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THERAPEUTIC POTENTIAL OF LIGAND-ACTIVATED PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR β/δ IN PANCREATIC CANCER

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ABSTRACT

Pancreatic cancer is the fourth leading cause of cancer-related deaths with a five-year survival rate of approximately 6%. Due to its asymptomatic nature until late stages of disease, less than 10% of patients have the option for surgical resection. Smoking, alcoholism, chronic pancreatitis, and metabolic disorders such as obesity, diabetes, and atherosclerosis are associated risk factors for pancreatic cancer development. The proto-oncogene *Kras* is mutated in 90% of pancreatic ductal adenocarcinoma (PDAC) cases, resulting in constitutive activation. The complexity of *Kras* makes it a difficult therapeutic target with dysregulation affecting several downstream signaling pathways. Candidates for early prognosis detection and therapeutic targeting need to be elucidated.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors involved in terminal differentiation, glucose and lipid metabolism. *Pparb* is ubiquitously expressed with highest levels in the gastrointestinal tract, kidneys, and skeletal muscle. PPARβ/δ associates with the transcriptional repressor BCL6, and may be a potential target for metabolic disorders due to its ability to modulate insulin sensitivity. Furthermore, PPARβ/δ also plays a role in protecting against oxidative stress, a contributing factor towards chronic inflammation and cancer. PPARβ/δ's role in cancer is controversial.

This dissertation sought to substantiate the role of PPARβ/δ in inflammation and metastasis and to investigate its potential as a therapeutic target and prognosis marker for pancreatic cancer. In a human pancreatic cancer cell line, Mia PaCa-2, over-expression of *Pparb* inhibited basal and tumor necrosis factor α (TNFα)-induced nuclear factor kappa b1 (*Nfkb*) activity. A specific agonist of PPARβ/δ, GW501516, suppressed TNFα-induced *Nfkb* reporter activity. RNAi knockdown of *Pparb* attenuated the GW501516 effect on *Nfkb* luciferase activity, while knockdown of *Bcl6* enhanced TNFα-induced *Nfkb* activity. PPARβ/δ activation induced

expression of several anti-inflammatory genes in a dose-dependent manner, and GW501516 inhibited monocyte chemotactic protein 1 (*Mcp1*) promoter-driven luciferase in a BCL6-dependent manner. Pro-inflammatory genes were suppressed in a BCL6-dependent manner. GW501516-treated pancreatic cancer cell conditioned media suppressed pro-inflammatory expression in THP-1 macrophages as well as reduced invasiveness across a basement membrane. Similarly, GW501516 reduced TNFα-induced expression of several genes involved in metastasis, including *Mmp9* mRNA and protein and *Pparb* knockdowns resulted in lower *Mmp9* mRNA and protein expression. The ability of Mia PaCa-2 cells to invade an artificial basement membrane was reduced by GW501516 in a PPARβ/δ- and BCL6-dependent manner. Thus, the PPARβ/δ-BCL6 signaling pathway has an anti-inflammatory and –metastasis role in Mia PaCa-2 cells that is enhanced upon treatment with GW501516.

The expression of PPARβ/δ, BCL6 and several of their target genes were examined in human PDAC, relative to matched normal pancreas tissue, and in a mouse model that mimics the human progression of disease (*Pdx1*-cre/LSL-$Kras^{G12D}$). *Pparb* mRNA was over-expressed in human tumor tissue compared to normal tissue, while *Bcl6* remained unaltered. Inflammatory genes that are controlled by the PPARβ/δ-BCL6 pathway were increased in the tumor tissue, including the important BCL6 target genes and pancreatic cancer prognostic indicators *Mcp1* and *Mmp9*. Similar observations were made in the $Kras^{G12D}$ mice where pancreas of mice carrying this constitutively active oncogene had higher expression of *Pparb* mRNA, unaltered *Bcl6* expression and increased *Mcp1* and *Mmp9* mRNA. These *in vivo* associations were further examined in the Mia PaCa-2 cell line. Over-expression of PPARβ/δ increased proliferation of pancreatic cancer cells, an effect that was ameliorated by GW501516. Over-expression of *Pparb* also increased basal and TNFα-induced *Mcp1* reporter activity, which was diminished by transfection of BCL6 or treatment with GW501516. Taken together, these data strongly support a

sequestering of BCL6 by PPARβ/δ that occurs during the progression of PDAC when dysregulation results in over-expression of PPARβ/δ.

The data presented herein supports a dichotomous role for the PPARβ/δ-BCL6 in the pancreatic cancer. Over-expression of PPARβ/δ, through sequestering of the transcription repressor BCL6, enhances the expression of genes known to be involved in PDAC progression such as *Mcp1* and *Mmp9*. However, upon treatment with the PPARβ/δ ligand GW501516, these same genes are repressed by the dual action of PPARβ/δ and BCL6. Taken together, this has important implications for pancreatic cancer therapy as well as diagnosis. Treatment with a specific PPARβ/δ ligand, such as GW501516, decreases inflammatory signaling and reduces macrophage recruitment, thereby limiting tumor progression. Also, via release of BCL6, the expression of metastatic proteins such as *Vcam1* and *Mmp9* is reduced, thereby decreasing metastatic potential. From a prognostic standpoint, it would be expected that a patient with higher expression of PPARβ/δ, and hence a repression of BCL6 signaling, in tumor tissue would benefit more significantly from GW501516 treatment. Combined with drugs that inhibit pancreatic cancer cell proliferation, GW501516 has the potential to be an effective tool in the fight against pancreatic cancer.

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18S;	18S ribosomal RNA
4-HNE;	4-hydroxynonenal
ADRP;	Adipose differentiation-related protein
ANGPTL;	Angiopoietin-like
BCL;	B-cell lymphoma
BSA;	Bovine serum albumin
CBR;	Carbonyl reductase
CD20;	B-lymphocyte antigen cd20
ChIP;	Chromatin immunoprecipitation
COX;	Cyclooxygenase
DLBCL;	Diffuse large B-cell lymphoma
DMEM;	Dulbecco's modified eagle's medium
DMSO;	Dimethyl sulfoxide
EC_{50};	Concentration of a drug required for half of max response
EDTA;	Ethylenediaminetetraacetic acid
ESEL;	E-selectin
FBS;	Fetal bovine serum
FGF;	Fibroblast growth factor
HEK;	Human embryonic kidney
HRM;	High resolution melting
HSD11B;	11-β-Hydroxysteroid Dehydrogenase
ICAM;	Intracellular adhesion molecule
KRAS;	V-ki-ras2 kirsten rat sarcoma viral oncogene homolog
Ig;	Immunoglobulin
IL1Ra;	Interleukin 1 receptor antagonist
LSL;	Lox-stop-lox
MCP;	Monocyte chemotactic protein
MMP;	Matrix metallopeptidase

NFκB;	Nuclear factor κ-light chain-enhancer of activated B cells
PanIN;	Pancreatic intraepithelial neoplasia
PBS;	Phosphate buffer saline
PDAC;	Pancreatic ductal adenocarcinoma
PDX;	Pancreatic and duodenal homeobox
PMA;	12-myristate 13-acetate
PPAR;	Peroxisome proliferator-activated receptor
PPRE;	Peroxisome proliferator response element
qPCR;	Quantitative polymerase chain reaction
RBP;	Retinol binding protein
RIPA;	Radioimmunoprecipitation assay
RNAi;	RNA interference
ROS;	Radical oxygen species
RPMI;	Roswell park memorial institute medium
RT-PCR;	Reverse transcriptase polymerase chain reaction
RXR;	Retinoid X receptor
SDS-PAGE;	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA;	Small hairpin RNA
siRNA;	Small interfering RNA
SNP;	Single nucleotide polymorphism
SOD;	Superoxide dismutase
STAT;	Signal transducer and activator of transcription
TAM;	Tumor associated macrophages
TE;	Tris + EDTA
TGFβ;	Transforming growth factor β
TNFα;	Tumor necrosis factor α
TSPAN;	Tetraspanin
VCAM;	Vascular cell adhesion molecule
VEGF;	Vascular endothelial growth factor

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Chapter 1

Literature review

1.1 Pancreatic cancer

The pancreas regulates two physiological processes, digestion and glucose metabolism. The exocrine pancreas consists of acinar and ductal cells and makes up approximately 95% of all pancreatic cancers, the remaining 5% being endocrine in nature [1]. Acinar cells produce digestive enzymes and constitute a large portion of the pancreatic tissue [9]. These cells form clusters called acini that attach to the ends of the branches of the pancreatic duct that runs down the body of the pancreas, of which consists of ductal cells that contribute mucous and bicarbonate to enzymatic secretions that release into the duodenum [58]. The ductal cells that are located within the acinar gland are targets of inflammation and is where carcinoma may occur [9].

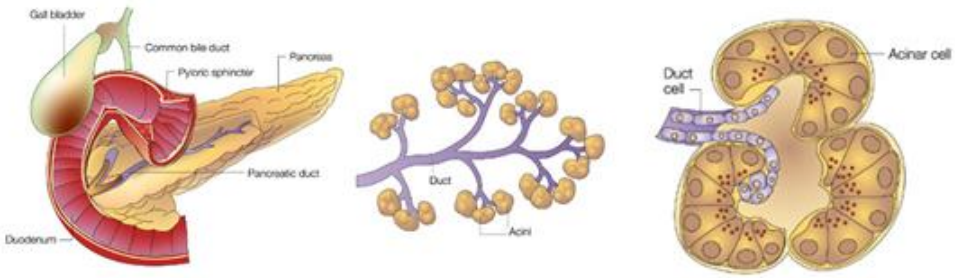

Figure 1.1 Illustration of the pancreas surrounded by duodenum and gall bladder, the pancreatic duct with acini lobes travel down the body of the pancreas, and the acinus gland where secretion of digestive enzymes occurs [196].

