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ROLE OF PROSTAGLANDIN E2 IN THE REGULATION OF PANCREATIC STELLATE CELLS HYPER ACTIVITY ASSOCIATED WITH PANCREATIC CANCER

Chantale Charo

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**ROLE OF PROSTAGLANDIN E2 IN THE REGULATION OF
PANCREATIC STELLATE CELLS HYPER ACTIVITY ASSOCIATED WITH
PANCREATIC CANCER**

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

M. D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

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Houston, Texas

May, 2011

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DEDICATION

I dedicate this thesis to my parents Samia and Joseph Charo who taught me the value of education and hard work. Mom, Dad, I can't thank you enough for your endless support, unconditional love and faith in me.

This thesis is also dedicated to the memory of every patient who lost their battle against pancreatic cancer and to every patient who is currently fighting this deadly disease. I hope that my contributions to the field will add to the knowledge and understanding of pancreatic cancer and will help in bringing us one step closer to finding a cure.

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Publication No. _____

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Supervisory Professor: Craig D. Logsdon, Ph.D.

Pancreatic cancer is one of the most lethal type of cancer due to its high metastasis rate and resistance to chemotherapy. Pancreatic fibrosis is a constant pathological feature of chronic pancreatitis and the hyperactive stroma associated with pancreatic cancer. Strong evidence supports an important role of cyclooxygenase-2 (COX-2) and COX-2 generated prostaglandin E2 (PGE2) during pancreatic fibrosis. Pancreatic stellate cells (PSC) are the predominant source of extracellular matrix production (ECM), thus being the key players in both diseases. Given this background, the primary objective is to delineate the role of PGE2 on human pancreatic stellate cells (PSC) hyper activation associated with pancreatic cancer.

This study showed that human PSC cells express COX-2 and synthesize high levels of PGE2. PGE2 stimulated PSC migration and invasion; expression of extra cellular matrix (ECM) genes and tissue degrading matrix metallo

proteinases (MMP) genes. I further identified the PGE2 EP receptor responsible for mediating these effects on PSC. Using genetic and pharmacological approaches I identified the receptor required for PGE2 mediates PSC hyper activation. Treating PSC with Specific antagonists against EP1, EP2 and EP4, demonstrated that blocking EP4 receptor only, resulted in a complete reduction of PGE2 mediated PSC activation. Furthermore, siRNA mediated silencing of EP4, but not other EP receptors, blocked the effects of PGE2 on PSC fibrogenic activity. Further examination of the downstream pathway modulators revealed that PGE2 stimulation of PSC involved CREB and not AKT pathway.

The regulation of PSC by PGE2 was further investigated at the molecular level, with a focus on COL1A1. Collagen I deposition by PSC is one of the most important events in pancreatic cancer. I found that PGE2 regulates PSC through activation of COL1A1 expression and transcriptional activity. Downstream of PGE2, silencing of EP4 receptor caused a complete reduction of COL1A1 expression and activity supporting the role of EP4 mediated stimulation of PSC. Taken together, this data indicate that PGE2 regulates PSC via EP4 and suggest that EP4 can be a better therapeutic target for pancreatic cancer to reduce the extensive stromal reaction, possibly in combination with chemotherapeutic drugs can further kill pancreatic cancer cells.

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CHAPTER ONE: INTRODUCTION

INTRODUCTION TO THE PANCREAS

A brief overview of the development, anatomy, function and histology of the pancreas is discussed below. For a more thorough description please refer to references 1-3.(1)

Anatomy and physiology

The human digestive system is composed of the salivary glands, the liver and the pancreas. The pancreas is an elongated organ is situated in the center of the abdomen, behind the stomach. The pancreas is composed of a head, neck, body and tail. The head of the pancreas is the widest part and it lies in the duodenum while the tapered left side of the pancreas referred to as the body ends near the spleen. It is surrounded by numerous structures and important blood vessels.

In humans, the pancreas weighs on average 80 g and is 15 to 20 cm long (1).

During embryogenesis, the dorsal and ventral buds join to form the pancreas.

The duct that forms from the duodenum becomes the duct of Santorini, whereas the duct forms from the hepatic diverticulum and gives rise to the duct of

Wirsung. The duct of Wirsung collects pancreatic juice and empties it in the

duodenum. With gut rotation, the ventral bud becomes the pancreatic head and fuses with the dorsal bud, which becomes the body and the tail of the pancreas.

The ducts of both buds fuse together and drain in the pancreatic head where the

duct of Wirsung becomes the main pancreatic duct. Enzymes needed for the digestion of carbohydrates, fat and proteins continuously flow through the ducts. The ducts fail to fuse in a small percentage of patients which results in mal function of the pancreas.

Function and histology

The pancreas is composed of two glands: The exocrine gland and the endocrine gland. In adults, most of the activity of the pancreas is dedicated to the exocrine gland. The exocrine pancreas and salivary glands share structural similarities and is controlled by both hormonal and neural signals. The exocrine component of the pancreas is composed of acinar cells, which makes up most of the exocrine pancreas. When the acinar cell is stimulated, it releases the enzymatic component of the pancreatic juice from the zymogen granules into the lumen of the acinus where it mixes with the secretions of the centroacinar cells. As the name indicates the centroacinar cell is the cell that is in the center of the acinus cell and is part of the ductal system. Under the microscope, the acinar cells look like blind ended tubules surrounded by polygonal acinar cells, which resembles a cluster of grapes, hence the name acinar cells which means grape in latin. The tiny ducts that drain the acini are called intercalated ducts. The intercalated ducts empty into larger intralobular ducts which in turn drain into an extralobular duct that empties into larger ducts and then into a main large duct that drains the pancreas and enters the duodenum. During this trajectory, the

pancreatic juice accumulates water, chloride and bicarbonates that are secreted by the duct cells to help neutralize the pH of the duodenal content (2). Each day the pancreas secretes 1L, more than 10 times its weight of pancreatic fluids. (3) These secretions contain amylases, proteases and lipases which aid in the digestion process of carbohydrates, proteins and fats.

The endocrine cells of the pancreas form islets in the parenchyma called the Islets of Langerhans. There are five types of cells in the Islets of Langerhans; about 75% of the cells in each islet are insulin producing beta cells. The remaining 25% of cells consists of alpha, delta and F cells which secrete glucagon, somatostatin and pancreatic polypeptide and are located at the periphery of each islet. The hormones secreted by these cells are important in glucose homeostasis and GI functions such as bile secretion and nutrient storage (4).

PANCREATIC DUCTAL ADENOCARCINOMA

Epidemiology

Pancreatic ductal adenocarcinoma (PDAC) is the most occurring type of pancreatic cancer and it affects the exocrine cells of the pancreas. Less than 1% of pancreatic tumors occur in the endocrine system (5). PDAC is now the fourth leading cause of cancer related death in the United States although it is ranked as 10th in the list of most occurring cancers (6). For the year 2010, it is estimated

that more than 43,000 individuals would have been diagnosed with pancreatic cancer and 36,800 have died from the disease (6).

Pancreatic cancer is a disease of old individuals. Based on the SEER cancer statistics, only 13% of individuals are diagnosed before the age of 60 and more than 50% of the patients are 75 years old and older at the time of diagnosis(3). Men are 30% more likely to develop pancreatic cancer than women. Blacks and Jewish people have a higher incidence of pancreatic cancer compared to Caucasians (7). At the molecular levels, blacks have more frequent k-ras mutations than Caucasians and Chinese people have a different expression of Ki-ras and p53 than Caucasians (8).

There are many genetic and environmental risk factors for pancreatic cancer. Smoking is the strongest environmental factor for pancreatic cancer. Carcinogens from cigarette smoking enter the bloodstream and reach the pancreas after leaving the lungs. There is a strong correlation between smoking and pancreatic cancer(9). Studies show that the risk of pancreatic cancer increases by two folds in individuals that smoke compared to non smokers (10, 11). An Important risk factor for pancreatic cancer is the diet. Dietary factors can either increase or decrease the risk of pancreatic cancer. Studies have linked increased caloric intake and obesity to increased risk of pancreatic cancer. On the other hand, increased intake of fruits and vegetables reduce the risk (12, 13). The lack of physical activity has also been reported as a risk factor for pancreatic cancer (14). Alcohol is a major risk for chronic pancreatitis however studies have failed to link alcohol consumption to pancreatic cancer. One of the problems

trying to link pancreatic cancer to dietary intake and environmental factors is due to the rapid progression of the disease and the lack of methods for early detection. Most studies are case control studies and at the time of diagnosis, the patients are in an advanced stage of the tumor that unable them to respond to changes in the diet usually incorporated early on as a chemo preventive measure. However, studies have shown a strong correlation between preexisting conditions and the risk for pancreatic cancer (15, 16). Alcoholic and non alcoholic pancreatitis results in an increase of up to 20 folds for pancreatic cancer. For tropical and hereditary that develops early on the risk for pancreatic cancer is much higher, up to 40% for patients with hereditary pancreatitis. It is not clear whether diabetes is a risk for pancreatic cancer since it can be one of the earlier symptoms of the disease, however many studies have shown that diabetics have a 2 fold increased chance of developing pancreatic cancer (17).

Mutations in genes such as BRCA1 (18), BRCA2 (19, 20), p16/CDKN2A(21) and others (20, 22) that are commonly altered in many cancers, are associated with an increased risk for developing pancreatic cancer . The most important genetic alteration is that of BRCA2 which is found in up to 10% of patients with sporadic pancreatic cancer and up to 20% of patients with a family history (23). Most diseases that increase the risk of pancreatic cancer are autosomally dominant inherited, however, autosomal recessive diseases such as ataxia-telangiectasia (24), fanconi anemia (25) and cystic fibrosis (26, 27) have been reported to be linked to the disease.

Detection and diagnosis of pancreatic cancer

Pancreatic cancer is called “the silent disease” and one of the major reasons why pancreatic cancer survival is low, is because the symptoms don’t start to appear until more advanced and incurable stages. Most patients show sudden weight loss. Jaundice is also seen in a number of patients, when the tumor blocks the bile duct and prevents bile passing to the digestive system, usually these patients have a better prognosis because the tumor is located at the head of the pancreas and is resectable. However, tumors that develop at the tail of the pancreas are usually harder to detect and are asymptomatic therefore patients have a lower survival rate (28). The most common symptom reported in around 80% of patients with locally advanced pancreatic cancer is epigastric pain when eating or lying down. As with other types of cancers, nausea, loss of appetite and fatigue are reported. Even though it is uncertain whether diabetes is directly correlated with pancreatic cancer, patients over 50 years old that develop diabetes are usually tested for pancreatic cancer (29).

Currently, two imaging regimes are used to detect pancreatic cancer MRI (magnetic resonance imaging) and EUS (endoscopic ultrasonography). Standard radiological testings and CT scans are used for the detection of pancreatic cancer; however, they are not sensitive enough to detect small early staged tumors (30). Other methods like PET (positron emission tomography) are used to detect metastases. Commercially available tumor marker for pancreatic cancer CA19-9, is used to monitor the therapeutic response of patients. However, limitations such as the percentage of patients with pancreatic cancer do not

secrete CA19-9 (10-15%) and the normal range of CA19-9 in patients with small local tumors prevents CA19-9 from being used as a screening marker(31). Even though CA19-9 is not used for diagnosis and detection of pancreatic cancer, it strongly correlates with the progression of the disease and the response to treatment. In many cases, even when the physician suspects pancreatic cancer imaging tests fail to detect the tumor therefore biomarkers are needed to facilitate diagnosis. For example, mutant K-ras is found in the pancreatic juice and in the stool of patients with pancreatic cancer however, K-ras cannot be used as a biomarker because of its presence among smokers and patients with chronic pancreatitis. K-ras is also associated with late stages of the disease therefore it is not an ideal biomarker (32).

Management and staging of pancreatic cancer

Pancreatic cancer staging is the most important factor in determining treatment methods and management. The American Joint Committee on Cancer (AJCC) use the TNM system to stage pancreatic cancer. T is the size of the primary tumor and whether it has spread within the pancreas or locally- the tumor is usually resectable, only 10-15% of patients belong to this category and are candidates for surgery. The tumor is usually located at the head of the pancreas and is detected early because of extreme pain. The Whipple procedure is performed to remove the tumor. The procedure is highly invasive and requires the removal of the duodenum, distal part of the stomach, gallbladder and the

common bile duct, therefore this procedure is associated with mortality ranging from 4-16%(33).The overall survival for patients with resectable pancreatic cancer is 20% over 5 year. All patients who undergo surgical removal of the tumor receive chemotherapy and radiation therapy. Recent studies show at least 10% long term survival benefit when chemotherapy and radiation follow surgery. N describes the stage where the tumor has spread to regional lymph nodes- the tumor is locally advanced. M is the stage where the tumor has metastasized to other organs of the body. The numbers 0-4 indicate the severity of each stage- the tumor is metastatic(34). Tumors in the tail of the pancreas usually belong to the N or M stage because of the late detection. This tumor is usually not subject to surgery and chemotherapy slightly improves survival (35). Patients with locally advanced tumors and patients who are not candidates for surgery undergo palliative care to alleviate the pain. Jaundice is the most common symptom of pancreatic cancer and is due to bile obstruction. To alleviate symptoms and clear biliary obstruction associated with jaundice, endoscopic biliary stent insertion is used. This method is the primary method for non surgical palliation of jaundice in patients with pancreatic cancer. Incapacitating pain is another symptom of pancreatic cancer. There are many causes of tumor associated pain that could be caused by tumor infiltration, obstruction of the GI tract, tumor pressure and more. Pain is usually treated by the administration of oral analgesics and at later stages by morphine (36). In several cases, palliative surgery is used to alleviate biliary obstruction, duodenal obstruction, and pain and to improve quality of life of patients.

Chemotherapy

Up to 85% of patients are diagnosed with locally advanced pancreatic cancer and metastatic cancer, therefore cannot be treated with surgery (37). Adjuvant therapy with 5-fluorouracil (5FU) combined with radiation had shown a survival benefit in a study done in the 1980s (38). Recent studies have shown greater long term survival in patients treated with 5FU after surgery compared to patients who did not receive chemotherapy, however studies show that 5FU combined with radiation showed no benefit (39). Nowadays, Gemcitabine is the standard of care for patients with metastatic pancreatic cancer. Patients treated with Gemcitabine have shown higher survival than patients treated with placebo or 5FU (35). Therefore, Gemcitabine is considered first line therapy for advanced pancreatic cancer (40). Several new agents have been tested alone or in combination with Gemcitabine, however, the benefits were dismal. Gemcitabine (29,29-difluoro 29-deoxycytidine, dFdC) is a cytidine analog. Like its analog Ara-C, Gemcitabine is a prodrug and is activated by cellular uptake and intracellular phosphorylation by deoxycytidine kinase (dCK) into gemcitabine monophosphate (dFdCMP) which is then converted into gemcitabine di (dFdCDP) and triphosphate (dFdCTP), the active metabolites(41). Gemcitabine has many antitumor effects (42); it inhibits DNA synthesis(43), DNA polymerase, induces apoptosis and is incorporated in the DNA. Its location in the DNA chain prevents its detection by DNA repair enzymes (44). Pancreatic cancer cells are highly resistant to Gemcitabine. Resistance is either acquired after treatment or the

patient is resistant from the beginning. The response to Gemcitabine varies between patients and this is mostly due to changes in the intracellular levels of the metabolites and in the activities of drug transporters and enzymes involved in the metabolism of the drug (45). An important determinant of a patients response to Gemcitabine treatment is the intracellular retention of gemcitabine nucleotides particularly gemcitabine tri phosphate.

Resistance to chemotherapy

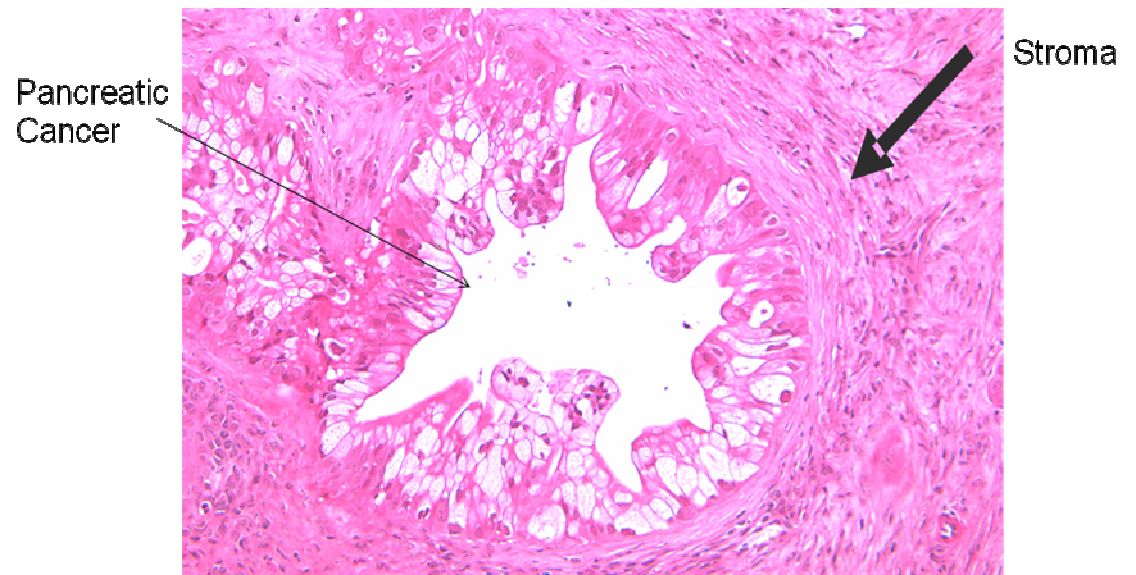
As mentioned previously, most new trials have not shown significant survival advantages and Gemcitabine is still the first line therapy for unresectable pancreatic cancer. Preclinical investigations have identified many molecular mechanisms that contribute to the resistance of pancreatic cancer to therapy. A recent study shed the light on the tumor vasculature of pancreatic cancer (46). Pancreatic cancer is hypovascular and poorly perfused. The poor network of blood vessels impedes proper delivery of chemotherapeutic agents and increase drug resistance (46). Another aspect of pancreatic cancer chemoresistance is the alteration of genes involved in gemcitabine transport and metabolism. The human equilibrative nucleoside transporter -1 (hENT-1) is the major gemcitabine transporter in cells. In the absence or malfunction of hENT-1 gemcitabine cannot exert its antiproliferative effects (47, 48). Low expression of hENT-1 In patients correlates with low survival and bad prognosis (49). One of the most studied mechanisms of pancreatic cancer resistance are related to ABC (ATP binding

cassette) highly expressed in cancer cells and cancer stem cells (50). As discussed previously, deficiency in dCK which phosphorylates gemcitabine into its active form increases resistance. It has been reported that resistant pancreatic cancer cell lines have reduced activity of dCK (51). Anti-apoptotic molecules are overexpressed in pancreatic cancer which causes the cancer cell to evade apoptosis thus reduces their chemosensitivity. For example, bcl-XL expression levels are high in 82% of patients (52). Upregulation of bcl-2 has also been correlated with increased resistance to gemcitabine (53) On the other hand, pro-apoptotic molecules are downregulated, for example BNIP3 expression is low in almost 90% of patients (54) . The transcription factor nuclear-factor kappa B (NF-kappaB) is constitutively expressed in pancreatic cancer and promotes tumor growth, invasion, angiogenesis and chemoresistance (55, 56). The inhibition of NF-KappaB increases chemo sensitivity and improves the outcome of pancreatic cancer (55, 57).

Accumulating evidence shows that the dense tumor associated desmoplasia plays a central role in chemo resistance. A recent study that uses gemcitabine in combination with IPI-926 a hedgehog inhibitor that depletes the stroma show increased survival and drug delivery compared to gemcitabine treatment alone (46) therefore, desmoplasia hinders proper drug delivery and might cause pancreatic cancer resistance to chemotherapy.

Pancreatic desmoplasia

The hallmark of both chronic pancreatitis and pancreatic cancer is the extensive fibrosis that develops in the pancreas. Pathological fibrosis is the formation or development of excess fibrous connective tissue as a reparative or reactive process. The fibrotic reaction that surrounds the pancreatic tumor tissue is called desmoplastic reaction (Figure 1). This dense desmoplasia characterizes the majority of pancreatic cancers (58), with the cancer cells constituting only a minor population of the whole tumor mass. The stroma of pancreatic cancer is composed of interstitial connective tissue mostly made of collagen I and fibronectin, growth factors, inflammatory cells, new blood vessels, endothelial cells, nerve cells, immune cells such as macrophages, dendritic cells and stellate cells. The pancreatic stellate cell (PSC), which will be discussed in the next section in more detail, is the major cell responsible for the production of the desmoplastic reaction (59). Pancreatic tumor-stroma interactions are bidirectional. Formation of new blood vessels in the stroma has been shown to facilitate pancreatic tumor cell invasion (60). The stroma has also been correlated with the degree of aggressiveness of pancreatic tumors; tumors that have a less prominent stromal reaction are less likely to be aggressive (61). On the other hand, the tumor also influences the stroma. One example is the secretion of TGF- β by tumor cells which increases the production of Collagen I and Connective Tissue Growth Factor (CTGF) by the stroma (62). Understanding the molecular and cellular mechanisms of the stroma and how the tumor-stroma interactions are influenced will be critical for identifying potential therapeutic targets for pancreatic cancer.



Logsdon CD. unpublished data

**Figure 1. Desmoplastic reaction surrounding the tumor mass in
PDAC**

Pancreatic Stellate Cells (PSC)

Initiation

Pancreatic stellate cells are the principle source of fibrosis in both chronic pancreatitis and pancreatic cancer (59, 63). Stellate cells are resident cells of the pancreas, located at the basolateral aspect of acinar cells and constitute approximately 4% of pancreatic cells (64). In the normal pancreas, stellate cells are quiescent, identifiable by the presence of vitamin-A containing lipid droplets in the cytoplasm (65) and positive immunostaining for cytoskeletal proteins such as desmin and glial acidic fibrillary protein, which are the adopted way to identify quiescent PSC. One of the normal physiological roles of PSC is in extracellular matrix turnover via their ability to synthesize matrix proteins as well as matrix-degrading enzymes, MMPs. It has been suggested that PSC are involved in maintaining pancreatic acinar cells because of their location around the acini. Because of their periductal and perivascular location, PSC might be involved in normal duct and vascular regulation in the pancreas (66).

Activation

During inflammatory injury, PSC undergo various changes in their morphology and behavior, they become activated and assume a myofibroblast-like phenotype characterized by the loss of vitamin A droplets, the production of α -SMA and extracellular matrix proteins such as collagen Ia1 and III, fibronectin and laminin (64). In vitro studies have identified several factors involved in the activation of

PSC such as alcohol (ethanol), cytokines (IL-1, IL-6), growth factors (platelet derived growth factor; PDGF, TGF- β 1, activin A), oxidative stress and pressure, and changes in extra cellular matrix (67, 68). Other cells in the pancreatic microenvironment such as macrophages, platelets and endothelial cells as well as acinar cells and cancer cells are the source of PSC activating factors (67, 68).

