Q-PCR

Reverse transcription was conducted as previously described (153). Briefly, RNA was isolated from tissue samples of normal pancreas, chronic pancreatitis and pancreatic cancer obtained from the MD Anderson Cancer Center tissue bank. DNase treatment of isolated RNA was performed to remove traces of genomic DNA. The primers for EphA2 were the following: forward, 5'-CCC CCT CCG CCC CAC ACT ACC TC-3' and reverse, 5'-ACA CGG CCC GCA TTC CCC AGA CTC-3'. 18-S was used as an internal control with the following primers: forward, 5'-GAG CGG TCG GCG TCC CCC AAC TTC-3' and reverse, 5'-GCG CGT GCA GCC CCG GAC ATC TAA-3'. DNA amplification was visualized using SYBR green dye. Bio-Rad iCycler iQ multicolor real-time PCR detection system was used.

Lentivirus preparation and infection

To study the effect of EphA2 silencing in pancreatic cancer cells, lentiviral vector pLKO.1-puro-shRNA for EphA2 (Open Biosystems) was cotransfected with packaging constructs into 293T cells. Lenti pLKO.1 virus 200 µl was mixed with polybrene (4 µg/ml of medium) and added directly to the cultured cells. EphA2 silencing was confirmed by Q-PCR.

Cell growth assay (DAPI immunocytochemistry)

DAPI staining for fluorometric measurement of cell density and proliferation has been previously described (154). Briefly, cells were seeded on 96-well plates at 2×10^6 cells per plate. At the time of plating, cells were infected with either Control shRNA (shCtrl) or EphA2 shRNA (shEphA2) lentiviruses. The next day cells were treated with gemcitabine (1 µM) or PBS for 8 hrs. The time of gemcitabine treatment was taken for 0 hrs time-point. At 24, 48, 72 and 120 hrs, cells were washed in cold PBS and fixed in 2% paraformaldehyde at 4° C for at least 24 hrs. When cells for all time-points were fixed, their nuclei were stained with DAPI at the same time, by adding DAPI solution (10 µg/ml) for 2 hrs at 4° C. At the end of incubation, DAPI solution was replaced with PBS and whole well signal was measured with the following settings: excitation, emission. Three wells were used for each condition and for each cell line.

Propidium iodide fluorescence activated cell sorting analysis (PI-FACS).

Cells were seeded onto 6-well plates at 2×10^6 cells/plate and infected with shCtrl or shEphA2 lentiviruses. The next day cells were treated with gemcitabine (1 μ M) or PBS for 8 hrs. Seventy-two hrs after gemcitabine treatment, media and cells were collected and fixed in 75% cold ethanol. Cells were stained with PI (50 μ g/ml) and analyzed by flow cytometry (Beckman Coulter, Inc)

Small interfering RNA constructs and in vitro delivery.

siRNA against a target sequence 5'-AATGACATGCCGATCTACATG-3' has been previously shown to effectively silence the EphA2 receptor (115, 126). A nonsilencing siRNA was used as a control (Ambion). For in vitro silencing of EphA2 receptor in MPanc96 cells HiPerfect Transfection Reagent (Qiagen) was used according to the manufacturer's protocol. Briefly, 150 ng of siRNA and 12 μ l of HiPerfect reagent were mixed with serum free DMEM for a final volume of 100 μ l. Then, the mixture was allowed to incubate for 10 minutes. siRNA in complexes with the transfection reagent were added to 60% confluent cells in 2.3 mls of DMEM with 10% FBS and incubated overnight. The next day the media was replaced with fresh DMEM with 10% FBS. The protein was collected 48 hrs after transfection.

Liposomal preparation.

siRNA was complexed with liposomes as described previously(115). Briefly, DOPC and siRNA were mixed in the presence of excess tertiary butanol at a ratio of 1:10 (w/w) siRNA/DOPC. To this mixture, Tween 20 was added in a ratio of 1:19 Tween 20:siRNA/DOPC. After the mixture was vortexed, it was frozen in an acetone/dry ice bath, and lyophilized. Before in vivo administration, this preparation was resuspended with normal 0.9% saline to achieve the final concentration of 50 µg/ml. 100 µl of liposomal siRNA preparation was used for each injection.

Tumor growth and invasion studies in nude mice

The Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center reviewed and approved all mouse experiments performed in this work. Role of EphA2 in orthotopic pancreatic tumor biology was explored using 2 different mouse experiments, which employed 2 methods of RNA interference.

In the first experiment, MiaPaca-2 cells expressing luciferase reporter were infected with either control shRNA or EphA2 shRNA lentivirus and implanted orthotopically at 100,000 cells per mouse. Mice were divided into 4 groups (i) control shRNA, (ii) EphA2 shRNA, (iii) control shRNA + gemcitabine treatment (100 mg/kg body weight/weekly, IP), and (iv) EphA2 shRNA + gemcitabine. Treatment continued for 4 weeks.

In the second experiment, MPanc96-Luc cells expressing endogenous levels of EphA2 were injected orthotopically at 2×10^5 MPanc96-Luc cells per mouse. One week

later, mice were imaged using the IVIS bioluminescent imaging system, and the light emission values were used to divide the mice into four groups having similar average tumor volumes. Mice in the “control” group were treated with control siRNA alone (5 µg/animal), mice in the “siEphA2” group were treated with EphA2 siRNA alone (5 µg/animal), mice in the “gemcitabine” group were treated with gemcitabine (100mg/kg.b.wt) combined with control siRNA and mice in the “combination” group were treated with gemcitabine combined with EphA2 siRNA. All treatments were performed by intraperitoneal injections twice a week, with siRNA injection preceding gemcitabine by 1 day. Tumor burden and mouse weight was assessed weekly using the IVIS bioluminescent system and scale, respectively. At the end of the experiment the mice were killed and the primary tumor was removed, weighed and preserved for further studies. After the primary tumor was excised mice were imaged for metastases, which were counted using Living Image Software (Xenogen Corp).

Bioluminescent Imaging

Bioluminescent imaging was conducted as previously described (152). Image was obtained with the help of cryogenically cooled IVIS 100 imaging system. It was further analysed with the Living Image Software (Xenogen Corp). Before imaging, mice were injected with 15 mg/ml of luciferin in PBS (150 mg/kg.b.wt, intraperitoneally). Seven minutes after luciferin injection mice were sedated with 1.5% isoflurane-air mixture. Twelve minutes after luciferin injection a digital image of a mouse was acquired. Detected photons emerging from the active luciferase enzyme within the mouse were measured and a

pseudocolor representation of the photon spatial distribution was overlaid on the image. Tumor volume was quantified from a whole-mouse photon flux, which is a sum of all detected photons emitted by the mouse per second.

Western Blot

Cultured cell lysates were prepared by washing cells in PBS, followed by cell lysis with modified RIPA buffer for 10 minutes on ice. Cells were scraped from the plates and centrifuged at 12,000xg for 15 minutes in 4°C. Supernatant was stored in -80°C. Tumor tissue lysates was prepared from the resected pancreatic tumors after they were snap-frozen in liquid nitrogen. The frozen tissue was dropped into a tube with RIPA buffer and homogenized (Polytron). Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad). Protein lysates were run on SDS-PAGE gel and transferred to nitrocellulose membrane by semidry electrophoresis (Bio-Rad). Membrane was blocked in 5% milk in TBS for 1 hr and then immunoblotted with primary antibody overnight. The next day the membrane was washed in PBS-T and incubated in secondary antibody (Li-Cor Biosciences) for 1 hour. The signal was detected by Odyssey IR imaging system (Li-Cor Biosciences). The following primary antibodies were used: EphA2 (Upstate), luciferase, β -actin (Abcam).

Statistics

Unless specified otherwise, all experiments were performed on 3 or more separate occasions. Data presented are means of 3 or more independent experiments \pm SE. Statistically significant differences between two groups were determined by two-tailed unpaired Student's *t* test and were defined as $p < 0.05$. When four different groups were compared, a one-way ANOVA was performed followed by a Bonferroni's post-test analysis. Statistically significant difference was defined as $p < 0.05$.

Due to a high interexperimental variability of the effect of EphA2 silencing on cell apoptosis, a two-tailed paired Student's *t* test was performed to compare the percentage of apoptotic cells in groups treated with shCtrl and shEphA2 lentiviruses. Significant difference was defined as $p < 0.05$. For all statistical analyses GraphPad Prism software was used.

RESULTS

EphA2 is specifically expressed in pancreatic cancer

We previously identified EphA2 mRNA as being highly expressed in pancreatic cancer compared to tissues isolated from healthy pancreas or chronic pancreatitis in microarray studies (149). In order to confirm these findings in the current study, we performed real-time quantitative PCR on cDNA isolated from samples of human tissues of healthy pancreas (n=5), chronic pancreatitis (n=5) and PDAC (n=6). We observed that EphA2 expression in PDAC was 7 times as high as in normal pancreas and nearly 9 times as high as in chronic pancreatitis (Figure 2.1). No significant difference in EphA2 expression was found between normal pancreas and chronic pancreatitis.

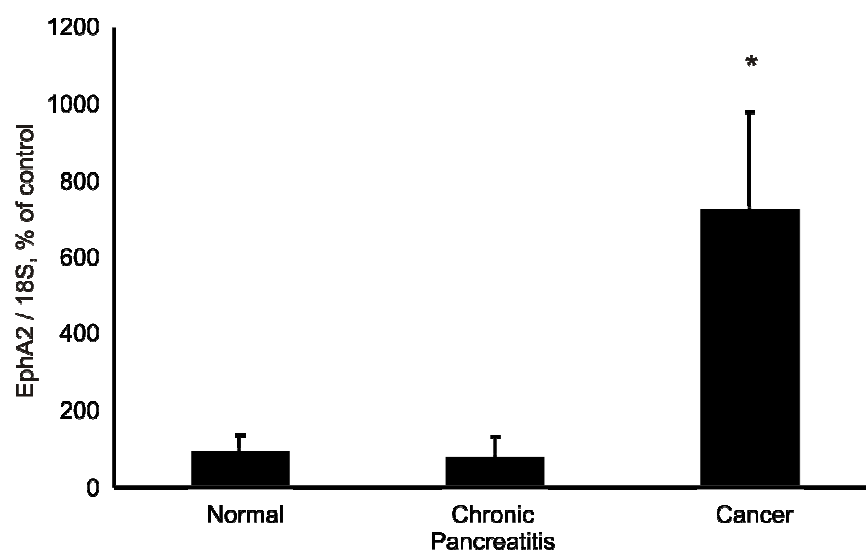


Figure 2.1. EphA2 is overexpressed in pancreatic adenocarcinoma. Quantitative PCR of EphA2 normalized to 18S in cDNA isolated from human tissues: normal pancreas (n = 5), chronic pancreatitis (n = 5) or pancreatic adenocarcinoma (n = 6). *, p<0.05 vs. normal pancreas.

EphA2 silencing decreases growth of pancreatic cancer cells and increases their sensitivity to gemcitabine in vitro.

To examine the role EphA2 receptor plays in pancreatic cancer, we silenced the receptor in 3 different pancreatic cancer cell lines by infecting them with shRNA against EphA2. MPanc96, MiaPaca and Panc-1 cells were selected for their known resistance to chemotherapeutics. They were previously shown to be resistant to gemcitabine, 5-FU and cisplatin (40). EphA2 expression in these three cell lines was measured at 96 hours by Q-PCR which indicated about a 60% decrease in EphA2 mRNA expression as compared to cells infected with non-targeting shCtrl lentivirus (Figure 2.2A).

The effect of silencing EphA2 on cell number was then assessed after a short exposure to gemcitabine. As expected with these cell lines, gemcitabine alone had no effect on MiaPaca-2 cells growth and demonstrated a cytostatic effect on MPanc96 and Panc-1 cells. Silencing of EphA2 had a small effect on MPanc96 and MiaPaca-2 cell numbers but did not influence Panc1 cells (Figure 2.2B). Importantly, consistent with these cells being gemcitabine resistant, no decrease in cell number from the beginning of the experiment was observed in any of the cell lines that received gemcitabine treatment. In contrast, silencing of EphA2 in combination with gemcitabine caused an obvious decrease in cell numbers in all treated cell lines.

Next, we examined the effect of shEphA2 on cell cycle and apoptosis using propidium iodide FACS analysis (Figure 2.2C). All 3 cell lines showed basal levels of apoptosis around 5%, which is consistent with our previous studies. This also indicates that

the infection with shCtrl lentivirus did not increase the basal level of apoptosis in these cells. Consistent with the results on cell numbers, EphA2 silencing increased the basal level of apoptosis to the greatest extent in MPanc96, while having lesser effects in MiaPaca-2 and Panc-1 cells. Furthermore, the combination of EphA2 silencing and gemcitabine increased the rate of cell apoptosis more than gemcitabine alone in all tested cells ($p < 0.05$). No effect of EphA2 silencing was observed on the fraction of cells undergoing DNA synthesis stage of the cell cycle (data not shown).

A.

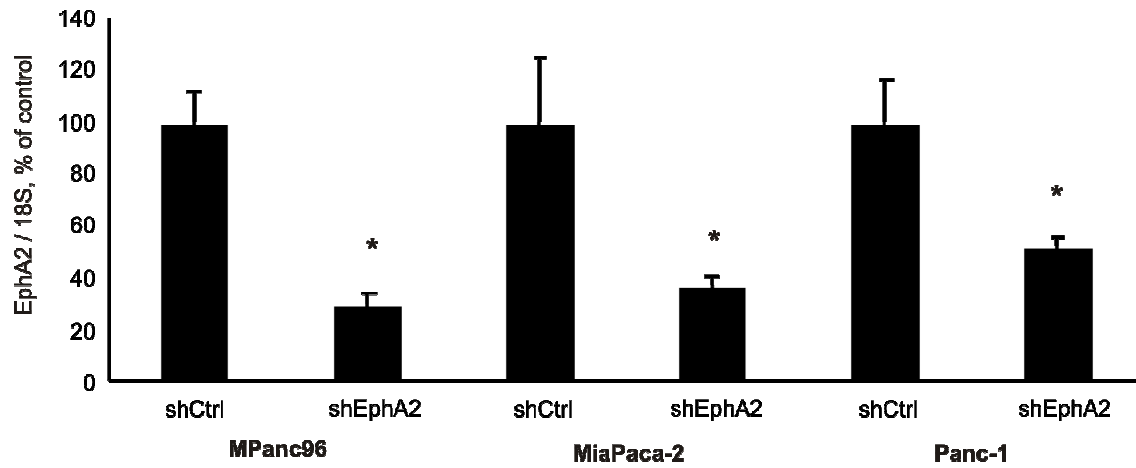


Figure 2.2. Silencing of EphA2 in vitro decreases cell growth by increasing apoptosis. A. MPanc96, Miapaca-2 and Panc-1 cells were infected with lentivirus expressing Control shRNA (shCtrl) or EphA2 shRNA (shEphA2). EphA2 expression was measured by Q-PCR and normalized to 18S. *, $p < 0.05$ vs shCtrl

B.