Pancreatic ductal adenocarcinoma (PDAC) is the predominant form of pancreatic cancer with a 5-year survival rate of approximately 6% [1] and is estimated to be the second leading cause of cancer death by the year 2020 [2]. Out of 46,420 people that have been diagnosed, approximately 39,590 people have died [3]. The number of new cases are predicted to increase by two-fold, and the number of deaths to increase by 2.4-fold by 2030 [2]. The average life expectancy after diagnosis is three to six months [1]. Ninety percent of patients develop tumors sporadically while familial development represents approximately 7% and inherited forms represent 3% of reported cases [3]. PDAC usually remains asymptomatic until late stages leading to less than 10% of patients qualifying for surgical resection at time of diagnosis [4], surgical resection being the only viable option for extending survival among patients. Late diagnosis is due to the lack of feasible tools for early detection when surgery is still an option.

Pancreatic cancer is diagnosed after patients report vague symptoms that may also indicate several other less severe conditions within the gastrointestinal tract. Symptoms include nausea, loss of appetite, weight loss, and pain in the abdomen or lower back caused by pressure of the tumor growing into the nerve network of the celiac plexus [9]. Jaundice may also be a symptom [9]. The inability to diagnose these symptoms eventually leads to imaging of organs or surrounding tissues by MRI or CT scan which is usually the first step in tumor discovery. Over the past 20 years the advancement in science and technology have not resulted in any significant improvements in therapeutics for pancreatic cancer.

1.1.1 Chemotherapy

Gemcitabine has been an important development in chemotherapy treatment for PDAC ever since the discovery that the 6, 9, and 12 month survival rates were significantly higher than treatment with 5-fluorouracil, the previous standard form of chemotherapy [5]. Since then the developments in single drug therapies have yielded disappointing results. Drug combinations have been used with minimal improvement, gemcitabine/oxilaplatin [6], gemcitabine/cisplatin [7], and gemcitabine/capecitabine [8] have improved progression-free survival as well as response rate compared to gemcitabine alone but failed to improve 1 year survival rate passed 5%. Recently, a significant survival advantage has been found with gemcitabine regimes including FOLFIRINOX (folinic acid, fluorouracil, irinotecan hydrochloride, oxaliplatin) and nab-paclitaxel that have extended overall survival from 6 months at 20% to 9-11 months at 35-48% [9]. Maintenance chemotherapy regimens has been found to be most effective in inhibiting PDAC progress and extending survival, however, combination with a multi-target molecular approach has been gaining momentum and may prove to be beneficial in pancreatic cancer therapeutics. Thus, proposed in this dissertation will be the potential of molecular pathway, PPARβ/δ and BCL6, in mitigating tumorigenic cell signaling with the surrounding microenvironment in concordance with the cytotoxic effects of chemotherapy agents.

Gemcitabine, or “Gemzar,” is a nucleoside analog anti-metabolite that is given intravenously and can inhibit tumor growth by two mechanisms [5]. The triphosphate analogue replaces cytidine, causing a disruption in the DNA replication process, and resulting in apoptosis [182]. Alternatively, the diphosphate analogue binds to the active site

of ribonucleotide reductase, an enzyme that catalyzes the formation of deoxyribonucleotides from ribonucleotides during DNA synthesis, irreversibly attenuating DNA replication and repair resulting in apoptosis and inhibition of tumor growth [182]. Cisplatin ("Platinol") and oxilaplatin ("Eloxatin") bind to DNA causing intra- and inter-crosslinks resulting in inhibition of DNA replication and apoptosis [183]. As described above, oxilaplatin is a component in FOLFIRINOX, a regimen of chemotherapy drugs such as fluorouracil, a pyrimidine analogue similar in function to that of gemcitabine, irinotecan, a topoisomerase inhibitor that prevents uncoiling of DNA, and folinic acid, a vitamin B derivative that is meant to reduce the side effects caused by fluorouracil [184]. Paclitaxel bound to a delivery vehicle, albumin (nab-paclitaxel or "Abraxane"), prevents the breakdown of microtubules that occur during cell division [185]. Paclitaxel is often used as a less toxic, as well as less effective alternative to FOLFIRINOX [9].

1.1.2 Etiology

As stated previously, about 90% of pancreatic cancer cases are sporadic [3], meaning it is difficult to determine on a case-to-case basis what exactly causes pancreatic cancer to develop, however, there are common risk factors associated with development of PDAC that will be the focus of this chapter. There are a small percentage of cases that are inherited as well, with one allele from a parent containing a mutated form of a gene. Having only one healthy copy and a mutated copy may increases risk of pancreatic cancer, in particular *Brca2* (breast cancer 2) or *Palb2* (partner and localizer of *Brca2*), genes involved in development of breast cancer [10], *Cdkn2a* (cyclin-dependent kinase

inhibitor 2A) involved in familial atypical multiple mole melanoma syndrome (FAMMM) [3,11], several DNA repair genes involved in hereditary nonpolyposis colorectal cancer (HNPCC) [12], and tumor suppressor *Stk11* (Serine/Threonine Kinase 11) involved in Peutz-Jeghers syndrome [13]. Inherited polymorphisms in these genes are associated with increased development of pancreatic cancer.

Several behavior risk factors have been identified as well. The most established risk factor is cigarette smoking making up a large portion of sporadic cases [14], accounting for approximately 25-30% of the cases. Cigarette smoking contains N-nitrosamines that are carcinogenic by damaging DNA through adduct formation [15,16]. Respiratory intake of tumorigenic N-nitroso compounds circulate to the pancreatic ductal epithelium [17]. Several animal models develop pancreatic cancer through administration of these compounds [18], and reducing exposure has been linked to reduction in PDAC development risk [16]. Dietary intake of nitrites may also lead to exposure to N-nitrosamines through metabolism in the stomach, this may lead to alteration in secretion in the exocrine pancreas which may also be carcinogenic [17].

Metabolic disorders have been linked to pancreatic cancer risk. High fat, high calorie diets, consisting mostly of red meat, have been linked to development of PDAC [19–21], while diets high in fruits and vegetables have been significantly beneficial [22,23]. Previous studies have shown a strong link between obesity and pancreatic cancer [24]. High alcohol consumption may also be linked to incidence of PDAC, since there is a strong correlation with pancreatitis, about 80% of chronic pancreatitis is caused by high alcohol consumption [16,25]. Positive lifestyle habits such as regular exercise and well-rounded diets have also been associated with reduction in PDAC incidence [26]. Patients with long term

diabetes mellitus showed an increased risk of pancreatic cancer [27–29], the link between abnormal glucose metabolism may be a major contributory factor [19,30].

Chronic pancreatitis has long been linked to pancreatic cancer [31,32]. Hereditary pancreatitis plays a role in transitioning to cancer making up approximately 3-6% of reported cases. [33] Risk of developing pancreatic cancer by 70 years of age due to hereditary pancreatitis was approximately 40% and paternal inheritance increases the risk to 70% [34]. Repeated episodes of acute pancreatitis may lead to irreversible tissue damage that can progress to chronic pancreatitis, and the overwhelming malfunction of DNA repair mechanisms can initiate transition from chronic pancreatitis phenotype to pancreatic cancer. Despite this strong link, only approximately 5% of patients diagnosed with chronic pancreatitis over the past 20 years usually progress to pancreatic cancer [35].

1.1.3 Epidemiology

Data out of the United States, Europe, and Japan show the incidence rate of acute pancreatitis ranges from approximately 13 to 45 cases out of 100,000 people [36], while chronic pancreatitis ranges from 5 to 12 cases out of 100,000 people a year [37] There are variations in disease estimates due to different methodologies in gathering data, variation in diagnoses, and different behavior risk factors based on region [32]. Western cultures tend to have a higher incidence of alcohol-related pancreatitis compared to Asian countries [38]. Pancreatitis tends to be much higher in obese people and obesity increases risk of gallstone formation [39]. Though gallstones do not seem to be associated with pancreatic cancer risk [40], they are causative of pancreatitis by blocking the bile duct [41]. The approximate incidence rate of pancreatic cancer is 8 cases out of 100,000 people [26]. The

majority being exocrine tumors such as adenocarcinoma, while endocrine tumors make up a very small percentage. In 2008, approximately 279,000 people were diagnosed with pancreatic cancer making up 2.2% of total cancer incidences [38].

Pancreatitis based on age and sex is highly dependent on behavioral risk factors such as diet and alcohol consumption, however chronic pancreatitis does seem more commonly discovered in middle aged men. Risk of chronic pancreatitis is equal among men and women when in beyond middle aged. Likeliness of acute pancreatitis increases with age [32]. Occurrences of pancreatitis that does not fall into these categories are regarded as from genetic origin, especially if the patient is of pre-adult age [42,43]. Men have a higher incidence rate of pancreatic cancer than women, and the disease presents itself usually older than 55 with a median age of 71 [32].

Despite there being no link between blood type and pancreatitis [44], people with a non-O blood type have a higher risk of pancreatic cancer [45]. Since the majority of pancreatic cancer incidences are sporadic and behavioral-dependent, the mechanism is unknown, however, approximately 20% of all pancreatic cancer cases will be of non-O blood type patients [46].

1.1.4 Molecular biology

Pancreatic ductal adenocarcinoma is a complex disease that is still relatively poorly understood, however, there are still some generalities characteristic of pancreatic cancer. Nearly every case reported contained mutations in *Kras* (kirsten rat sarcoma viral oncogene homolog)[47,48], and is generally understood to be one of the earliest steps in development of pancreatic cancer[49]. *Kras* encodes a proto-oncogene involved in cellular

growth, differentiation, and survival. Point mutations in this gene occur at three positions- G12, G13, Q61, and can result in dysregulation of GTPase activity [50]. G12 and G13 mutations result in constitutive activation caused by steric hindrance that prevents interaction with Ras-related GTPase-activating proteins [189]. The Q61 position interferes with the coordination of a water molecule necessary for GTP hydrolysis [189]. There are eight different mutations found at the G12 location, but the predominant one found in pancreatic cancer is the G12D mutation (glycine for aspartic acid) [189].