Activated PSC proliferate, migrate, increase the production of extra cellular matrix molecules as well as ECM related molecules and secrete cytokines and chemokines (66, 69). Chemokines produced by PSC recruit inflammatory cells to the pancreas. Cytokines and growth factors produced by PSC themselves and other neighboring cells (acini, tumor, immune cells, platelets or any other cell in the microenvironment) activate PSC and the reaction is bidirectional. Activation occurs in both paracrine and autocrine manner (70). It has been suggested that autocrine activation of PSC by cytokines like TGF- β , sustain the fibrotic response and allows it to perpetuate after stimulation by the initial signal, which promotes desmoplasia (33, 63). PDGF and TGF- β are believed to be the most potent activators of PSC. PDGF induces the proliferation and migration of PSC (71, 72), whereas TGF- β induces the expression of the activation marker α SMA and ECM proteins to sustain the activation of PSC (73, 74). As mentioned previously, PSC are involved in maintaining normal tissue architecture and ECM turnover, and were shown to secrete MMPs (matrix metalloproteinases) such as MMP2, MMP9 and MMP13, and TIMPs 1 and 2 (tissue inhibitors of matrix metalloproteinases) (69) which could impact the invasion and metastatic potential of pancreatic cancer. MMP2 is known to degrade collagen IV, an essential

component of the basement membrane, thereby facilitating the deposition of fibrillar collagen type I observed in pancreatic fibrosis. PSC promote the angiogenic potential of cancer cells, so far two mechanisms have been identified. The first mechanism involved the secretion of proteolytic matrix degrading MMP-2 enzyme. Increased MMP2 expression by PSC has been shown to accelerate pancreatic tumor progression (75). The second known mechanism by which PSC increase angiogenesis is by the production of proangiogenic factors such as VEGF. When active, PSC constitutively produce vascular endothelial growth factor (VEGF) and other angiogenic molecules such as VEGF receptors (flk-1, flt-1) and others (76). Therefore, PSC play a profibrogenic and proangiogenic role, and tumor induced PSC activation lead to increased angiogenesis, which could promote pancreatic cancer progression by delivering oxygen and nutrients to the hypovascular tumor in order to increase growth, invasion and metastasis.

Postactivation

Following activation, PSC have one of two fates depending on the severity of the injury. If the injury is limited, activated PSC lose their active phenotype and become quiescent again. PSC might also undergo apoptosis. In the latter two cases, fibrosis does not occur. If the inflammatory is severe and repeated, PSC activation is sustained and perpetuated and pancreatic fibrosis develops. Repeated and sustained injuries to the pancreas are important for the development of fibrosis. From this point on, pancreatic fibrosis is defined as the pathological changes of extra cellular matrix composition, both in quality

(collagen IV is replaced by collagen I and quantity (excessive collagen I deposition) , caused by the irreversible activation of PSC.

Origin of PSC

There are many speculations about the embryonic origin of PSC. PSC share similarities with their hepatic counterpart, hepatic stellate cells (HSC). Both cells express alphaSMA, GFAP, and transcriptome analysis studies revealed 99.9% homology at the mRNA level (77). This might suggest that PSC and HSC share a common origin but have organ specific variations that could be due to the different microenvironmental factors the cell is exposed to. A different study demonstrated that PSC are derived from a pancreas-specific precursor (78). Activated PSC are thought to arise from quiescent PSC in the pancreas. However, studies done on mice with sex-mismatched bone marrow (BM) transplantation from a male mouse carrying enhanced GFP to female mouse suggest that a small subgroup of PSC, approximately 5%, is derived from the BM (79).

Molecular regulation of PSC

PPAR- α is a member of the PPAR family of nuclear hormone receptors. Upon dimerization with RXR, PPAR- α binds PPAR response elements in the 5' flanking region of target genes, therefore controlling cell proliferation, macrophage function and immunity (80). Negative regulation of PPAR- α is associated with PSC activation and overexpression of PPAR- α results in the loss

of PSC activation (81). Therefore, PPAR- α is involved in maintaining the quiescence of PSC.

Rho and Rho kinase are also involved in the regulation of PSC. Incubation of quiescent PSC with Rho kinase inhibitors (HA-1077 and Y-27632) blocks the activation of freshly isolated PSC in culture on plastic (74). Rho kinase inhibitors also inhibit stress fiber formation associated with activated PSC migration and contraction in response to endothelin-1 (82).

In activated PSC, a variety of stimuli activate the MAPK pathway including ERK and p38MAPK (83). Activation of MAPK increases the production of cytokines and chemokines by PSC. Studies show that inhibition of p38MAPK blocks the activation of PSC (84) therefore, p38 MAPK mediates PSC activation. ERK activation precedes α SMA expression and mediates PSC proliferation to different stimuli in response to PDGF (83).

Phosphatidyl inositol 3 kinase (PI3K)/ Akt pathway is also involved in the regulation of PSC. Activation of PSC correlates with activation of PI3K/Akt pathway (74). PDGF stimulates PSC migration via a PI3K/Akt dependent pathway mediates however it was not found to be involved in PSC proliferation (47, 85).

TGF- β is a potent regulation of PSC; it can regulate PSC in SMAD dependent and SMAD independent mechanisms. The extra cellular matrix production by PSC is regulated by TGF- β in a Smad2 dependent manner (86) while TGF- β inhibits PSC proliferation by activating Smad3 dependent

downstream signaling. Treatment of PSC with MEK1 inhibitors results in a decrease in the mRNA levels of TGF- β 1 thus demonstrating the involvement of ERK pathway in the regulation of TGF- β .

Prostaglandin E2 (PGE2)

Prostaglandins (PG) are 20-carbon fatty acid derivatives found in all tissues and organs. Diverse stimuli trigger PGs synthesis by the release of arachidonic acid (AA) from the plasma membrane by the action of phospholipase-A2 (PLA2). Upon its release from the plasma membrane, AA is converted to an unstable endoperoxide intermediate prostaglandin G2 (PGG2) by cyclooxygenases; this is rate limiting enzymatic reaction. PGG2 is then converted to an oxygenated intermediate prostaglandin H2 by the peroxidase activity of COX- enzymes. PGH2 is then metabolized by the action of cell specific synthases and is the precursor of several prostaglandins; PGE2, PGD2, PGF2 α , PGI2 and TXA2 (87). Prostaglandins are released outside the cell immediately following their biosynthesis. PG actions are dependent upon their interactions with prostanoid specific G-coupled protein receptors, the focus will be on PGE2 and its four receptors EP1-4. The actions of prostaglandins could also be terminated when they are transported across the plasma membrane into the cytoplasm by prostaglandin transporters (88) where they are reduced or oxygenated by PG catabolizing enzymes (88, 89).

Currently, there are three known COX isoforms COX-1, COX-2 and COX-3 (a splice variant of COX-1). COX-1 is a ubiquitously and constitutively

expressed isoform that is postulated to have “housekeeping” functions with basal production of prostaglandins under homeostatic conditions. In contrast, COX-2 is encoded by an early-response gene and can be rapidly induced by growth factors, cytokines, inflammatory mediators and tumor promoters (20, 90). By immunohistochemistry (IHC), COX-2 was found to be highly expressed in chronic pancreatitis (91) and pancreatic adenocarcinoma (92-94) and is also found in precursor lesions associated with pancreatic cancer referred to as pancreatic intraepithelial neoplasia (PanIN) (95) .

As mentioned previously, COX-2 is frequently overexpressed in many types of cancer including breast, colon, lung and pancreatic cancer (96-98). PGE2 has been identified as the major prostaglandin behind COX-2 proneoplastic functions [100]. Many tumors that overexpress COX-2 have high intramural levels of PGE2. In pancreatic cancer, COX-2 overexpression was reported in 74-100% of patients, irrespective of the grade and histological type of the tumor (92, 95, 99). Furthermore, PGE2 promotes cell growth, angiogenesis, migration, invasion and survival in pancreatic cancer (100-102). Many studies have implicated the role of PGE2 in carcinogenesis for a wide range of cancers including pancreatic cancer. However, the downstream targets by which PGE2 mediate these processes are not fully understood.

EP receptors subtypes

PGE2 exerts its effects by binding to four membrane bound E-prostanoid (EP) receptors. In humans, there are four EP receptors EP1, EP2, EP3 and EP4.

EP receptors are G-protein coupled receptors (GPCRs) that are activated by the actions of PGE₂. PGE₂ binding to EP receptors produces a change in receptor conformation, exposing intracellular sites involved in the interaction with the G protein, consisting of α , β , and γ subunits. The interaction with the receptor causes the guanosine diphosphate (GDP) to be freed from the α subunit and replaced by guanosine triphosphate (GTP). The binding of GTP activates G α which dissociates from the G $\beta\gamma$ dimer and triggers a G α -specific pathway. The G $\beta\gamma$ complex can also activate a downstream pathway. Even though EP receptors share common signaling mechanisms however, each receptor has different biological effects (103, 104). All EP receptors are expressed on the membrane (105) with EP3 and EP4 also exhibiting nuclear membrane localization (106).

EP1 receptor is a 42-kDA protein that is particularly abundant in the kidney and on smooth muscle associated with vessels. It has the least affinity for PGE₂. PGE₂ binding to EP1 converts GDP G α_q to GTP G α_q which then activates PLC β . Upon its activation, PLC β hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP₂) and generates diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃) which is associated with the release of CA⁺⁺ from intracellular stores. The activation of EP1 downstream signaling alters the activity of many proteins including activation of PKC (107) . Studies implicate EP1 receptor in colon carcinogenesis (108).

EP3 receptor is unique among other EP receptors because of its 7 alternative splicing variants defined by their C-terminal cytoplasmic tails. EP3

activation usually inhibits cAMP production by inhibiting adenylate cyclase via the activation of Gi (104). However, EP3 receptor has been shown to couple to Gi, Gs and Gq protein therefore, the role of EP3 receptor may vary depending on the cell type.

EP2 receptor is a 53 kDa protein that upon coupling to Gs increases cAMP levels and activation of protein kinase A (PKA) signaling. Activation of cAMP/PKA pathway is associated with growth and proliferation. Phosphorylation of PKA activates Akt which inhibits GSK-3. Inhibition of GSK-3 relieves the phosphorylation of β -catenin, therefore allowing it to translocate to the nucleus, which results in an increase in cell proliferation (109). EP2 receptor can also activate proliferation by association with Axin which inactivates and releases GSK-3 β causing β -catenin activation and translocation to the nucleus. EP2 receptor is heavily involved in tumor development and progression in many organs and tissues such as breast, prostate, skin and pancreas (110, 111) (112, 113).

EP4 receptor also increases cAMP levels in a similar manner to EP2 receptor. In addition to cAMP/PKA pathway, EP4 receptor activates PI3K/AKT signaling. Activation of EP4 receptor leads to the phosphorylation of extra cellular signal regulated kinases (ERKs) by PI3K (114) which activates early growth factor-1 (EGR-1). EGR-1 is known to regulate many genes important in cancer and inflammation such as PGE2 synthase, TNF- α and cyclin D1. Knock out studies of EP4 receptor has shown a role for EP4 in cancer and inflammation (115-117).

Targeting COX-2/PGE2

COX-2 and PGE2 inhibition have been considered a potential chemotherapeutic target for pancreatic cancer. Several drugs that target COX-2 have been developed in the past decade, the most notorious of which is the COX-2 inhibitor Celecoxib. Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) and is a highly selective COX-2 inhibitor. Traditional NSAIDs inhibit both COX-1 and COX-2, while celecoxib is more selective for COX-2 inhibition. COX-1 inhibits prostaglandin and thromboxanes, while COX-2 inhibits prostaglandin production alone. COX-2 inhibition thus spares thromboxane without any effect on platelet aggregation or blood clotting. Selective inhibition of COX-2 by celecoxib was effective in suppressing the growth of pancreatic cancer cells in vitro (118) and in inhibiting tumor growth and angiogenesis in orthotopic implantation tumor models (119). But the treatment of pancreatic cancer patients with celecoxib alone or in combination with standard chemotherapeutic drugs was not effective in reducing the pancreatic tumor in advanced staged but also was highly toxic to patients (120). Patients treated with COX-2 inhibitors particularly, celecoxib had a high risk for heart attack and strokes. In a clinical trial for colon cancer prevention, patients treated with celebrex, a COX-2 inhibitor, showed a high risk for cardiovascular diseases compared to the placebo (121). The toxicity behind COX-2 inhibition is mainly due to the unspecific inhibition of all prostaglandins. Inhibition of COX-2 causes a shift in the PGI₂/TxA₂ balance. Prostacyclin I₂ (PGI₂) has anti proliferative, anti aggregating

and vasodilating functions, whereas Thromboxane (TxA₂) is a vasoconstrictor that has smooth muscle proliferating effects and causes platelet aggregation. The PGI₂/TxA₂ balance is essential to maintain vascular homeostasis and the disruption of the balance, which could be caused by COX-2 inhibition, causes thrombosis (122). COX-2 inhibitors have also been reported to increase blood pressure because of the high levels of TxA₂. Therefore, emphasis has been on finding an alternative route to block PGE₂ production, downstream of COX-2, to avoid the toxic side effects seen when COX-2 is inhibited. Inhibition of PGE₂ could be achieved by either increasing its inactivation by 15-PGDH or by targeting the inhibition of EP receptors. 15-PGDH is an enzyme that catalyzes the oxidation of the 15(S)-hydroxyl group of prostaglandins leading to the formation of metabolites with decreased activities compared to PGE₂ (123), therefore, biologically inactivating PGE₂. 15-PGDH has been shown to be a tumor suppressor gene that is down regulated in breast, lung, colon and bladder cancers (124-127). A study done in colon cancer shows that reintroducing 15-PGDH to CD11b myeloid cells inhibits PGE₂ inflammatory, immunosuppressive functions and increased survival. 15-PGDH inhibition of PGE₂ was also associated with inhibition of inflammatory cytokines such as IL-6, IL-10, IL- β and of immunosuppressive cytokine IL-13 (128).

An alternative way to block PGE₂ production is by the inhibition of one of more EP receptor. As discussed previously, PGE₂ exerts its actions through binding to one of its four receptors EP1-4. A number of studies support the role of each EP receptor in tumorigenesis and inflammation. Recently, EP receptors

have been targeted for the treatment and/or prevention of cancer. Synthetic drugs, that act as antagonists or agonists for EP receptors, have been developed by pharmaceutical companies, and tested in vitro for their anti tumor effects (129). Treatment of breast cancer with an EP4 antagonist shows a reduction in metastasis compared to the untreated control (130). In another study done in colon cancer, inhibition of EP1 and EP4 receptor showed a decreased number and size of polyps (131).

PGE2 and fibrosis

COX-2 expression is considered a major factor linking chronic inflammation with metaplastic and neoplastic changes in pancreas (96). The expression of COX-2 in chronic pancreatitis was localized in the cytoplasm of pancreatic acinar cells, islet cells, and ductal cells but not in the surrounding stromal cells or infiltrating Inflammatory cells, while in pancreatic adenocarcinoma it was localized only to cancer cells and not to the stromal or inflammatory cells (92). In the pancreas the fibrotic process is associated with Inflammation and high levels of COX-2 activity (91, 132). COX-2 activity leads to increased production of the prostaglandin PGE2 at sites of inflammation in human tissues (91). PGE2 has both pro-inflammatory and cell-protective activities, but its specific roles in pancreatic disease are unknown. PGE2 mediate a wide away of physiological and pathological functions in different organs and tissues, such as inflammation.

PGE2 and the tumor microenvironment

It is now well known that the stroma surrounding the tumor cells aids in the development and progression of cancer. A number of tumor associated stromal cells express high levels of COX-2, however, not much is known on the role of PGE2 and its receptors in the stroma. In colon cancer, over expression of COX-2 in the stromal cells increased the proliferation of colon cancer cells and VEGF production. Blocking EP4 receptor on the stromal cells blocked those effects completely. These results suggest that regardless of the status of COX-2 in cancer cells, blocking COX-2 or EP receptors could block tumor –stroma interactions (133). Another study done in colon cancer identifies a role for stromal EP4 receptor in metastasis (134). EP4 knock out in the stroma showed a reduction in tumor growth and metastasis. In a study done on sarcoma cells, EP3 receptor knock out in the stromal cells reduced angiogenesis and tumor growth, further supporting the role of PGE2 produced by the stroma in tumor development and angiogenesis (135). Studies where EP receptors are inhibited and studies that target the increase of 15-PGDH (128) activity demonstrate a vital role for PGE2 in the stroma of tumors.

Stromal PGE2 in pancreatic cancer

The role of PGE2 in the stroma of pancreatic cancer is not fully understood, as only a limited number of studies have aimed to determine the role

of PGE2 in the stroma of pancreatic cancer. As discussed previously, pancreatic and hepatic stellate cells share many similarities and studies from hepatic stellate cells are used to understand the behavior of pancreatic stellate cells. In a study done on HSC in vitro, treatment with NS-398, a COX-2 inhibitor, reduced the expression of alpha smooth muscle actin, which is an activation marker for stellate cells. Therefore indicating that COX-2 might play a role in the activation of HSC (136). In another study, celecoxib induced HSC apoptosis through the inhibition of Akt activation which resulted in a reduction of fibrosis (137). One of the few studies that examine the role of COX-2 in pancreatic stellate cells shows that COX-2 is expressed and required for PSC to respond to inflammatory cytokines (138). However, this study focuses more on the role of TGF- β in activating COX-2 expression in PSC and does not investigate the role of stromal COX-2/PGE2. The role of COX-2/PGE2 in the stroma of PDAC is not understood. There are no studies examine the role of PGE2 or its receptors in the regulation of stromal activation in pancreatic cancer. As mentioned previously, COX-2 expression is high in both chronic pancreatitis and pancreatic cancer which suggest that COX-2/PGE2 play a role in the regulation of pancreatic stellate cells, however the precise mechanism is unknown.

EXPERIMENTAL RATIONALE AND HYPOTHESIS

As discussed above, pancreatic ductal adenocarcinoma, also called the “silent killer”, is a highly lethal disease that is often asymptomatic, with a reported 5-year survival rate of less than 5%. In western countries, PDAC is the fourth most common cause of cancer related death. Currently, the therapies available for pancreatic cancer show only a modest increase in survival rate; patients have a 6 month median survival rate. A distinct feature that characterizes PDAC is the extensive desmoplastic reaction that surrounds the tumor. PSCs produce the majority of the fibrosis associated with PDAC and most lines of evidence indicate that PSC promote tumor development and progression. Therefore, it is clear that more research to understand the stroma of pancreatic cancer is needed in order to develop novel therapies with the hope of improving patients’ prognosis.

Based on the available evidence, the hypothesis for this dissertation was that PGE₂ regulates PDAC stromal activation by increasing the activity of PSC in an invitro model using immortalized pancreatic stellate cells from a patient with pancreatic cancer. This dissertation addresses the regulation of PSC by PGE₂ and determines the receptor critical for mediating PGE₂ dependent functions in fibrosis. In order to accomplish this, in the first aim, stromal activation in pancreatic cancer was “dissected” in vitro, and the effects of PGE₂ on each step were determined by measuring changes in PSC functions. This aim assessed the levels of COX-2/PGE₂ produced by PSC and examined the effects of exogenous PGE₂ on proliferation, migration, invasion potential of PSC as well as changes in extra cellular matrix after treatment with PGE₂.

The second aim of this dissertation explores the mechanism by which PGE2 regulates PSC, focusing primarily on determining which EP receptors are involved in mediating PSC activity by PGE2 and what signaling pathways activated downstream of PGE2 in PSC. In addition, this aim addresses modulation of PSC “fibrotic” functions by blocking EP4 receptor activity in vitro to determine the role of EP4 as a potential therapeutic target.

The third aim of this dissertation examines the mechanisms of PGE2 on stromal activation in pancreatic cancer at the molecular level by determining the EP4 dependent downstream signaling pathways activated and by determining the role of PGE2 in regulation Collagen I, focusing on the expression and transcriptional activity of Collagen I gene. This aim also determines the role of blocking EP4 receptor on Collagen I gene. The last part of this chapter aimed at identifying the area in the promoter of collagen I regulated by PGE2.

**CHAPTER TWO: PROSTAGLANDIN E2 REGULATES THE ACTIVATED
PHENOTYPE OF PANCREATIC STELLATE CELLS**

INTRODUCTION

Pancreatic fibrosis is the formation or development of excess fibrous connective tissue in pancreas as a reparative or reactive process (139). The hallmark of chronic pancreatitis and pancreatic cancer is pancreatic fibrosis. It is the inflammation of the pancreas. Inflammation is associated with a high increase of COX-2 activity (140). COX-2 activity leads to increased production of the prostaglandins PGE₂ at sites of inflammation in human tissues. PGE₂ has both pro-inflammatory and cell-protective activities. COX-2 and PGE₂ are correlated with worse prognosis in many cancers like lung, gastric and pancreas (141).

Prostaglandin endoperoxide synthase, commonly referred to as cyclooxygenase (COX), catalyzes the double oxygenation and reduction of arachidonic acid (AA), after its release from membrane glycerophospholipids by phospholipase A₂ (PLA₂), to the intermediate form prostaglandin H₂ (142) which is further metabolized to form prostaglandin E₂ catalyzed by microsomal PGE₂ synthases (143). Currently, there are three known COX isoforms COX-1, COX-2 and COX-3 (a splice variant of COX-1). COX-1 is a ubiquitously and constitutively expressed isoform that is postulated to have “housekeeping” functions with basal production of prostaglandins under homeostatic conditions. In contrast, COX-2 is encoded by an early-response gene and can be rapidly induced by growth factors, cytokines, inflammatory mediators and tumor promoters (144). By immunohistochemistry (IHC), COX-2 was found to be highly expressed in chronic pancreatitis (91), pancreatic adenocarcinoma (92-95) and

pancreatic intraepithelial neoplasia (PanIN) (145). The expression of COX-2 in chronic pancreatitis was localized in the cytoplasm of pancreatic acinar cells, islet cells, and ductal cells but not in the surrounding stromal cells or infiltrating inflammatory cells (91), while in pancreatic adenocarcinoma also it was localized only to cancer cells and not to the stromal or inflammatory cells (92). COX-2 overexpression is an established factor linking chronic inflammation with metaplastic and neoplastic changes in pancreas (96). Transgenic overexpression of COX-2 in the pancreas has been shown to lead to the development of pancreatic fibrosis (96). Despite the correlation between COX-2 and fibrosis, the mechanisms involved in COX-2 mediated stromal activation in pancreatic cancer are unclear.

Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) and is a highly selective COX-2 inhibitor. Traditional NSAIDs inhibit both COX-1 and COX-2, while celecoxib is more selective for COX-2 inhibition. COX-1 inhibits prostaglandin and thromboxanes, while COX-2 inhibits prostaglandin production alone. COX-2 inhibition thus spares thromboxane without any effect on platelet aggregation or blood clotting. Selective inhibition of COX-2 by celecoxib was effective in suppressing the growth of pancreatic cancer cells in vitro (146) and in inhibiting tumor growth and angiogenesis in orthotopic implantation tumor models (119). But the treatment of pancreatic cancer patients with celecoxib alone or in combination with standard chemotherapeutic drugs was highly toxic to patients (120).