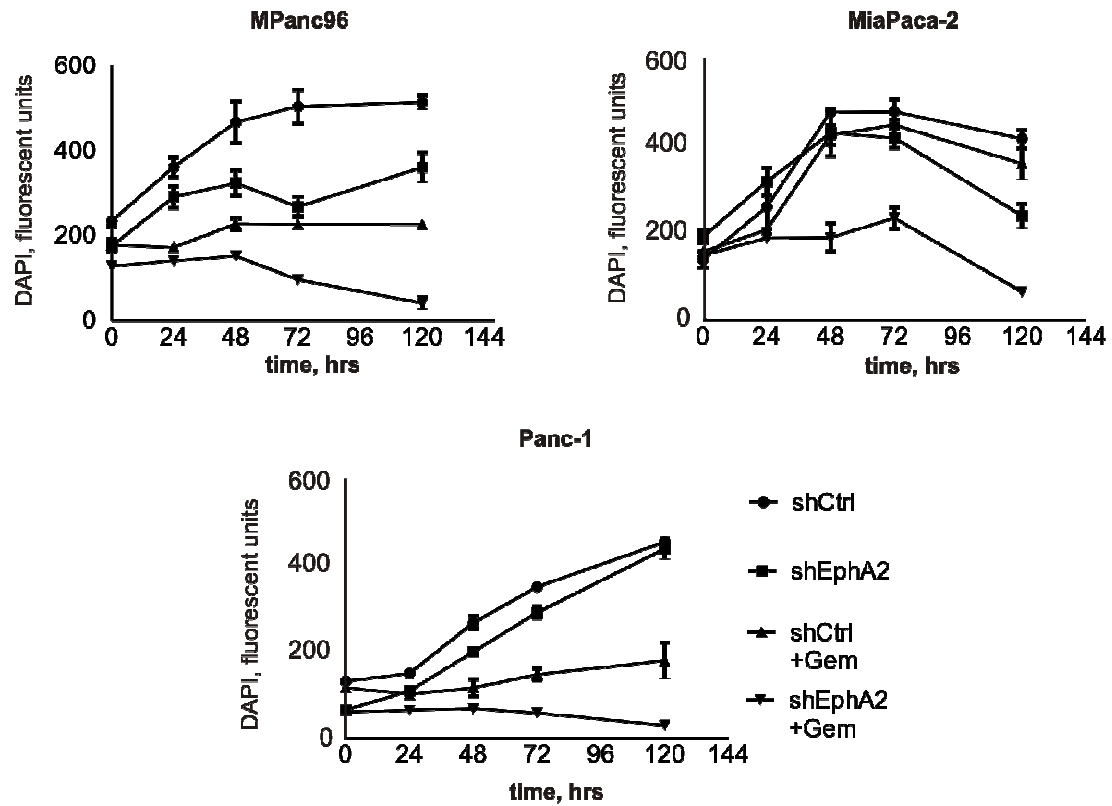


Figure 2.2, continued. B, Cell growth assay of shCtrl and shEphA2 infected cells with or without exposure to gemcitabine (1 μ M). Cell nuclei were stained with DAPI; fluorescence measurement is shown. Experiment was done in triplicates, shown as average \pm SE. This experiment was repeated with a similar result.

C.

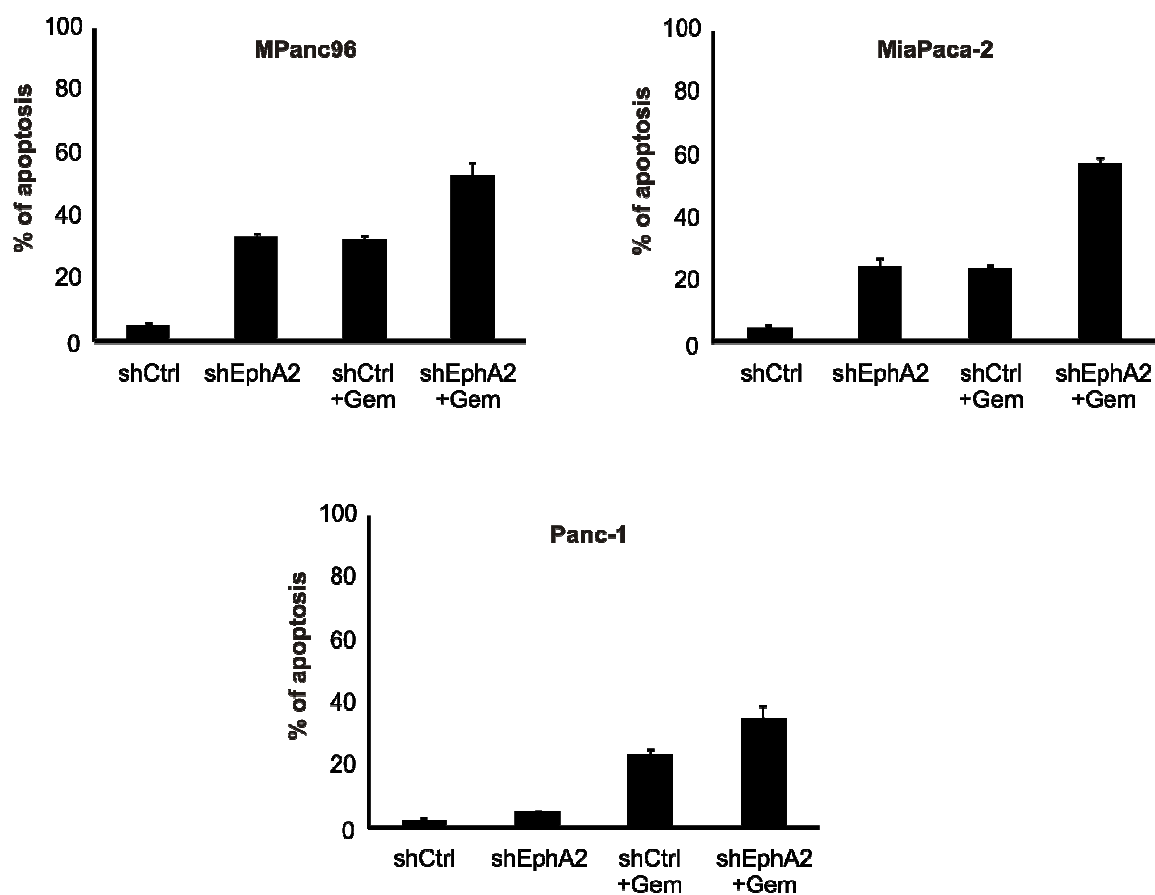


Figure 2.2, continued. C, shCtrl and shEphA2 infected cells were treated with gemcitabine and subjected to propidium iodide staining. FACS was performed and cell apoptosis was measured. Representative experiment is shown. Statistics was performed as described in Methods. shEphA2 and shEphA2 + Gemcitabine was statistically different from shCtrl and shCtrl + Gemcitabine, respectively ($p < 0.05$).

Silencing of EphA2 receptor in combination with gemcitabine decreases pancreatic tumor growth and invasion.

To confirm our observations in vivo, orthotopic tumors were formed in mouse pancreases with luciferase reporter-expressing MiaPaca-2 cells that were previously infected with shCtrl or shEphA2 lentiviruses. Imaging was performed 2 days later to confirm successful implantation. One week after cell injection, mice were placed on a regimen of gemcitabine (100 mg/kg of mouse body weight, weekly) or saline. Tumor burden was measured every week for 4 weeks using luciferase light emission (Figure 2.3). Mice with EphA2 silenced cells showed a significant regression of the initial tumor after 3 weeks regardless of gemcitabine treatment and demonstrated no increase in tumor in the 4 weeks of the experiment. In contrast, as expected mice that received shCtrl infected cells showed an exponential increase in tumor burden over the time-course of the study. Gemcitabine treatment alone did not significantly reduce tumor growth in these animals.

To demonstrate a more clinically relevant approach, we also tested in vivo delivery of siRNA for EphA2 in a pre-established orthotopic model of pancreatic cancer using another gemcitabine-resistant cell line, MPanc96. First, we confirmed silencing of the EphA2 receptor by siRNA in MPanc96 cells in vitro. In vitro transfection with EphA2 siRNA resulted in $60 \pm 9\%$ silencing (Figure 2.4A). Next, mice were injected orthotopically with 2×10^5 MPanc96 cells stably expressing firefly-luciferase gene (MPanc96Luc) and tumor burden was measured one week later using bioluminescence imaging. Mice were allocated to 4 groups having similar average tumor burdens. The groups were then treated

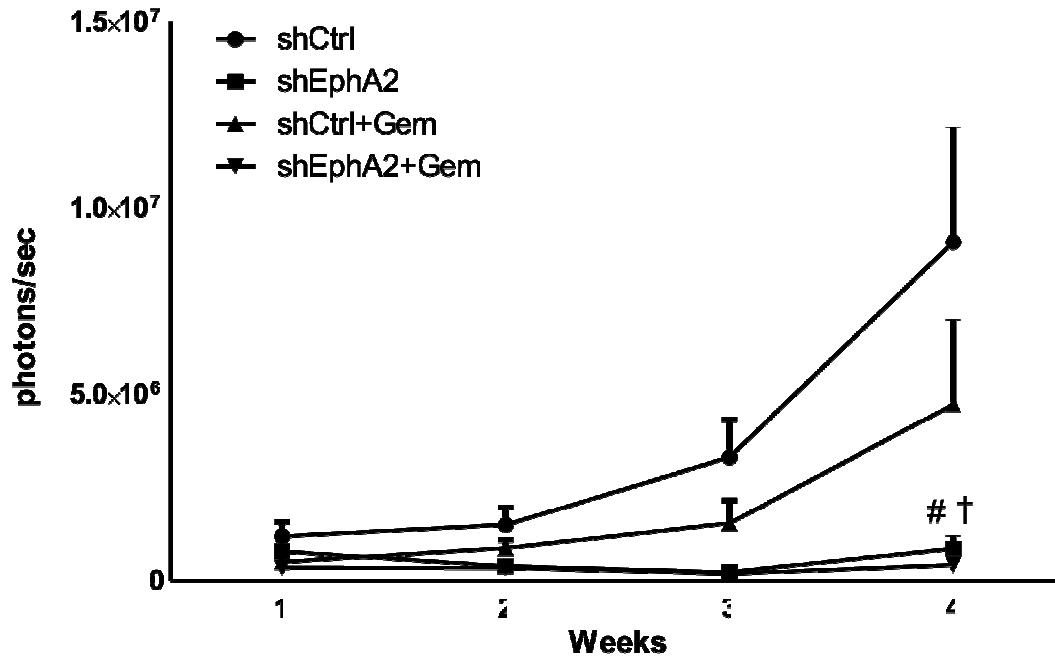


Figure 2.3. Silencing of EphA2 in Miapaca-2 cells decreases tumor growth, regardless of gemcitabine. Miapaca-2-Luciferase cells infected with shCtrl or shEphA2 lentiviruses were injected into mouse pancreas. Mice were treated with gemcitabine or PBS and tumor growth was measured by luminescence. #, † - $p < 0.05$ vs. shCtrl and shCtrl + Gemcitabine, respectively.

with control siRNA alone, EphA2 siRNA alone, combination of control siRNA with gemcitabine, or a combination of EphA2 siRNA with gemcitabine. Tumor growth was monitored for 6 weeks (Figure 2.4C). Neither EphA2 nor gemcitabine produced any significant reduction of tumor growth in this model. In contrast, beginning from week 3 and till the end of the experiment, the combination treatment significantly decreased MPanc96 cell tumor growth. At the end of 6 weeks, animals were sacrificed and pancreases were excised and weighed (Figure 2.4D). Tumor weight measurements were consistent with bioluminescent data, confirming that the combination treatment of EphA2 siRNA with gemcitabine led to a significant inhibition of pancreatic tumor growth. In contrast, neither EphA2 siRNA or gemcitabine treatments alone were effective.

After mice were sacrificed, liver and lungs were dissected out and the number of metastatic foci was counted using bioluminescence and Living Image software (liver: Figure 2.4E, lung: Figure 2.4F). Combination treatment of EphA2 siRNA and gemcitabine significantly reduced the number of metastatic foci in liver and lung compared to control siRNA alone or control siRNA plus gemcitabine treatment groups. EphA2 siRNA treatment alone tended to decrease the number of liver and lung metastases; however it failed to achieve statistical significance.

To confirm that EphA2 siRNA inhibited EphA2 expression in vivo, protein lysates from tumor tissues of animals treated with control siRNA and EphA2 siRNA were isolated and subjected to western blotting for EphA2. Luciferase was used as a loading control. Western blots showed that liposomal delivery of EphA2 siRNA decreased EphA2 expression in mouse orthotopic human pancreatic tumors by $61 \pm 11\%$ (Figure 2.4B).