CDKN2A, a tumor suppressor that inhibits the S phase of the cell cycle, is inactivated in most pancreatic cancer cases [51]. Another tumor suppressor gene and transcription factor, p53 (Tumor Protein 53), is inactive in approximately 50-75% of PDAC cases [52], and is responsible for regulating several proteins involved in apoptosis, cell cycle, and DNA repair [53]. SMAD4 (mothers against decapentaplegic homolog 4) is a cell cycle protein that is inactivated in 55% of PDAC [51]. *Tgfb* (transforming growth factor β) is an anti-inflammatory gene that is associated with *Smad4* in the inhibition of cellular proliferation. It is postulated the inactivation of *Smad4* allows cancer cells to escape the anti-inflammatory effects of *Tgfb* [54]. EGFRs (epidermal growth factor receptors) are tyrosine kinases involved in proliferation that tend to be over expressed in PDAC. Tumor progression is exacerbated when ligands of these receptors become over-expressed as well [55]. Another example is *Vegf* (vascular endothelial growth factor) which has been associated with angiogenesis and is also commonly over-expressed [53]. *Mucin*, over-expressed in many cancers, is involved in secretion of a gel-like protective barrier over epithelial cells and the modulation of survival-signaling contributes to the protection of

tumors from immune response [186]. Cyclin D1, cell cycle protein involved in the G1 to S transition, is dysregulated as well [51].

Kras mutations and constitutive activation of EGFRs and their ligands may lead to downstream activation of several pathways, these signaling cascades play a role in the dysregulation of proliferation and PDAC development [50]. Constitutive activation of *Kras* may lead to activation of serine/threonine *Raf* kinases, which leads to phosphorylation of *Map2ks* (mitogen-activated protein kinase kinases) and then downstream *Erks* (extracellular signal-related kinases) [55]. *Pi3k* (phosphoinositide 3-kinase) may also become activated through *Kras* and activate downstream *Pkb* (protein kinase B) and *Mtor* (mammalian target of rapamycin) leading to dysregulation of cell cycle and proliferation [56]. *Nfkb* (nuclear factor κ-light-chain enhancer of activated B cells), over-expressed in most reported pancreatic cancer cases, is a transcription factor involved in cell survival, angiogenesis and metastasis [57]. One thing that is abundantly clear is the need for identification of more molecular targets. Targeting *Kras* has been difficult and ineffective in PDAC therapeutics, however, recent studies suggesting targeting one or several of its downstream effectors may prove more useful in preventing progression of the disease.

1.1.5 Inflammation

Inflammation is the protective immune response against injury or infection. Neutrophils are the first cells to migrate to the site of inflammation, modulated by localized macrophages and mast cells [190-192]. Leukocytes, lymphocytes, and other inflammatory cells are subsequently activated and migrate to the inflamed site using a complex signaling network of growth factors, cytokines, and chemokines (ex. *Mcp1*) [191].

These cells cooperatively breakdown tissue in efforts to destroy the source of infection [190,192]. An eventual shift occurs during these events, caused by pro- and anti-inflammatory cell signaling such as prostaglandin E2 (*Pge_2*, also *Cox2*) [188] and *Tgfb* [54] respectively, to terminate inflammatory response. Macrophages, dendritic cells, and phagocytes terminate inflammation by initiating apoptosis as well as phagocytosis [191]. This inflammatory program may become dysregulated which may lead to chronic inflammation. Macrophages and other inflammatory cells produce more growth factors, cytokines, and ROS that may continue breakdown of tissue [191,192]. The continuation of this response un-attenuated creates a microenvironment that may promote neoplasia [192]. Macrophages and T lymphocytes may release inflammatory TNFα and migration inhibitory factors that further contribute to damaging of DNA and accumulating oncogenic mutations [192].

Macrophages, neutrophils, eosinophils, dendritic cells, mast cells, and lymphocytes have all been found to play a significant role in sustaining a pro-tumorigenic microenvironment through infiltration of leukocytes and other immune cells to the site of inflammation [188,191,192]. In clinical studies it was discovered an increasing population of tumor associated macrophages (TAMs) was correlated with poor prognosis [188,193]. TAMs may contribute to cancer growth by release of IL10 and COX2 that suppress anti-tumorigenic response [191], and by increased expression of genes involved in angiogenesis and metastasis such as *Vegf*, *Fgf*, and *Mmp9* [194]. TAMs have also been found to release EGFR ligands that promote proliferation, and TNFα involved in cancer-related inflammation [190]. Mast cells and neutrophils have also been implicated in tumor angiogenesis, invasion, and metastasis in similar manner to that of TAMs [195]. Knockout of T cells resulted in a decrease in leukocytes infiltration and reduced expression of

Mmp9 in premalignant skin lesions [191]. It is clear the tumor microenvironment plays a critical role in sustaining tumorigenesis by not only protecting the tumor cells from the immune response but even using it to its advantage. Inhibiting this feedback loop by targeting one or more molecular pathways may provide an effective method in chemoprevention, or synergistic anti-cancer treatment in combination with chemotherapy agents.

1.1.6 Physiology

Pancreatic adenocarcinoma originates from three types of precursor lesions: pancreatic intraepithelial neoplasia (PanIN), macroscopic intraductal papillary mucinous neoplasm [58], and mucinous cystic neoplasm [59]. PanIN are the most common lesion and are classified as stage 1 (PanIN1) to stage 3 (PanIN3) based on severity of genetic alterations and damage to cellular structure and function [60]. (Figure 1.2) This has been a widely accepted morphological progression model for PanIN to PDAC, however, another model has also been proposed describing cancerous ductal cells originating from metaplastic acinar cells [61]. PanIN1A consists of flat tall columnar cells with nuclei still physiological similar to that of normal nuclei, though a small percentage may be misshapen. PanIN1B is characterized by a more pseudo-stratified architecture and a higher percentage of dysmorphic nuclei [187]. This stage is when *Kras* mutations begin to occur along with dysregulation of mucin [47,48,186]. PanIN2 is characterized by papillary mucinous epithelial lesions with nuclear abnormalities such as loss of polarity and enlarged nuclei [187]. Dysregulation of tumor suppressors and cell signaling proteins like p16 and cyclin D1, respectively, lead to this stage [51,52], and over-expression of EGFRs

occur [50]. Macrophages are recruited to the site of the tumor as well which will be discussed further in this review [188]. PanIN3 are all papillary lesions with total loss of polarity, goblet cells contain nuclei oriented toward the lumen and cytoplasm toward the basement membrane [187]. Dysregulation of metastasis genes *Mmp9* and *Tspan8*, and genes involved in cell migration, *Vcam* and *E-selectin* are reported [54,57].

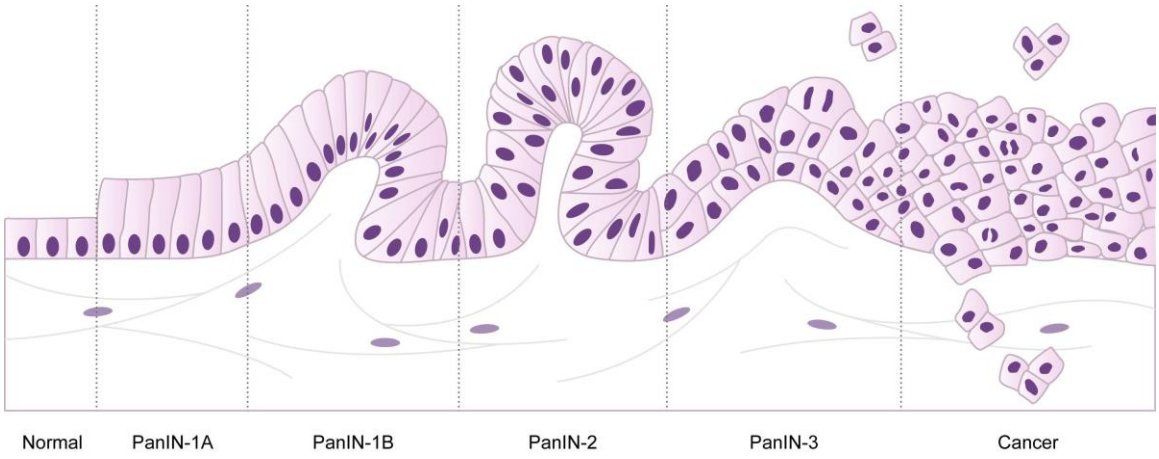

Figure 1.2 An illustration of the physiological changes that occur during PanIN progression to PDAC [62,179,180,181]. PanINs are staged from PanIN1A/B to 3. A series of physiological and molecular changes occur that allow development of metastatic tumors.

1.1.7 Stages

The American Joint Commission on Cancer (AJCC) has created a system entitled the Tumor-Node-Metastasis staging system to determine stage of development for pancreatic cancer. PDAC has been divided into four stages (Figure 1.3). Stages are qualified based on several considerations, including the size and extent of the primary tumor (T), regional lymph node metastasis (N), and distant metastasis (M). Stages 1 (T1) and 2 (T2) are defined by their confinement in the pancreas, while T3 and T4 are distinguished by their resectability. T4 is unresectable because of their progression into

the celiac axis or superior mesenteric artery [63]. Tumors that are extrapancreatic without reaching either of those two points are considered T3 stage. N0 indicates a lack of regional lymph node metastasis while M0 signifies a lack of distant metastasis, while N1 and M1 indicate the presence of metastasis. Stage I are tumors confined to the pancreas that are resectable. Stage II are tumors confined to the pancreas and are still resectable despite angiogenesis and N1 metastasis. Stage III are extrapancreatic but unresectable due to their involvement in the celiac axis and superior mesenteric artery. Stage IV are unresectable due to M1 metastasis. Furthermore, the progression into the superior mesenteric artery is also ranked by R0 to R2 stages of advancement, calling into question the nature of resectability and what qualifies as operable and inoperable tumors [64]. Due to its asymptomatic nature, approximately 68% of newly diagnosed patients are late stage cancers [63]. Tumors may metastasize through infiltration of lymph nodes or blood vessels such as the hepatic and/or superior mesenteric artery, portal vein, or celiac axis, and depending on the nature of metastasis may reach several distal organs like the lungs, liver, or peritoneal cavity [63,64].