Stellate cells are resident cells of the pancreas, located at the basolateral aspect of acinar cells (147). In the normal pancreas, stellate cells are quiescent, identifiable by the presence of vitamin-A containing lipid droplets in the cytoplasm and positive immunostaining for cytoskeletal proteins such as desmin and glial acidic fibrillary protein. In health, PSC play a role in extracellular matrix turnover via their ability to synthesize matrix proteins as well as matrix-degrading enzymes, MMPs. During necroinflammatory injury, PSC become activated and assume a myofibroblast-like phenotype characterized by the loss of vitamin A droplets, the production of α -SMA and extracellular matrix proteins such as collagen I and III, fibronectin and laminin (148). Factors known to be upregulated during pancreatic injury such as TGF- β , platelet-derived growth factor (PDGF) and proinflammatory cytokines, stimulate PSC proliferation and production of extracellular matrix proteins (149). Notably, activated PSC also produce increased amounts of matrix metalloproteinase-2 (MMP2) (69), known to degrade collagen IV, an essential component of the basement membrane, thereby facilitating the deposition of fibrillar collagen1a as observed in pancreatic fibrosis. But, the exact mechanisms regulating stellate cells and their development of pancreatic desmoplasia are not fully understood.

In the current study, I sought to further understand the role of PGE2 on stromal activation in pancreatic cancer in vitro using human pancreatic stellate cells from a patient with PDAC. I determined that PGE2 might be a useful target for novel therapies and that targeting prostaglandin pathways may be a strategy to interfere with stromal activation in pancreatic cancer. Hence, I have

investigated the effects of PGE₂ on pancreatic stellate cells. As a measure to study stromal activation in pancreatic cancer, primary human pancreatic stellate cells (PSC) from a patient with pancreatic cancer, were isolated and immortalized as mentioned in previous publications (68). These studies showed that, PSCs cells express COX-2 and secrete PGE₂ and PGE₂ has autocrine effects on stellate cells. Exogenous addition of PGE₂ on PSC stimulated the migration and invasion of stellate cells and also production of extra cellular matrix (ECM) proteins – fibronectin, collagen 1a, HSPG2, vimentin and elastin; and production of matrix metalloproteinases (MMP) – 2 & 9. Thus, this study provides a better understanding on regulation of stellate cells by PGE₂ and further the role of stellate cells on pancreatic stromal activation in pancreatic cancer. This study suggests that PGE₂ could be an alternative potential therapeutic target to reduce COX-2 mediated stromal hyperactivation associated with chronic pancreatitis and pancreatic cancer.

MATERIALS AND METHODS

Materials

Culture media and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Rockville, MD). PGE₂ was obtained from Cayman Chemicals.

Cell Culture

Primary human pancreatic stellate cells (PSC) were isolated using the outgrowth method from pancreatic adenocarcinoma samples from patients undergoing surgical resection and were immortalized (68)(Hwang RF, 2008). PSCs were maintained at 37°C in a humidified atmosphere of 5% CO₂ and were grown in 10% DMEM containing 1% antibiotic. PGE₂ was purchased from Cayman Chemicals (Ann Arbor, MI).

Invasion and Migration assays

For studies of cell invasiveness, BIOCOAT Matrigel invasion chambers (BD Biosciences, Chicago, IL) were used. Briefly, 2×10^5 cells in 100 μ l of serum-free medium were added to the upper chamber and different concentrations of PGE2 (1–1000 nM) in 0.5% serum containing DMEM were added into the lower chamber. The cells were allowed to invade the Matrigel for 22 h at 37°C in a 5% CO₂ atmosphere. DMEM containing 0.5% serum was used as control. The non-invading cells on the upper surface of the membrane were removed with a cotton swab and the invading

cells on the lower surface of the membrane were fixed and stained with a Diff-Quick stain kit (BD Biosciences), washed twice with water and air-dried. Invading cells in three adjacent microscope fields for each membrane were imaged at 20x magnification. To assess cellular migratory potential, the protocol described above was used, except that migration chamber devoid of matrigel was used (BD Biosciences, Chicago, IL). Experiments were performed at least in triplicate, and the results were shown as mean \pm SD of three independent experiments

MTS proliferation assay

MTS was obtained from Promega. MTS is a tetrazolium salt that undergoes a color change caused by its bio-reduction of MTS into a water-soluble formazan.

The conversion of MTS into the aqueous-soluble formazan is accomplished by dehydrogenase enzymes found in active mitochondria and is such that the reaction occurs only in living cells. The quantity of formazan product measured by the amount of 490-nm light absorbance is directly proportional to the number of living cells in culture. MTS (2 mg/ml; pH 6.5) was dissolved in PBS and filter sterilized. A 3-mM PMS solution was also prepared (in PBS) and filter sterilized. These solutions were stored at -20°C in light-protected containers. MTS (100 μ l) was added to each well. After incubation at 37°C in a humidified atmosphere with 5% CO_2 for 1 hours, the optical density was measured at 490 nm by means of spectrophotometry. Cell growth was analyzed by means of MTS assay after 0, 24, and 48 hours of culture. Cell proliferation was analyzed with a hemocytometer and a cell counter (Coulter, Hialeah, FL).

Apoptosis detection

To define the level of apoptosis, PSC cells were trypsinized, pelleted, fixed, and propidium iodide (PI) stained. Propidium iodide fluorescence activated cell sorting analysis (PI-FACS). Cells were seeded onto 6-well plates at 2×10^6 cells/plate. The next day, media was aspirated and replaced with low serum 0.5% FBS containing media. After 24 hours of starvation, PSC were treated with PGE2 (50 or 100 nM) or DMSO for 24 hrs. Twenty four hrs after PGE2 treatment, media and cells were collected and fixed in 75% cold ethanol. Cells were stained with PI (50 $\mu\text{g/ml}$) and analyzed by flow cytometry (Beckman Coulter, Inc).

Reverse transcription-PCR

Total RNA was isolated from PSC with and without siRNA transfection and the quality of RNA was tested as mentioned previously. DNase was used to remove contaminating genomic DNA and RNA purification. Quality of the RNA was confirmed by running on a denaturing gel, and we have observed clear 28S and 18S rRNA bands. A non reverse transcribed control was used to assure that no genomic DNA was amplified. Primers were designed for human

MMP-2 (5' CTTCTTGTGCGGGTCGTAGTCCTC3') (3' TGGCGATGGATACCCCCTTGA5') ,

MMP-9 (5' GCGCTGGGCTTAGATCATTCTCA 3') (3' GCAGCGCGGGCCATTGTC 5') ,

MMP-1 (5' ATTCTACTGATATCGGGGCTTTGA3') (3' TGTCTTGGGGTATCCGTGTGTAG5') ,

MMP-7 (5' AAACTCCCCGCGTCATAGAAATAAT 3') (3' TGAGTTGCAGCATACAGGAAGTT 5'),

MMP-11 (5' CTGGCGGGCGCTGGGAGAAGAC 3') (3' CAGGGCTGGCCATATAGGTGTTGA5'),

TIMP-1 (5' CGTCATCAGGGGCCAAGTTCGTG 3') (3' GAGGCAGGCAGGCAAGGTGAC 5'),

TIMP-2 (5'CTGGCGGGCGCTGGGAGAAGAC3')
(3'CAGGGCTGGCCATATAGGTGTTGA3')

Fibronectin (5' CCGCCACGTGCCAGGATTACC 3') (5'
AGGGGCTCGCTCTTCTGATTATTC 3') ,

Vimentin (5' GGTCCGTGTCCTCGTCCTCCTAC 3') (3'
CGCGGGCTTTGTCGTTGGTTA 5') ,

Elastin (5' GGACCCCTGACTCACGACCTC 3') (5'
ACTTGGCCGCTCCCCTCTTGTTTC 3'),

HSPG2 (5' CCGCCAGGGCAGGTCA 3') (3'
GGTGGGCAGCGGTAGGAAGGAGTA 5'),

COX-2 (5'GGTCTGGTGCCTGGTCTGATGATG3') (5'
GTCCTTTCAAGGAGAATGGTGC 3').

The amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide. Primers designed for β -actin (Genbank BC_016045), which was used as a loading control for the PCR reactions, were forward 5' ATG ATA TCG CCG CGC TCG TCG TC 3' and reverse 5' CGC TCG GCC GTG GTG GTG AA 3'.

Immunohistochemical Staining for COX-2

Unstained 4uM tissue sections from human patients were deparaffinized with xylene and rehydrated with ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol and non specific binding sites were blocked with protein blocking solution (5% normal horse and 1% normal goat serum). Primary antibody against COX-2 (1:800 dilution; cat # HPA001335) was added and the samples were incubated overnight at 4°C. The secondary antibody was added and incubated for 1 hour at room temperature. Finally, slides were developed with 3,3 diaminobenzidine substrate (DAB) and counterstained with hematoxylin. Then the slides were dehydrated with ethanol, fixed with xylene and mounted. Immunohistochemistry was analyzed using an inverted light microscope (Olympus, Center valley, PA). Images were captured using a chilled, charge coupled device camera (Hamamatsu, Bridgewater, NJ) and smartcapture software (Digital Scientific, Cambridge, UK).

Statistical analysis

All experiments were conducted in triplicates and carried out on three or more separate occasions. Data presented are means of the three or more independent experiments +/- standard error mean (SEM). Statistical analysis was done using GraphPad Prism (GraphPad Software). Comparisons were made using two-tailed Student's *t* test and significant difference was defined as $P < 0.05$. Data are shown as mean \pm SE.

RESULTS

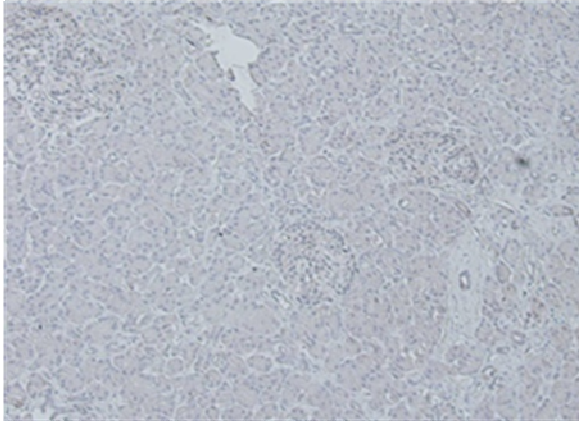
COX-2 is overexpressed in PDAC and PSC produce high levels of PGE2

The immunohistochemical analysis of COX-2 expression in tissue sections from pancreas resected from human pancreatic tumors showed high levels of staining intensity of COX-2 compared to normal pancreas tissues (Figure 2.1.1). PDAC

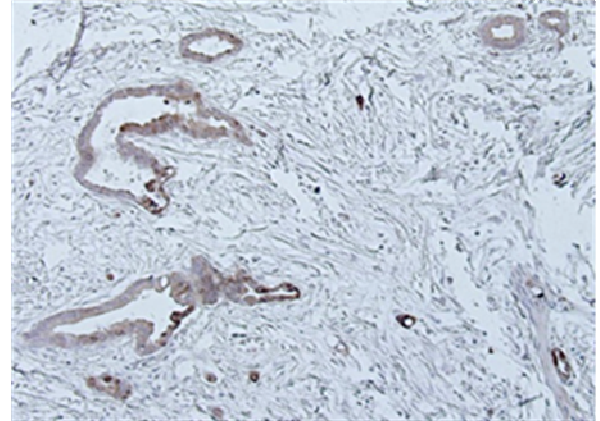
showed high patterns of expression from low staining to high staining. In order to determine whether COX-2 plays a role in the pancreatic stroma, I examined if PSC express COX-2. mRNA was amplified from immortalized PSCs (line 1 and 2) and RT-PCR showed that all PSC examined express COX-2 (Figure 2.1.2). Furthermore, to determine whether PGE2 is secreted by the PSCs, I quantified the levels of PGE2 both intracellularly and media bathing the stellate cells. I employed Liquid Chromatography- tandem mass spectrometry, which is able to distinguish geometrical isomers and accurately measure levels of different prostaglandins produced. PGE2 had an intracellular concentration of 20 ng/ml, being the highest among all other eicosanoids examined. Even at the secreted levels of eicosanoids, PGE2 levels were the highest among the prostaglandins examined (260 ng/ml) (Figure 2.1.3). PGE2 was found in the media bathing the cultured PSCs, indicating that this mediator is released by the PSCs into the microenvironment. This data indicates that PGE2 is highly present and produced by PSC. It is already known that PGE2 are secreted by pancreatic cancer cells. Therefore, there are at least two potential sources that account for the production of PGE2 in the stroma. This also suggests that PGE2 plays a role in the regulation of the tumor microenvironment and not only the cancer cells.

1.

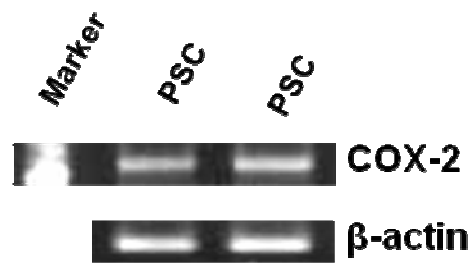
Normal



PDAC



2.



3.

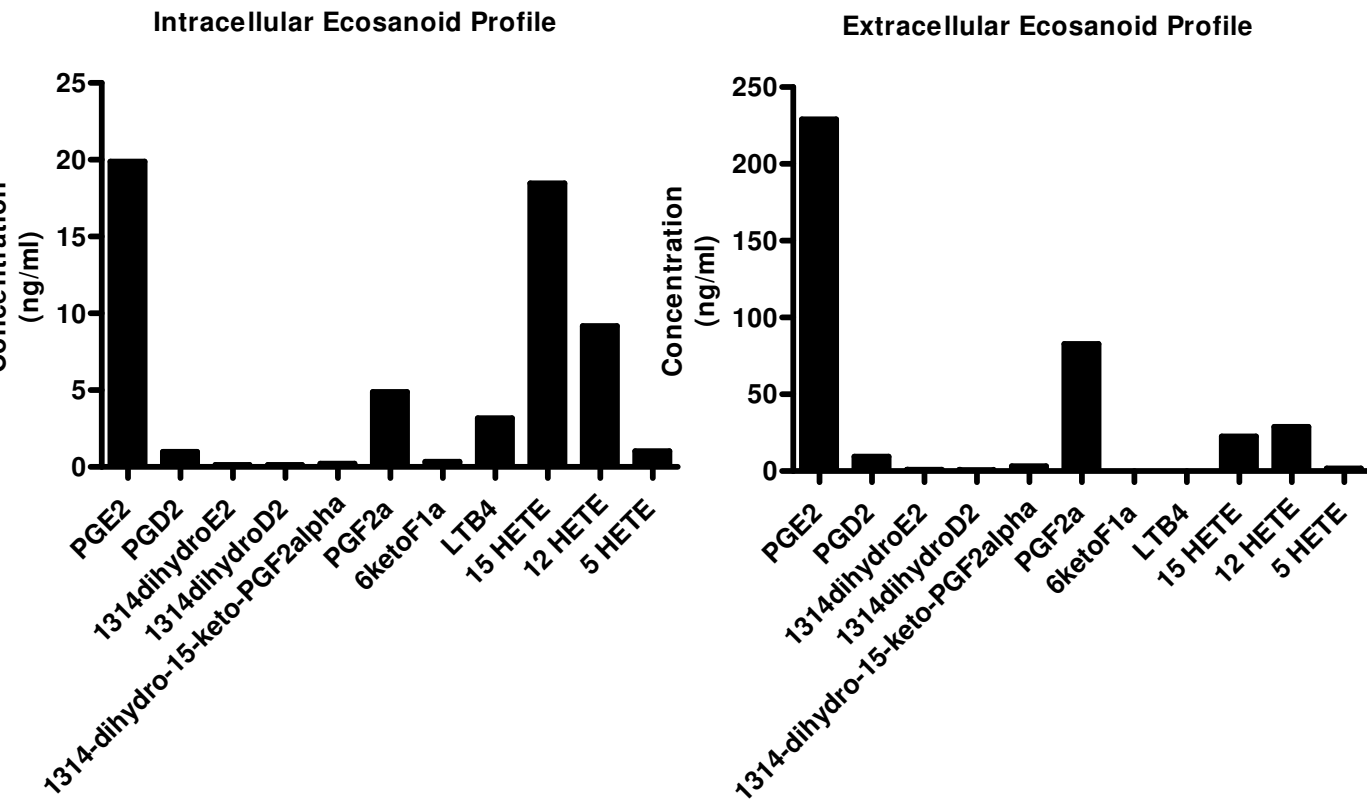


Figure 2.1 Eicosanoid levels in PDAC and PSC (1) Human tissue sections were immunostained for COX-2 and their respective serial sections with H&E. IgG was used as a control and showed no immunoreactivity (data not shown). COX-2 expression was not detected in the sections containing normal pancreas. In cancer sections, COX-2 is upregulated when compared to the normal pancreas and positive staining is found in the ductal compartment of the pancreas (x400 magnification). (2) RT-PCR showing the expression of COX-2 in PSC. (3) Liquid Chromatography tandem mass spectrometry showing the levels of eicosanoids intracellularly; cell pellet was collected and subjected to LC MS MS to determine the concentration of eicosanoids. The media bathing PSC was collected and subjected to LC MS MS to determine the levels of eicosanoids extracellularly.

PGE2 stimulates migration, invasion in PSC

To determine the effects of PGE2 on the functions of stellate cells, I looked at phenotypic changes in PSCs caused by PGE2 treatment *in vitro*. First, the migration potential of PSCs was assessed using a conventional Boyden Chamber assay, with the control or PGE2 in different concentration was added to the bottom chambers to determine if PGE2 acts as a chemoattractant that induces PSC migration. I treated PSCs with PGE2 *in vitro*. PSC re-suspended in 0.5% serum containing media (2×10^4) was plated on migration chambers with and without PGE2 (0-1000nM). After 22hrs, cells migrated to the bottom side of the chamber were fixed with methanol and stained with hematoxylin and number of cells migrated or invaded was counted on at least 10 fields looking under the microscope. Exogenous addition of PGE2 induced a dose dependent significant increase in PSC cell migration (Figure 2.2) *in vitro* with an optimal concentration being 100nM of PGE2. Next, I looked at the effects of PGE2 on the invasion potential of PSC and its ability to penetrate through Matrigel, using a modified Boyden Chamber assay. PSCs were plated at a confluency of 2×10^4 cells per well (upper chamber) after being incubated in near starvation conditions (0.5% serum containing media) for 24 hours. The bottom chambers contained different concentrations of PGE2 or the vehicle. 10% FBS was used as a positive control. After 22hrs, cells invaded through the matrigel matrix to the bottom side of the chamber were fixed with methanol and stained with hematoxylin and number of cells invaded was counted on at least 10 fields looking under the microscope.

The wells that did not have PGE2 showed no PSC invasion. However, PGE2 increase PSC invasion at concentrations ranging from as little as 25nM of PGE2 to 1000nM PGE2 after 22 hours (Figure 2.3). The maximum invasive effects were detected at 100nM PGE2. Therefore, PGE2 stimulates both PSC migration and invasion in a dose dependent manner compared to the untreated control.

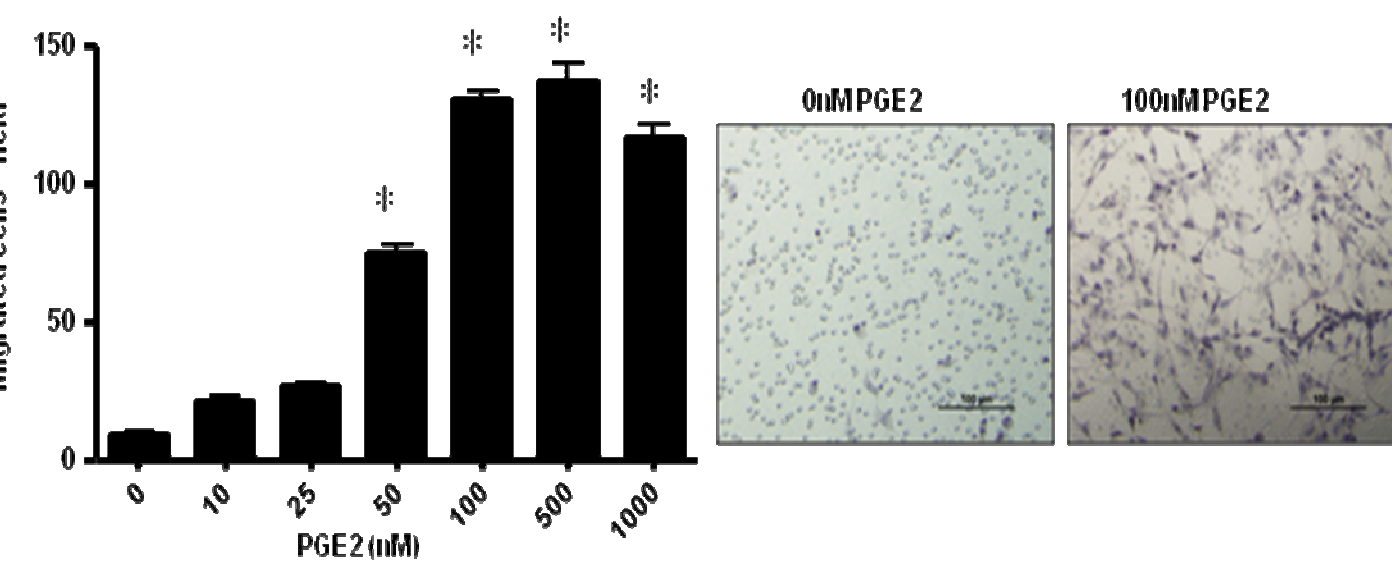
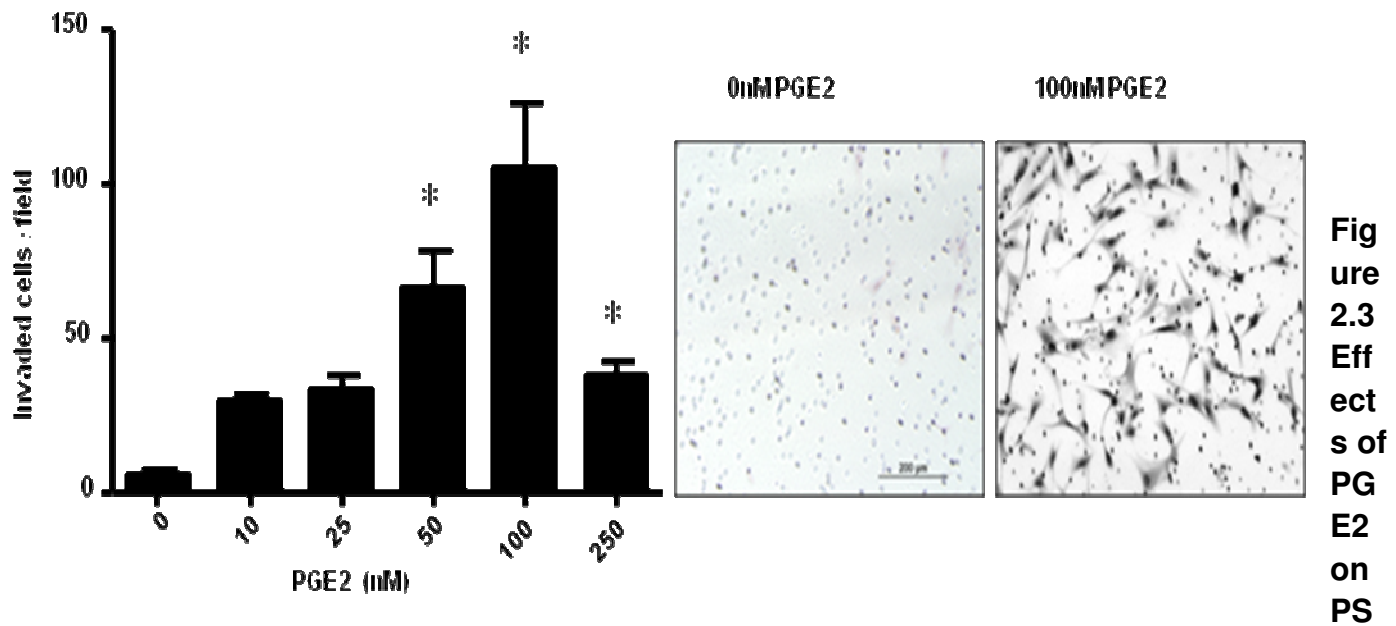


Figure 2.2 Effects of PGE2 on PSC migration PSC migration was evaluated using a Boyden chamber assay. PSC (20×10^3 cells/well) were plated in the upper Boyden Chamber in serum free media. The lower wells contained 0,10,25,50,100,500,1000nM) of PGE2. Cells were stained with H&E and the number of cells migrated was calculated. PSC stimulated with PGE2 for 6 hours (data not shown *, $P < 0.05$)



C invasion PSC invasion was assessed by modified Boyden chamber assay. PSC (20 x 10³ cells/well) were plated in the upper chamber of the apparatus and different concentrations of PGE2 were added to the lower chambers. Cells were stained by H&E and cell invasion through matrigel was calculated. P<0.05 versus control.