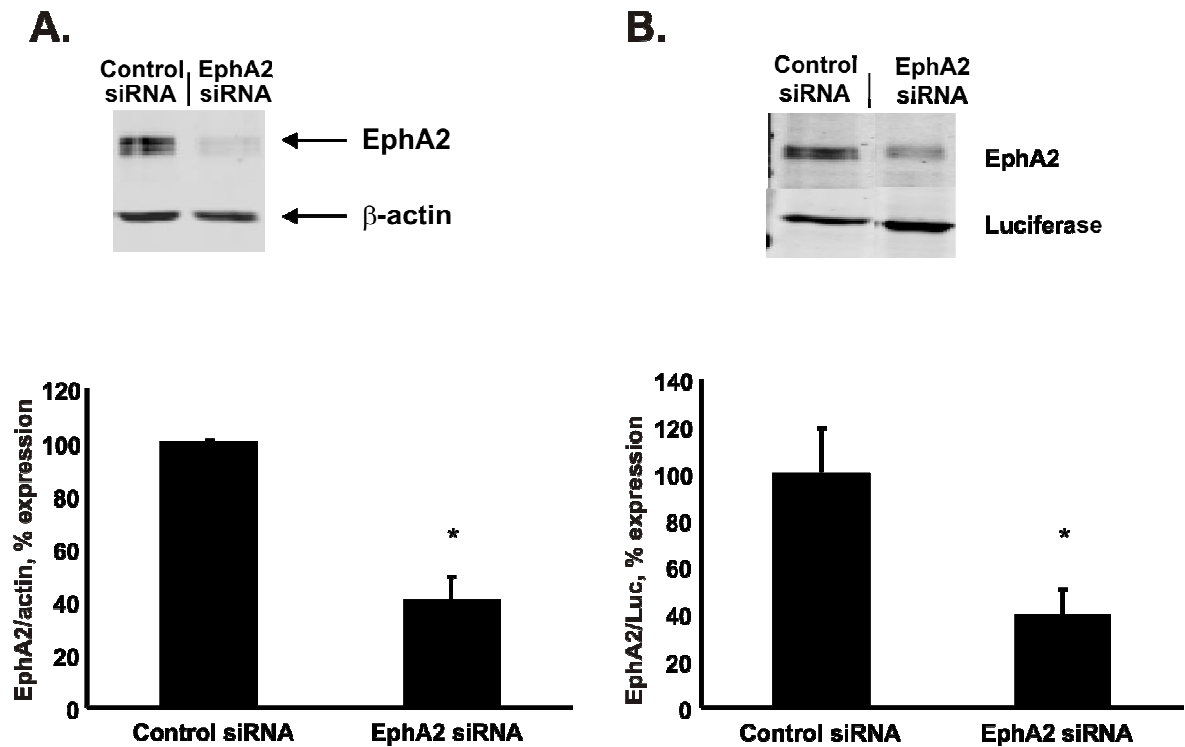


Figure 2.4. Silencing of EphA2 sensitizes gemcitabine-resistant MPanc96 cells. EphA2 siRNA decreases EphA2 expression in MPanc96Luc cell line A. in vitro and B. in vivo. A, MPanc96Luc cells were transfected with either control siRNA or EphA2 siRNA. Cell lysates were blotted for EphA2. β-actin was used as a loading control. Band intensity was measured and graphed (EphA2 expression in cells transfected with control siRNA was taken for 100%). B, mice with MPanc96-Luc orthotopic tumors were treated with Control or EphA2 siRNA for 6 weeks. Tumor lysates were blotted for EphA2, and luciferase was used as a loading control. Band intensity was measured and graphed. *, $p < 0.05$

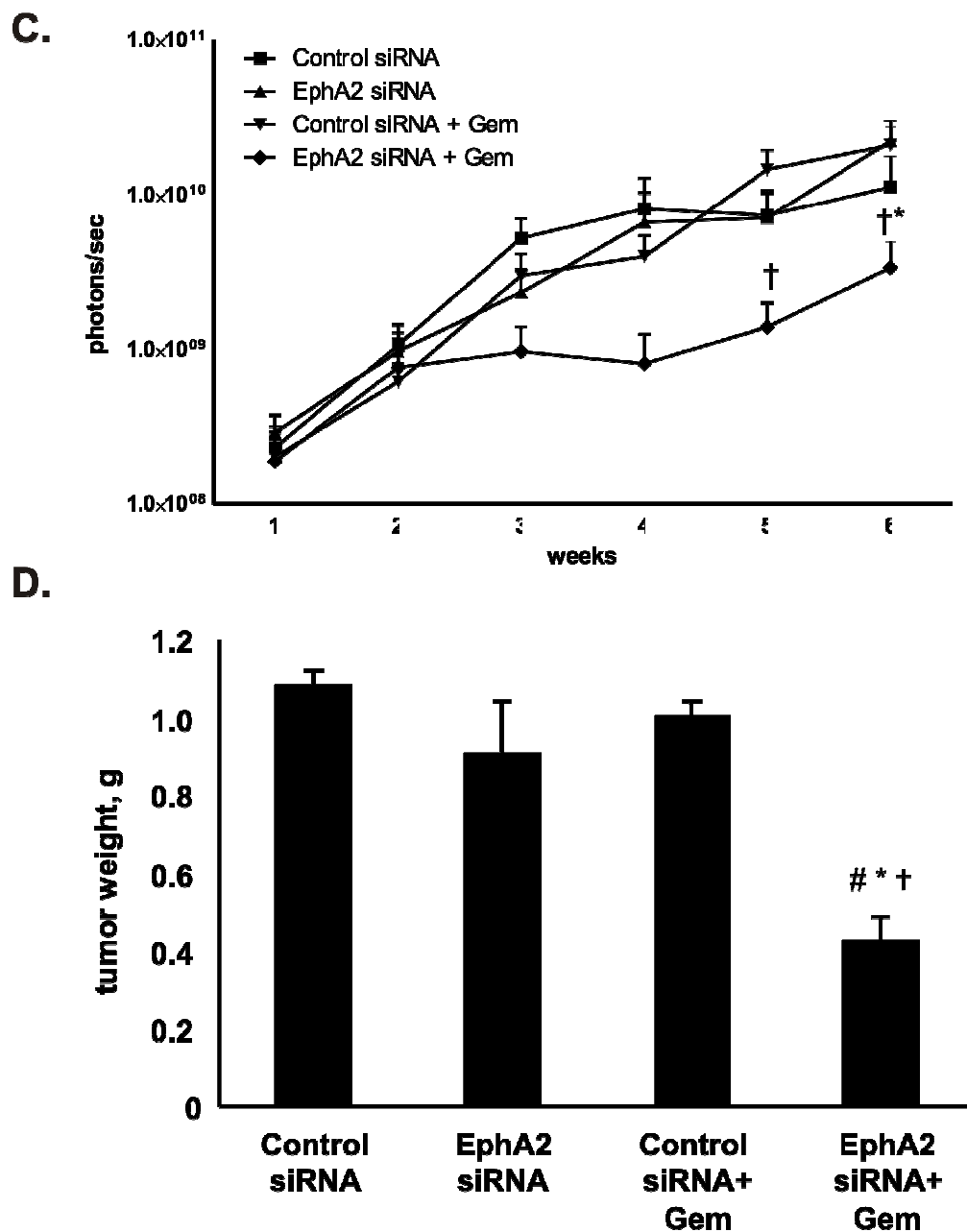
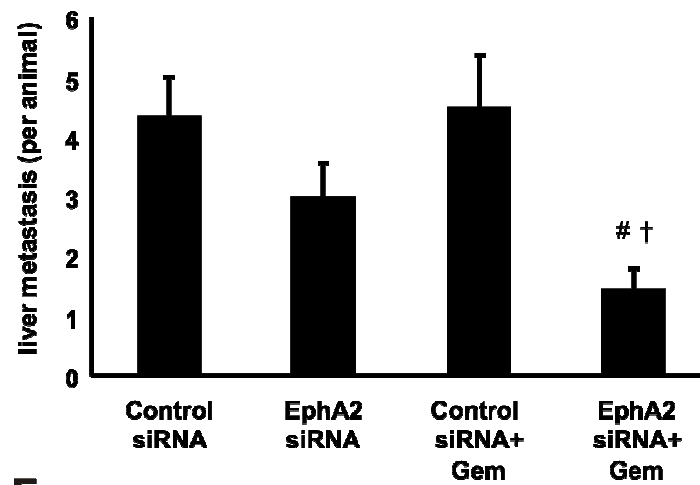


Figure 2.4, continued. C. Luminometer reading of light emission from MPanc96-Luc orthotopic tumors was measured. D. Mouse pancreata containing MPanc96-Luc tumors were excised and weighed. #, *, † - $p < 0.05$ as compared to Control siRNA, EphA2 siRNA and Control siRNA + Gemcitabine, respectively.

E.



F.

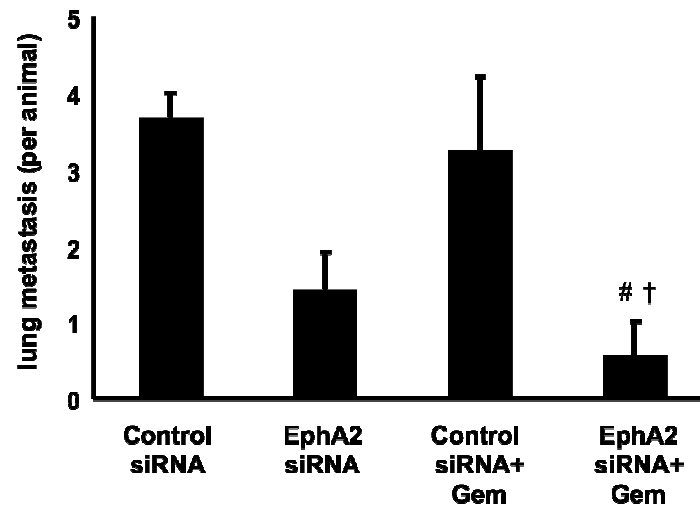


Figure 2.4, continued. E, liver and F, lung were excised and the number of metastatic foci was counted based on their bioluminescence. #, † - $p < 0.05$ as compared to Control siRNA and Control siRNA + Gemcitabine, respectively.

DISCUSSION

Pancreatic cancer remains one of the most lethal malignancies. Gemcitabine is the current front-line treatment for pancreatic cancer. However, its effectiveness is marginal, because most tumors are initially resistant to gemcitabine or develop resistance soon after the initiation of therapy. Clearly, treatments that can improve the effectiveness of gemcitabine would be very valuable in the clinic. In this study, we evaluated the effect of EphA2 targeting on gemcitabine-resistant pancreatic cancer cell growth in vitro and in vivo. We demonstrated that silencing of EphA2 increases cell apoptosis and enhances cytotoxic effect of gemcitabine on drug resistant cells. We also demonstrate for the first time that systemic delivery of siRNA against EphA2 greatly improves the effectiveness of gemcitabine in drug-resistant orthotopic pancreatic cancer in mice. Therefore, these studies support the pursuit of EphA2 directed combination therapies for the human disease.

Despite a growing body of literature on high EphA2 expression in different cancer tissues, there is only one study comparing expression of the EphA2 receptor in normal pancreas to pancreatic cancer (99). Using immunohistochemistry staining of human pancreatic cancer tissue, Mudali and colleagues showed that EphA2 expression increases as the disease develops (99). Our Q-PCR data supports that finding and shows that EphA2 expression is increased in pancreatic cancer compared to both normal pancreas and chronic pancreatitis. Chronic pancreatitis tissues possess an inflammatory stroma, which resembles histology of pancreatic cancer. Our data support that high expression of EphA2 is specific to neoplastic transformation and not inflammation. It has been reported that EphA2 expression may be driven by activation of Ras-MAPK pathway (106, 107). Therefore, high level of EphA2 expression in pancreatic cancer may be explained by the fact that K-Ras is mutated

and constitutively active early in pancreatic cancer development (155, 156) and 90% of pancreatic cancers patients have K-Ras mutations (157).

The literature on the role of EphA2 in cancer is complex and sometimes contradictory. Both tumor suppressing (108, 109, 158) and tumor promoting (87, 159) roles of EphA2 have been described. Likewise, EphA2 has been found to stimulate (130) and suppress (107, 129) the same oncogenic pathways. This apparent duality of EphA2 receptor function may be dependent on cellular context, receptor/ligand ratio and ligand clustering (119, 160, 161), as well as receptor intracellular localization and phosphorylation (120). However, an apparent difference in EphA2 function may also stem from the variety of technical approaches that have been used. The methods of interference with receptor function can be divided into 2 categories: 1) posttranslational and 2) pretranslational. Posttranslationally, EphA2 expression on the cell surface has been manipulated either by employing agonistic antibodies (116, 147) or soluble ephrin-A1 (101, 118) in order to stimulate the receptor. Receptor stimulation causes its subsequent internalization and degradation. These methods, however, accept temporary increase in EphA2 signaling due to receptor phosphorylation, before receptor is degraded. This method also relies on the assumption that the receptor is available for ligand binding on the cell surface. However, studies showed that unlike in normal cells, cancer cells keep EphA2 receptor localized to the cytoplasm (87, 120). In addition, due to receptor-ligand promiscuity, soluble ephrin-A1 is not specific for EphA2 and can bind to other EphA receptors. In an approach to prevent EphA2 receptors from interacting with ephrins, soluble EphA2-Fc receptors are often used as ligand traps (104, 145). However, similar to soluble ephrins, EphA2-Fc chimeras bind multiple ephrin-As and are not specific for ephrin-A1. In addition, EphA2 is capable of

ligand independent signaling that can be activated through interaction with other receptors (160). For these reasons, pretranslational manipulation using RNAi technology as demonstrated in this study that blocks the EphA2 receptor by decreasing receptor synthesis avoids confounding variables, such as changes in receptor signaling or receptor-ligand promiscuity, and therefore is more specific. This is the first study of orthotopic pancreatic cancer, in which EphA2 receptor expression was manipulated pretranslationally using siRNA.

EphA2 has been implicated in increasing survival and drug resistance of cancer cells. Several studies showed that decreasing EphA2 expression levels decreases cell survival (111) and drug resistance (115, 162), while increasing its expression or activity increases cell survival (87, 112). Although the mechanism of EphA2 regulation of gemcitabine resistance specifically is beyond the scope of the current study, we speculate that EphA2 increases activity of the proteins involved in cell survival, such as Src and FAK. In several studies, EphA2 has been shown to associate with phosphorylated Src (116) and FAK (123). Src phosphorylation and kinase activity have been correlated to gemcitabine resistance, while targeting Src has been shown to increase gemcitabine sensitivity (47, 163-165). Similarly, FAK phosphorylation has been found to correlate with intrinsic gemcitabine resistance of pancreatic cancer cells and inhibition of FAK increases gemcitabine cytotoxicity and tumor regression (48, 166). Moreover, EphA2 is also known to directly interact with EGF receptors. Even though EGFR importance in pancreatic cancer is still controversial, the EGFR inhibitor, Erlotinib, has been shown to slightly increase survival of pancreatic cancer patients (73).

As part of the current study we utilized systemic delivery of liposomal siRNA for EphA2 as a treatment for PDAC in mice. RNAi technology has a tremendous potential for clinical use. However, systemic delivery of siRNA remains a difficult problem, due to low stability of siRNA, lack of cell penetration and potential toxicity. Pancreatic cancer is hypovascularized (39) and therefore is even more challenging. Previously we and other groups have reported successful delivery of siRNA into the pancreatic cancer tissue after systemic administration of neutral DOPC liposome-coated oligonucleotides (167-169). In this study, siRNA delivered to pancreatic cancer tissue through intraperitoneal administration of a liposomal preparation achieved approximately 60% gene knockdown. Development of new approaches to siRNA delivery in patients may hold the promise of more effective treatments. The current study suggests that the combination of EphA2 silencing with gemcitabine treatment should be considered once these approaches are available.