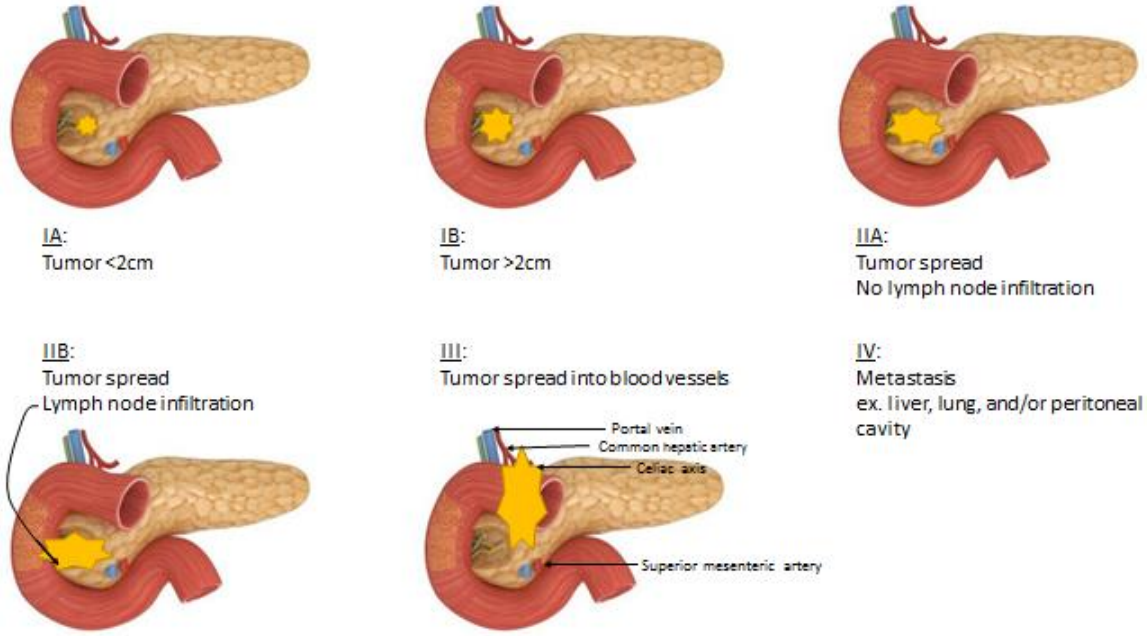

Figure 1.3 Stages of pancreatic cancer as determined by the American Joint Commission on Cancer (AJCC) [63]. Tumors below 2cm in size is staged 1A, and above is considered 1B. Tumors staged IIA have spread further without infiltration into lymph nodes, IIB has lymph node infiltration. Stage III advanced into the blood vessels and adjacent organs. Stage IV have metastasized into distal organs.

1.1.8 Cell lines

It has been discovered that cell lines for colon and breast cancer properly recapitulate the genomic events leading to the physiological changes and neoplasia found in patients, and this is also true for pancreatic cancer with multiple cell lines exhibiting common mutations found in patients with similar phenotypes [204]. Cell line work, *in vitro* and *in vivo*, have become a convenient starting point for proof of principle studies for early stages of clinical drug development. A better understanding of the specific molecular mechanisms involved in pancreatic cancer development and progression is paramount in establishing early detection and preventative strategies, as well as

therapeutic options. Two pancreatic cancer cell lines were utilized in the studies presented in this dissertation, Mia PaCa-2 (*Cox2*-negative) and BxPc-3 (*Cox2*-positive) cells.

Mia PaCa-2 cells were derived from adenocarcinoma of a 65-year-old man. The tumor extended the body of the pancreas into the tail and was infiltrating into the periaortic artery. The cells do not produce measurable amounts of carcinoembryonic antigen [205]. BxPc-3 cells were derived from adenocarcinoma of a 61-year-old woman. This cell line is not characterized as metastatic, and tumors grown in xenograft mice resemble primary tumors of patients. These cells produce carcinoembryonic and pancreas-specific antigen, and mucin [206].

BxPc-3 cells have a higher adhesion ability compared to Mia PaCa-2 cells exhibiting greater adhesion to collagen I, fibronectin, and laminin [207], however, Mia PaCa-2 cells have higher adhesion to collagen IV [208]. It was important for the studies in this dissertation to assess the effects of GW501516-activation of PPARβ/δ pathways on inflammation and metastatic potential in a highly aggressive cell line (Mia PaCa-2) and a non-metastatic cell line (BxPc-3). Additionally, another key component of metastasis is cell migration, and the migratory ability of these cell lines are a point of contention in the literature. A study in Matrigel, collagen, and laminin showed BxPc-3 cells to have more invasive potential than Mia PaCa-2 cells [209], however, several other studies indicate the opposite [207,210].

Cox2, usually over-expressed in malignancies, promotes angiogenesis by metabolism of arachidonic acid into activating factors that contribute to angiogenic signaling [211], and is also activated by *Nfkb* by prostaglandins. BxPc-3 cells were chosen

as an alternative pancreatic cancer cell line in these studies for their expression of *Cox2* to determine if the absence of *Cox2* significantly affected biomarker expression of angiogenesis. Mia PaCa-2 cells have very low angiogenic marker expression such as *Il8*, *Il1a* is undetectable, and consistent with these observations is the increase in capillary-like structures in co-culture of BxPc-3 and HUVEC cells, an observation not present with Mia PaCa-2 cells [212].

1.2 Mouse models of pancreatic cancer

Since mutations causing activation in *Kras* occurs within over 90% of PDAC cases most genetic mouse models are based on this premise [65]. Mice expressing a mutant form of *Kras* usually develop early and advanced forms of pancreatic cancers found in humans, this provides preclinical models to analyze the molecular biology of disease progression and quantify the effects of new therapies [66]. Several mouse models of pancreatic adenocarcinoma exist (Table 1.1). In this dissertation we have utilized the Cre-mediated activation of *Kras(G12D)* mouse model known for full recapitulation of human PanIN to PDAC progression. As stated previously, mutations at the G12 position of *Kras* are most common in pancreatic cancer in humans (approximately 90%), and the predominant mutation is the replacement of a glycine with an aspartic acid (G12D) [65,189]. Despite varying inconsistent tumor development time and the tendency for tumors to grow throughout the pancreas, we chose this model due to its accurate physiological recapitulation of pancreatic cancer progression making it an excellent model for early molecular prognostic studies. Progeny results from the mating of two mouse models: *Pdx1*-Cre mouse and the LSL-*Kras(G12D)* mouse (described below).

Table 1.1 List of mouse models for pancreatic cancer including time of expression and tumor development, phenotype, and survival [67].

Genotype	Time of expression	Time to tumor development (mo)	PC phenotype	Survival (mo)
Pdx1-cre, LSL *Kras*^{G12D}	E8.5	6	PDAC, penetrant PanIN, age-dependent severity	16
P48$^{+/-}$cre, LSL *Kras*^{G12D}	E9.5	8	PDAC, penetrant PanIN, age-dependent severity	16
Pdx1-cre, LSL *Kras*^{G12D}, LSL *Trp53*$^{R172H/-}$	E8.5	2-3	PDAC	5-6
Mist1$^{KrasG12D/+}$	E10.5	2	Accelerated acinar-derived PanIN, Differentiated	10.8
Kpcb$^{wt/wt}$	E8.5	2-3	PDAC	5.6
Kpcb$^{Tr/wt}$	E8.5	3	PDAC	4.8
Kpcb$^{Tr/\Delta 11}$	E8.5	1.5	PDAC	2.8
Ckb$^{wt/\Delta 11}$	E8.5	6	PDAC	12
Ckb$^{wt/wt}$	E8.5	6	PDAC	13.5
Cpb$^{\Delta 11/\Delta 11}$	E8.5	3-5	PDAC	10
Pdx1-cre, *Kras*^{G12D} *Ink4A/Arf*$^{flox/flox}$	E8.5	2	PDAC, accelerated PanIN, Poorly differentiated	2-3
Pdx1-cre, *Kras*^{G12D} *Smad4*$^{flox/flox}$	E8.5	2-3	Intraductal papillary mucinous neoplasia	2-6
Ptfla$^{Cre/+}$, LSL *Kras*$^{G12D/+}$, *Tgfbr2*$^{flox/flox}$	E9.5	1	PDAC, accelerated PanIN	2

1.2.1 *Pdx1* Cre Mouse

Pancreatic and duodenal homeobox 1 (*Pdx1*) Cre mice express high levels of Cre recombinase in the pancreas. *Pdx1* is a critical transcription factor in the development program of the pancreas [70]. When mating this strain with Tyler Jacks' LSL *Kras(G12D)* strain, the *Pdx1*-activated Cre recombinase causes ductal lesions that recapitulate the full spectrum of PanINs. Mice are usually infected via intranasal injection of an adenovirus that encodes Cre [69].

1.2.2 LSL *Kras(G12D)* mouse

This mouse model contains a latent point mutation in the *Kras2* allele that leads to a glycine being substituted for an aspartic acid that activates constitutive downstream signaling of ras pathways [68]. A lox-stop-lox (LSL) termination cassette was inserted into the *Kras* locus upstream of locus 1. When crossed with the *Pdx1* Cre mouse, activation of the *Pdx1* gene within the progeny of these mice results in Cre-mediated recombination that removes the transcriptional termination sequence [69], and results in pancreas epithelium-specific *Kras*(*G12D*) expression at physiological levels by the native *Kras* promoter [68]. Mice that are homozygous for this mutation end up dying *in utero* [69].