PGE2 stimulates changes in PSCs gene expression

In order to see if stimulation of PSC with high levels of PGE2 affects stromal gene expression, PSCs were incubating in 0.5% serum containing media for 24 hours for serum starvation, next RNA was extracted from PSC treated with PGE2 (100nM) and control treated with the vehicle (DMSO) and converted to cDNA. Using RT-PCR with specific primers for each gene, I looked at changes in the expression of the following genes: HSPG2, vimentin, fibronectin and elastin (Figure 2.4.1) which are known to be involved in fibrosis and were found in microarray studies from our laboratory to be high in the stroma, therefore called “stromal genes”. This data shows that PGE2 addition to PSCs stimulated the expression of the above mentioned genes therefore indicating the stimulatory effects of PGE2 on stromal gene expression.

To further investigate the role of PGE2 in PSCs activation, changes to the expression pattern of genes involved in matrix turnover were assessed. I looked at changes in the expression of a panel of MMPs and TIMPs after PGE2 treatment compared to the untreated control (Figure 2.4.2). I found that PGE2 addition, increases PSC gene expression of MMP-2, MMP-3, MMP-9, and TIMP-1 whereas the expression of MMP-11 and TIMP-2 remained unchanged.

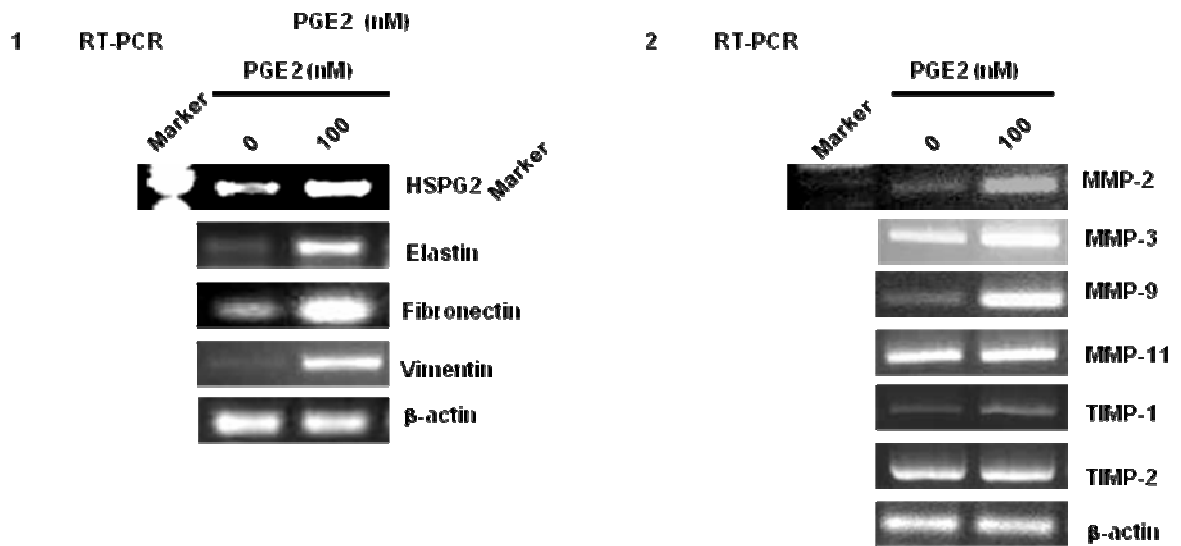


Figure 2.4 Effects of PGE2 on PSC gene expression: (1) RT-PCR showing the expression of HSPG2, Elastin, Fibronectin, Vimentin before and after stimulation with PGE2. 100nM PGE2 was added to PSC after over night serum starvation and left for 24 hours. B-actin was used as a control. (2) RT-PCR the expression of MMP-2, 3, 9, 11 and TIMP-1, 2 before and after stimulation with PGE2. 100nM PGE2 was added to PSC after over night serum starvation and left for 24 hours. B-actin was used as a control. *, $P < 0.05$

PGE2 stimulates the proliferation of PSC

To examine the role of PGE2 on PSC proliferation and growth, PSC were incubated in near starvation conditions for 24 hours and treated with PGE2 in doses ranging from 10nM to 1000nM. As a negative control, the vehicle for PGE2, DMSO was added to PSC separately. PSC proliferation was analyzed by means of a non radioactive MTS assay after 0, 24, 48 hours of PGE2 treatment. PGE2 addition to PSC induced an increase in proliferation seen at 100nM PGE2 added for 24 hours (Figure 2.5). Results were confirmed by the cell counter and hemocytometer proliferation assays (data not shown here). There was no significant difference between addition of PGE2 for 24 hours or 48 hours (data not shown).

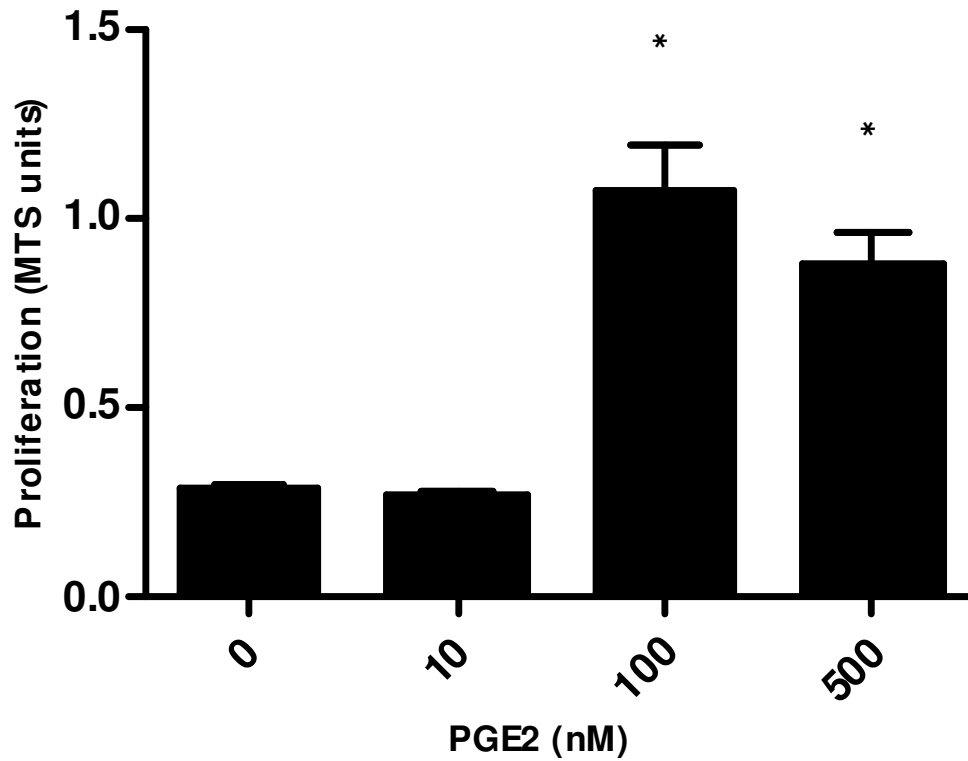


Figure 2.5 Effects of PGE2 on PSC Proliferation PSC proliferation was measured by MTS assay. PSC (1000 cells) were plated on a 96-well plate and serum starved overnight. PGE2 (10,100,500 nM) were exogenously added to the plates and estimated after 24 hours by MTS assay. PGE2 stimulates the proliferation of PSC*, $P < 0.05$

PGE2 did not affect PSC apoptosis

To determine the effects of PGE2 on PSC apoptosis, I looked at PGE2 induced PSC apoptosis by propidium iodide (PI) staining. I analyzed by FACS the level of apoptosis of PSC treated with 0, 50 and 100nM PGE2 over 24 and 48 hours (data for 48 hours is not shown). The percentage of PSC stained with PI and analyzed by FACS did not change when PGE2 was added to the cells, therefore PGE2 did not affects the apoptosis level of PSC (figure 2.6). No effects of PGE2 treatment was observed on the fraction of the cells underdoing DNA synthesis stage of the cell cycle (data not shown).

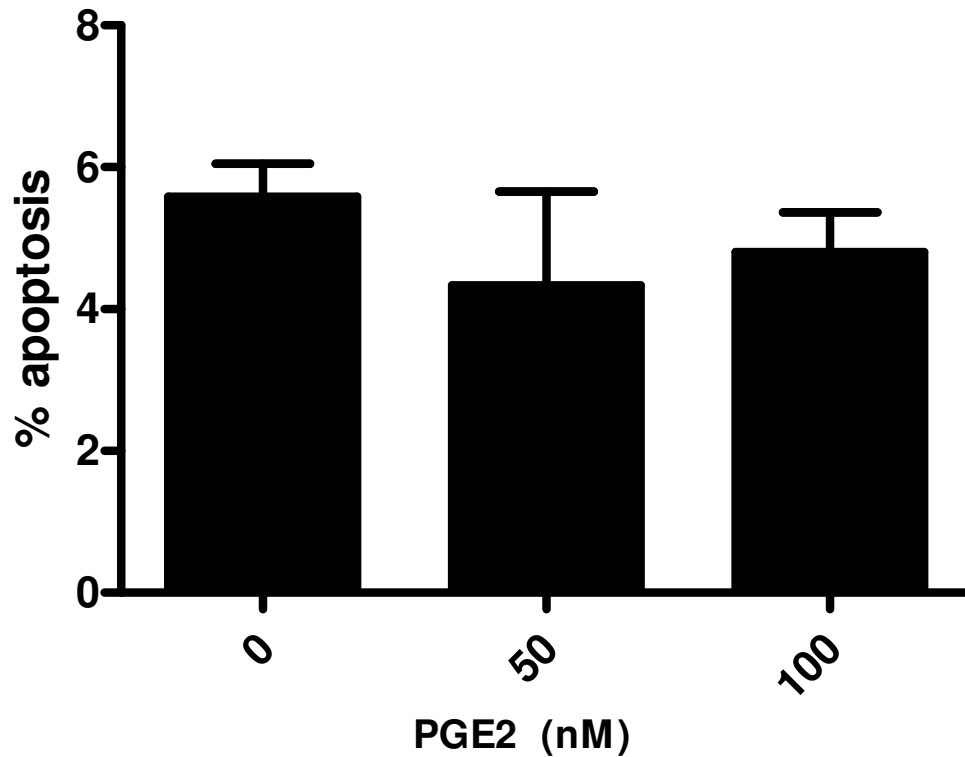


Figure 2.5. Effects of PGE2 on PSC survival and apoptosis PSC apoptosis and survival was assessed by FACS analysis and PI staining. PSC were treated with 0 and 100 nM PGE2 for 24 hours and subjected to propidium iodide staining. Representative experiment is shown. Statistics were performed as described in methods. No statistical difference between PGE2 treated cells and the negative untreated control. *, $P < 0.05$

DISCUSSION

PSCs have recently become the focus of much attention due to their importance in PDAC. However, these cells also play a critical role in CP. In both diseases PSCs become activated and produce an abundant desmoplastic response. Many factors are known to regulate PSC activity including regulatory molecules such as TGFb, PDGF, CTGF and others. Studies have shown that addition of TGFb results in an increase in PSC activation and subsequent increase in contractility, matrix degradation, proliferation and MMP and TIMP production(150, 151) Furthermore, studies have shown that COX-2 is high in both CP and PDAC therefore suggesting a potential role in stromal hyperactivation related associated with PDAC. Many studies have shown that COX-2 levels positively correlates with the aggressiveness of PDAC (141) (96) and that blocking COX-2 (152) significantly reduces the tumor size and metastatic potential. There have been suggestions that the activity of COX-2 and prostaglandins influence PSC (153). However, the role of COX-2 and PGE2 has not been fully assessed in the stroma of PDAC. This study aimed at identifying the functional role of COX-2 and its downstream effector PGE2 in the stroma, particularly by examining PGE2 dependent alterations in the profibrogenic phenotype of PSC. I have dissected the stromal hyperactivation related associated with PDAC into five major responses that contribute to the development of the fibrogenic phenotype that follows quiescent PSC activation in response to inflammation; proliferation, migration, penetration (invasion),

alteration of matrix composition (over expression of stromal genes) and apoptosis. A major aspect of fibrosis is the changes in extra cellular matrix composition. During fibrosis, the matrix composition shifts from a normal matrix rich in collagen IV rich basal lamina to a fibril forming collagen I. These findings show that upon stimulation with PGE2 serum starved PSC highly increase their expression of several genes involved in matrix formation such as HSPG2, fibronectin and elastin. Matrix MetalloProteases (MMPs) are also involved in fibrosis, particularly MMP-2 and MMP-9. MMP-2's primary function is the degradation of Collagen IV. The following step in the fibrotic cascade is the migration of PSC to the newly formed basement membrane rich in collagen I and the penetration, or invasion through the matrix. Our data show a timely increase in both PSC migration and invasion following stimulation with PGE2. MMP-2 and MMP-9 degrade the extra cellular matrix thereby facilitating the penetration of the cells through the basement membrane. Our data also show that PGE2 increases the expression of MMP-2 and MMP-9 in serum starved PSC. PGE2 mildly stimulated the proliferation of PSC, however, the role of proliferation in already activated PSC is debatable. It has been suggested that stellate cells that make up the fibrotic reaction do not all derive from quiescent stellate cells. Stellate cells also derive from bone marrow and epithelial mesenchymal transitions(154) .

Until now the specific effects of prostaglandins on PSC has not been identified. I observed that there are several potential sources of prostaglandins within the microenvironment of PDAC including PDAC cells as well as PSC

themselves. I also found that PGE2 stimulated PSC functions including migration, proliferation, invasion and expression of genes such as MMPs and TIMPs which are likely to determine whether fibrosis recedes or proceeds (155, 156).

This study also examined the role of PGE2 in apoptosis of PSC. I found that PGE2 does not stimulate apoptosis of PSC. Furthermore, treatment with PGE2 caused an increase in cell proliferation. The importance of PSC- PDAC has recently been the focus of many studies. Recent studies have shown that PSC promoted the progression of PDAC (157). The co injection of PSC and low numbers of PDAC cells in a nude mouse model resulted in 100% tumor incidence, as opposed to 57% when the cancer cells were injected alone. Although the role of importance of the stroma in PDAC is well known, the molecular mechanisms that regulate stromal activity and the downstream signaling involved are poorly understood. This study provides evidence for the first time that PGE2 can induce major changes in the “fibrogenic response” associated with PDAC and CP. These changes include contribution to the formation of the “fibrotic” matrix by changing its composition, migration, invasion as well as changes in gene expression. Therefore, prostaglandin E2 is responsible for inducing pro fibrogenic changes in pancreatic stellate cells.

Furthermore, it has been shown that TGF- β secreted by the cancer cells activates quiescent stellate cells. (158) Candidate genes that mediate tumor stroma interactions have been identified by gene expression profiling. Cyclooxygenase-2 (COX-2) was one of the genes that was found to be high in

both PDAC and CP. Studies have shown the COX-2 pathway to be an important target in the treatment and prevention of many cancers including pancreatic cancer (159). A study done has shown COX-2 gene and its primary and most important metabolite prostaglandin E2 (PGE2) to be expressed in over 90% of pancreatic (160) suggesting that COX-2 maybe a target for chemoprevention and treatment of PDAC. Many studies support this theory, however, the limited success of COX-2 inhibitors in the clinic mainly because of the cardiovascular and renal side effects (152, 161), urges us to look for a more specific target in the COX-2 pathway.

**CHAPTER THREE: EP4 RECEPTOR IS REQUIRED FOR PGE2 MEDIATED
PSC HYPERACTIVITY**

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related death in the U.S. Currently no effective treatments for PDAC and the dense desmoplasia surrounding it exist. The prognosis of PDAC is dismal with a 5 year survival rate of 5%. The dense desmoplastic reaction around the tumor mass is a major characteristic of pancreatic cancer. Numerous reports in the literature suggest that the tumor-stroma interactions in the pancreas contribute to progression and metastasis. Until now, the focus has been on identifying therapy potentials that targeting the actual tumor disregarding the importance and contribution of the stroma. No therapies against pancreatic desmoplasia are available. Numerous reports had shown that cyclooxygenase-2 (COX-2) was upregulated in the majority of pancreatic cancer patients (92), this over expression was also associated with advanced tumour stage and correlates with poor prognosis. In the pancreas, the fibrotic process is associated with inflammation and high levels of COX-2 activity. Microarray data from our laboratory identify COX-2 gene as one of the genes that might play a role in stromal hyperactivation. COX-2 levels were low in normal pancreas tissue however; COX-2 levels increase in chronic pancreatitis and increase further more in PDAC samples therefore, suggesting a role of COX-2 in the fibrotic compartment of PDAC. Treatment with NSAIDs like

celecoxib, which block COX-2 activity have shown efficient reduction of tumorigenicity in vitro. Furthermore, pancreatic cancer patients treated with selective COX-2 inhibitors have presented significant reduction in tumor size and increased survival. While many studies confirm the chemopreventive effects of COX-2 inhibitors, the side effects caused by long term therapy with COX-2 inhibitors such as cardiovascular and renal events are even clearer (118, 120, 146).

The chemopreventive and anti tumorigenic functions of NSAIDs are due to their inhibition of prostaglandin synthesis by COX mostly, PGE₂. PGE₂ has been reported to be the COX-2's most biologically active product. Most of COX-2 functions have been attributed to PGE₂. In humans, four receptors called EP receptors have been identified as mediators of PGE₂ activity. EP_{1,2,3} and 4 are localized to the plasma membrane and the binding of PGE₂ to each receptor activates specific downstream signaling pathways (162).

EP₁ receptor binds a G-protein which increases intracellular Ca²⁺ levels and IP₃ levels. EP₂ and EP₄ receptors are G_s protein-linked that increase cyclic AMP (cAMP), on the other hand, EP₃ receptor is G_i linked and blocks cAMP increase. Therefore, the effects of PGE₂ greatly depend on downstream signaling that starts with second messenger response (104). Recently, studies have shown that EP receptors play a role in colon carcinogenesis (163) and in carcinomas of endometrial, breast, and lung (113, 135, 164).

Despite of the importance of EP receptors in cancer progression and even though EP-2 receptor has been identified to be crucial for pancreatic cancer cells, the role EP receptors in pancreatic desmoplasia or PSC biology has not been identified. PGE2 modulates a wide array of biological functions essential to tumor growth, progression and survival (90, 165, 166). Therefore, there is a strong rationale to understand the mechanisms behind PGE2 functions and the downstream targets of COX-2/PGE2. Selective COX-2 inhibitors have shown great promise in treating pancreatic cancer but their adverse side effects are not suitable for long term use (121, 167) therefore, it is clear that the attention must shift from focusing on COX-2/PGE2 to targeting more downstream effectors such as EP receptors as a potential therapeutic target. Based on these findings, I sought to determine the functional role of EP receptors on the stromal compartment of pancreatic cancer in vitro by looking at the biology of PSC.

The aim of this study was to identify the functional role of each EP receptor in mediating PGE2 dependent stimulation of PSC. I examined the mechanism behind PGE2 stimulation of PSC activity by silencing each receptor to identify the role each plays in mediating the changes inflicted by PGE2. Furthermore, I explored blocking EP receptors by treating PSC with specific antagonists against each receptor and measured changes in migration, invasion and gene expression. This study uses two different approaches to identify the functional role of each receptor in PSC biology; siRNA silencing and receptor antagonism. The results of this study were very promising as blocking only EP4 receptor resulted in a dramatic reduction of PSC activity to a near basal level. In

this study, I have identified for the first time the receptors that mediate the effects of PGE₂ on pancreatic stellate cells. By siRNA mediated silencing, I have identified EP4 as the receptor responsible for PGE₂ mediated effects on PSCs. Knowledge of the roles of the specific receptors will aid in the identification of appropriate targets for therapeutic development against pancreatic fibrosis. Thus, this study provides a better understanding on regulation of stellate cells by PGE₂ and further the role of stellate cells on pancreatic stromal hyper stimulation. This study also identifies EP4 as a mediator of PGE₂ activity that is required for PSC hyper activation therefore, EP4 could be an alternative potential therapeutic target to reduce COX-2 mediated fibrosis associated with chronic pancreatitis and pancreatic cancer.

MATERIALS & METHODS

Materials

Culture media and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Rockville, MD). PGE₂, was obtained from Cayman Chemicals.

Cell Culture

Primary human pancreatic stellate cells (PSC) were isolated using the outgrowth method from pancreatic adenocarcinoma samples from patients undergoing surgical resection and were immortalized (68) (Hwang RF, 2008). PSCs were maintained at 37°C in a humidified atmosphere of 5% CO₂ and were grown in 10% DMEM containing 1% antibiotic. PGE₂, EP1 antagonist (cat # SC18220) and EP2 antagonist (cat # AH 6809) were purchased from Cayman Chemicals (Ann Arbor, MI). The EP4 antagonist (ONOAE3208) was obtained from ONO pharmaceuticals (Osaka, Japan).