**CHAPTER THREE: THE RAS/MEK2/ERK PATHWAY
REGULATES EPHA2 EXPRESSION IN PANCREATIC CANCER**

INTRODUCTION

Pancreatic cancer remains the fourth major cancer killer in the US (5). The hallmark of pancreatic cancer is its aggressive invasiveness and metastasis, which renders surgical treatment unavailable in 80% of the cases. Median survival of pancreatic cancer patients is 6 months, with 80% dying within the first year of diagnosis. In the last 30 years, patient 5-year survival after diagnosis only increased from 3% to 5% (5). It is apparent that more research focusing on pancreatic cancer progression and metastasis is absolutely essential.

EphA2 has been shown to play a major role in cancer progression. EphA2 is highly expressed in a number of cancers, including pancreatic ductal adenocarcinoma (99, 126). Moreover, EphA2 levels have been correlated with increased tumor aggressiveness and decreased patient survival in ovarian and lung cancer (170, 171). In pancreatic cancer, EphA2 is also highly expressed and increases pancreatic tumor growth and invasiveness (118, 126, 145). Targeting of EphA2 receptor in pancreatic cancer cells decreases tumor growth and metastasis and increases the rate of cell death [reference].

In spite of the importance of EphA2 receptor in cancer progression, few studies have focused on EphA2 regulation. Dohn and colleagues reported that p53 regulates EphA2 transcription, while Jin et al have found that there is a p53 regulatory region in the EphA2 promoter, 180 base pairs upstream of the transcription initiation site (108, 109). However, 40%-75% of pancreatic cancer patients have a loss or a mutation in p53 (30). Therefore, it seems unlikely that p53 is responsible for the high level of EphA2 expression in PDAC. In lung and breast cancer models, it has been suggested that EphA2 is regulated by Ras-MAPK pathway although the detailed pathway was not elucidated (106, 107).

In the current study we examined the involvement of p53 and the Ras/MAPK pathway in transcriptional regulation of EphA2. We observed no influence of p53, but found that Ras activity up-regulated EphA2 in a MEK2, RSK and AP-1 dependent manner. We also explored means of regulation of EphA2 in pancreatic cancer through manipulating the output of MAPK pathway in the presence of constitutively active mutant K-Ras.

MATERIALS & METHODS

Cell lines and reagents

Pancreatic cancer cell lines BxPC3, Hs766T, MiaPaca-2, Su86.86, Panc1, HPAC and Human Pancreatic Nestin-positive Epithelial (HPNE) cells were obtained from American Type Tissue Collection. Pancreatic cancer cell line MPanc96 and human pancreatic ductal epithelial (HPDE) cells were provided by Dr. Timothy J. Eberlein (Washington University, St. Louis, MO) and Dr. M. Tsao (Ontario Cancer Institute, Toronto, Ontario, Canada), respectively. L3.6pl cells were derived from pancreatic cancer cell line COLO357 and were provided by Dr. Isaiah J. Fidler (MD Anderson Cancer Center, Houston) (172). Pancreatic cancer cell line SW1990 was generously shared by Drs. Eric Collisson, Joe Gray, and Martin McMahon (University of California and Lawrence Livermore Laboratory, San Francisco, CA). DanG cells were received from Deutsches Krebsforschungszentrum (Heidelberg, Germany). Adenoviruses adPEA15 and adGFP were a generous gift from Drs. Ueno Naoto and Chandra Bartholomeusz (MD Anderson Cancer Center, Houston).

Transient transfection of small interfering RNA

Cells were transfected in a 6-well plate format, using HiPerfect reagent (Qiagen) and following reverse transfection protocol. The final siRNA concentration was 10nM. Protein and RNA collection were performed 48 hours after transfection. On-Target plus smart pool control siRNA (Dharmacon) was used as a control transfection. Target siRNA sequences used were:

MEK1 – 5'-TTGTGAATAAATGCTTAATA-3' (Qiagen),

MEK2 – 5'-CAGCATTTGCATGGAACACAT-3' (Qiagen),

c-Jun – 5'-AGATGGAAACGACCTTCTA-3' (173),

K-Ras G12D – 5'-GTTGGAGCTGATGGCGTAG-3' (61).

RNA extraction, reverse transcription and real-time quantitative PCR

RNA extraction from cells in 6-well plates was performed using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells were collected with 1 ml of Trizol reagent. RNA was separated from the rest of the cell extract with chloroform and precipitated out of the aqueous fraction using with isopropanol. RNA was washed with 70% ethanol, dried and resuspended in water. RNA concentration was measured with spectrophotometer. 1µg of RNA was subjected to DNase treatment and used for reverse transcription reaction. Both DNase and reverse transcription was performed with the help of Quantitech kit (Qiagen) following the company's protocol.

Quantitative PCR was performed using iCycler (Bio-Rad). Amplification product was detected using SYBR green dye. For each primer, a standard curve was constructed and the values were obtained by extrapolating the threshold counts onto the standard curve. Each sample was run in triplicate. The average value from the triplicates for each sample was normalized to corresponding average value of 18S for the same sample. The primers used in this study were:

K-Ras – forward, 5'-CCGCACAAGGCACTGGGTAT-3',

reverse, 5'-GCATCGTGTTATCTCTGGGTCGTA-3'

MEK1 - forward, 5'-GACTCCATGGCCAACTCCTTCGTG-3',

reverse, 5'-CCTCCCAACCGCCATCTCTACCAG-3'

MEK2 – forward, 5'-TGCTGCCGGCGCTCACCATC-3'

reverse, 5'-GCCGCTTCTTCTGCTGCTCGTCAA-3'

EphA2 – forward, 5'-CCCCCTCCGCCCCACACTACCTC-3'

reverse, 5'-ACACGGCCCGCATTCCCCAGACTC-3'

18S – forward, 5'-GAGCGGTCGGCGTCCCCCAACTCC-3'

reverse, 5'-GCGCGTGCAGCCCCGGACATCTAA-3'

The primer for c-Jun was purchased from Applied Biosystems. Each primer set was checked for producing one melting peak and one band on agarose gel electrophoresis.

Protein extraction and western blot

For protein extraction, adherent cells in culture were washed twice with ice-cold PBS. Modified RIPA buffer, with addition of protease inhibitors (Sigma) and phosphatase inhibitors (Pierce) was used to lyse the cells. The protein lysate was collected by scraping. Lysates were subjected to brief sonication and spun down. Protein concentration of the supernatant was measured with Bio-Rad reagent. 30-50 µg of protein was loaded per well of the SDS-PAGE gel. Electrophoresis was performed and the proteins were transferred onto a

nitrocellulose membrane and immunoblotted. Precision plus dual color protein standards served as a molecular weight marker.

The following antibodies were used: Ras, EphA2 (Upstate), phospho-ERK, total JNK, HA-tag (Santa Cruz), total ERK, phospho-JNK, phospho-Akt, phospho-Fra1, phospho-RSK (Cell Signaling), tubulin (Abcam). Fluorescent goat anti-rabbit 800 and goat anti-mouse 680 (LiCor) secondary antibodies were used. The bands were visualized, using Odyssey scanner and quantified with the manufacturers software.

RSK and MEK inhibition, time courses and dose curves.

For all inhibition studies, two days before the experiment, MPanc96 cells were seeded onto a 6-well plate at 2×10^6 cells per plate. The day before the experiment, the cell culture media was replaced with serum-free DMEM.

For RSK inhibition studies, cells were treated with a specific RSK inhibitor, FMK (3 μ M, courtesy of Dr. Jack Taunton, Howard Hughes Medical Institute, San Francisco, CA), for 0, 3, 6, and 12 hours before the protein was collected.

To investigate the effect of MEK inhibition on EphA2 mRNA expression, MPanc96 cells were treated with either DMSO, PD98059 (50 μ M, Cell Signaling), or U0126 (10 μ M, Cell Signaling). Four hours after treatment, RNA was collected as quantitative RT-PCR performed.

For the time course, MPanc96 cells were treated with PD98059 (50 μ M) or U0126 (10 μ M), 0, 3, 6, 12 and 24 hours before the protein was collected.

For the dose curve, MPanc96 cells were treated with DMSO or 6.25, 12.5, 25 and 50 μ M of PD98059. After 24 hours, protein and RNA were collected as described above.

Adenovirus infection

MPanc96 cells, grown at 60% confluency, were infected with adGFP or adPEA15 adenoviruses at 50 MOI. Media was replaced the next day and GFP expression confirmed under fluorescent microscope (Olympus). Forty-eight hours after infection, RNA and protein were collected.

EGF stimulation

MPanc96 or MiaPaca-2 cells were grown to 60% confluency. On the day of the experiment the media was replaced with 10% FBS DMEM. Cells were treated with either PBS or EGF (20 nM, Sigma). RNA was collected 2 hrs after the treatment. To confirm that changes in EphA2 mRNA level were due to transcription, a transcription blocker, actinomycin D (Sigma), was added 30 minutes before addition of PBS or EGF. Protein was collected 2 hrs after EGF treatment.

EGFR inhibition

For the pilot experiment, HPNE cells were seeded at 1×10^6 cells per 10 cm plate the day before the experiment. On the day of experiment cells were treated with gefitinib (10 μ M) at 0, 3, 6, 12 and 24 hours before protein collection.

To screen the cell lines for EGFR inhibitor sensitivity, 12 cell lines were treated with gefitinib for 0, 6 and 12 hours. Protein was collected and western blot was performed as described above. The experiment was repeated and the lysates from 0 and 6 hours were used for RPPA analysis as described elsewhere (174, 175).

Statistics

Unless specified otherwise, all experiments were performed on 3 or more separate occasions. Data presented are means of 3 or more independent experiments \pm SE. Statistically significant differences between two groups were determined by two-tailed unpaired Student's *t* test and were defined as $p < 0.05$.

RESULTS

K-Ras regulates EphA2 protein and RNA expression in pancreatic cancer.

To explore the role of K-Ras in regulation of EphA2 levels in pancreatic cancer, we examined EphA2 expression in tumor tissues of transgenic mice with mutant K-Ras-driven pancreatic cancer (Figure 3.1A). Microarray profiling demonstrated that EphA2 mRNA levels were increased ~10-fold in pancreatic cancer tissues as compared to a normal mouse pancreas. Deletion of p53 in the mutant Ras expressing cells of the transgenic mice did not affect EphA2 mRNA levels. These data were confirmed by Q-RT-PCR (Figure 3.1B).

To further investigate the relationship between mutant K-Ras and EphA2 in pancreatic cancer, MPanc96 pancreatic cancer cells which possess mutant active K-Ras were transfected with K-Ras siRNA. Quantitative RT-PCR examination of EphA2 mRNA demonstrated significant reduction of EphA2 levels in K-Ras silenced cells (Figure 3.1C). Western blot of protein lysates isolated from K-Ras silenced cells confirmed the significant decrease in K-Ras protein levels, which was paralleled by a 60% reduction of EphA2 (Figure 3.1D; quantification Figure 3.1E).

Downstream mediators of K-Ras activity including the phosphorylation status of Erk, Akt, and JNK were examined after K-Ras silencing. Surprisingly, no decrease in phospho-Akt or phospho-JNK was observed. However, a dramatic decrease in phospho-Erk band was evident in K-Ras silenced MPanc96 cells (Figure 3.1D).

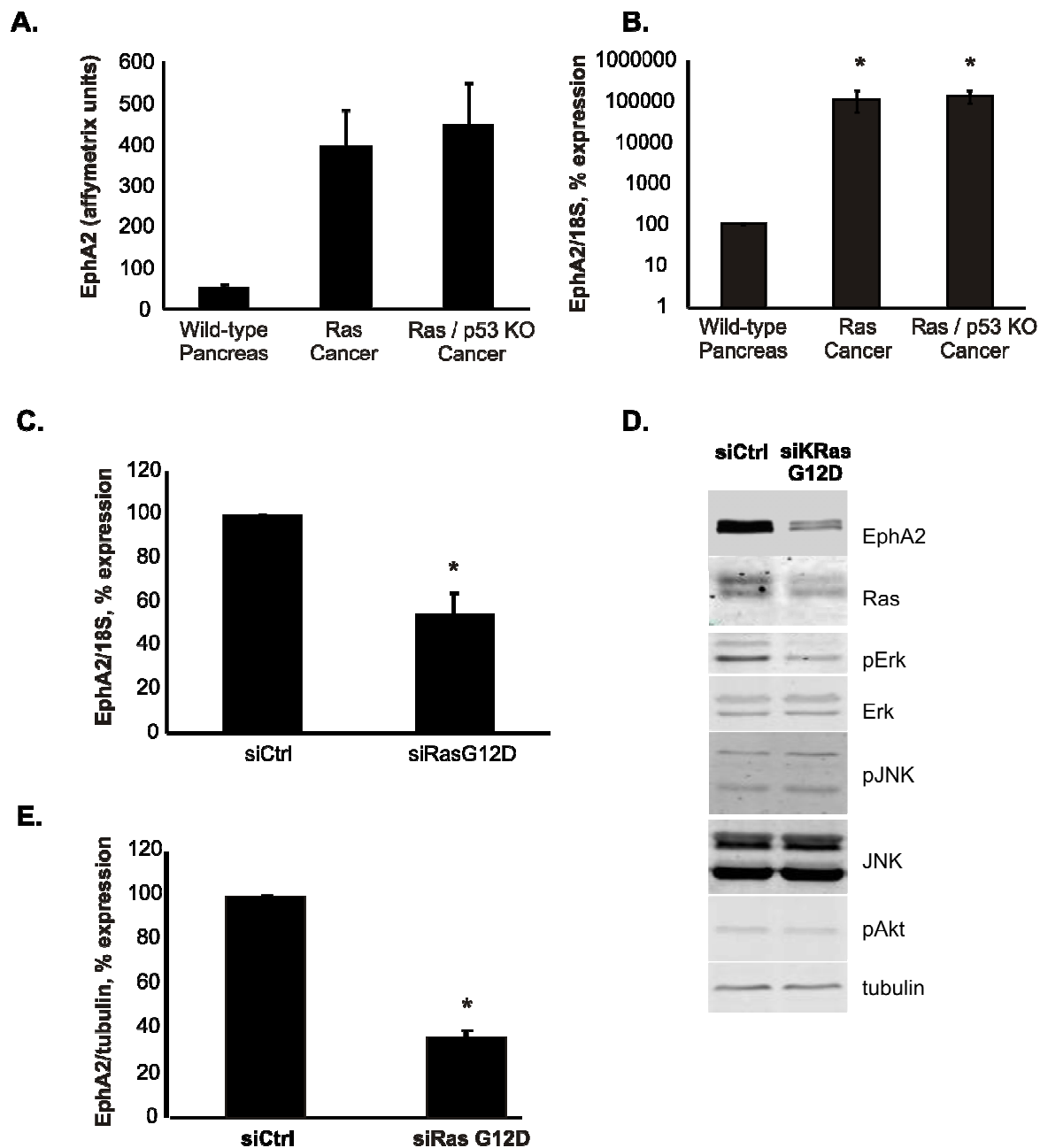


Figure 3.1. EphA2 expression levels are regulated by K-Ras. RNA was isolated from pancreata of transgenic mice with K-Ras-driven pancreatic cancer. A, microarray data for EphA2, which was confirmed by B, Q-PCR. C, Q-PCR of EphA2 after silencing of KRasG12D in MPanc96 cells. EphA2 expression was normalized to 18S and is shown as a percentage of EphA2 levels from control siRNA transfected samples. D, Western blot of EphA2, Ras and Ras effectors, ERK, JNK, and Akt, after transfection of MPanc96 cells with control siRNA and siRNAs for KRasG12D. E, Quantification of EphA2 bands, normalized to tubulin, is shown as percentages of control siRNA transfected cells. *, $p < 0.05$

MEK2 regulates EphA2 expression.