1.2.3 Cre-activated *Kras(G12D)*

Pancreas from mice containing the heterozygous *Kras(G12D)* mutation are larger than their control counterparts. Ductal lesions identical to the stages of human PanINs develop over time. Approximately two-week-old mice develop PanIN-1A lesions and higher grade lesions develop as the mice ages in higher frequencies. Latter stages begin

to show presence of stromal or desmoplastic fibroblasts and inflammatory cells which is characteristic of the beginning stages of cancer. Notch signaling, a pathway modulating quiescence, is highly activated well as inflammatory pathways such as Hes family BHLH transcription factor 1 (*Hes1*) and cyclooxygenase 2 (*Cox2*), and metastasis genes such as matrix metallopeptidase 7 (*Mmp7*) [67]. Some of these lesions will progress to invasive and metastatic PDAC within a year that replicate the PDAC found frequently in humans making this mouse model an invaluable tool [69].

1.3 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated type II nuclear receptor transcription factors that are involved in regulation of genes that control differentiation, glucose and lipid metabolism [71], and tumorigenesis [72]. These transcription factors consist of three subtypes, PPARα, β/δ, and γ. PPARα is expressed mainly in the hepatocytes and skeletal muscle with trace amounts in cardiomyocytes, kidney, intestine, and brown adipose tissues [73]. PPARγ, through differential splicing, yields four different isoforms that are mainly expressed in adipose tissue. PPARγ1 is ubiquitously expressed while PPARγ2 is expressed mostly in adipose tissue, PPARγ3 is found in macrophages and intestine, and PPARγ4 is expressed solely in endothelial cells [74]. PPARβ/δ is ubiquitously expressed, but at highest levels in the gastrointestinal tract, kidneys, and skeletal muscle. PPARβ/δ is the least studied isotype, and the only PPAR known to associate with tumor suppressor, B-cell lymphoma 6 (BCL6) [73,75].

PPARs dimerize with the retinoid X receptor (RXR) subfamily, more specifically with the α subtype (RXRα). The function of the PPAR is dependent upon the conformation of its ligand-binding domain, which can be altered based on the presence or absence of coactivator or corepressor proteins [76]. Several natural and synthetic ligands are capable of activating all PPARs or be isotype-specific. Unsaturated fatty acids and their metabolites tend to activate all isotypes of PPARs (PPAR agonists). Fenofibrate, clofibrate, and gemfibrozil are PPARα-specific synthetic ligands. PPARγ is activated by several synthetic ligands, such as the anti-diabetic drug rosiglitazone [77]. The main synthetic compounds that are PPARβ/δ-specific and the main focus of this dissertation, GW501516 and GW0742, are approximately 1000-fold selective for the PPARβ/δ subtype with an EC_{50} of 1nM and 1.1nM, respectively [78,79]. The studies in this dissertation will use concentrations of 500nM and 250nM for optimal induction of a PPARβ/δ response, effects on PPARα and γ aren't seen until approximately 1000nM. A list of PPARβ/δ ligands is shown in Table 1.2 and chemical structures of those most used in research and clinical trials shown in Figure 1.4. Upon ligand activation, a conformational change causes dissociation of the corepressor complex, translocation to the nucleus and binding to the peroxisome proliferator response element (*PPRE*) of the target gene, the liganded complex then recruits coactivators to initiate the transcriptional machinery [80].

Table 1.2 A list of PPARβ/δ ligands [77].

Ligands	
Synthetic agonists	L165014 GW501516 GW0742 GW9578 L783483 L796449 L165041
Fatty acids/metabolites	Docahexanoic acid Arachidonic acid Linoleic acid C6-C8
Eicosanoids	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 PGJ_2 PGB_2 $PGA_{1/2}$
Vitamins	trans-retinoic acid
Synthetic antagonists	GSK0660 Sulindac
Fatty acid inhibitors	2Br-C16 Tetradecylglycidic acid
Other	Sphingolipid analogues Perfluorooctanoic acid Perfluorooctane sulfonate

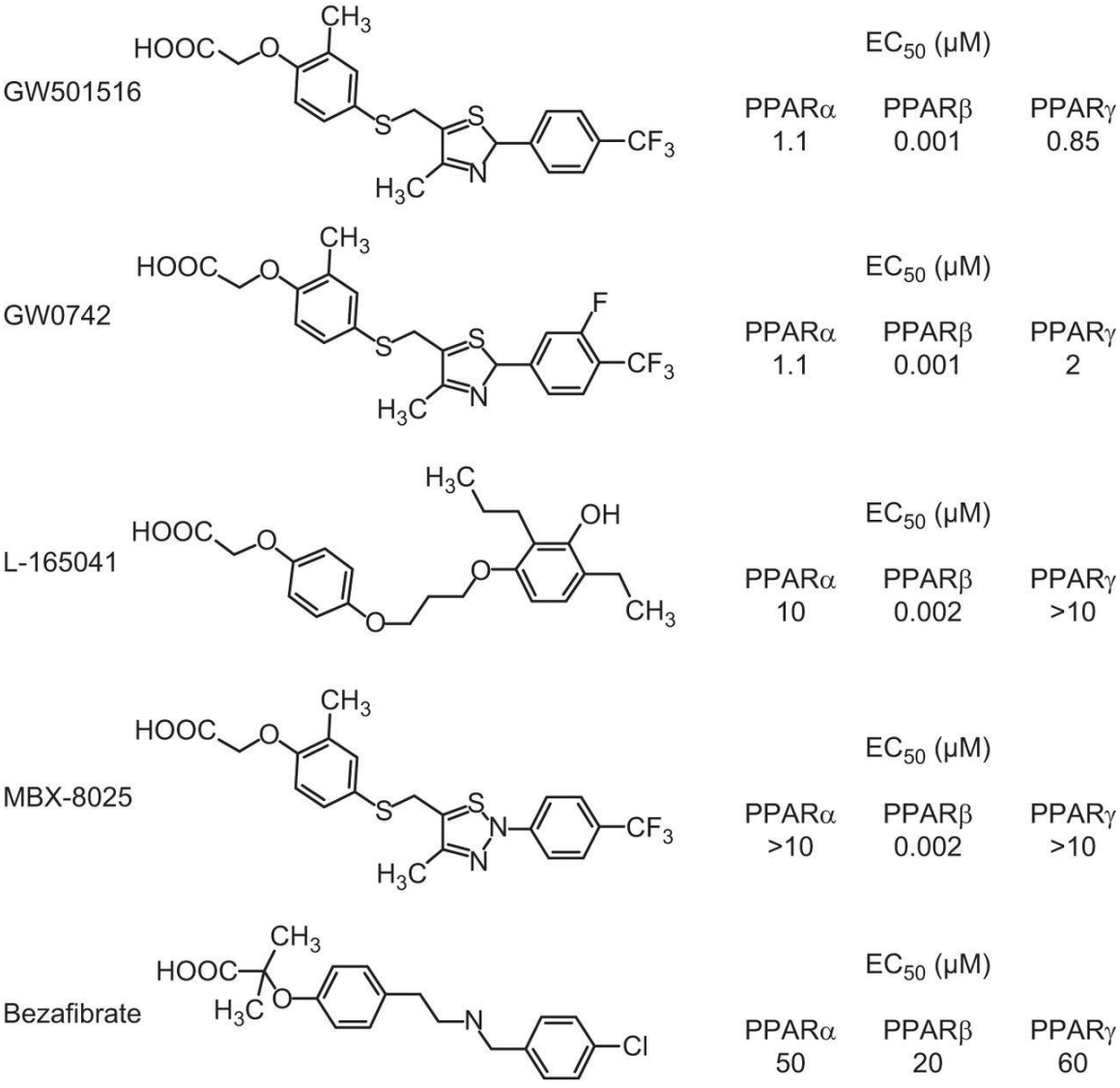

Figure 1.4 Chemical structures of PPAR ligands and the efficacy for each subtype. Ligands are highly selective for PPARβ/δ [178].

The three types of PPARs are transcribed from different genes. *Ppara* is located on chromosome 22q12-13.1, *Pparb* from 6p21.2-21.1, and *Pparg* from 3p25 [73,81]. Similar to other nuclear receptors, PPARs have six distinct regions: an N-terminal region containing a ligand-independent AF1 transactivation domain (A/B), a DNA-binding

domain (DBD) consisting of two zinc finger motifs that bind to *PPRE*s (C), a flexible hinge region that contains the Nuclear Localization Signal (NLS) that tags the protein for transport into the nucleus (D) [82], a dimerization domain and ligand-binding domain (LBD) consisting of 13 α helices and a four-stranded β sheet (E), and, a C-terminal region containing the ligand-dependent AF2 transactivation domain (F) [73] (Figure 1.5).

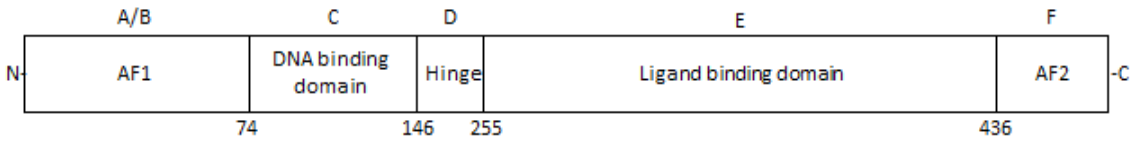

Figure 1.5 The structure of PPARs [83]. The A/B domain contains the ligand-independent activation function 1 (AF1), the C domain is the DNA binding domain, the D domain is the hinge region, the E domain is the ligand binding domain, and the F domain contains the ligand-dependent activation function 2 (AF2).

1.3.1 PPARβ/δ

PPARβ/δ, the least studied of the PPAR types, has been considered a potential drug target for treatment and prevention of metabolic syndrome due to its ability to modulate insulin sensitivity and increase fatty acid catabolism [84]. There is also strong evidence it can control gastrointestinal function and mitigate metabolic disease [85]. PPARβ/δ plays a context-specific role in adipose tissue mass, skin inflammatory responses, would healing, and neural cell myelination [84]. However, whether or not PPARβ/δ could be a potential target in cancer prevention and treatment is still yet to be seen and is not without controversy, as will be discussed below.