Invasion and Migration assays

For studies of cell invasiveness, BIOCOAT Matrigel invasion chambers (BD Biosciences, Chicago, IL) were used. Briefly, 2×10^5 cells in 100 μ l of serum-free medium were added to the upper chamber and different concentrations of PGE2 (1–1000 nM) in 0.5% serum containing DMEM were added into the lower chamber. The cells were allowed to invade the Matrigel for 22 h at 37°C in a 5% CO₂ atmosphere. DMEM containing 0.5% serum was used as control. The non-invading cells on the upper surface of the membrane were removed with a cotton swab and the invading cells on the lower surface of the membrane were fixed and stained with a Diff-Quick stain kit (BD Biosciences), washed twice with water and air-dried. Invading cells in three adjacent microscope fields for each membrane were imaged at 20x magnification. To assess cellular migratory potential, the protocol described above was used, except that migration chambers devoid of matrigel was used (BD Biosciences, Chicago, IL). Experiments were performed at least in triplicate, and the results were shown as mean \pm SD of three independent experiments

Reverse transcription-PCR

Total RNA was isolated from PSC with and without siRNA transfection and the quality of RNA was tested as mentioned previously. DNase was used to remove contaminating genomic DNA and RNA purification. Quality of the RNA was

confirmed by running on a denaturing gel, and we have observed clear 28S and 18S rRNA bands. A non reverse transcribed control was used to assure that no genomic DNA was amplified. Primers were designed for human

EP1 (5' ATCGCTTCGGCCTCCACCTTCTTT 3') (3' GCCAGCGCCACCAACACCA 5'),

EP2 (5' CTCGCTGCCGCTGCTGGACTATGG 3') (3' GCAGGCGAGCACCGAGACAATGAG 5'),

EP3 (5' GGCGCTGGCGATGAACAACGAG 3') (3' GGCGCTGGAGATGAACAACGAG 5'),

EP4 (5' CCGCCCCCAGGTAGCCAGGAG 3') (3'TGCGGGAGGACAGCGTTCAGGT 5'),

MMP-2 (5' CTTCTTGTCGCGGTCTAGTCCTC3') (3' TGGCGATGGATACCCCCTTGA5') ,

MMP-9 (5' GCGCTGGGCTTAGATCATTCTCA 3') (3' GCAGCGCGGGCCATTGTC 5') ,

The amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide. Primers designed for β -actin (Genbank BC_016045), which was used as a loading control for the PCR reactions, were forward 5' ATG ATA TCG CCG CGC TCG TCG TC 3' and reverse 5' CGC TCG GCC GTG GTG GTG AA 3'.

Transient transfection of small interfering RNA

PSC was plated on 100-mm dishes and transiently transfected with siRNAs- siControl (siRNA ID #4611 Ambion INC Austinm TX) and siEP2 and EP4 (siRNA IDs # 5732, # 5734, Qiagen, Valencia, CA) at a final concentration of 10 nmol/L (Dharmacon, Inc.) with Hiperfect transfection reagent (Qiagen, Inc.), and lysates were prepared for RT-PCR after 72 h.

Statistical analysis

All experiments were conducted in triplicates and carried out on three or more separate occasions. Data presented are means of the three of more independent experiments +/- standard error mean (SEM). Statistical analysis was done using GraphPad Prism (GraphPad Software). Comparisons were made using two-tailed Student's *t* test and significant difference was defined as $P < 0.05$. Data are shown as mean \pm SE.

RESULTS

Evaluation of EP receptors subtype expression in PSC

There are four potential receptors that mediate PGE2 activity that are known to bind PGE2 with high affinity, EP1-4. For a preliminary screen of the EP receptor profile in PSC, a set of novel primers for EP1-4 were designed using Primerselect software, to amplify fragments of each EP receptor by reverse

transcription-PCR (RT-PCR; each according to Genbank annotations nm-000955, nm-000956, nm-000957, and nm-000958 for EP1-4, respectively. The expression of EP receptors in PSC cells was determined by RT-PCR using specific oligonucleotide primers. Each of the four bands in Figure 3.1 corresponds to the expected PCR product sizes. EP1, 2, 3 and 4 are clearly expressed in PSCs (Figure 3.1).

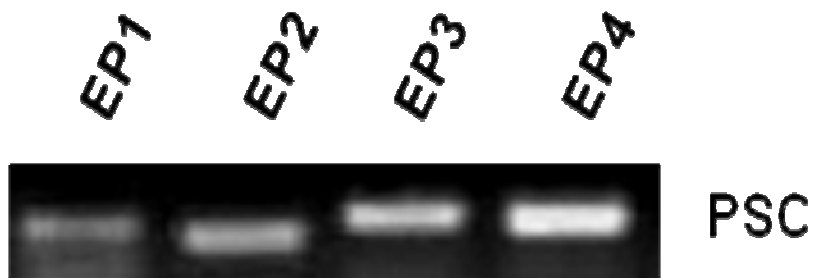


Figure 3.1. EP Receptor expression. RT-PCR showing the expression of EP1,2,3,4 in PSC. All four EP receptor subtypes are expressed in PSC

EP4 receptor is required for maintaining PSCs migration and invasion

Having shown that all four EP receptors are expressed in PSCs, it was of interest to determine how silencing of each EP receptor, starting with EP2 and EP4, affects PSC profibrogenic phenotype. To elucidate the functional role of EP receptors and the mechanism behind the phenotypic changes in PSC caused by exogenous PGE₂, I silenced each of the EP receptors EP2 and EP4. RT-PCR shows that cells treated with siEP2 or siEP4 respectively show a reduction in mRNA levels of EP2 and EP4 respectively compared to the siCONTROL (Figure 3.2.1, Figure 3.2.2). Therefore, siRNA treatment against either EP2 or EP4 receptor is successful in inducing silencing and that the transfection with siRNA significantly reduced the levels of either EP2 receptor or EP4 receptor on PSC.

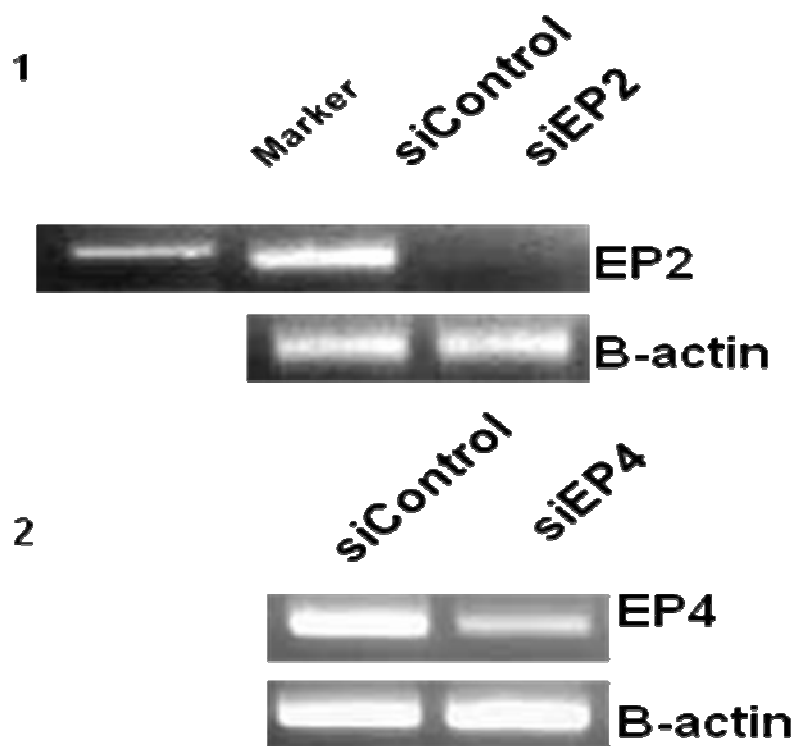


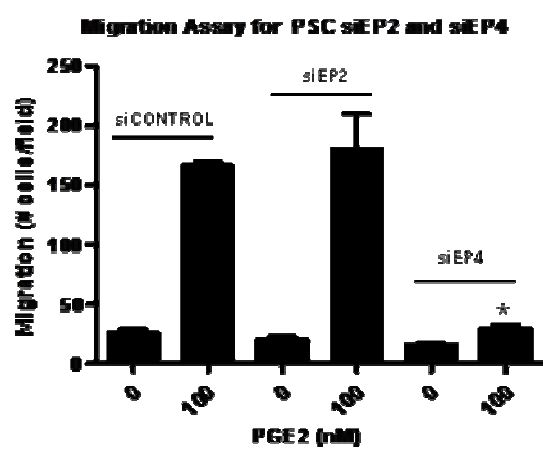
Figure 3.2 EP silencing by siRNA: PSC were transiently transfected with human siControl and siEP2 (1) or siEP4 (2) using Hiperfect reagent and after 72 hours cells were harvested and RNA was extracted. RT-PCR shows the effects of silencing EP2 and EP4 receptors in PSC. B-actin served as a control for RT-PCR.

To identify the role of EP receptor signaling in mediating PGE₂ actions in PSC, I investigated the effects of silencing of EP2 and EP4 receptor on PGE₂ mediated functions. To examine the effects of independently silencing EP2 and EP4 receptor, I looked at the migration of PSC. PSC were plated in serum containing media, one day later, the siRNA complexes specific for EP2, EP4 and siCONTROL were added to the cells. After a total of 48 hours after silencing, PSC were trypsinized and counted. 20 000 cells were plated in the top chamber of the Boyden Chamber migration apparatus in serum free media. Either PGE₂ or the vehicle, DMSO were added to the bottom chambers to assay the migratory potential of PSC in response to PGE₂ after silencing EP2 or EP4. After 22hrs, cells migrated to the bottom side of the chamber were fixed with methanol and stained with hematoxylin and number of cells migrated or invaded was counted on at least 10 fields looking under the microscope. PSC treated with siRNA against EP2 receptor did not show significant reduction in the rate of migration (Figure 3.3.1). The migrated cell number was not significantly different from the control. However, PSC treated with siRNA against EP4 receptor showed a dramatic reduction in PSC migration (82 ± 2.906 N=3 migrated cells per area) compared to the siCONTROL (166.0 ± 4.359 N=3 migrated cells per field) ($P < 0.05$) (Figure 3.3.1). Therefore, EP4 silencing completely blocked the migratory effects of exogenous PGE₂ treatment. Thus, migration of human pancreatic stellate cells induced by the addition of PGE₂ is predominantly mediated through EP4.

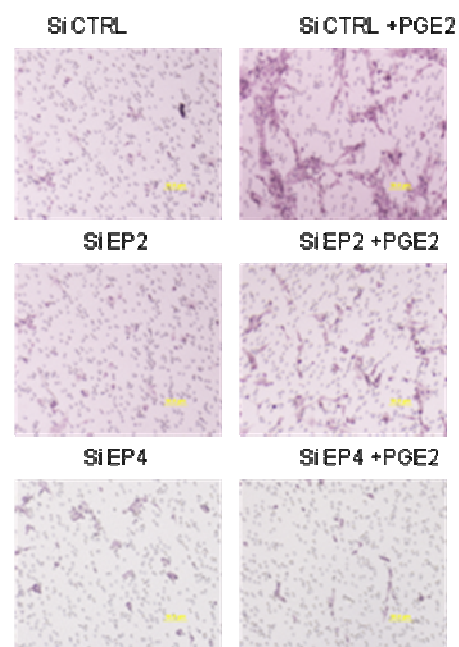
In order to determine the functional role of EP2 and EP4 receptors on PSC invasion, a Boyden Chamber apparatus that uses Matrigel as the matrix was used. PSC were transfected with a scramble siRNA (siCONTROL), siRNA against EP2 or EP4 receptors as described in the methods section. After a total of 48 hours, PSC were trypsinized, counted and plated at a rate of 20 000 cells/well in the upper chamber of the modified boyden chamber apparatus. The bottom wells contained 100nM of PGE2 or DMSO as a control. After 22hrs, the cells invaded to the bottom side of the chamber were fixed with methanol and stained with hematoxylin and number of cells migrated or invaded was counted on at least 10 fields looking under the microscope. Transfection of PSC with siRNA against EP2 receptor or siCONTROL did not affect PSC invasion (Figure 3.3.2). However, silencing of EP4 receptor greatly reduced PSC cell invasion through matrigel (41.33 ± 10.41 N=3 invaded cells per field) compared to the control (118.3 ± 13.02 N=3 invaded cells per field) ($P<0.05$) (Figure 3.3.2). Silencing of EP4 receptor but not EP2 receptor reduced both PSC migration and invasion

These data collectively suggest that the effects of PGE2 on PSC migration and invasion are mediated by the EP4 receptor and not by EP2 receptors. Silencing of EP4 receptor reduced both migration and invasion of PSC to a near basal level.

1



2



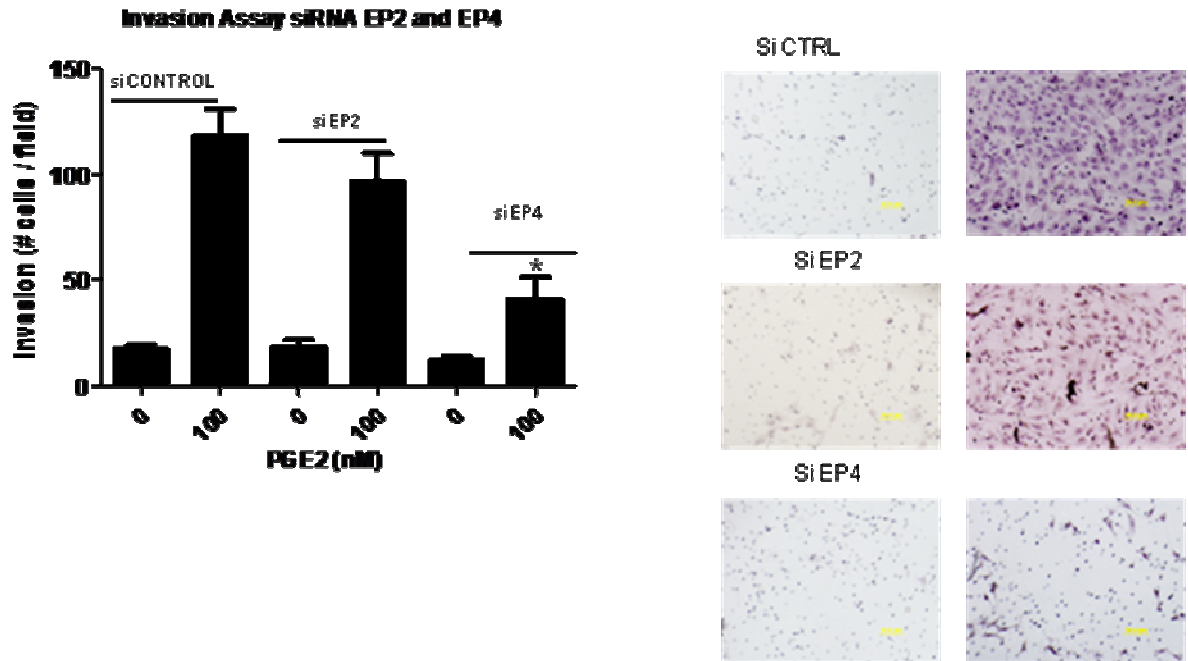


Figure 3.3 Effects of EP2 and EP4 silencing on PSC PSC were transfected with siCONTROL, siEP2 or siEP4 serum starved and plated in the top chamber of (1) a migration boyden apparatus, (2) invasion apparatus (30 x 10³ cells/well). 100nM PGE2 or the control were added to the bottom chambers. Cells were counted and cell migration (1) and invasion (2) were assessed. *, P<0.05

Enhanced expression of MMP-2 and MMP-9 is mediated by EP4 receptor

To further examine the functional role of EP2 and EP4 receptors in PSC, changes in the expression of stromal genes, identified by our microarray data as genes highly expressed in the stroma of PDAC, were studied. PSC were treated with siCONTROL, siEP2 and siEP4 separately, for 48 hours. After a total of 48 hours of siRNA treatment, 100nM PGE2 or DMSO (control) was added to the cells. PSC were incubated for an additional 24 hours with PGE2 and RNA was harvested. I compared cDNA from PSC treated with siCONTROL, siEP2 and siEP4 both treated with PGE2. Results show that PSC treated with siCONTROL or siEP2 showed no change in gene expression of MMP2 or MMP9. However, PSC treated with siEP4 had a strong reduction in the mRNA levels of MMP2 and

MMP9 (Figure 3.4). Silencing of EP2 did not have a measurable effect on expression of these genes in the presence or absence of PGE2. These data generated using siRNA silencing of EP2 and EP4 receptor identify EP4 receptor as the sole mediator of PGE2 activity in PSC. Furthermore, it presents EP4 receptor as “ the” receptor required by PSC to maintain the PGE2 dependent profibrogenic phenotype which included increased migration, invasion and gene expression.

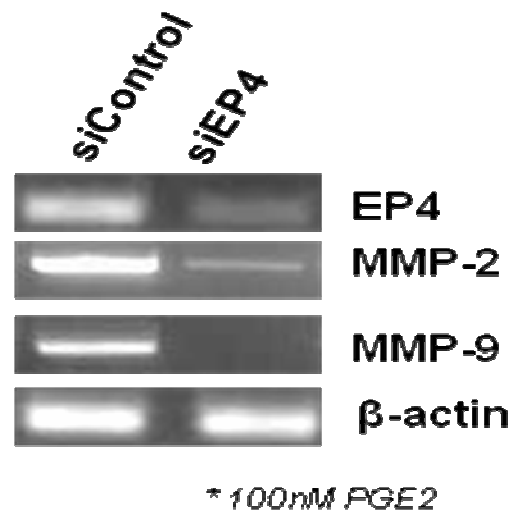


Figure 3.4 RT-PCR showing the silencing effects of siEP2 and siEP4 on PSC gene expression. PSC transfected with siRNAs (5nM) showed a significant reduction in the expression of COL1a1, MMP2 and MMP-9 genes as shown by RT-PCR using specific oligonucleotide for MMP-2 and MMP-9 respectively. B-actin was used as a loading control

Treatment of PSC with EP4 antagonist reduces the hyper activation of PSC

To evaluate the role of EP1, EP2 as well as provide further confirmation for the role of EP4 receptor, specific antagonists for EP1, EP2 and EP4 receptors were used. PSC were plated in the migration chamber of the Boyden Apparatus then 10uM of each antagonist was added to the lower chambers 1 hour prior to PGE2 treatment in serum free media. Treatment of PSC with antagonists for either EP1 or EP2 receptor did not modify cell migration as seen quantitatively and qualitatively by DAPI staining (Figure 3.5.1). Treatment of PSC with a specific antagonist against EP4 receptor show a significant reduction in cell migration from 239.3 ± 30.99 N=3 cells per field to 12.00 ± 5.292 N=3 cells per field ($P<0.05$). To examine whether EP1 and EP2 receptors affect PSC invasion, PSC were treated independently with specific antagonists against either EP1, EP2 receptors. Cell invasion was measured and addition of either antagonist for EP1 or EP2 did not modify cell invasion, Addition of EP4 antagonist strongly reduced PSC invasion from 188.0 ± 16.17 N=3 cells per field to a near basal level with only 20.67 ± 2.333 N=3 cells per field ($P<0.05$) (Figure 3.5.2). These results clearly demonstrate by two independent methods that EP4 receptor is required for PGE2-dependent PSC stimulation. Therefore, blocking stromal EP4 receptor could be of therapeutic value for both chronic pancreatitis and the desmoplasia associated with PDAC.

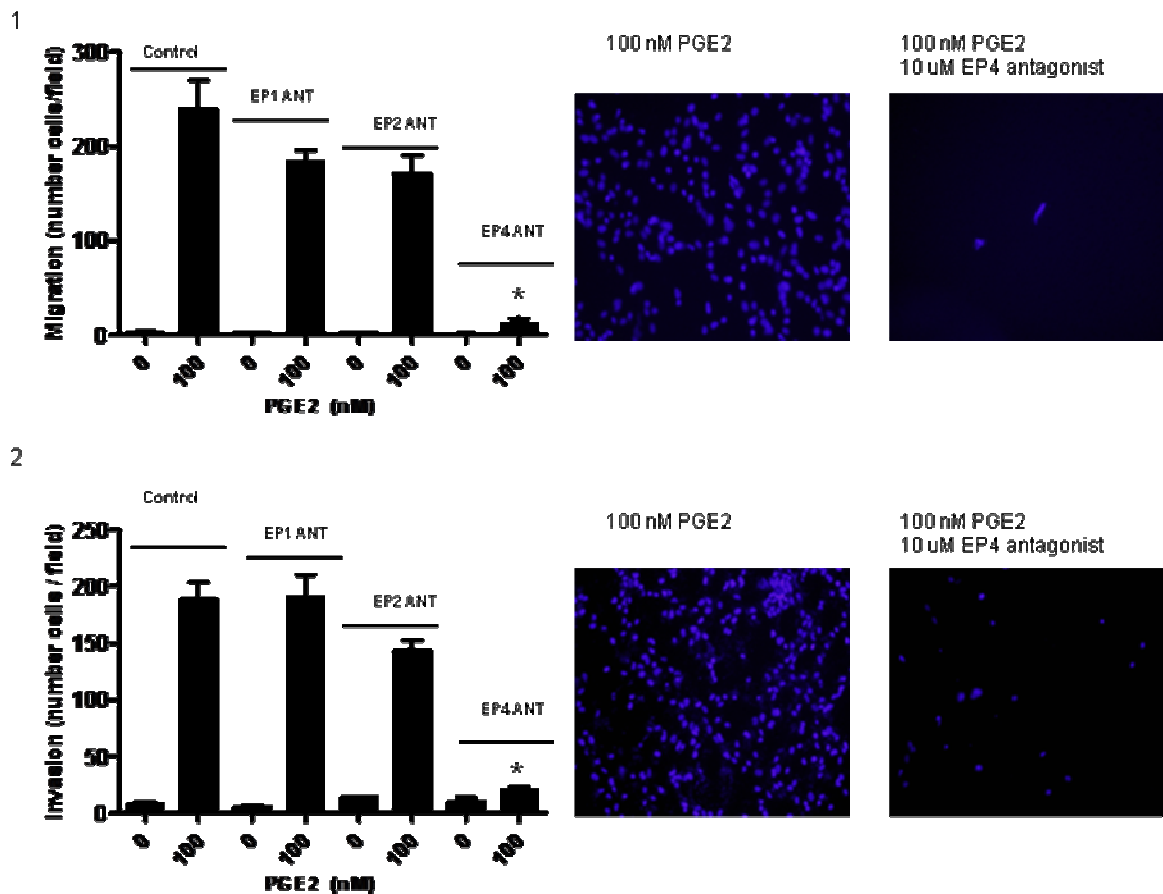


Figure 3.5 Effects of specific antagonists for EP1, EP2 and EP4 on PSC

PSC plated at 70% confluency and allowed to settle overnight. Serum containing media was replated with serum free media for 24 hours before addition of EP1, EP2 or EP4 antagonists at a dose of 10uM. 1 hour after the antagonist addition PGE2 was added to the cells for 24 hours. Cell migration and invasion were assessed by counting the number of cells that penetrated the migration membrane (1) or matrigel (2). *, $P < 0.05$

DISCUSSION

COX-2 over expression in pancreatic tumors is both biologically and clinically important (42, 46, 53) and the use of selective COX-2 inhibitors have shown great promises (168, 169). However, because of the long term side effects associated with COX-2 inhibitors, there is now an urgent need to identify targets downstream of COX-2 that mediate COX-2 activity in the hopes of identifying safer and more effective strategies to improve the overall prognosis of pancreatic cancer patients. Studies have attributed the majority of COX-2 activity and biological functions to PGE₂ (170). Four subtypes of membrane PGE₂ receptor have been identified. They are called prostanoid receptors EP_{1,2,3} and 4. The relative contribution of each of these receptors to key signaling events has not been fully elucidated. The role of the COX-2/PGE₂ pathway in the regulation of the stroma of PDAC has not been studied. This study provides evidence for the first time that PGE₂ EP receptors play a major role mediating pancreatic stromal hyperactivation and aims at targeting downstream effectors of COX-2 as an alternative way to reducing stromal hyperactivation associated with CP and PDAC while avoiding the unwanted effects of COX-2 inhibitors.