To further analyze the signaling pathways involved in EphA2 expression in pancreatic cancer cells, MPanc96 cells were treated with a specific MEK chemical inhibitors: PD98059 (50 μ M). A strong reduction of phospho-ERK, the known downstream target of MEK, was observed at the earliest measured time point, indicating the effectiveness of the inhibitors. Inhibition of the Erk pathway was followed by a delayed but obvious reduction in the levels of EphA2 (Figure 3.2A). This results were confirmed using a different MEK inhibitor U0126 (10 μ M, data not shown). Likewise, EphA2 mRNA levels decreased by 90% after 4 hours of MEK inhibition with either inhibitor as compared to DMSO treated controls (Figure 3.2B). MEK inhibition had no effect on the level of total Erk. Further investigation indicated that a significant drop in EphA2 mRNA was observed at the lowest attempted concentration of PD98059 (6.25 μ m) (Figure 3.2D). The dose-dependence of the effects of inhibitors on inhibition of phospho-ERK and reduction of EphA2 protein levels was similar (Figure 3.2C). These results were further confirmed in Miapaca-2 cells (data not shown)

Several studies have shown non-redundant roles of MEK1 and MEK2 (66-68). To distinguish between the 2 proteins and also to confirm the data obtained with chemical inhibitors, MPanc96 cells were transfected with siRNA against MEK1, MEK2, or a combination of both (Figure 3.2E). Silencing of MEK1 and MEK2 at the protein level was confirmed and quantitated to be 93% and 88%, respectively (Figure 3.2F). No significant decrease in EphA2 mRNA was observed after silencing of MEK1. In contrast, MEK2

siRNA transfected cells showed 50% reduction in EphA2 (Figure 3.2E). Reduction of EphA2 expression was not further enhanced by the combination of MEK1 and MEK2 siRNA. Similar results were observed in the MiaPaca-2 pancreatic cancer cell line (data not shown).

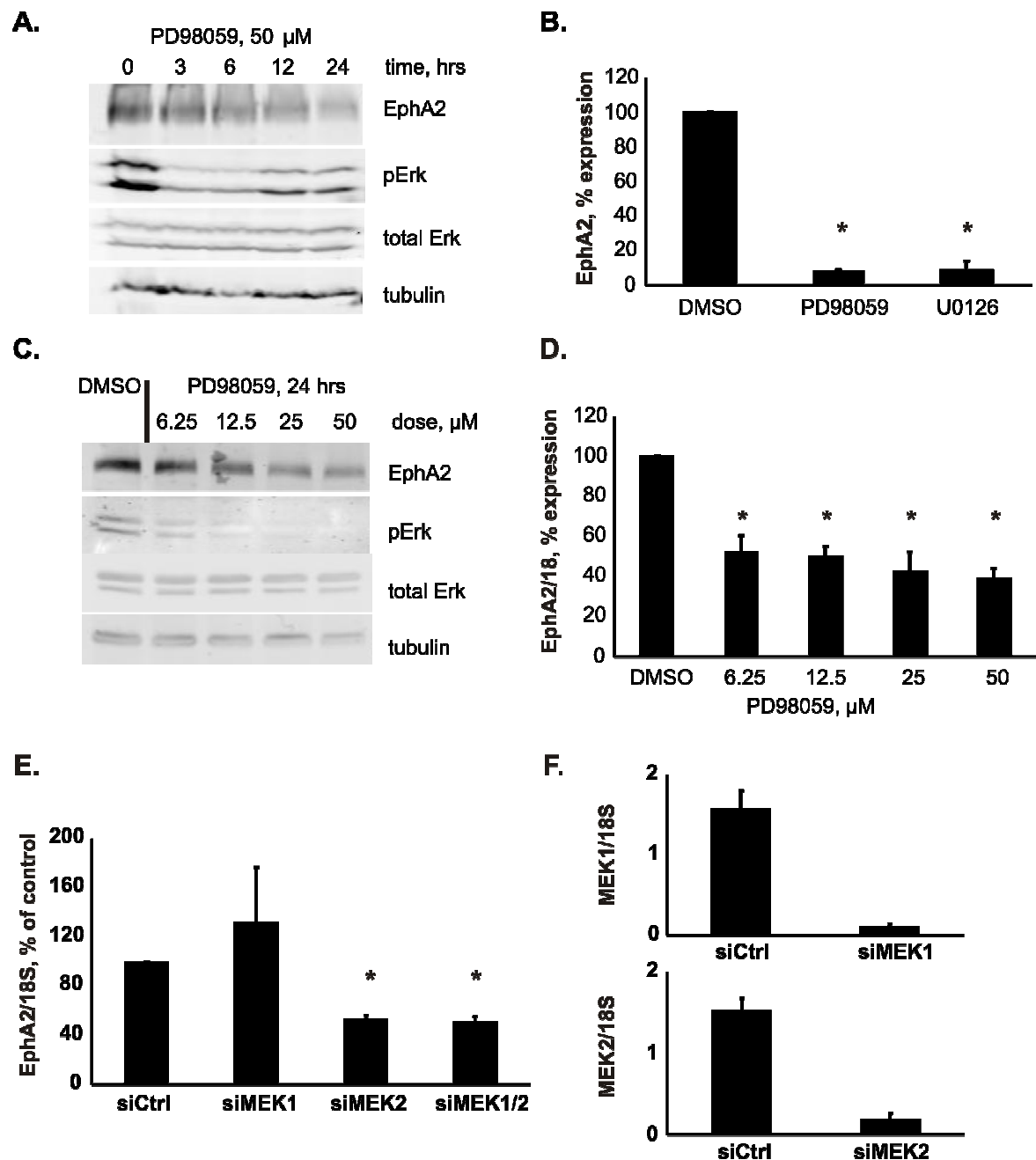


Figure 3.2. MEK2 and not MEK1 regulates EphA2 expression. A, Western blot of EphA2 at 0, 3, 6, 12 and 24 hrs after treatment with PD98059 in MPanc96 cells. B, Q-PCR of EphA2 normalized to 18S after 4 hours of treatment of MPanc96 cells with MEK inhibitors PD98059 and U0126. Values shown are normalized to DMSO-treated controls. C, EphA2 protein (western blot) and D, mRNA (Q-PCR) in MPanc96 cells after 4 hours of treatment with DMSO or 6.25, 12.5, 25, and 50 μ M concentrations of PD98059. E, Q-PCR of EphA2 normalized to 18S after silencing of MEK1, MEK2 or both in MPanc96 cells. F, Q-PCR was performed for MEK1 (top) and MEK2 (bottom) to confirm silencing. *, $p < 0.05$

PEA-15 modulates the effects of the Ras-MAPK pathway on EphA2 expression.

The majority of pancreatic cancer patients have a K-Ras mutation that renders the protein constitutively active. Nevertheless, other downstream and upstream modulators of MAPK pathway can regulate the extent of the effect of active Ras. Phosphoprotein enriched in astrocytes 15 (PEA-15) is an adaptor protein that binds ERK and prevents it from translocating to the nucleus (74, 176). Although, it does not prevent ERK enzymatic activity, it effectively blocks phosphorylation of nuclear targets of phospho-ERK (74). We examined the effect of PEA-15 on EphA2 levels by overexpressing PEA-15 in MPanc96 cells using an adenovirus vector (adPEA15). PEA-15 expression significantly reduced EphA2 mRNA levels (Figure 3.3A) and protein levels (Figure 3.3B) compared to GFP infected controls. To confirm the ability of PEA-15 to reduce nuclear localization of ERK, we analyzed phosphorylation of Fra-1, a known nuclear target of ERK (69). A decrease in phospho-Fra1 protein levels was observed in adPEA15-infected cells (Figure 3.3B), confirming a decrease in nuclear ERK activity. To confirm the data obtained with adPEA15 in vitro, tissues and cell lines from genetic mouse models of pancreatic cancer induced by mutant K-Ras were examined for PEA-15 and EphA2 mRNA levels by microarray (Figure 3.3C). A strong statistically significant negative correlation existed between endogenous levels of expression of PEA-15 and EphA2 in these samples. Although all the samples have constitutively active K-Ras, nonetheless, EphA2 expression decreases with increasing endogenous PEA-15.

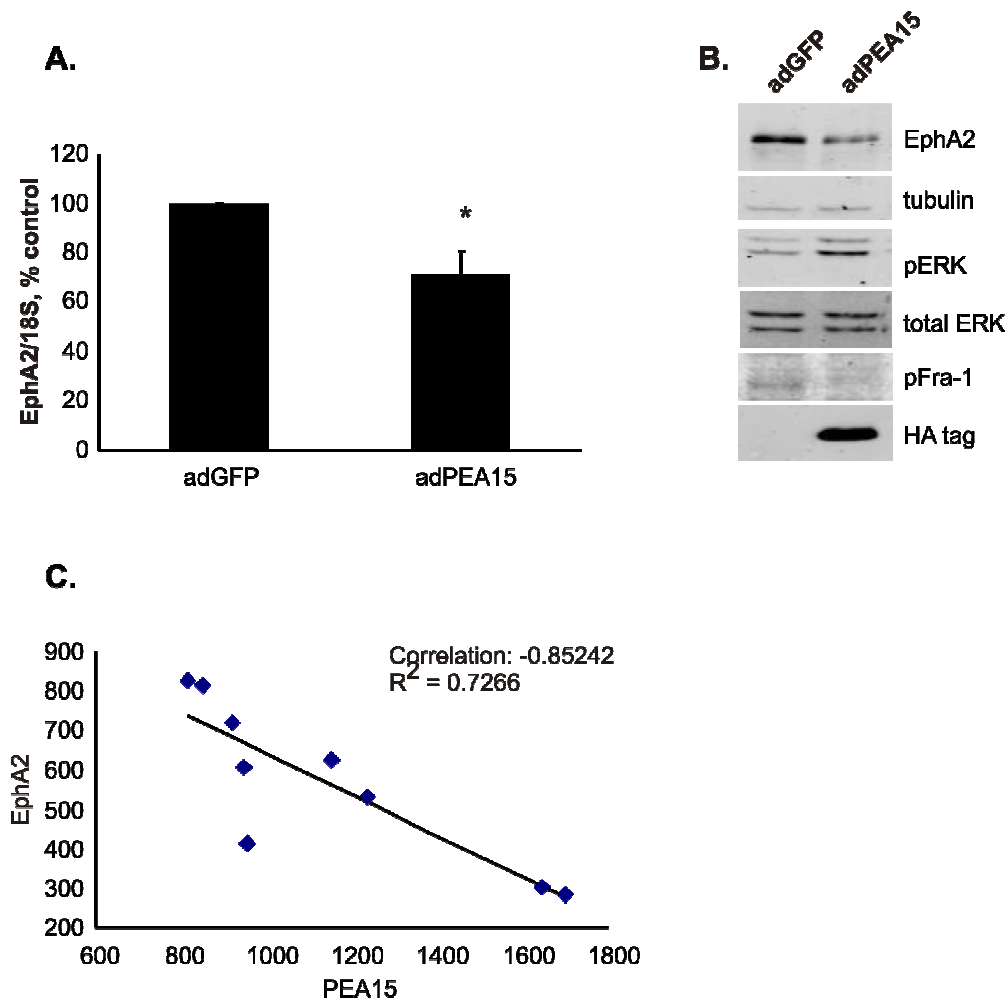


Figure 3.3. PEA15 decreases EphA2 expression in vitro and in vivo. A, left, Q-PCR of EphA2 in MPanc96 cells infected with PEA15 adenovirus (adPEA15). Values are normalized to 18S and shown as a percentage of control adGFP infected cells. B, western blot of EphA2, phospho- and total ERK, phospho-Fra1 and PEA-15 (HA tag) in adGFP and adPEA15 infected MPanc96 cells. C, scatter plot of PEA-15 vs. EphA2 mRNA in mouse models of pancreatic cancer driven by mutant K-Ras. Values are expressed in affymetrix units. *, $p < 0.05$

EGF stimulates EphA2 expression.

EGF is a well known stimulator of Ras-MAPK pathways (177). However, the influence of EGF in the presence of downstream constitutively active mutant K-Ras is uncertain. Nonetheless, recently, EGF receptor inhibitors have been used to achieve a slight benefit in the treatment of pancreatic cancer (73). Therefore, we explored the relationship between EGFR activity and EphA2 expression. Stimulation of serum-starved MPanc96 cells with EGF (20 ng/ml) resulted in a 2.5 fold increase in EphA2 mRNA levels by 2 hours (Figure 3.4A). EGF-mediated induction of EphA2 was almost completely blocked by pretreatment of cells with a transcription inhibitor, actinomycin D (Figure 3.4B). A time-dependent increase in EphA2 protein level was also observed after EGF stimulation (Figure 3.4C). Conversely, inhibition of EGF receptor activity with gefitinib (Iressa, 10 μ M) caused a time-dependent decrease in EphA2 levels in immortalized Human Pancreatic Nestin-positive Epithelial cells (HPNE), which are used as a control pancreatic duct-like cell which possesses wild-type K-Ras (Figure 3.4D). To further investigate the effect of EGFR inhibition in the presence of mutant K-Ras, a panel of pancreatic non-transformed and cancer cells were treated with Iressa for 0, 6 and 12 hours. Immunoblotting of EphA2 revealed that 5 out of 12 cell lines down-regulated EphA2 after treatment with Iressa. In the other 7 cell lines EphA2 levels either remained constant or increased (ie, MiaPaca-2; Figure 3.4E). Interestingly, phospho-ERK levels followed a similar pattern showing decrease in the same 5 out of 12 cell lines (Figure 3.4F).