PPARβ/δ is ubiquitously expressed and generally lower in tumor tissue compared to adjacent non-transformed tissue, though higher expression in tumors have been reported in lung and colon [72]. PPARβ/δ is moderately expressed in the pancreas relative to other tissues, expression is slightly higher in liver, thyroid, and lung, and much higher in placenta [83]. According to Segara *et al*., *Pparb* is among the top 10% of genes found over-expressed in pancreatic ductal tumors [197]. We sought to confirm this in human tumor tissue with corresponding adjacent normal tissue obtained from Penn State Hershey.

1.3.2 DNA binding

PPARβ/δ may exert its function by four mechanisms: (1) Increasing target gene transcription in response to ligand activation via a *PPRE*-dependent process (canonical PPAR activation, see Figure 1.6). A conformational change occurs leading to the release of the corepressor complex and auxiliary proteins, and recruitment of coactivators that contain histone acetylase (HAT) [73]. Histone acetylation frees the DNA from around the histones allowing RNA polymerase II to bind leading to gene transcription [81]. (2) Binding with other transcription factors such as NFκB and suppressing inflammatory signaling in a *PPRE*-independent pathway, sometimes called "squelching". (3) Repressing gene expression when bound to an antagonist by recruiting histone deacetylase (HDAC) activity of the corepressor silencing mediator for retinoic receptor and thyroid-hormone receptor (SMRT) [85]. Finally, (4) release of the transcriptional repressor protein BCL6 upon ligand binding (Figure 1.7). The last pathway (regulation of BCL6 activity) will be discussed in more detail below.

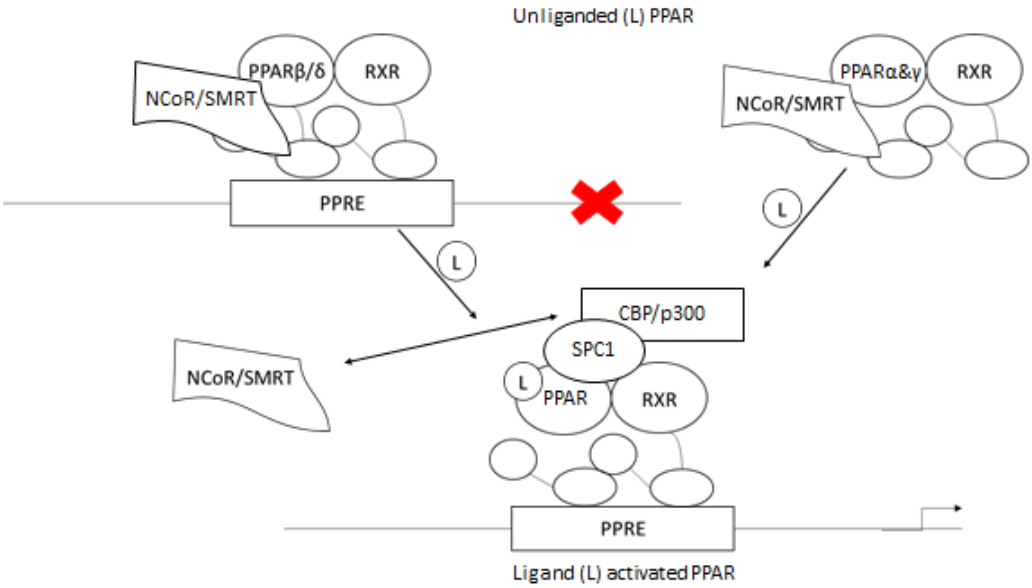

Figure 1.6 Mechanism of PPARβ/δ activation [83]. Unliganded PPARβ/δ is dimerized with RXR and is bound to response elements of target genes with the corepressor complex. When activated dissociates the corepressor complex, translocates to the response elements of target genes, and recruits coactivators that allows recruitment of the transcriptional machinery to the TATA box.

1.3.3 Association with BCL6

PPARβ/δ is the only PPAR that associates with the tumor suppressor BCL6 [86] (Figure 1.7). Immunoprecipitation in human embryonic kidney cells demonstrated the interaction between PPARβ/δ and BCL6 in the absence of ligand, treatment with GW501516 lead to dissociation of the two proteins. A mutant construct of *Pparb* lacking the *Af2* transactivation domain maintained BCL6 interaction with or without ligand. Glutathione S-transferase pull-down assays and mutant constructs of BCL6 lacking the zinc finger domain showed this region was necessary in its interaction with PPARβ/δ.

PPARα and PPARγ were also analyzed and found to have no affinity for BCL6 [87]. BCL6, when dissociated from PPARβ/δ, binds to and represses transcription of *Mcp1*, *Mcp3*, and *Mip1b*, and has anti-inflammatory properties in cooperation with PPARβ/δ [87,88].

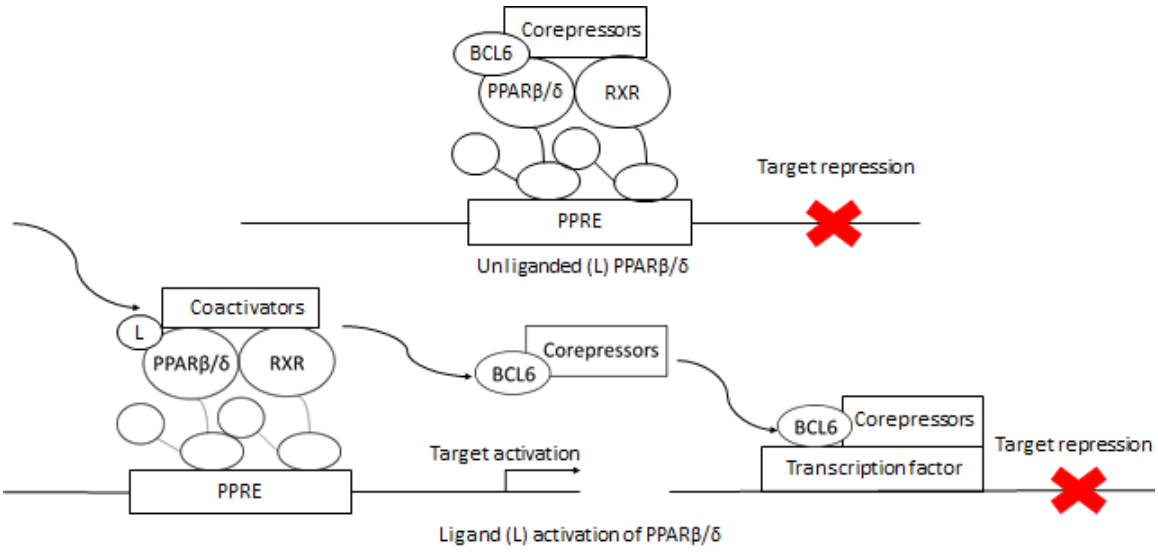

Figure 1.7 PPARβ/δ transrepression via BCL6 [89]. Ligand activation of PPARβ/δ causes a conformational change that allows dissociation of BCL6 where it translocates to the promoter region of several target genes involved in inflammation and metastasis.

1.3.4 Gene regulation

The *Pparb* gene is without a TATA box, a promoter sequence where transcription traditionally begins. Instead, a promoter sequence was discovered approximately 380bp upstream of the transcription initiation ATG codon where transcription regulation occurs [90]. This region is rich in specificity protein-1 (*Sp1*) binding elements, a zinc finger

transcription factor with the ability to be an activator or a repressor of transcription, involved in cellular differentiation, growth, apoptosis, and chromatin remodeling, as well as post-translational modifications such as acetylation, glycosylation, and phosphorylation [91]. The presence of *Sp1* binding elements are characteristic of housekeeping genes, as well as prevalent in *Nfkb* [92,93]. Multiple 5' untranslated region (UTR) splice variants in *Pparb* mRNA may affect protein translation efficiency, the dysregulation of which may be a factor in its role in disease. The role of PPARβ/δ 5' splice variants is yet to be elucidated. Furthermore, a 3' splice variant has been linked to differential expression of a dominant negative PPARβ/δ that is expressed in several cell lines [94]. Secondary or multiple promoters may be present adding more complexity to the regulatory mechanisms of *Pparb* [95]. Activator protein 1 (AP1) is a transcription factor involved in increased expression of genes in response to cytokines, growth and stress factors, and infection, by binding to 12-O-tetradecanoylphorbol-13-acetate (TPA) DNA response elements [96]. These binding sites are present in the promoter region of *Pparb* inducing expression in response to the kinase cascade in keratinocytes [97]. PPARβ/δ also interacts with steroid receptor coactivator 1 (SRC1) [98] and the MAPK pathway involved in phosphorylation of SRC1 [99]. SRC1 is recruited to promoters via nuclear receptor activation where it provides histone acetyltransferase activity to allow transcription to occur of the target gene [98]. Protein kinase A (PKA), in the presence of cyclic AMP, phosphorylates the DNA binding domain of PPARβ/δ modulating protein:DNA binding affinity [100]. It is due to this that ligand binding with elevated levels of cAMP may increase PPARβ/δ transactivation activity adding an additional layer of trans-regulation of transcription factors [101].

1.4 Roles of PPARβ/δ

1.4.1 Oxidative stress

Oxidative stress is not only a contributing factor of aging but also a causative factor of many diseases resulting from molecular damage caused by reactive oxygen species (ROS) and oxygen-derived free radicals [102]. Oxidation of low-density lipoprotein (LDL) has been linked to hardening of arterial walls and causing atherosclerosis and heart disease [103]. Low blood antioxidant levels is associated with other metabolic disorders such as arthritis [104] and diabetes [27,105]. Diets low in antioxidants, such as lacking fruits and vegetables, have been linked to chronic inflammation leading to development of cancer [16,20,61].