Since there are currently no studies that investigate the role of EP receptors in the stroma of pancreatic cancer, the first part of this study was to determine the expression of EP receptors in PSC. I found that all of the EP receptor subtypes are expressed in PSC.

The four EP receptors that mediate PGE2 functions have been shown to control many aspects of cancer such as invasion, migration, and growth (163). Numerous reports have shown that blocking EP receptors as an alternative way to blocking COX-2 could prove to be beneficial in the clinic (163). There are no studies on the role of EP receptors in pancreatic desmoplasia or in PSCs. The proneoplastic roles of EP2 and EP4 receptors have been reported in several cancers (103, 110, 112, 117, 130, 134). Therefore, to evaluate the role of each EP receptor subtype in PSCs, I looked at EP2 and EP4 receptors first.

Using siRNA mediated gene silencing I determined the receptor that mediates the profibrogenic functions of PSC. The data from this study shows that transfection of PSC with siEP2 did not show a significant reduction in PSC migration. On the other hand, silencing of EP4 receptor causes a dramatic drop in the migratory rate of PSC. Next, I assessed the role of EP2 and EP4 in PSC invasion. Similarly, silencing of EP2 receptor did not reduce PSC invasion through matrigel. However, when EP4 was silenced, PSC invasion was reduced to a near basal level even in the presence of PGE2. I also looked at the expression of MMP-2 and MMP-9. Silencing of EP4 only and not EP2 receptor reduced the mRNA gene expression of MMP-2 and MMP-9. Therefore, PGE2 mediated MMP-2 and MMP-9 over expression is EP4 dependent. These results clearly identify EP4 receptor as required for PSC to be able to migrate, invade and alter the matrix composition. To examine the role of EP1, EP3 and further confirm these finding, I treated PSC with specific antagonists against EP1, EP2 and EP4 receptors and found that inhibiting EP4 receptor only diminished PGE2

stimulation of PSC. EP1 or EP2 antagonists were added to PSC prior to PGE2 treatment, and no changes in PSC migration, invasion or MMP-2/MMP-9 expression was detected. However, treatment of PSC with the EP4 antagonist greatly reduced migration, invasion and gene expression of PSC. These data provide for the first time EP4 receptor as a potential target for the regulation of pancreatic stroma formation that may be useful in treatments of pancreatic diseases including CP and PDAC.

A study done in lung cancer has reported that PGE2 through EP3 and EP4 receptor regulate the stromal formation and angiogenesis (171), and that inhibition of COX-2 and EP3/EP4 receptors results in reduction of the stroma and of CXCL12/CXCR4 axis important in mediating lung cancer metastasis. In pancreatic cancer, it has been shown that it is the EP2 receptor that regulates pancreatic cancer cells (172). PGE2 actions are mediating by its receptors EP1-4. EP receptors are G-protein coupled receptors that mediate a variety of biological functions. Four EP receptor subtypes have been identified and are designated EP₁, EP₂, EP₃, and EP₄. EP₁ signals via increased Ca²⁺, which leads to vasoconstriction. EP₃ can also serve to stimulate vasoconstriction and inhibits the generation of cAMP, whereas EP₂ and EP₄ are known to mediate vasorelaxation by stimulating an increase in cAMP levels. A recent interest in studying the role of EP4 receptor in colon cancer has emerged. EP4 receptor targeting (117) was found to decrease foci formation, metastasis and tumor incidence. The role of EP receptors in the regulation of the stroma of PDAC has not yet been shown. Therefore, this study attempted to determine the role of EP

receptors in the regulation of PSC. I looked at EP2 and EP4 receptors and silenced by siRNA each receptor. These results show a strong inhibition of migration, invasion, MMP-2 and MMP-9 expression when EP4 and not EP2 receptor is silenced. These findings suggest that EP4 receptor pathway tightly regulates pancreatic stellate cell activity.

Although the role of importance of the stroma in PDAC is well known, the molecular mechanisms that regulate stromal activity and the downstream signaling involved are poorly understood. This study provides evidence for the first time that EP4 receptor can control major changes in the fibrogenic response associated with PDAC and CP. These changes include contribution to the formation of the fibrillar matrix, migration, invasion as well as changes in gene expression. I found that prostaglandin E2 EP4 receptor is responsible for the PGE2 induced changes in pancreatic stellate cells. Pancreatic fibrosis associated with either PDAC or CP is untreatable. Evidence showing that fibrosis of the pancreas is tightly regulated by EP4 receptor presents it as a potential target for the prevention of pancreatic fibrosis or as adjuvant treatment administered along with treatments for PDAC.

**CHAPTER FOUR: PGE2 ACTIVATES MULTIPLE SIGNALING PATHWAYS
AND REGULATES COL1A1 EXPRESSION AND TRANSCRIPTIONAL
ACTIVITY IN PSC**

INTRODUCTION

Pancreatic fibrosis is a characteristic of both chronic pancreatitis and the dense desmoplastic reaction associated with pancreatic cancer. The cellular and molecular mechanisms that control pancreatic fibrosis are not fully understood, mostly because of the lack of in vitro models. In 1998, pancreatic stellate cells (PSC) were discovered. They are now known to be the cells that produce the majority of the fibrotic reaction in the pancreas (65). In normal physiological conditions, PSC are quiescent and are identified by Vitamin A fat droplets and positive staining for desmin and GFAP. Upon activation, PSC lose their Vitamin A droplets and increase their expression of alpha smooth muscle actin (α -SMA). Activated stellate cells express cytokines, chemokines and cell adhesion molecules. They also produce large amount of extra cellular matrix (ECM) components, mostly collagen I (65). Collagen I is the most abundant protein in the ECM and hyper accumulation is a characteristic of pancreatic fibrosis. Collagen I is formed by the polymerization of pro- α 1(I) chain and pro- α 2(I) which are generated by COL1A1 and COL1A2 genes respectively (173). COL1A1 and COL2A1 exist in a 2:1 ration. Pro collagen molecules have a distinct triple stranded rope like structure. Once they are processed outside the cell, Collagen I

fibrils rearrange into long fibrils that cross link to each other in the ECM, which gives the tensile strength and hardness to the fibrotic tissue (174). In cancers, study show that both the cells of the microenvironment and the collagen I matrix are needed for the tumor to progress. An earlier study done by Ronnov Jessen in 1995 supports the role of collagen in tumor progression and demonstrates the need for both the stromal cells and the collagen I matrix for a primary breast cancer cell to progress to a more advanced malignant phenotype (175). Primary breast cancer cells were plated on a plastic petri dish and compared to the same primary breast cancer cells that were plated on a collagen matrix with myofibroblasts. Over a period of two weeks, the primary breast cancers cells that were plated on a collagen matrix in the presence of stromal cells progressed into an advanced stage whereas the primary cells plated on a Petri dish retained their primary structure therefore indicating that in order for a tumor to progress, a collagen rich matrix is required in addition to the myofibroblasts.

In pancreatic cancer, the content of collagen I is three times higher compared to the normal pancreas (176, 177). Many studies have tried to understand the role of collagen I in pancreatic cancer. In this study, they assessed the behavior of BXPC3 plated on different ECM molecules coated dishes. They found that BXPC3 plated on laminin or fibronectin coated plates no change in morphology was noted. However, when BXPC3 cells were placed on a collagen I coated plate, they became more spread out and aggressive (178). Also, pancreatic cancer cells plated on a collagen I matrix presented a reduction in E-cadherin,

enhanced proliferation and migration compared to the cells plated on matrigel. These results show that collagen I triggers the EMT of pancreatic cancer cells (179). In the same study, collagen I was also shown to increase the invasion and metastasis of pancreatic cancer. In vivo and in vitro data from a recent study, show that collagen I up regulation of MT1-MMP contributes to gemcitabine resistance of pancreatic cancer (180). The clinical relevance of the collagen I overproduction was assessed in a study done using human PDAC samples. Collagen I staining of sections taken from human patients with pancreatic cancer demonstrates that collagen I levels in patients' correlates with stage and prognosis of the disease (181). Collagen I has been well studied in pancreatic cancer because of the extensive desmoplasia surrounding the tumor. However, the majority of collagen I comes from PSCs and not the tumor. Since the molecular regulation of Collagen I gene in PSC is not fully understood, it is therefore necessary to understand the molecular mechanisms that regulate Collagen I in PSCs.

In this study, I present novel evidence for the regulation of Collagen I gene expression and activity by Prostaglandin E2 (PGE2). I show that PGE2 stimulates the gene expression of COL1A1 and that this stimulation is mediated by EP4 receptor. I also demonstrate for the first time by doing promoter analysis assay that PGE2 regulates the promoter of COL1A1. Blocking EP4 receptor reduced the hyperstimulation of COL1A1 therefore, EP4 receptor could be a therapeutic target for the treatment of pancreatic fibrosis associated with chronic pancreatitis and pancreatic cancer.

MATERIALS AND METHODS

Materials

Culture media and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Rockville, MD). PGE₂, was obtained from Cayman Chemicals.

Cell Culture

Primary human pancreatic stellate cells (PSC) were isolated using the outgrowth method from pancreatic adenocarcinoma samples from patients undergoing surgical resection and were immortalized (68)(Hwang RF, 2008). PSCs were maintained at 37°C in a humidified atmosphere of 5% CO₂ and were grown in 10% DMEM containing 1% antibiotic. PGE₂ was purchased from Cayman Chemicals (Ann Arbor, MI). EP4 antagonist was obtained from ONO pharmaceuticals (Japan).

Protein extraction and western blot

For protein extraction, PSC 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 cell scraping. Lysates were sonicated 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: phospho CREB ser 133 and total CREB (Upstate), phospho-AKT, total AKT (Cell Signaling), Actin (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.

Reverse transcription-PCR

Total RNA was isolated from PSC with and without siRNA transfection and the quality of RNA was tested as mentioned previously. DNase was used to remove contaminating genomic DNA and RNA purification. Quality of the RNA was confirmed by running on a denaturing gel, and we have observed clear 28S and 18S rRNA bands. A non reverse transcribed control was used to assure that no genomic DNA was amplified. Primers were designed for human

Collagen 1A1 (5' TGTCCACCGAGGCTCCCAGAAC 3') (5' CCCAGGCTCCGGTGTGACTCGTG 3')

The amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide. Primers designed for β-actin (Genbank BC_016045), which

was used as a loading control for the PCR reactions, were forward 5' ATG ATA TCG CCG CGC TCG TCG TC 3' and reverse 5' CGC TCG GCC GTG GTG GTG AA 3'.

Promoter cloning of COL1A1

PCR was done to amplify the full length COL1A1 promoter region using the following primers forward 5' CTG CCC ACG GCT AGC CGG CCA GCC GAC 3' and reverse 5' GCC GGA GGT CCA CAGA TCT GAA CAT GTC 3'. The PCR product was then cut using NheI and BglIII enzymes to get the full length promoter. In order to get ligate the promoter to luciferase, DNA ligase enzyme was used.

Functional Promoter Analysis of COL1A1

Various lengths of DNA fragments upstream of the initiating ATG codon were PCR amplified and inserted into luciferase reporter vector pGL3, a promoter vector (Promega) to analyze the promoter of COL1a1. First, the FL COL1A1 promoter was cut with several restriction enzymes to generate plasmids with different sizes. StuI was used to cut the FL1853 bp COL1A1 promoter and generate a 1067 bp which was cut with NheI and AvrII restriction enzymes. The generated fragment of 868 bp was cut with StuI and EcoRV enzymes which

resulted in a 564 bp fragment. Cutting the 564 bp fragment with pvull generated a 376 bp fragment.

Transient DNA transfection

Transient transfection of luciferase reporter plasmids was performed using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD), according to the protocol recommended by the manufacturer. Briefly, cells were seeded at about 70% confluency in a 24 or 6 tissue culture dish in serum containing media for 24 hours. Then, PSCs were treated with transfection mixtures containing 400 ng and 2ug for 24 and 6 well plates respectively, of luciferase reporter plasmids and 0.5 mg of promoterless as a negative control vector for 7 h at 37°C. Then, 3 ml of growth media were added to the cells, followed by incubation for an additional 16 h. The cells were then serum starved for 16-18 hours before PGE2 or the vehicle addition and harvested 72 h after the transfection. As a positive control, PSC were transfected with GFP-luc vector in the same experimental conditions and GFP expression was assessed by fluorescent microscope (Olympus).

Luciferase Assays

After a total of 70 hours since transfection the media was replaced with luciferin containing media. 15ug/ml luciferin in PBS was added to each well and luciferase activity was assessed by measuring the signal using IVIS machine using the automatic settings.

Statistical analysis

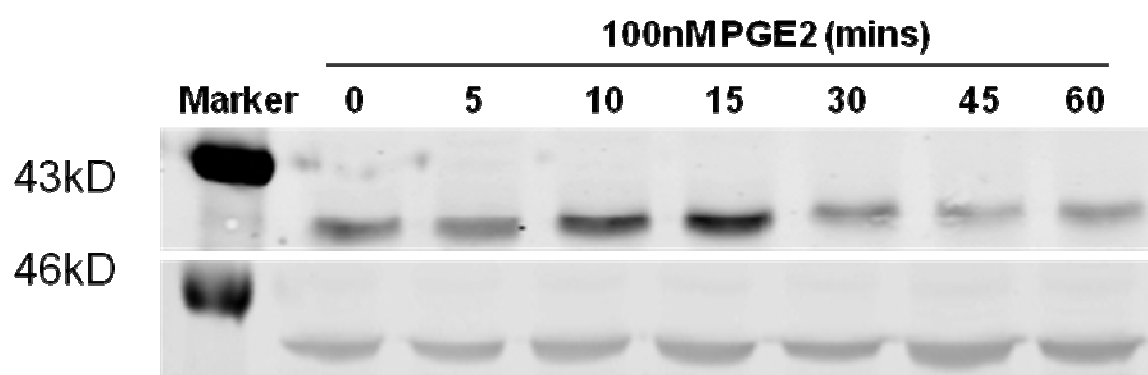
All experiments were conducted in triplicates and carried out on three or more separate occasions. Data presented are means of the three or more independent experiments \pm standard error mean (SEM). Statistical analysis was done using Graph Pad Prism (GraphPad Software). Comparisons were made using two-tailed Student's *t* test and significant difference was defined as $P < 0.05$. Data are shown as mean \pm SE.

RESULTS

PGE2 regulates CREB levels

PGE2 is reported to increase cAMP levels and activate PKA/CREB signaling pathway. Furthermore, CREB has been shown to be involved in migration and metastasis. Therefore, to determine whether the CREB pathway is activated by PGE2 in PSC, cell lysates treated with several time points of PGE2 were isolated. Using a specific antibody against phosphorylated CREB (ser 133), western blot analysis determined that PGE2 increases the phosphorylated levels of CREB at 10 and 15mins (Figure 4.1). Therefore, suggesting that there might be EP receptor specificity for mediating PGE2 functions in PSC (162).

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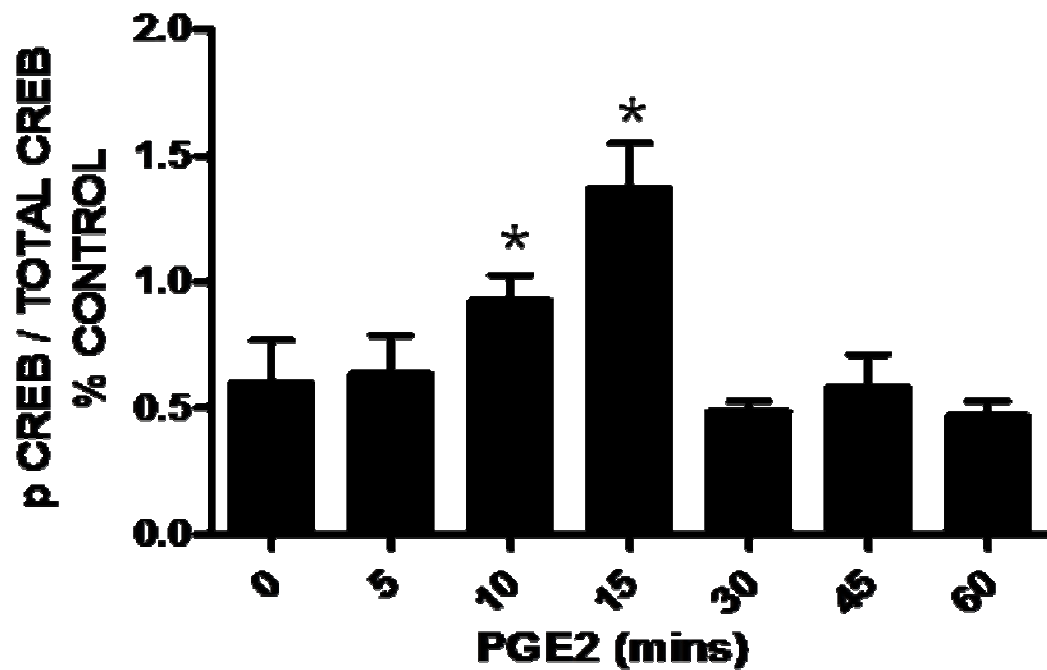


Figure 4.1 PGE2 induces cAMP response element binding protein (CREB) in PSC (1) Western Blot of phosphor and total CREB at 0,5,10,15,30,45,60 mins after stimulation with PGE2 in PSC. PGE2 induces phosphorylation of CREB at ser 133 residue (2) Densitometry analysis of the relative amounts of total CREB and p-CREB from 3 separate experiments (n=3). Values are the mean and SEM. *, P<0.05

Regulation of AKT by PGE2

A number of signaling pathways is reported to regulated cell motility. The PI3K/AKT pathway has been shown to stimulate invasion, migration, and MMP-2 and MMP-9 expression in many cell types (182, 183). Therefore, the PI3K/AKT signaling pathway was evaluated following PGE2 treatment. PSC were serum starved and treated with 100 nMPGE2. The levels of phosphorylated (ser 473) AKT were determined by western blotting analysis. The levels of phosphorylated AKT did not change following PGE2 treatment (Figure 4.2).

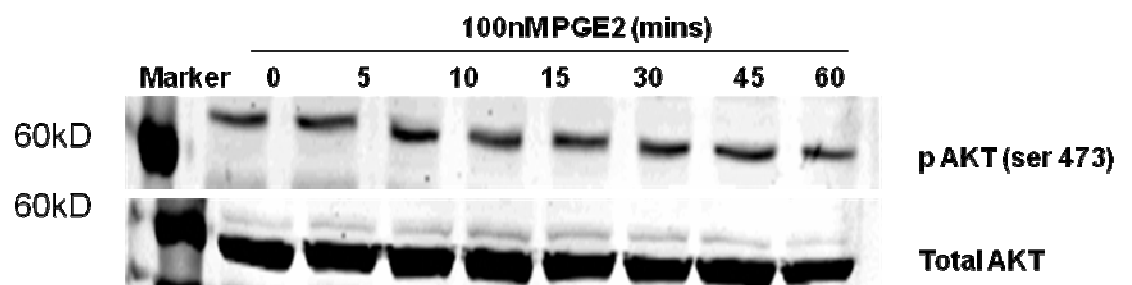


Figure 4.2 PGE2 does not activate Akt pathway in PSC (1) Western Blot of phospho and total Akt at 0, 5, 10, 15,30,45,60 mins after stimulation with PGE2 in PSC. PGE2 does not induce phosphorylation of Akt

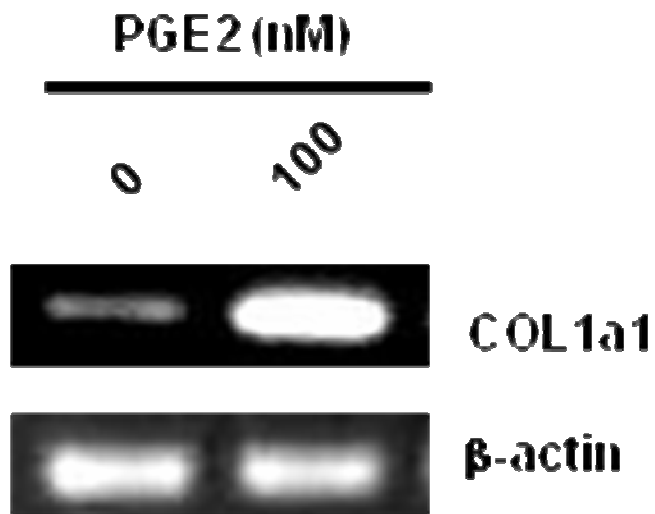
PGE2 regulates COL1A1 mRNA expression and activity

The majority of pancreatic cancers have high levels of COL1A1 mostly produced by PSC, which facilitates their invasion and metastatic potential. In addition, previous data shows that PGE2 stimulates the expression of ECM molecules in PSC. Therefore, to examine the role of PGE2 in the regulation of COL1A1 gene expression in PSC, PSC were serum starved for 24 hours before PGE2 addition, RNA was extracted and RT-PCR was performed on the cDNA obtained. The results from the RT-PCR demonstrate a strong increase in the mRNA levels of COL1A1 gene after 24 hours treatment with PGE2 compared to the negative control treated with DMSO. In the absence of PGE2, serum starved PSC showed little expression of COL1A1 gene. These results demonstrate that PGE2 increase COL1A1 gene expression in PSC (Figure 4.3.1).