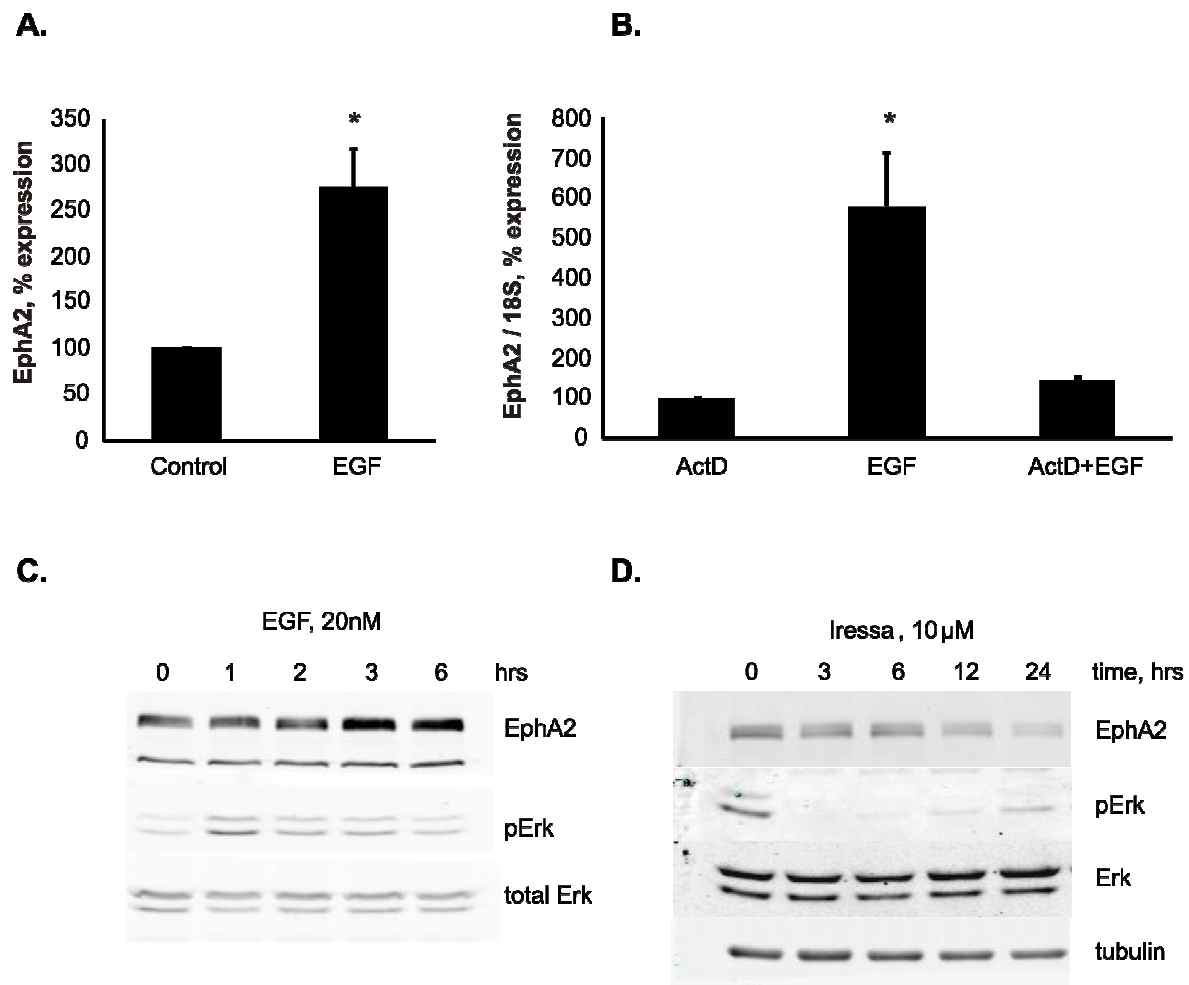
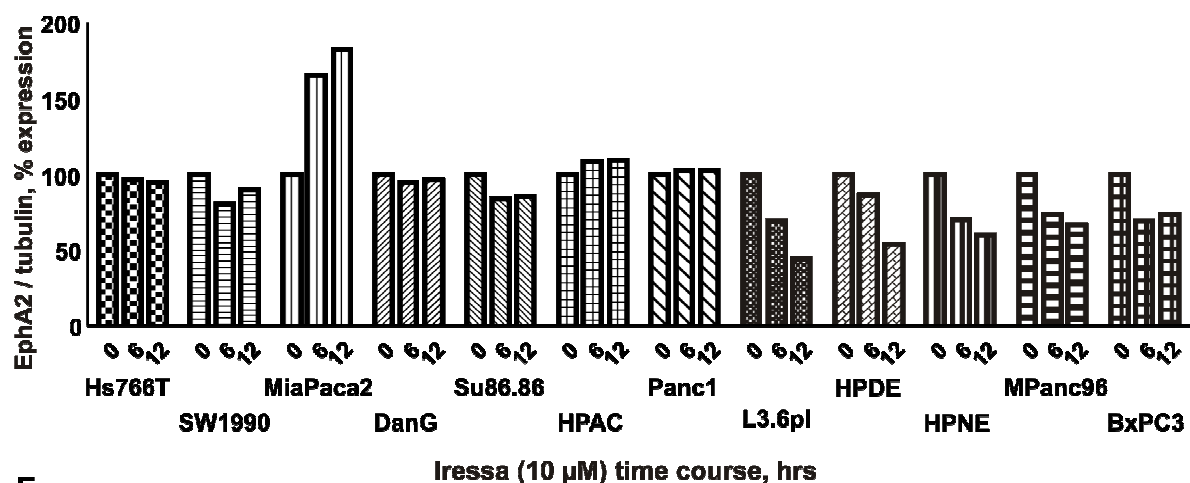


Figure 3.4. EGF regulates EphA2 expression through MAPK pathway in a subset of pancreatic cell lines. A, Q-PCR of EphA2 at 2 hours after stimulation with 20 ng/ml of EGF. EphA2 values were normalized to 18S and shown as a percentage of EphA2 in unstimulated MPanc96 cells. B, MPanc96 cells were treated with actinomycin D (ActD), EGF (20 ng/ml) or both for 2 hours and EphA2 mRNA was measured by Q-PCR and normalized to 18S. Data shown as a percentage of actinomycin D- treated samples. C, western blot of EphA2 at 0, 1, 2, 3 and 6 hours after stimulation with 20 ng/ml of EGF on MPanc96 cells. D, western blot of EphA2 at 0, 3, 6, 12, and 24 hours after treatment with EGFR inhibitor, Iressa (10 μ M), in HPNE cells.

E.



F.

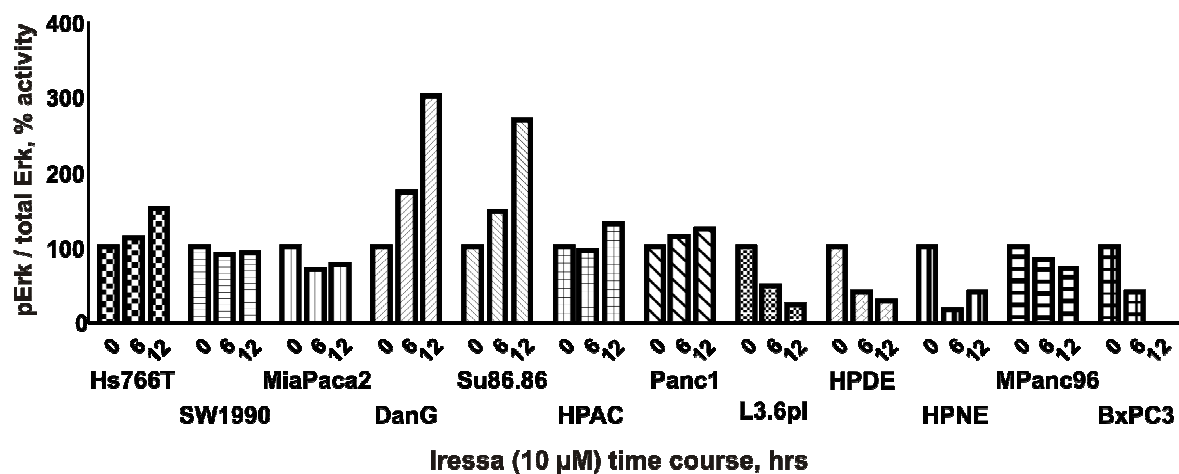


Figure 3.4, continued. E, quantification of EphA2 and F. phospho-Erk at 0, 6, and 12 hours after treatment with Iressa (10 μ M) in multiple cell lines. EphA2 was normalized to tubulin and phospho-Erk to total Erk. Values shown as percentages of untreated samples.

EphA2 gene expression is regulated by c-Jun and RSK.

To identify further downstream targets of phospho-ERK involved in regulation of EphA2 we utilized reverse protein phase arrays (RPPA) performed on cell lysates of the previously mentioned 12 cell lines after treatment with Iressa. Changes in expression or phosphorylation of all proteins included in RPPA were correlated to the changes in phospho-ERK. A graphic representation of the proteins that correlated to phospho-ERK with an absolute correlation coefficient of 0.5 or more are shown in Figure 5A. Some of the correlated proteins are known components of the Ras-MAPK pathway, such as phospho-MEK, phospho-ERK (pMAPK), and phospho-RSK. We also observed that levels of the transcription factor c-Jun correlated with Iressa-induced phospho-ERK changes, as expected (178).

To explore the relationship between c-Jun and EphA2, MPanc96 cells were transfected with c-Jun siRNA, achieving 60% knockdown (Figure 5B, gray bars). EphA2 levels were reduced by 40% in c-Jun siRNA transfected samples compared to nontargeting control siRNA treated samples (Figure 5B, black bars). To investigate the relationship between RSK and EphA2, MPanc96 cells were treated with a RSK inhibitor, FMK, for various times and the levels of EphA2 RNA and protein were measured (Figure 5C and 5D, respectively). FMK decreased the level of phospho-RSK within 1 hour and levels remained low for the whole duration of the experiment (Figure 5D). A time-dependent decrease was observed in EphA2 mRNA, which reached its nadir 4 hours after FMK treatment and remained significantly lower than untreated samples for at least 24 hours (Figure 5C). Similarly, a decrease in EphA2 protein level was observed after FMK treatment (Figure 5D).

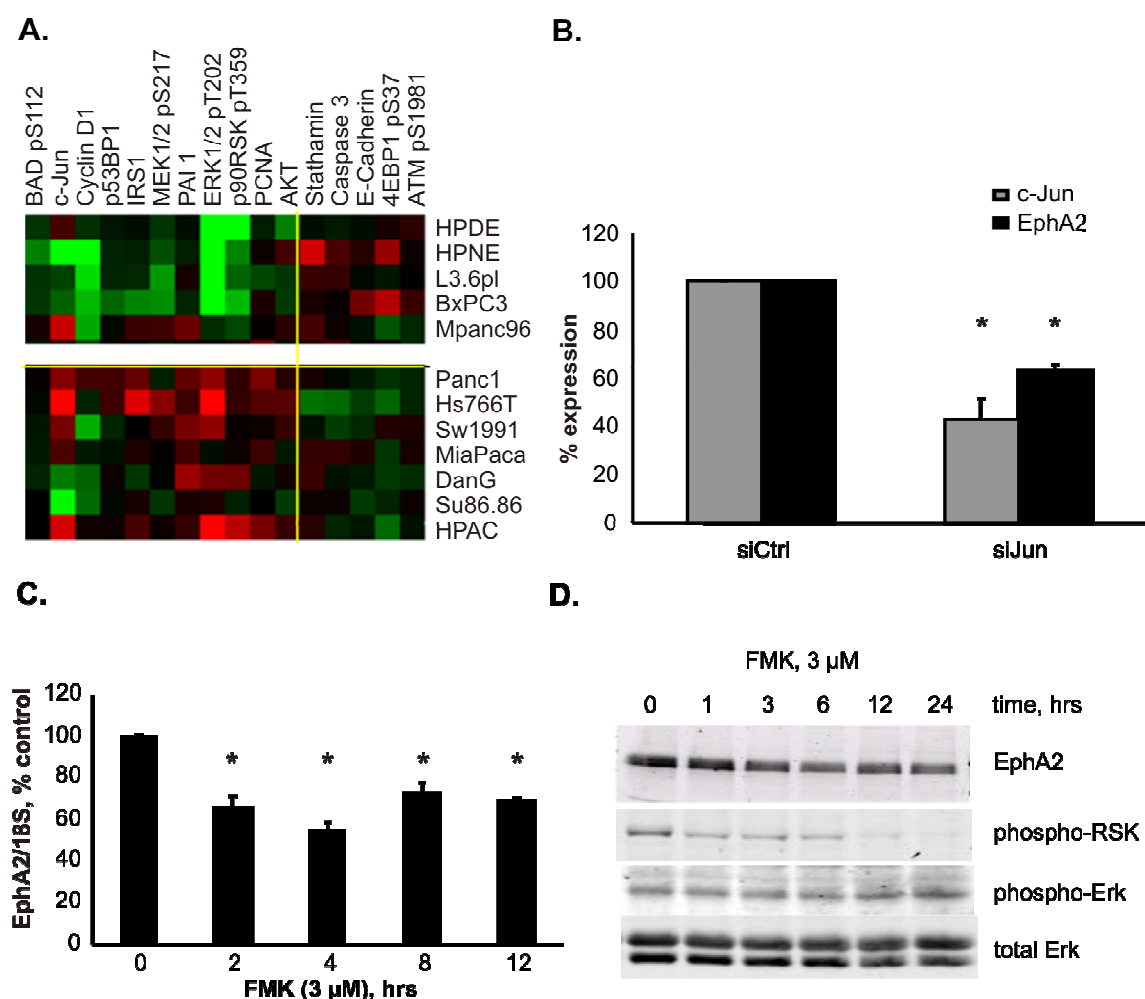


Figure 3.5. EphA2 is regulated by c-Jun and RSK. A, Reverse protein phase array was performed on multiple pancreatic cancer and ductal cell lines treated with Iressa (10 μ M) for 6 hrs. Values were normalized to protein loading and untreated controls. Proteins that are correlated to EphA2 levels with absolute value of more than 0.5 are shown. B, Q-PCR of EphA2 in Jun-silenced MPanc96 cells. Values are normalized to 18S and shown as a percentage of control siRNA transfected cells. C, Q-PCR of EphA2 expression at 0, 2, 4, 8, and 12 hours after treatment with RSK inhibitor, FMK (3 μ M). EphA2 mRNA values were normalized to 18S and shown as an average percentage of untreated control. D, stern blot of EphA2 at 0, 3, 6, 12, and 24 hours after treatment with FMK (3 μ M). *, $p < 0.05$

DISCUSSION

EphA2 plays an important role in pancreatic cancer progression. EphA2 protein levels have been reported to be highly expressed in pancreatic cancer tissue samples (99). However, this is the first study to show that this elevation of EphA2 is related to regulation by Ras acting specifically through MEK2. We did not find any evidence for a role of p53 in EphA2 expression in pancreatic cancer cells. Rather, the current results suggest that c-Jun, down-stream from Ras/MEK2/ERK is the likely transcription factor. Additionally, we demonstrate that despite the presence of constitutively active mutant K-Ras, EphA2 expression is still influenced by activity within this pathway which can be elevated by EGF receptor activity and inhibited by PEA-15 expression levels.