A target gene of PPARβ/δ, *Catalase*, is involved in the inhibition of oxidative stress in a PPARβ/δ-dependent manner [106]. 8-iso-prostaglandin-F2α, a marker of systemic oxidative stress, was reduced by 30% with treatment of GW501516, a PPARβ/δ-specific ligand, no reduction was observed with activation of other PPAR subtypes, although PPARs have been implicated in the reduction of oxidative stress [107,108]. An increase in glucose concentration inhibited the expression of *Pparb* in a dose-dependent manner suggesting that ROS-mediated cell hypertrophy occurs through the reduction of *Pparb* [109]. 4-hydroxynonenal (4-HNE) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) are mediators of oxidative stress that were reduced in the livers of rats treated with alcohol diets containing GW501516 compared to DMSO control diets. 8-OHdG production was significantly reduced by PPARβ/δ activation but was unable to completely protect from DNA damage [110]. 4-HNE has been discovered to bind and activate PPARβ/δ, protecting

against oxidative damage in hepatocytes [111]. Furthermore, PPARβ/δ induced angiopoietin-like protein 4 (*Angptl4*), a protein involved in the reduction in fatty acid-induced oxidative stress in cardiomyocytes [112].

1.4.2 Metabolic disorders

As previously stated, oxidative stress is a key step in the development of metabolic disorders such as diabetes, atherosclerosis, and arthritis. Oxygenase-1 (*Ho1*) is a target for PPARβ/δ, and a constitutively active *Ho1* induces *Pparb* in a positive feedback loop while inhibition of *Pparb* reduces the protective effect in vascular endothelium [113]. Multiple studies have shown PPARβ/δ to be beneficial against atherosclerosis [114,115]. Ligand-activated PPARβ/δ prevents weight gain and suppresses macrophage-derived inflammation, indicating it may be a promising therapeutic target for treatment of obesity and dyslipidemia [116]. Mice expressing a constitutively active *Pparb* had higher levels of lipid metabolism than control mice, control mice increased in body weight when given high fat diets relative to those with constitutively active *Pparb* [117]. Dyslipidemia is defined by high levels of triglycerides and low levels of high density lipoprotein (HDL) and is a very common metabolic disorder. GW501516 was able to increase HDL in mice by approximately 50% regardless of their body mass index (BMI) [118,119]. Significant reduction in Niemann-pick C1-like 1 (*Npc1l1*) in intestine led to decreased cholesterol absorption in wild-type mice and increased expression of cholesterol efflux regulator protein (*Cerp*) [119]. GW501516 also reduced diabetic symptoms in ob/ob mice by reducing blood glucose and insulin levels [120]. Further ob/ob

mouse studies have shown that PPARβ/δ activation in the liver increased fatty acid oxidation while reducing gluconeogenesis [121].

1.4.3 Inflammation and cancer

NFκB is a ubiquitously expressed transcription factor that is a key regulator of inflammation by modulating expression of several genes. NFκB/Rel proteins consist of class I: p105/p50, p100/p52, and class II: RelA (p65), RelB, and c-Rel [199]. The most abundant heterodimer being p50 and p65 [141]. *Nfkb1* transcribes the p105 protein, a Rel-specific inhibitor that through the 26S proteasome goes through cotranslational processing and is altered to p50, the DNA-binding subunit of *Nfkb* [199]. When in an inactive state, NFκB is sequestered by IκB inhibitory proteins in the cytoplasm. When stimulated with pro-inflammatory cytokines, like TNFα and IL1, NFκB is freed by phosphorylation of IκB. NFκB then translocates to the nucleus where it interacts with a wide arrange of promoters [200]. The p65-p50 and RelB-p50 dimers are considered transcriptional activators of inflammation. The p50 homodimer is a transcriptional repressor, however, have been known to be activators in complex with BCL3 [201]. PPARβ/δ exerts its anti-inflammatory function, partially, by binding to and suppressing the p65 subunit in endothelial cells and cardiomyocytes [198], as well as inhibit activity indirectly via BCL6 [141]. Additionally, PPARβ/δ agonist, GW501516, reduced TNFα-induced p65 acetylation through an increase in AMPK and p300 phosphorylation reducing p300 activation of p65 [203]. The interaction between PPARβ/δ and NFκB is still relatively unexplored in the pancreas.

Inflammation has long been linked as a major causative factor to cancer initiation and progression [1]. It has been established PPARβ/δ and its ligands have anti-inflammatory activity [106,108,122–124]. Signal transducer and activator of transcription 3 (STAT3) is over-expressed in many cancers and is anti-apoptotic [125], the anti-inflammatory properties of PPARβ/δ may be attributed in part by its inhibition of STAT3 [31,126] and extracellular-signal-regulated kinase 5 (ERK5) [127]. An anti-inflammatory role for PPARβ/δ has been found in the gastrointestinal tract [128,129], specifically inflammatory bowel disease [130,131], however some research suggests PPARβ/δ may exacerbate inflammation [132]. The biggest controversy lies within PPARβ/δ's role in colon cancer with some attributing PPARβ/δ to be anti-inflammatory [133–135] and some pro-inflammatory [136,137], aiding in the progression of the disease [138]. PPARβ/δ is over-expressed in colon cancer, and genetically modified mice disrupting expression attenuates incidence of colorectal cancer through reduction of *Vegf* [128]. Wnt/β-catenin has also been shown to stimulate *Pparb* expression, and activation of *Cox2* resulted in the production of prostaglandin E2 activating PPARβ/δ via the PI3K-AKT pathway [129]. Hypotheses have been proposed to explain this contradiction from ligand-activation to promote tumorigenesis to ligand-activation inhibiting tumorigenesis, all consisting of various proposed mechanisms, but the verdict is still out as the underlying pathways are still poorly understood.

Another model in which PPARβ/δ is poorly understood is the pancreas, although initial reports speculate the transcription factor to be anti-inflammatory [139], especially in cooperation with its associated transcriptional suppressor, BCL6 [75,140,141]. PPARβ/δ

activated by GW0742 reduced acute pancreatitis caused by cerulean and taurocholate [202]. Further studies are necessary to delineate the role of the PPARβ/δ/BCL6 complex in the pancreas, specifically ductal adenocarcinoma. PDAC not only makes up the majority of pancreatic cancer cases but they express *Bcl6*, whereas β cells of endocrine tumors lack BCL6 protein [75].

1.5 B-Cell lymphoma 6

1.5.1 Structure

Bcl6 is expressed moderately in the pancreas compared to other tissues with vastly higher levels in blood and skeletal muscle, it is a nuclear phosphoprotein that is prominently expressed in mature B cells during germinal center differentiation [87]. It is made up of six kruppel-type C-terminal zinc-finger motifs that recognize consensus DNA sequences in vitro, indicating it plays a role in transcriptional regulation, and an N-terminal *Btb/Poz* (Pox virus and zinc finger) autonomous transrepression domain of approximately 120 amino acids involved in homo- and heterodimerization [142,143] (Figure 1.8). The *Poz* domain is made up of α-helices flanked by β-sheets and is responsible for transrepression of nuclear receptor corepressor 2 (NCOR2)-SIN3 transcription regulator family member A (SIN3A)-HDAC corepressor complex [144], and may be a dimer partner with other *Poz*-expressing transcriptional regulators [145]. Transrepression of DNA binding sites by BCL6 can occur regardless of 5' or 3' orientation and distance from the promoter sequence of target genes. Amino acids 11-121 in the *Poz* domain and 267-395 have been found necessary for transrepression [144]. BCL6 contains several PEST motifs rich in

proline (P), glutamic acid (E), serine (S), and threonine (T) [146]. PEST motifs contain MAPK phosphorylation sites that are required for ubiquitin-proteosome-mediated phosphorylation and degradation [147,148]. BCL6 is also subject to CBP-mediated acetylation by p300 [93].

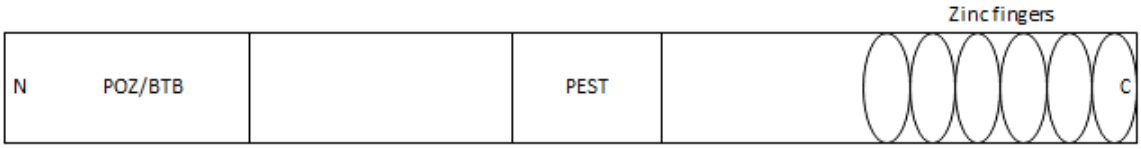

Figure 1.8 Structure of BCL6 [149]. Pox virus and zinc finger (POZ)/BR-C, ttk and bab (BTB) domain near the N-terminus mediates homo- and heterodimerization. A group of α helices flanked by short β sheets make up the central scaffolding. PEST sequences may play a role in protein degradation. The zinc finger domain at the C-terminus provides a transcription factor its binding strength. The DNA-binding loop appear to form finger-like looking projections.

1.5.2 Physiological function

Bcl6 is involved in the regulation of B lymphocyte development and growth [150,151] and altered expression of *Bcl6* has been linked to development of hematologic malignancies [87,152,153]. It is a potent inhibitor of senescence in mice [154] and plays a role in primary human B cell replication [155]. BCL6 modulates the transcription of signal transducers and activators of transcription (*Stat*)-dependent IL4 responses of B cells [156], and can bind to DNA elements similar to that of *Stat* recognition sites [157,158]. Analysis of the murine germline ε promoter involved in immunoglobulin class switching has shown three BCL6 binding sites, cooperative binding of at least two being required for

repression, that are also competitive binding sites for STAT6 and CCAAT/enhancer-binding protein β (C/EBPβ) [158]. At least one BCL6 recognizes the DNA sequence- 5' GAAAATTCCTAGAAAGCATA that is flanked by several residues that increases the transcription suppressor's binding affinity [150]. Chromatin Immunoprecipitation assays have detected BCL6 bound to promoter regions of vascular cell adhesion molecule 1 (*Vcam1*) [159]. NFκB motifs are found near STAT6 motifs, suggesting another gene suppressed by BCL6 both *in vivo* and *in vitro* [88,141]. *Bcl6* cDNA retrovirally-infected into epithelial cells resulted in cells that were resistant to apoptosis and showed cell survival [160], indicating that this protein has effects on cell survival pathways.