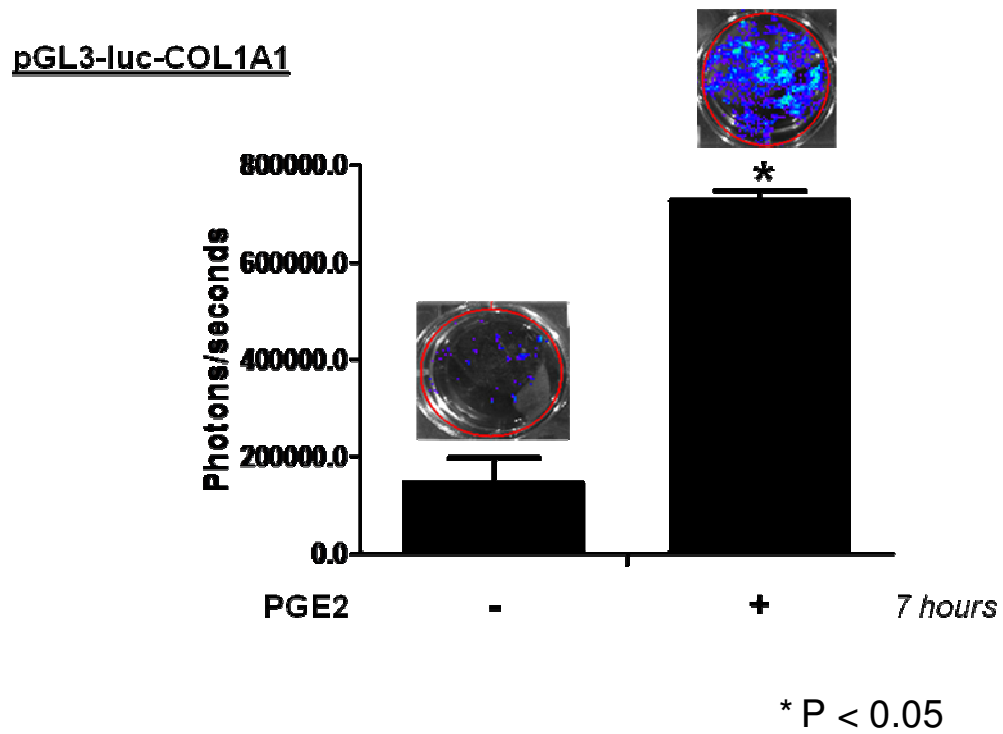
To further investigate the role of PGE2 in the regulation of COL1A1 gene, transient transfection of PSC with a construct prepared from the full length promoter of COL1A1 gene ligated to a luciferase reporter gene was performed (Figure 4.3.2, 4.3.3). The results of the transfection indicate that treatment with PGE2 for 7 hours induced a ~2.5 fold increase in transcription (Figure 4.3.2). In order to evaluate the promoter activity of COL1A1 gene further, PSC were transfected with the full length COL1A1 promoter for 24 hours and PGE2 was added to serum starved PSC for 17 hours. Treatment of PSC with PGE2 for 17 hours showed a steady increase in the promoter activity of COL1A1 which was not seen in the negative control (Figure 4.3.3). Treatment with DMSO (negative

control) did not stimulate COL1A1 promoter activity. These results confirmed the data from the previous time point done at 7 hours that PGE2 treatment causes a strong induction of COL1A1 promoter luciferase activity.

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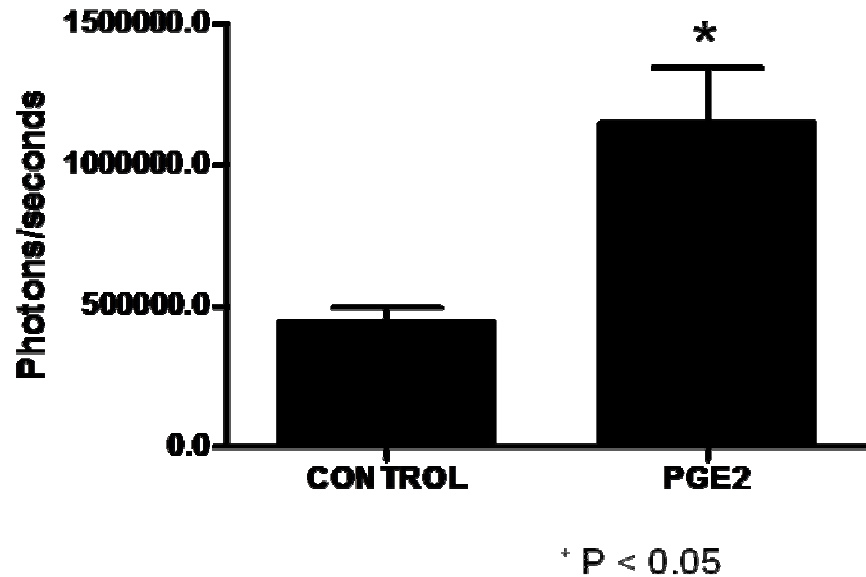
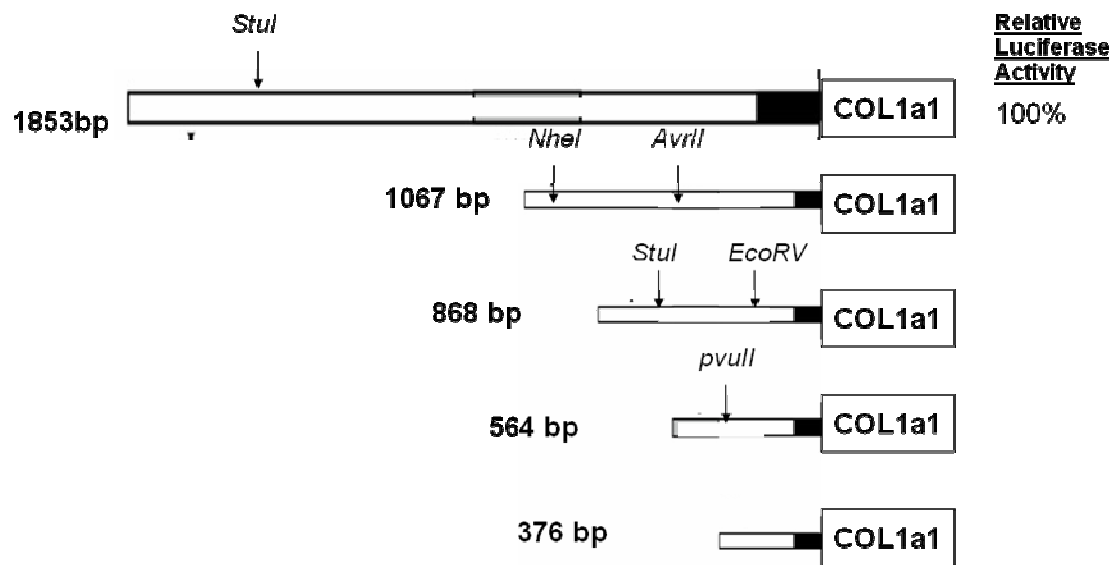
pGL3-luc-COL1A1

Figure 4.3. PGE2 induces the mRNA expression and transcription of COL1A1. (1) 0 or 100 nM PGE2 was added to PSC after overnight serum starving and RNA was extracted after 24 hours of treatment. As shown by RT-PCR conducted with respective human primers, PGE2 stimulated the expression of COL1A1. b-actin was used as a loading control.(2),(3) PGE2 induction of the transcriptional activity of COL1A1. PSC were transiently transfected with pGL3-luc-COL1A1 promoter construct. PGE2 (0, 100nM) was added 7 hours (2) and 17 (3) hours before measuring luciferase activity. Luciferase activity was measured in photons/seconds using IVIS software. *, $P < 0.05$

Analysis of the COL1A1 promoter constructs demonstrates that PGE2 regulates several areas in the COL1A1 promoter

To identify the core promoter essential for transcriptional activation, 5' truncations of the ~1.8kb full length COL1A1 promoter were prepared and analyzed by luciferase assay (Figure 4.4.1). Various sized constructs ligated to a luciferase reporter gene were transiently transfected in PSC. PGE2 was added for 24 hours and luciferase activity was assessed in the presence of PGE2 for each construct and compared to the full length COL1A1 construct. COL1A1 promoter activity of the 1067 bp, 868 bp, 564 bp and 376 bp constructs were determined after PGE2 treatment and compared to the full length COL1A1 promoter activity and the promoterless negative control. Data obtained shows that PGE2 stimulates all four constructs. No statistically significant difference was noted between the full length COL1A1 promoter and the various sized constructs. As shown in Figure 4.4.2, transcriptional activity in PSC was not significantly different between the various truncated fragments compared to the full length fragment. Minimal change in activity was detected; however the 868 bp fragment exhibited the minimal activity. Nevertheless, these activities are still significant because they were several folds the activity of the promoterless reporter plasmid (negative control). This data suggests PGE2 regulates one or more areas in the COL1A1 promoter.

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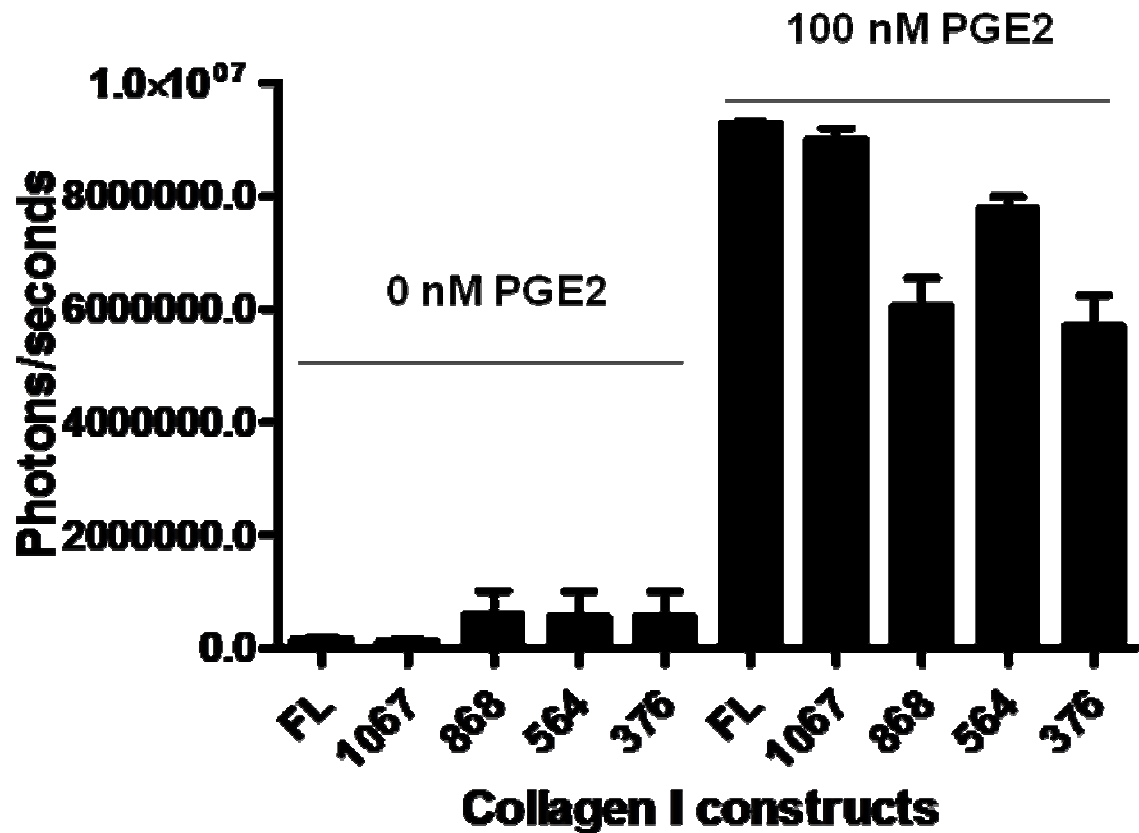


Figure 4.4 Functional analyses of regulatory regions in the COL1A1 promoter responsible for mediating PGE2 activation of transcription. (1) Schematic representation of the various constructs generated by restriction enzyme digestion and ligated to a luciferase reporter gene. (2) PGE2 induction of the transcriptional activity of COL1A1 constructs. PSC were transiently transfected with each of the generated pGL3-luc-COL1A1 promoter constructs shown in (1). PGE2 (0, 100nM) was added for 17 hours before measuring luciferase activity. Luciferase activity was measured in photons/seconds using IVIS software. *, $P < 0.05$

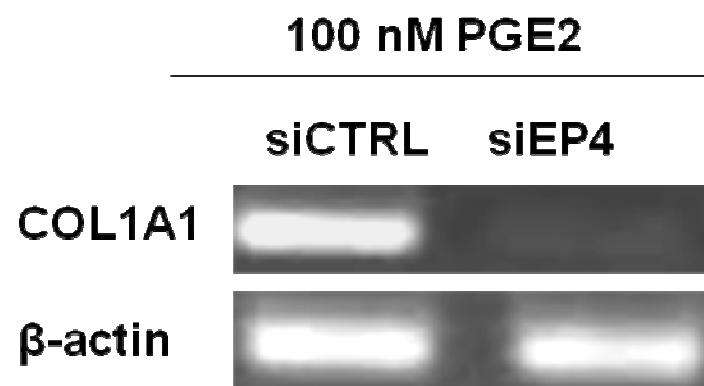
Inhibition of EP4 receptor reduces COL1A1 expression and activity

PGE2 signaling through EP4 receptor has been associated with tumorigenesis and I have previously found that PGE2 mediated PSC hyperactivation is EP4 dependent. I have also shown that blocking EP4 receptor reduces the profibrogenic phenotype of PSC by decreasing migration, invasion and MMP-2, MMP-9 expression that accompany high levels of PGE2. Therefore demonstrating that blocking EP4 receptor could be used as an anti fibrogenic therapy to reduces PSC activity. To further examine the role of EP4 receptor in hyper activation of PSC, I evaluated the role of EP4 receptor in mediating PGE2 dependent COL1A1 stimulation. First, to determine whether PGE2 increases COL1A1 expression via EP4 in PSC, siRNA silencing was performed. siRNA silencing of EP4 receptor decreased COL1A1 gene expression in PSC treated with PGE2 compared to the negative control (Figure 4.5.1). To complement the siRNA approach, a pharmacological approach was used. A selective EP4 antagonist (ONO) was added to serum starved PSC 1 hour prior to PGE2 treatment and total RNA was collected. Using RT-PCR, I found that treatment of PSC with the antagonist prior to PGE2 blocked PGE2 stimulation of COL1A1 mRNA (Figure 4.5.2). These results suggest that blocking EP4 receptor by either siRNA silencing or pharmacologically using EP4 antagonism reduces COL1A1 expression.

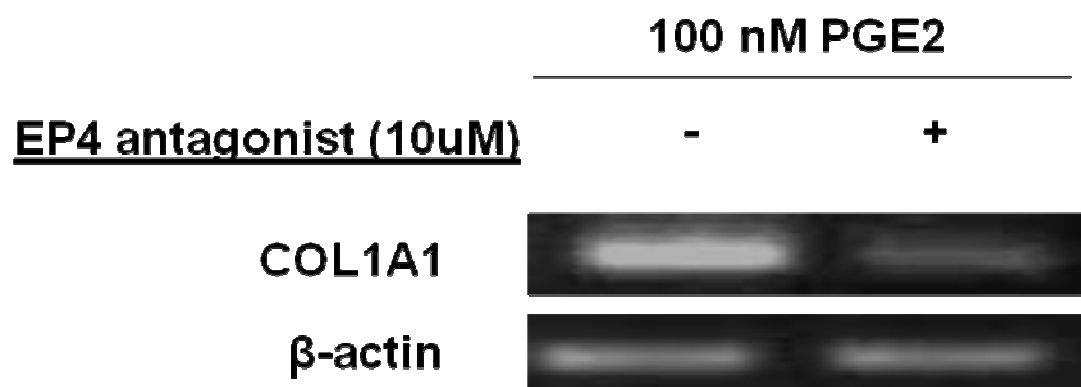
To determine the role of EP4 in mediating the transcriptional activation of COL1A1 by PGE2, PSC were transiently transfected with the full length COL1A1 promoter construct. After 24 hours of serum starvation, PSC were treated with EP4 antagonist 1 hour prior to addition of PGE2 for 17 hours. Luciferase activity was measured after a total of 70 hours after the transfection. The increase in COL1A1 transcriptional activity caused by PGE2 addition was reduced to a near basal level when EP4 antagonist was added compared to the DMSO treated negative control. Therefore, EP4 antagonist nullifies the actions of PGE2 hyper activation of COL1A1 promoter (Figure 4.5.3).

These results demonstrate that EP4 receptor is required for PGE2 to increase COL1A1 activation and that blocking EP4 receptor by both siRNA and EP4 antagonist reduces both the expression and the transcriptional activity of COL1A1 caused by high levels of PGE2.

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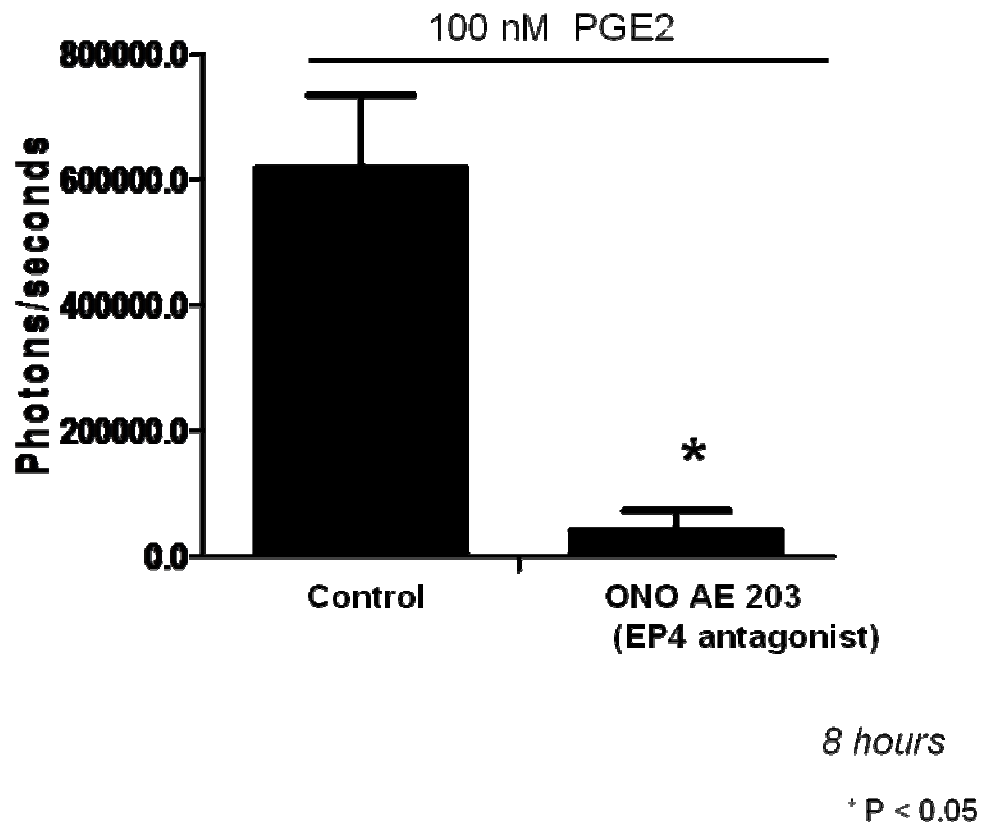


Figure 4.5 Effects of blocking EP4 receptor on COL1A1 expression and transcription (1) PSC transfected with siRNAs (5nM) showed a significant reduction in the expression of COL1A1 gene as shown by RT-PCR using specific oligonucleotide for COL1A1. B-actin was used as a loading control. (2) PSC treated with 10uM of the EP4 antagonist 1 hour prior to PGE2 (100nM) addition showed a significant reduction in the expression of COL1a1 gene compared to the control as shown by RT-PCR using specific oligonucleotide for COL1A1. B-actin was used as a loading control. (3) PGE2 induction of the transcriptional activity of COL1A1 was blocked by EP4 antagonism. PSC were transiently transfected the full length pGL3-luc-COL1A1 promoter construct. PGE2 (0, 100nM) was added for 8 hours before measuring luciferase activity. EP4 antagonism reduced PGE2 dependent activation of COL1A1 promoter. Luciferase activity was measured in photons/seconds using IVIS software. *, P<0.05

DISCUSSION

Pancreatic fibrosis is a common feature in chronic pancreatitis and desmoplasia associated with pancreatic cancer. Pancreatic fibrosis is associated with inflammation and high levels of COX-2/PGE2 (132). I have previously shown for the first time that PGE2 promotes the fibrogenic response by increasing the overall activity of PSC. To further understand the molecular mechanisms of PGE2-mediated pancreatic fibrosis, I looked at the downstream signaling pathways activated in PSC by PGE2 and at the role of PGE2 in Collagen I hyperstimulation. Since previous data show a strong role for EP4 receptor in mediating PGE2 hyperactivation of PSC functions, I looked at CREB and AKT pathways known to be activated by EP4 receptor (103). I found that PGE2 does not activate AKT pathway in PSC. AKT is known to be involved in survival and evasion of apoptosis. I have previously found that PGE2 does not affect PSC apoptosis and survival; therefore, this data further confirms that PGE2 is not involved in survival of PSC. I also looked at CREB and found that PGE2 induces the expression of phospho CREB (ser 133) in PSC; therefore, suggesting that PGE2 could activate the transcriptional potential of CREB. Collagen I is the most fibrous collagen and about 84% of the collagen synthesized by fibroblast (184). PSC produce the majority of Collagen I associated with fibrosis (63, 65). In pancreatic cancer, Collagen I overproduction has been correlated with increased tumor growth, metastasis, invasion, angiogenesis and EMT (178, 180, 181, 185,