The loss of the tumor suppressor p53 is common in cancers including pancreatic tumors of which at least 50% possess inactivating mutations in p53 (30). However, nearly all pancreatic tumors have been found to express high levels of EphA2 (99). Therefore, it was not surprising to observe that pancreatic tumors developed in mutant Ras-induced mouse models possessed elevated levels of EphA2 whether or not p53 was deleted. This observation is similar to that of Meritt and colleagues who found that EphA2 expression in tissues from ovarian cancer patients was actually elevated in the presence of inactivating mutations in p53 (179). Taken together, it seems unlikely that p53 is a major factor in the elevated levels of EphA2 observed in pancreatic cancer.

Another major pathway that is important in pancreatic cancer is mutant K-Ras. K-Ras mutations occur in more than 90% of pancreatic cancers compared to only 25%-30% in other adenocarcinomas (30). K-Ras mutations are found in early preneoplastic lesions and

are regarded as one of the earliest and most central mutations in pancreatic cancer (60). Moreover, mutant K-Ras alone in pancreatic acini is enough to induce development of pancreatic cancer and the efficiency of cancer development is dependent on Ras activity levels (50). Likewise, silencing of K-Ras in pancreatic cancer cells decreases their capacity to form tumors (61). Oncogenic K-Ras has been extensively studied and is known to signal through multiple intracellular pathways with different effectors, of which the most studied are: Raf-MAPK, PI3K-Akt, PLC β , and Ral (62). In the current study we observed that Ras activity had a major influence on EphA2 expression.

Specifically, we observed that the MAPK pathway dynamically influenced the levels of EphA2 in pancreatic cancer cells. When mutant K-Ras was silenced in pancreatic cancer cells in the current study, we noted a marked reduction in phospho-ERK but not phospho-Akt which correlated with a decrease in EphA2 expression. These data suggest that the ERK pathway is more sensitive to changes in Ras activity levels and is more likely to be involved in the regulation of EphA2. The role of the MAPK pathway in EphA2 regulation has previously been suggested in studies of lung and breast cancer (106, 107).

Down-stream effectors of K-Ras in the MAPK pathway include MEK1 and MEK2. Our studies indicate that MEK2 is specifically involved in the regulation of EphA2 levels. Although MEK1 and MEK2 have long been thought of as redundant proteins, recent studies showed that the signaling through these proteins is different and quite complicated. The differences between MEK1 and MEK2 were clearly observed in mouse gene knock-out studies. MEK1-null mice die in embryogenesis due to placental malformation, in spite of the presence of fully functional MEK2 (67). On the other hand, MEK2-null mice develop normally and do not show change in phenotype (66). MEK1 and MEK2 are also different in

2 conserved regions: N-terminal ERK binding domain and proline-rich regulatory region (PR). In this study, we observed that silencing MEK2, but not MEK1, decreased EphA2 expression. This data was not a result of total intracellular MEK1/2 levels, because MEK1 protein levels were higher than MEK2 levels. It was previously reported by Skarpen and colleagues that MEK1 and MEK2 influence intracellular localization of phospho-ERK. In their study they also demonstrated that MEK1 signaling largely influences cell proliferation, while MEK2 affects cell survival (68). Therefore, MEK2 regulation of EphA2 is consistent with the previously reported role of EphA2 as a protein involved in cell survival, but not proliferation (87, 111, 112, 126).

Down-stream from MEK2, ERK activation was found to correlate with EphA2 expression. ERK can act either in the cytoplasm or in the nucleus of cells. We observed that PEA-15, which possesses nuclear export sequence and upon binding confines ERK to the cytoplasm, significantly decreased EphA2 expression despite the presence of active Ras. PEA-15 has been shown to abolish phospho-ERK nuclear activity (74, 176). Our data suggests that EphA2 is regulated in part by the nuclear activity of ERK. In support of our in vitro studies, we also observed that PEA-15 mRNA levels were inversely correlated to EphA2 mRNA in K-Ras driven pancreatic cancer models. Since EphA2 expression is correlated to an aggressive phenotype, PEA-15 may be protective in pancreatic cancer. In support of this hypothesis, PEA-15 has been demonstrated to be protective in ovarian and breast cancers (75, 76, 180, 181). These are the first data linking PEA-15 and EphA2 expression and the first indicating a role of PEA-15 in pancreatic cancer. In the future it will be of interest to explore whether PEA-15 expression is correlated with improved pancreatic cancer patient survival.

In this study, we found that in a subset of pancreatic cancer cell lines possessing mutant K-Ras EGFR targeting decreased Ras-MAPK pathway activity, as evidenced by decreased ERK phosphorylation. Likewise, EGF was able to activate the pathway in many of the cell lines (data not shown). These data indicate that this pathway is not necessarily maximally stimulated by the presence of mutant K-Ras in pancreatic cancer cells. It is well known that the amplitude of intracellular Ras-MAPK signaling is not only dependent on Ras enzymatic activity but also on its intracellular location and the presence of other factors, for example the proximity and abundance of scaffold proteins, such as KSR (65, 182, 183). Importantly, EGFR targeting also decreased EphA2 expression in the cells which showed effects on MAPK signaling. These data suggest that EphA2 levels are an indication of the overall activity of the MAPK pathway in pancreatic cancer cells and that the presence of activating mutations in K-Ras are not sufficient for maximal induction.

EGFR and its multiple ligands are highly expressed in pancreatic cancer and their expression has been correlated to an increase in tumor aggressiveness (184, 185). EGFR targeting therapies have been approved for multiple cancer types. Unfortunately, a randomized Phase III study in colorectal cancer demonstrated that tumors possessing K-Ras mutations are resistant to a therapy of anti-EGFR antibody, panitumumab (71, 72). However, in another large Phase III trial, the EGFR inhibitor, erlotinib, in combination with gemcitabine conferred a modest increase in survival over gemcitabine treatment alone in pancreatic cancer (73). Based on these results, the FDA approved erlotinib for treatment of locally advanced, unresectable, or metastatic pancreatic carcinoma. Our data supports that in a subset of pancreatic cancers inhibition of EGFR regulates down-stream components of the ERK pathway and ultimately results in a decrease in EphA2 expression. Therefore, it may

be possible to use the influence of EGFR inhibitors on EphA2 levels as an indicator of the effectiveness of these drugs on individual patients.

Currently, biomarkers able to predict the clinical outcome of anti-EGFR therapy are an active area of research (186). It has been reported that pancreatic cancer patients that have a cutaneous rash after erlotinib treatment respond better to the therapy. The origin of rash and its relationship to EGF receptor is not clear and is being investigated. Our data suggest that EphA2 may be a good marker of anti-EGFR sensitivity. Not surprisingly, the cancer cell lines that responded by decreasing EphA2, have been previously shown to decrease proliferation and tumor growth in response to Iressa (187). Since EphA2 is a surface receptor, it may be possible to develop imaging approaches to assess changes of receptor expression levels in pancreatic cancer patient in response to the anti-EGFR therapy.

AP-1 transcription factor is a homo- or heterodimer made up of members of Jun family proteins (c-Jun, JunB, JunD) or a combination of Jun and one of the members of the Fos (eg, c-Fos, Fra-1), ATF (eg, ATF2), and the least studied Maf families (188, 189). AP-1 plays an important role in oncogenesis in different cancers (189). In pancreatic cancer, high AP-1 activity is regulated by ERK, JNK and Akt pathways (178, 190, 191). Also, AP-1 promotes anchorage dependent and independent growth of pancreatic cancer cells (190). In this study, we demonstrated that silencing of c-Jun decreases EphA2 expression, although the exact relationship between c-Jun and EphA2 remains to be characterized.

RSK is a well established effector of ERK and is responsible for 20% of ERK-regulated changes in mRNA (192). P90RSK family consists of four members, RSK1, RSK2, RSK3 and RSK4 (193). These proteins have been found to regulate important hallmarks of

oncogenesis, such as cell differentiation, survival, growth and motility (192, 193). We have observed that EphA2 mRNA levels decrease with the inhibition of RSK. Moreover, RSK has been shown to regulate expression and stability of two subunits of AP-1, c-Fos and Fra1 (192, 193). Further studies showed that 23% of RSK-induced transcription is regulated by Fra-1 (192). Therefore, it is possible that both RSK inhibition and c-Jun silencing decreases the activity of AP-1, thereby decreasing EphA2 transcription. In addition to Fra-1, RSK also regulates activity of the transcription factor CREB1 and its cofactors p300 and CREB binding protein (CBP) (193). Interestingly, a heterodimer Jun/ATF2 forms a CREB response element binding protein (CRE-BP), which can also bind to the same DNA sequences as CREB and initiate transcription (188). Therefore, there are several potential points of interaction between RSK and Jun that may be further investigated.

In summary, we observed that EphA2 levels are influenced by activity in the Ras/MAPK pathway. In fact, EphA2 levels are an indicator of overall activity within this pathway. Specifically, we found that MEK2 activation of nuclear phospho-ERK was required for EphA2 expression. These studies provide new insights into the regulation of EphA2 in pancreatic cancer and have identified new components of the regulatory system including MEK2 and PEA-15. This information may be useful for the development of prognostic tests and the identification of new targets for therapies aimed at pancreatic cancer.

CHAPTER FOUR: SUMMARY AND FUTURE DIRECTIONS

SUMMARY

Pancreatic cancer is the 4th leading cause of cancer related death in the US. The high mortality is believed to be caused by lack of early detection and therefore presence of advanced metastatic disease at the time of diagnosis. However, in rare cases when pancreatic cancer is diagnosed early with seemingly resectable tumor, 80% of patients will still die within 5 years of tumor resection due to tumor recurrence or presence of metastasis. Gemcitabine is the first-line therapy, but objective tumor response rate is low. Therefore, intervention that can increase pancreatic cancer diagnosis, prevent incidence of new metastasis, arrest tumor growth and/or reduce tumor burden will have tremendous impact on patient prognosis.

This dissertation is an attempt to bring the pancreatic cancer research field closer to achieving all of these goals. The hypothesis of the dissertation was that EphA2 is regulated by oncogenic Ras and may be an important therapeutic target for the treatment of pancreatic cancer.

To address this hypothesis, I first confirmed the finding by Mudali et al. that EphA2 is overexpressed specifically in pancreatic cancer, as compared to normal pancreas and chronic pancreatitis (99). Therefore, EphA2 receptor may potentially serve as a marker of pancreatic cancer. Since EphA2 is a transmembrane protein, it may be available for antibody or a peptide binding. Imaging agent conjugated to an antibody against EphA2 may permit physicians to detect high expression of this receptor in the human body, therefore allowing early pancreatic cancer detection. Also, soluble EphA2 receptor has been detected in plasma and serum of pancreatic cancer patients (194). The idea of EphA2 as a biomarker of

pancreatic cancer is currently being explored and has been presented at the recent meeting of American Association of Cancer Research (194).

To explore the biological role of EphA2 receptor in pancreatic cancer, I silenced EphA2 and observed increase in pancreatic cancer cell apoptosis. Also, combination of EphA2 and gemcitabine had a stronger cytotoxic effect than either treatment alone and successfully reduced the number of gemcitabine-resistant pancreatic cancer cells. I further demonstrated that in gemcitabine-resistant orthotopic mouse model, EphA2 silencing arrested tumor growth of MiaPaca-2 pancreatic cancer cells. In another orthotopic model, I examined systemic delivery of siRNA, coated in neutral DOPC liposomes, to an established orthotopic tumor. I observed significant reduction of EphA2 protein, which was comparable to the reduction observed in vitro. Moreover, silencing of EphA2 receptor sensitized orthotopically growing gemcitabine-resistant MPanc96 cells to gemcitabine treatment, causing decreased tumor mass and reduced the number of liver and lung metastasis. These results suggest that targeting of EphA2 either alone or in combination with gemcitabine may be a useful approach in treatment of pancreatic cancer. It may help overcome a prevalent problem of gemcitabine resistance and improve patient survival by decreasing tumor growth and metastasis.

In the second aim of this dissertation, I explored the link between mutant K-Ras and EphA2 expression. I demonstrated that K-Ras regulates EphA2 transcription through activation of MEK2 and phosphorylation of ERK. I further implicated AP1 as a transcription factor that may be responsible for EphA2 regulation. Considering the importance of EphA2 in pancreatic cancer biology, exploration of the mechanisms of EphA2 regulation may reveal new points of intervention in treatment of this disease.

In addition, this study addresses a question of Ras-MAPK pathway modulation in the presence of constitutively active mutant K-Ras and demonstrates that inhibition of EGFR receptor activity translates into a decrease of ERK phosphorylation and reduction of EphA2 expression in a subset of pancreatic cancer cell lines. EGFR therapy has proven to be beneficial, although slightly, in pancreatic cancer patients. It is possible that among the patients that do not show any response to anti-EGFR therapy, there is a small population that may have clinically significant response. Changes in EphA2 level correlates strongly to the changes in MAPK pathway activity, and therefore, a decrease in EphA2 may be used as a good marker of a positive response to anti-EGFR therapy. Identification of patients that respond to an anti-EGFR therapy can significantly improve their prognosis and prevent unnecessary expensive treatment of nonresponsive patients, thereby decreasing potential toxicity and medical cost.

Similarly, I implicated PEA-15 as an endogenous protein that may be responsible for regulation of MAPK pathway activity in pancreatic cancer. I also demonstrated that in K-Ras driven mouse models of pancreatic cancer expression of PEA-15 is inversely related to the expression of EphA2. These results indicate that PEA-15 is another important regulatory input of Ras-MAPK pathway that can be utilized as a potential point of therapeutic intervention.

In conclusion, this work demonstrates that EphA2 is highly and specifically expressed in pancreatic cancer, as compared to normal pancreas and chronic pancreatitis. It is regulated by K-Ras - MEK2 - ERK pathway, likely by employing AP1 transcription factor, and/or RSK. Moreover, silencing of EphA2 sensitizes chemoresistant cells to

gemcitabine and in combination with gemcitabine treatment decreases pancreatic cancer growth and metastasis.

FUTURE DIRECTIONS

How is EphA2 promoter regulated?

Despite the importance of EphA2 receptor in multiple cancers, the promoter of this protein is not well characterized. In this dissertation, several potential mechanisms of EphA2 transcriptional regulation were implicated; however, no direct link between any transcription factor and EphA2 was established.

Based on our observations in this study, AP-1 and CREB are suspected to be transcription factors regulating EphA2, although CREB involvement has not been confirmed. Interestingly, computer analysis of EphA2 promoter revealed that there AP-1 binding consensus sequences at ~300, 500 and 1000 bp upstream from the transcription initiation site (JASPAR and IFTI databases). Also, there are two CRE sites in EphA2 promoter ~700 and 100 bp upstream from the transcription initiation site (JASPAR and IFTI datasets). A CRE site ~100 bp upstream is conserved among species and is likely to be functionally relevant.

In a pilot experiment, a segment possessing ~1,800 bp upstream of EphA2 transcription initiation site was cloned into a pGL3 promoterless luciferase vector. Pancreatic cancer MPanc96 cells were transfected with this luciferase reporter and stimulated with EGF. As shown in Figure 4.1, we observed expected induction of luciferase activity, proving that this segment is sufficient to demonstrate EGF-mediated induction of EphA2. Further digestion of the promoter region, using restriction enzyme should provide us with a better understanding of the exact promoter sequence involved in EGF-mediated EphA2 induction. Also, this study provided enough evidence to perform a chromatin precipitation

(ChIP) of Jun in order to demonstrate the direct binding to the promoter at the suspected regions.

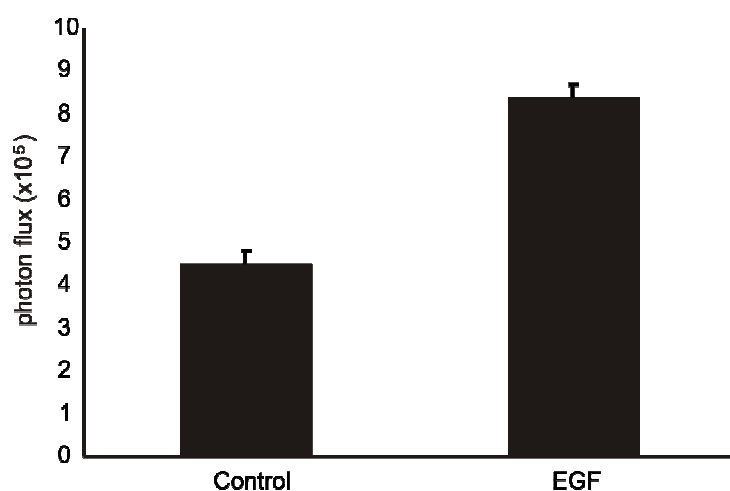


Figure 4.1. EGF increases EphA2 promoter activity. Mpanc96 cells transfected with EphA2-Luc reporter were treated with PBS or EGF for 4 hrs and the luciferase signal was measured. Experiment was performed in triplicates with bars. Average \pm SE is shown.

How does TNF α regulate EphA2 expression?

Presence of inflammatory stroma, called desmoplastic reaction, is a known feature of PDAC. The relationship between desmoplastic reaction and cancer is unclear, but it is known that chronic inflammation in the pancreas, such as chronic or familial pancreatitis, can often lead to development of pancreatic cancer (30). The stroma of pancreatic cancer possesses a number of inflammatory cells, such as macrophages, mast cells and lymphocytes (195). These cells secrete various proinflammatory cytokines, such as IL-6, IL-10, IL-8, and IL-1RA, which have been found in higher levels in pancreatic cancer patients (196).

TNF α was also found to be at higher levels in pancreatic cancer patients and has been associated with cancer stage and cachexia (197). In addition to stromal cells, TNF α is also secreted by pancreatic cancer cells themselves (198, 199). This cytokine increases pancreatic cancer cell resistance to chemotherapy in vitro (199), and in vivo, it increases tumor growth and metastasis (198).

A pilot study was performed, which showed that treatment of MPanc96 pancreatic cancer cells with TNF α leads to increased expression of EphA2, however, the exact mechanism mediating this effect is unknown (Figure 4.2). This experiment contradicts previously published findings (200), although the discrepancy may reflect the difference between the cell lines. Interestingly, TNF α stimulates the activity of a number of intracellular pathways, including MAPK and JNK, both of which activate AP-1 transcription factor (201). Inhibition of this pathway decreased total TNF α -induced EphA2 levels, but did not prevent induction of EphA2 over MAPK/JNK inhibited controls (Figure 4.2.)

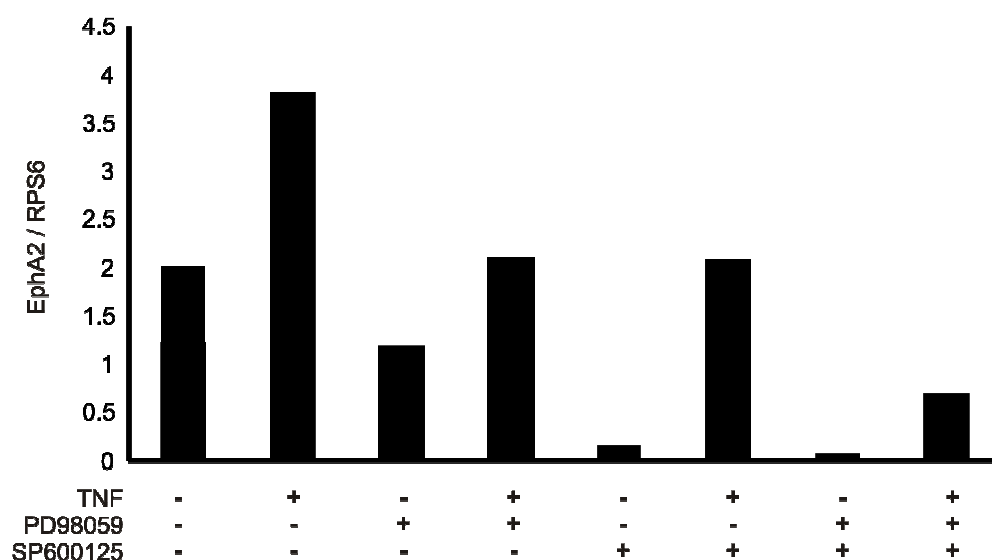


Figure 4.2. TNF α -mediated induction of EphA2 is regulated in part by MAPK and JNK pathways. Mpanc96 cell pretreated with MEK inhibitor Pd98059 (50 μ M) or JNK inhibitor Sp600125 (10 μ M) for 1 hr, were stimulated with TNF α for 1 hr. RNA was isolated and subjected to quantitative RT-PCR for EphA2. EphA2 mRNA values were normalized to RPS6.

How does EGF regulate MAPK pathway activity in the presence of constitutively active K-Ras?

Being a central player in pancreatic cancer development, oncogenic K-Ras has long been immune to therapeutic modulation. Therefore, our observation that inhibition of EGFR activity decreases MAPK pathway activity is very important and needs further investigation. Seufferlein and colleagues made a similar observation, demonstrating that stimulation of pancreatic cancer MiaPaca-2 and Panc-1 cells causes increase in H-Ras translocation to the cell membrane, increase in ERK phosphorylation and induction of AP-1 and RSK activity (191). However, effect of TGF α on K-Ras expression level has not been shown.

Serendipitously, we discovered that in MPanc96 and L3.6pl Ras protein expression level changes in response to EGFR activity. Briefly, to assess EGF effect on phospho-ERK in pancreatic cancer cells, MPanc96 and L3.6pl, either pre-treated or not with Iressa (10 μ M, 1 hr), were stimulated with EGF (20 nM) or PBS for 20-30 minutes. Protein was collected and WB was performed for pERK, Ras and tubulin (Figure 4.3A). As shown in Figure 4.3B, phospho-Erk levels changes as expected with a strong induction by EGF and almost complete block with Iressa. However, in L3.6pl increase in Ras level was observed with EGF treatment, which was completely blocked with Iressa (Figure 4.3C, left). In MPanc96, no increase in Ras was observed in response to EGF, however, Ras levels were decreased with Iressa treatment (Figure 4.3C, right). Since the experiment was set up to investigate the effect of EGF on Erk phosphorylation, the duration of EGF stimulation was very short, potentially diminishing the effect of EGF on Ras protein level. On the other hand, the short

duration of EGF stimulation also indicates that EGF-mediated Ras expression is unlikely to be transcriptional, but rather post-transcriptional (increasing RNA stability) or post-translational (increasing Ras protein stability). Nevertheless, EGF induction of Ras expression needs to be investigated further.

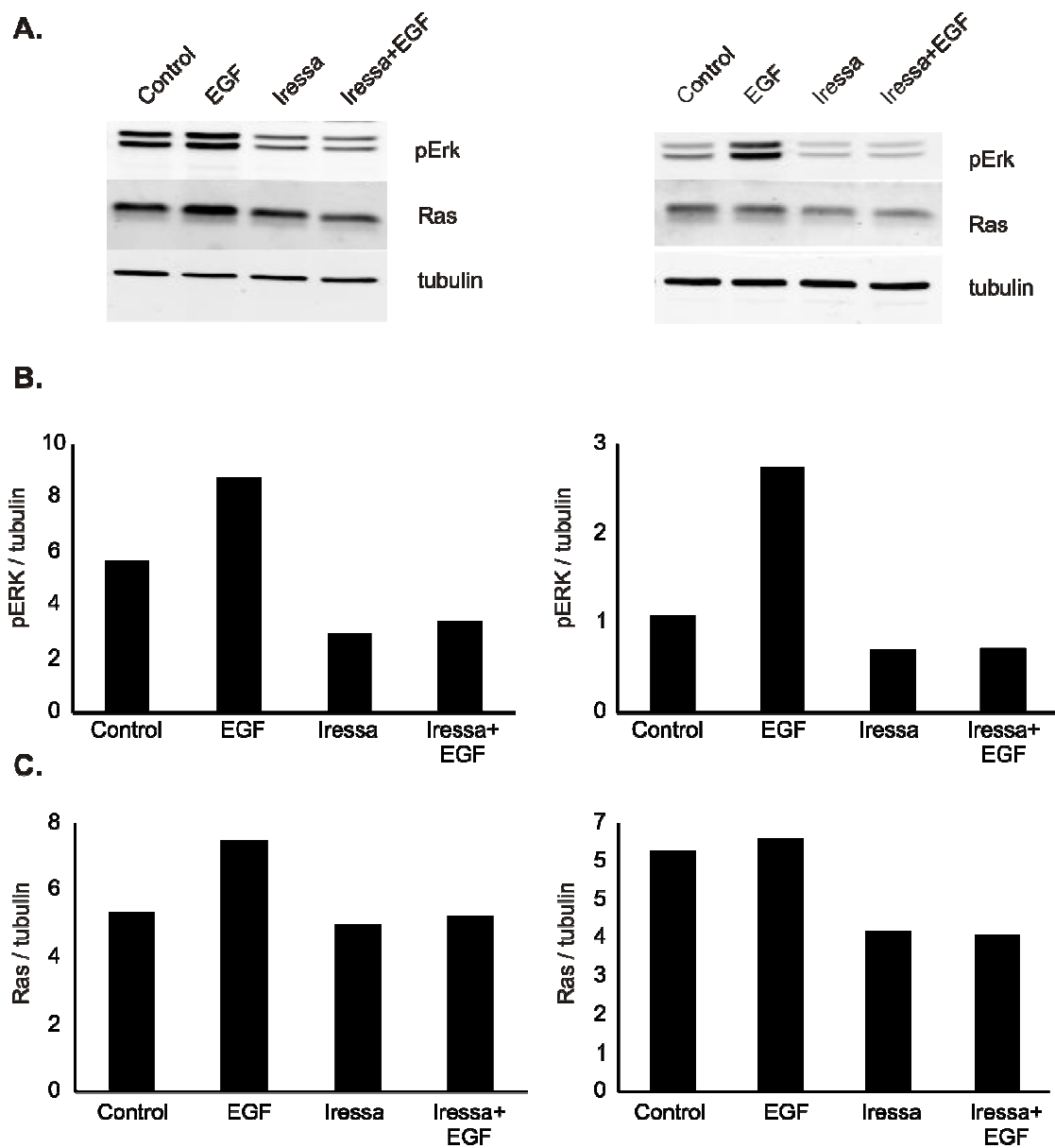


Figure 4.3. Ras protein expression in pancreatic cancer is regulated by EGF. L3.6pl (left) and Mpanc96 (right) pancreatic cancer cells were pretreated with Iressa (10 μ M) for 1 hr and then stimulated with EGF for 20-30 minutes. Protein lysates were collected and western blot was performed for phospho-ERK, Ras, and tubulin. Bands were quantified. A. Picture of western blots, B. phospho-Erk band quantification, normalized to tubulin, C. Ras, normalized to tubulin.

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VITA

Pavel Aleksander Levin, known to everyone as Pasha, was born on October 31, 1979, in Tashkent, Uzbekistan (a part of the former Soviet Union) to his parents Anna Isaakovna Levina and Aleksander Pavlovich Levin. In 1998, he immigrated to the United States (Houston, Texas) with his family. Within 1 year, he was accepted to The University of Houston, and after 3 years, he graduated *summa cum laude* in 2002 with a senior honors thesis and a B.S. in Biology. In addition, he received the distinction of valedictorian from the College of Natural Sciences and Mathematics. In the fall of 2002, Pasha matriculated to The University of Texas at Houston Medical School and began the MD/PhD program. After 3 years of medical school, Pasha joined Dr. Craig Logsdon's lab at The University of Texas MD Anderson Cancer Center to work on his doctoral dissertation. Within 1 year, Pasha's research on EphA2 in pancreatic cancer was presented at a national meeting, being awarded a "Poster of Distinction". Between 2007 and 2010, his research was awarded 2 additional Posters of Distinction, and he has had the opportunity to give 2 oral presentations across the country. He received multiple travel awards throughout his time in graduate school, and received a fellowship from 2007 – 2009 from the NIH-funded Center for Clinical and Translational Science training program. During his doctoral years, he also was a member of the Admissions Committee for the MD/PhD program, and was a member of the American Association of Cancer Research, the American Pancreatic Association, and the American Gastroenterological Association. Upon his dissertation defense, Pasha will join the 4th year medical students to complete his final year in medical school, and begin his internship and residency in 2011. Pasha is married to Maren Fuentes Levin and they have 1 son, Daniel Sebastian Levin, and 3 cats.