186). Collagen I has also been found to correlate with poor prognosis and gemcitabine resistance (180, 181). Therefore, Collagen I is clearly important for the development and progression of pancreatic cancer. It is known that active PSC deposit tremendous amounts of Collagen I which serves as a matrix where the tumor and the stroma communicate by exchanging factors. Studies have shown that the normal Collagen IV rich basement membrane is slowly replaced by a fibrotic Collagen I matrix which triggers PSC migration and proliferation in order to propagate the fibrotic response. Collagen I is needed for the formation of the fibrotic matrix which serves as an anchorage site for cells and as storage for cytokines and growth factors that provide signaling clue to the stroma and cancer cells. However, how Collagen I gene is regulated at the molecular levels is still not fully understood. Collagen I is composed of two polypeptide chains $\text{pro}\alpha 1$ and $\text{pro}\alpha 2$ in the ratio of 2:1. COL1A1 and COL1A2 genes control the synthesis of the two chains. Several studies have shown that the over production of Collagen I during the fibrotic process is largely due to an increase in the transcription rate (187, 188). The major cytokine reported to regulate the COL1A1 gene which is more abundant than COL1A2 is TGF- β (189). Numerous efforts have been made to identify the pathways that regulate the COL1A1 transcription and earlier studies identified TGF- β responsive sequences in the COL1A1 promoter which contain binding sites for Sp1, smad and AP1 (188). PGE2 was previously shown to stimulate PSC activity and alter the extra cellular matrix composition by increasing the expression of several ECM genes like fibronectin and elastin. Therefore, in order to further investigate the mechanism of PGE2 mediate PSC

activation at the molecular levels, I hypothesized that PGE2 stimulates hyper activation of PSC by increasing COL1A1 expression and transcriptional activation. The results of this study demonstrate that PGE2 has marked effects on Collagen I over production, by PSC. First, I show that PGE2 increases the mRNA expression of COL1A1 gene which is concurrent with the previous data that show that PGE2 stimulates ECM genes. In order to identify the role of PGE2 in the transcriptional regulation of COL1A1, the full length 1.8 kb promoter was cloned and ligated to luciferase reporter gene then transiently transfected in PSC. Luciferase readings show that PGE2 treatment induces the activation of COL1A1 transcription at several time points. This suggests that COL1A1 promoter might have important regulatory sequences controlled by PGE2. To perform a functional analysis of the upstream sequences of COL1A1, several chimeric constructs containing DNA fragments of various sizes were generated by restriction enzyme digestion. Fragments were ligated to luciferase reporter gene and their expression was examined after transient DNA transfection of PSC. Maximum transcriptional activity was noted when the full length promoter sequence was included. Minimal activity was detected with the transfection of the 868 bp segment. However, the differences were not significantly different and PGE2 induced the activity of all the segments generated. These results indicate that PGE2 regulate one or more region of the COL1A1 promoter. It could also indicate the PGE2 regulates the 376 bp region included in all five constructs. CREB (cAMP-response-element-binding protein) is a transcription factor that binds to cAMP-responsive element (CRE) promoter sites to regulate the

transcription of genes involved in a variety of physiological functions including cancer and inflammation(190). Previous data show that PGE2 stimulates the phosphorylation of CREB at ser133. The phosphorylation of CREB at this residue triggers the relocalization of CREB to the nucleus where it could act as a transcription factor or activator (190). Analysis of the COL1A1 promoter by TF search software showed that CREB has four binding sites. The four CREB binding sites are located within the 376 bp fragment generated by restriction enzyme digestion. Preliminary data based on siRNA studies show that silencing of CREB reduces the COL1A1 gene expression (data not shown) therefore, CREB could be mediating the PGE2 dependent transcriptional activation of COL1A1 by either being the transcription factor that binds to the 376 bp region or by being a co-activator. Nevertheless, the goal of this study is to identify the molecular mechanisms behind PGE2 mediates pancreatic fibrosis by looking at the mechanisms that govern COL1A1 hyper stimulation and identifying the transcription factor is beyond the scope of this study. To further investigate the mechanisms of COL1A1 stimulation by PGE2, I looked at EP4 receptor. Previous data show that EP4 mediates the majority of PGE2 dependent PSC profibrogenic activity. Similarly, the results of this study show that blocking EP4 receptor by either transfecting with siRNA against EP4 receptor or pharmacologically by using an EP4 antagonist, approved for the use on humans in Japan, reduces both the expression and the transcriptional activity of COL1A1 gene. Therefore, this study demonstrates for the first time a PGE2/EP4 dependent mechanism of COL1A1 regulation that has not been shown previously. TGF- β has been shown

to regulate COX-2/PGE2 and is the major regulator of COL1A1 transcription; therefore an argument could arise that the effects of PGE2 on COL1A1 could be that of TGF- β . However, TGF- β regulation of COL1A1 has been shown to be a SMAD dependent mechanism that is independent of PGE2. Therefore, blocking EP4 receptor would not reduce COL1A1 stimulation by PGE2 to a near basal level if TGF- β was the sole regulator of COL1A1. The reduction of COL1A1 activation observed by blocking EP4 receptor could be potentially used as a therapeutic approach to reduce fibrosis.

In summary, the data presented in this study indicate an important role for PGE2 in the regulation of hyper activation of PSC seen in PDAC. As PGE2 is overproduced by PSC, it could represent an important step in the development of pancreatic fibrosis during chronic pancreatitis and pancreatic cancer. This study also identifies EP4 receptor as a possible target for the selective inhibition of PGE2 dependent COL1A1 hyper stimulation that defines hyper activation of the stroma.

CHAPTER FIVE: SUMMARY AND FUTURE DIRECTIONS

SUMMARY

Pancreatic cancer is one of the most lethal diseases of our times. It is the 4th leading cause of cancer related death in western countries including the US. Also called the “silent killer” because of the absence of symptoms in patients, pancreatic cancer is detected at a late stage when patients already develop metastases. In rare cases, pancreatic cancer is detected early and is resectable however; approximately 80% of patients still succumb to his disease and die within 5 years of resection because of reoccurrence and metastasis. Pancreatic cancer is highly resistant to chemotherapy. Despite the extensive research done to identify novel therapies for PDAC, gemcitabine is still the first line therapy and the response rate is very low. Recent emphasis has been on the extensive desmoplastic reaction that surrounds pancreatic cancer. Pancreatic desmoplasia has been shown to impede proper drug delivery to the tumor and decrease survival in mice by acting as a barrier that shields the tumor. Tumor stroma interactions in pancreatic cancer are highly bidirectional. The stroma produces high levels of factors that aid tumor progression, such as MMP-2 which increases angiogenesis of the tumor, and vice versa the tumor produces cytokines and growth factors such as TGF- β and PDGF which activate the stroma. Inflammatory molecules such as COX-2/PGE2 have been reported to be high in both chronic pancreatitis and pancreatic cancer which share a common denominator; hyper activation of PSC. However, the role of COX-2/PGE2 in

hyper activation of the stroma is not known. The PSC has been identified as the major cell that produces the fibrotic reaction. Activation of PSC is followed by an increase in proliferation, migration, gene expression and collagen I deposition. Understanding how the stroma is regulated could lead to the identification of stroma specific targets that can reduce or block desmoplasia which can reduce chemoresistance, increase pancreatic cancer diagnosis, prevent metastasis, tumor growth and reduce the tumor burden which will increase patients' survival.

Therefore, more research to increase the understanding of the pancreatic stellate cell (PSC) to put us one step closer to finding the cure of pancreatic cancer and increasing patient lives. The hypothesis of this dissertation was that PGE₂ regulates stromal hyperactivation by amplifying PSC "profibrogenic" phenotype and COL1A1 activity via EP4 receptor which may be an important target for the treatment of this pathology.

To address this hypothesis, I first assessed the effects of PGE₂ on PSC biology. Since it is not known whether PSC produce COX-2/PGE₂ the first step was to determine the presence of COX-2 and PGE₂ in PSC. I found that COX-2 was over expressed in pancreatic cancer and that PSC express COX-2 mRNA. I also used liquid chromatography tandem mass spectrometry to quantify the levels of PGE₂ in PSC and found PSC to possess high levels of PGE₂ both intracellular and extracellular. PGE₂ levels were the highest among all eicosanoids profiled. Therefore, the high levels of stromal PGE₂ could have an effect on PSCs. To address this question, I dissected the "fibrotic reaction" associated with pancreatic cancer based on the literature. Upon activation, PSC

proliferate which causes an increase in gene expression. Once activated PSC migrate and penetrate through the membrane while depositing huge amounts of ECM mostly collagen I which forms a newly fibrotic matrix rich in collagen I. The collagen I rich matrix replaces the normal basement membrane made of Collagen IV. Collagen IV degradation by MMPs increases. To determine the effects of PGE2 on PSC, changes in each step mentioned above was studied after PGE2 treatment. I found that stimulation of PSC by PGE2 increases all of the above mentioned steps of the “fibrotic reaction” including migration, invasion, gene expression of ECM molecules such as fibronectin, elastin, HSPG2 and others. A panel of MMPs was also evaluated and PGE2 was shown to increase the mRNA expression of MMP-2, MMP-9 and TIMP-1 specifically. The proliferation of PSC was stimulated by PGE2. The idea of PGE2 as a profibrogenic molecule in pancreatic cancer is being explored currently and has been presented at the recent American Pancreatic Association.

To explore the mechanism behind PGE2 mediated PSC stimulation, I evaluated the role of each EP receptor subtype in PSC hyper activity in the second aim of this dissertation. All four receptors were expressed in PSC. Several reports emphasize the role of EP2 and EP4 in tumor development and progression therefore, the first part aimed at silencing EP2 and EP4 in PSC and measuring changes in migration, invasion and looking at the mRNA expression of MMP-2/MMP-9. I found that silencing only EP4 receptor and not EP2 resulted in a near complete blocking of PGE2 dependent stimulation of migration, invasion and gene expression. These results suggest that EP4 is required for

PSC activity. To assess the role of EP1 and EP3, and to further confirm the results of the siRNA silencing, I used a pharmacological approach. Specific chemical antagonists against each EP receptor were obtained. PSC treated with each antagonist were subjected to migration, invasion assays as well as gene expression studies. The data from this study confirm that only EP4 receptor is required for PGE2 to stimulate PSC activity. Blocking EP4 receptor resulted in a reduction to a near basal level of many of PSC profibrogenic functions such as migration, invasion and gene expression therefore, identifying EP4 receptor as a potential therapeutic target for hyper activation of the pancreatic stroma. Blocking EP4 may reduce the desmoplastic reaction surrounding pancreatic cancer and help overcome the problem of impeded drug delivery to the tumor caused by desmoplasia in pancreatic cancer.

The third aim of this dissertation is to identify the molecular mechanism by which PGE2 regulates PSC by studying COL1A1 gene regulation. I demonstrated that PGE2 regulates the expression of COL1A1. I also cloned the COL1A1 full length promoter and ligated it to a luciferase reporter gene to determine the transcriptional activation of COL1A1 by PGE2. I found that PGE2 induced the promoter activation of COL1A1 in as little as 7 hours. I also did functional studies to analyze the promoter of COL1A1 by generating various fragments of the COL1A1 promoter. Analysis of the 5' truncations of the promoter revealed that the transcriptional activity of all four truncations increased with PGE2 treatment. These findings suggest that PGE2 may regulate elements present in the 376 bp region that is common to all four truncations. Bioinformatics

analysis of the COL1A1 promoter identified several CREB binding sites in the COL1A1 promoter particularly in the 376 bp region. Furthermore, PGE2 was found to increase the phosphorylation of CREB at ser133 which triggers its entry to the nucleus to act as a transcription factor. Therefore, one possibility could be that CREB regulates the 376 bp region of COL1A1. However, identifying the transcription factor that regulates COL1A1 is beyond the scope of this study.

Since blocking EP4 reduced the overall fibrogenic phenotype of PSC, I explored the role of EP4 receptor in PGE2 mediated COL1A1 activation of transcription and expression. I demonstrated that EP4 receptor is required for PGE2 to induce COL1A1 expression and transcriptional activity by both silencing by siRNA EP receptor and by using a pharmacological antagonist. These results identify a novel mechanism by which COL1A1 is regulated.

In conclusion, this work demonstrates that PSC produce high levels of PGE2 and that PGE2 regulates the profibrogenic phenotype of PSC by stimulating migration, invasion, ECM production and degradation. Moreover, blocking each EP receptor showed that only EP4 receptor is required in PGE2 mediated stimulation of PSC. Furthermore, this study delineates for the first time that PGE2 regulates the expression and transcriptional activity of COL1A1 via EP4 receptor. Blocking EP4 receptor results in a complete reduction of PSC activation and COL1A1 activity therefore, this study identifies EP4 receptor as a potential therapeutic target for pancreatic fibrosis associated with chronic pancreatitis and pancreatic cancer.

FUTURE DIRECTIONS

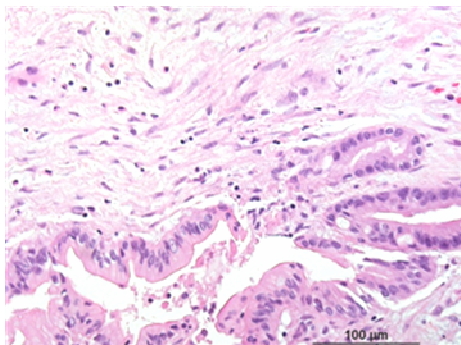
Will blocking EP4 receptor in PSC reduce desmoplasia and increase gemcitabine effectiveness in vivo?

In order to show the effect of blocking the production and function of PGE2 on pancreatic fibrosis by targeting EP4 receptor in vivo studies are needed. A mouse model of orthotopic implantation of pancreatic cancer (human) with associated stromal production (mouse) will be used.

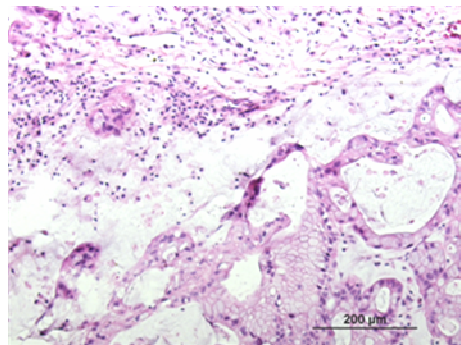
Preliminary data from experiments on 14 pancreatic cancer cells, CAPAN- 2 (ATCC) and primary pancreatic cancer cells freshly isolated from pancreatic cancer patients cultured in our laboratory (MDAPaCa-1) extensively produced pancreatic cancer associated fibrosis as seen by H & E staining (Figure 5.1). MDAPaCa-1 cells were further manipulated to express firefly luciferase to be

used for bioluminescence in vivo imaging. In this study, MDAPaCa-1 would be used orthotopically to develop pancreatic cancer and its associated fibrosis. Preliminary data indicate that liposomal delivery of siRNA is capable of sustaining silencing for 3 days (Figure 5.2).

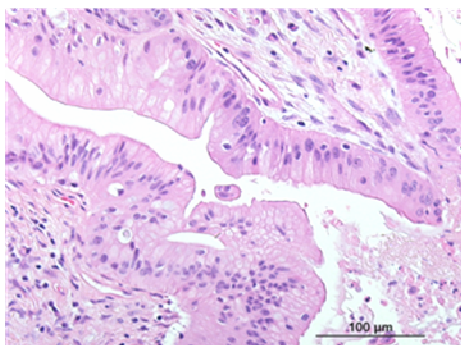
In this model, the effect of blocking EP4 on PSC will be studied by treating the mice every 3 days with either with neutral liposome couple siRNA [A] siCONTROL or with [B] mouse siRNA against EP4 receptor to specifically target the stroma. A and B will further be divided into two groups each, treated with Gemcitabine or Saline solution as a negative control. The rationale behind each treatment will be: Since the cancer formed will be human and the stroma will be mouse, administering mouse siEP4 will inhibit COX-2 production in the mouse stroma, thus reducing PGE2 production and hence will the effects on mouse stromal cells. After confirming that mouse siEP4 administration reduces PSC activation and the stroma, treatment with gemcitabine would answer the question whether reduction of the stroma would increase chemosensitivity. Survival, tumor growth and metastasis would be assessed.



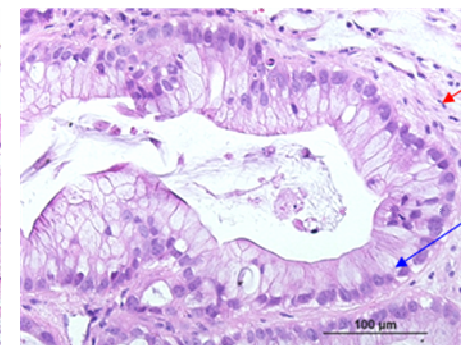
PDAC – Human



PDAC-PATC Cells



PDAC – Human



PDAC-PATC Cells

**Mouse
Stroma**

**Human
Tumor**

Logsdon CD, unpublished data

Figure 5.1 H & E staining of pancreatic tumor showing fibrosis. Primary pancreatic cancer cell line (MDAPaCa1) isolated from human pancreatic cancer patients was used to develop orthotopic pancreatic cancer. H & E staining showed extensive fibrosis formation.

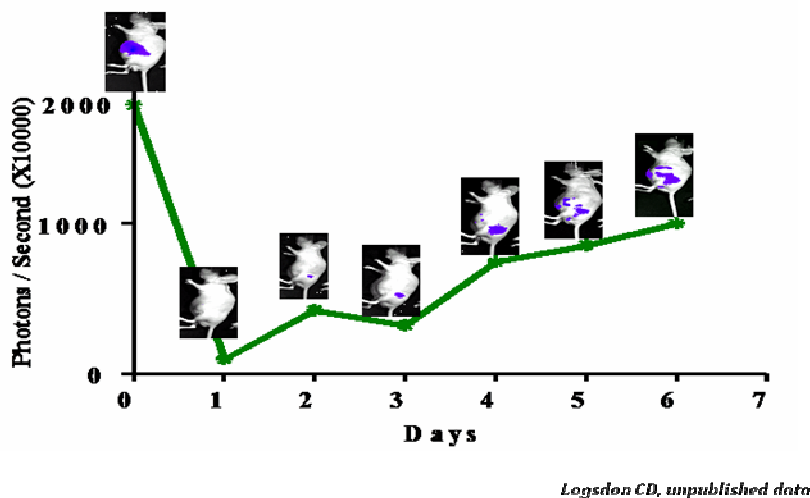


Figure 5.2 Silencing luciferase in vivo by neutral liposome coupled siRNA against luciferase gene. Panc-1 bearing firefly luciferase was orthotopically injected in mice and tumor was developed. Neutral liposomes coupled against siRNA luciferase were injected i.p. and bioluminescence imaging showed significant silencing at 1-3 days. Luciferase activity was expressed as photons/seconds.

How does CREB regulate COL1A1?

Pancreatic cancer is characterized by excessive deposition of Collagen Ia1 by PSC surrounding the tumor. The increase in stromal Collagen I in pancreatic cancer has been linked to EMT, survival, invasion and metastasis. Collagen Ia1 deposition by stellate cells is central for the development and progression of cancer associated fibrosis, however the molecular mechanism leading to the up regulation of collagen I levels by PSC in pancreatic cancer is unknown. Previous data show that PGE2 stimulates the production of extra cellular matrix genes via EP4. Particularly, PGE2 increases the expression of Collagen 1a1. a key component of the fibrillar matrix formed during pancreatic cancer. Silencing of PGE2 EP4 receptor only greatly reduces collagen Ia1 expression. Previous studies also show that PGE2 stimulates the expression of phosphor CREB ser 133. Once CREB is phosphorylated at that residue, its transcriptional potential is activated and CREB enters the nucleus to act as a transcription factor. Furthermore, analysis of the region upstream of Collagen Ia1 promoter showed multiple binding sites for CREB (Figure 5.3). Many of the cellular effects caused by the activation of cAMP/PKA pathway are mediated by the phosphorylation of CREB on Ser 133. This study would determine the potential role of EP4 mediated cAMP/PKA dependent transcriptional regulation of Collagen Ia1 by CREB.

The downstream signaling effectors that mediate PGE2 EP4 receptor up regulation of Collagen 1 will be determined. cAMP EIA assay will be performed on PSC with or without PGE2 treatment and siEP4 PSC treated with PGE2. This assay will be used to determine whether PGE2 activation of EP4 receptor will result in the activation of cAMP. The agonist Forskolin will be used to determine whether PGE2 stimulation of Collagen 1a1 will be cAMP dependent by Luciferase assay on PSC pGL3-E-COL1A1-Luc.L3.6 cell line will be used as a positive control. To determine whether Collagen 1a1 up regulation by PGE2 is dependent upon the transcriptional activation of CREB, a mutation of the ser 133 site to alanine will be done and Collagen 1a1 promoter luciferase assay would determine if CREB might transcriptionally regulate Collagen 1a1. Promoter analysis using restriction enzyme digestion of collagen 1a1 would be used to identify potential binding region for CREB that are PGE2/EP4 dependent. Point mutation in the CREB binding region of collagen 1 promoter would identify the precise CREB binding site that could be activated by PGE2. EMSA and ChIP assay will determine the direct binding of CREB to the site that will be identified as the CREB binding site of the Collagen 1a1 promoter that will be caused by PGE2 treatment and that will be EP4 dependent.

Mutant construct for CREB binding domains would identify a binding site for CREB in the promoter region of Collagen 1a1 that would be activated by PGE2 and EP4 dependent.

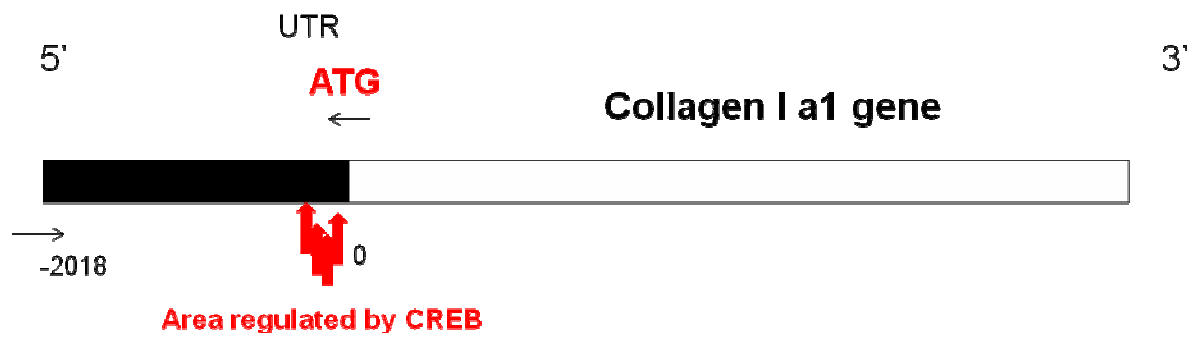


Figure 5.3 Bioinformatics analysis of COL1A1 promoter identifies four putative binding sites for CREB.

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VITA

Chantale Joseph Charo, was born in Beirut, Lebanon to her parents Samia Charo and Joseph Charo. In 2002, she joined the American University of Beirut to study biology and graduate with honors with a B.S in biology and a minor in nutrition in 2004. During her undergraduate studies, Chantale researched the chemopreventive effects of naturally occurring quinones on colon cancer. In 2003, Chantale was awarded an undergraduate research award from MD Anderson and did a summer rotation in Dr. Jean Pierre Issa where she studied epigenetics mechanisms and chemoresistance of cancer cells. In 2004, Chantale matriculated to The University of Texas at Houston MD Anderson Cancer Center and began the PhD program. After 3 years of research in Dr. Jean Pierre Issa's laboratory on epigenetic silencing in cancers, Chantale joined Dr. Craig Logsdon's lab at The University of Texas MD Anderson Cancer Center to work on her doctoral dissertation. Within 1 year, Chantale's research on PGE2 in pancreatic stellate cells was presented at the American Pancreatic Association meetings, and was awarded a "Poster of Distinction". During her graduate studies, her research was awarded 2 additional Posters of Distinction, and she has had the opportunity to give oral presentations across the country. Chantale received several travel awards throughout her Ph.D. During her Ph.D studies, she also was a member of the American Association of Cancer Research and the American Pancreatic Association. Upon her dissertation defense, Chantale will continue to research cancer as post doctoral fellow at the University of Miami.