BCL6 predominantly plays a role in the immune system. A $Bcl6^{-/-}$ mouse model was unable to develop germinal centers despite normal lymphoid development, perhaps due to a defect in T cell-dependent antibody response and commonly suffered growth retardation and died of severe myocardial injury [161]. Additionally, these mice exhibited an increase in the Th2 immune response such as eosinophil infiltration and immunoglobulin E (IgE), indicative of an inhibitory effect of the T helper lymphocyte Th2 response by BCL6 [162]. Protein coding 19 $(CD19)^{-/-}$ mice, deficient in B1 lymphocytes and have severely impaired T cell antigen response, failed to develop germinal centers as well but due to a defect in lymphoid organ development, unlike $Bcl6^{-/-}$ mice [163], therefore the mechanism by which BCL6 affects germinal center formation needed to be elucidated. Recombination activating gene 1 $(Rag1)^{-/-}$ mice, a mouse model unable to produce mature T and B cells, were given bone marrow from $Bcl6^{-/-}$ mice, showing differentiation of germinal center of B cells [164]. This indicates the $Bcl6^{-/-}$ mice contain a deficiency in B

cell development and BCL6 plays a role in regulation of germinal center formation. BCL6 targets are genes involved in lymphocyte activation and differentiation such as PR domain containing 1 (*Prdm1*) [165], chemokine CXC motif receptor 4 (*Cxcr4*)) [166], inflammation (*Nfkb, Mcp1*) [75,87], and cell cycle regulation (*Cyclin d2, P53*) [153,167]. Cytokine-induced cell cycle arrest and apoptosis was reversed by BCL6 direct repression of *Stat3* [168].

1.5.3 Proto-oncogene

Bcl6 gene rearrangement was identified in diffuse large B-cell lymphomas (DLBCL) on the chromosome location 3q27. Non-Hodgkin lymphoma represents many types of cancers of the lymphocytes that arise from either B- or T-cells [169]. Rearrangement affecting *Bcl6* was present in approximately 30-40% of DLBCL cases [170]. Although the coding region remains intact, the 5' non-coding region undergoes translocation, resulting in a promoter substitution that may alter the regulatory role of *Bcl6* [171]. Somatic point mutations in the regulatory region of *Bcl6* are most commonly found in B cell malignancies, DLBCL-derived *Bcl6* alleles have shown these non-coding mutations alter expression. This was substantiated in a mouse model expressing a mutant *Bcl6* mimicking the t(3,14)(q27,q32) translocation observed in DLBCL patients, incidence of B cell lymphoma development was significantly higher (30-60%) compared to control mice and introduction of carcinogens increased incidence more so compared to wild-type [172,173]. It is postulated that *P53* inactivation by BCL6 plays a significant role in lymphoma development [153]. BCL6-specific peptide inhibitors induce cell cycle arrest and

apoptosis in *Bcl6*-positive lymphoma cells [174,175], and may have a favorable clinical prognosis [176]. Examination of expression patterns of BCL6 have also aligned it with favorable clinical prognosis, with higher expression being linked to lower incidence [177].

1.6 Rationale and hypothesis

The role of PPARs in inflammation has been gaining notoriety for several years and have been largely accepted as anti-inflammatory, despite conflicting *in vitro* and *in vivo* studies in the colon. PPARβ/δ's role in the pancreas has remained unexplored. PPARβ/δ is involved in numerous cellular processes, more important of which appears to be beneficial roles in metabolic disorders such as diabetes, atherosclerosis, obesity, and several other inflammatory diseases. Several studies identify metabolic disorders as a significant causative factor of pancreatic cancer, and the link between inflammation and cancer has long been established. The role PPARβ/δ in chemoprevention is poorly understood. Studies presented in this dissertation will explore the hypothesis that the PPARβ/δ and BCL6 pathway plays a role in the progression of pancreatitis to the development of pancreatic cancer, and that the PPARβ/δ agonist GW501516 could be an effective therapeutic option for pancreatic cancer.

1.6.1 Sub-Hypothesis 1: PPARβ/δ and BCL6 are anti-inflammatory in pancreatic cancer cells and inhibit cell migration and metastasis

The data presented in the second chapter aims to substantiate the PPARβ/δ and BCL6 pathway as being involved in anti-inflammatory signaling in pancreatic cancer

cells. We demonstrated that PPARβ/δ inhibits NFκB directly and indirectly via tumor suppressor, BCL6. Indeed, PPARβ/δ induces anti-inflammatory target genes and inhibits pro-inflammatory genes via BCL6. Furthermore, the activation of PPARβ/δ in pancreatic cancer cells appeared to reduce inflammatory signaling in THP-1 differentiated macrophages in a BCL6-dependent manner. Suggesting this pathway may play a role in the crosstalk between cancer cells and TAMs within the surrounding microenvironment.

The third chapter is published research that focused on the PPARβ/δ and BCL6 pathway in cell migration and metastasis. We demonstrated that PPARβ/δ activation inhibited several genes involved in regulation of cell invasion, in part by BCL6, and that this also translated to the protein level for MMP9. Activation of this pathway also reduces invasion of multiple pancreatic cancer cell lines.

1.6.2 Sub-Hypothesis 2: Over-expression of PPARβ/δ exacerbate PDAC progression, potentially via sequestration of BCL6, an effect ameliorated by GW501516

The fourth chapter acknowledges a reoccurring theme untouched in the previous chapters that PPARβ/δ expression, in its unliganded state, appeared to be inflammatory. An effect reversed by treatment with PPARβ/δ-agonist GW501516. We sought to determine whether inactive PPARβ/δ sequestered BCL6 and could potentially play a tumorigenic role emphasizing the importance of GW501516 as a therapeutic option in pancreatic cancer. We demonstrated, in human and mouse tumor tissue sets: *Pparb* to be significantly elevated in tumor tissue compared to adjacent normal tissue (human), and *Pparb* to increase in tumor tissue in a time-dependent manner (mouse). Increased expression of *Pparb* promoted proliferation in pancreatic cancer cells in a dose-

dependent manner, this was significantly reduced by GW501516. This was substantiated in an *Mcp1* promoter-driven reporter assay showing an elevation of *Mcp1* luciferase activity corresponding with an increase in *Pparb* expression, again, an effect improved by treatment with GW501516.

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Chapter 2

Therapeutic potential of GW501516 and the role of Peroxisome proliferator-activated receptor β/δ and B-Cell Lymphoma 6 in inflammatory signaling in human pancreatic cancer cells

2.1 Abstract

Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) is a member of the nuclear receptor superfamily and a ligand-activated transcription factor that is involved in the regulation of the inflammatory response via activation of anti-inflammatory target genes and ligand-induced disassociation with the transcriptional repressor B-cell lymphoma 6 (BCL6). Chronic pancreatitis is considered to be a significant etiological factor for pancreatic cancer development, and a better understanding of the underlying mechanisms of the transition between inflammation and carcinogenesis would help further elucidate chemopreventative options. The aim of this study was to determine the role of PPARβ/δ and BCL6 in human pancreatic cancer of ductal origin, as well as the therapeutic potential of PPARβ/δ agonist, GW501516. Over-expression of PPARβ/δ inhibited basal and TNFα-induced *Nfkb* luciferase activity. GW501516 induced physical interaction between PPARβ/δ and the p50 subunit of NFκB in mammalian two hybrid assays, and suppressed TNFα-induced *Nfkb* reporter activity. RNAi knockdown of *Pparb* attenuated the GW501516 effect on *Nfkb* luciferase, while knockdown of *Bcl6* enhanced TNFα-induced *Nfkb* activity. PPARβ/δ activation induced expression of several anti-inflammatory genes in a dose-dependent manner, and GW501516 inhibited *Mcp1*

promoter-driven luciferase in a BCL6-dependent manner. Several pro-inflammatory genes were suppressed in a BCL6-dependent manner. Conditioned media from GW501516-treated pancreatic cancer cells suppressed pro-inflammatory expression in THP-1 macrophages as well as reduced invasiveness across a basement membrane. These results demonstrate that PPARβ/δ and BCL6 regulate anti-inflammatory signaling in human pancreatic cancer cells by inhibiting NFκB and pro-inflammatory gene expression, and via induction of anti-inflammatory target genes. Activation of PPARβ/δ may be a useful target in pancreatic cancer therapeutics.

2.2 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease, ranking as the fourth leading cause of cancer death with a 5-year survival rate of 6% [1]. Behavioral risk factors associated with PDAC include smoking and alcoholism, as well as metabolic disorders obesity and diabetes mellitus. Treatment with gemcitabine and fluorouracil yielded 20% overall survival of PDAC to 6 months, to more current regimes of nab-Paclitaxel/Gemcitabine and FOLFIRINOX yielding to 35%-48% overall survival to 9-11 months [2]. Despite these regimes, substantial improvements in therapeutics over the last 20 years have been lacking, identification of predictive biomarkers and therapeutic targets are sorely needed.

There is a strong correlation between chronic pancreatitis and pancreatic cancer development [3]. Molecular changes have been observed during the transition from pancreatitis to pancreatic cancer including mutations in in inflammatory signaling molecules kirsten rat sarcoma viral oncogene homolog (*Kras*)[4], serine protease inhibitor

kazal type 1 (*Spink1*)[5], cyclooxygenase-2 (*Cox2*), and nitric oxide (*No*)[6,7]. Nuclear factor κ-light chain enhancer of activated B cells (*Nfkb*) is master regulator of inflammatory response and genes involved in cell cycle, angiogenesis, and apoptosis [3]. Several pancreatic cancer cell lines and tissue have shown constitutive activation of *Nfkb* [8,9]. Autocrine secretion of interleukin 1α (IL1α) activates *Nfkb* in pancreatic cancer, concurrently, NFκB enhanced expression of *IL1α* resulting in a positive feedback loop for this constitutive activation[10]. NFκB signaling dysregulates downstream targets involved in angiogenesis and metastasis such as vascular endothelial growth factor (*Vegf*) and interleukin 8 (*Il8*)[11]. Clearly, *Nfkb* plays an essential role in cancer progression, and with the complexity of KRAS pathways making targeting therapeutics difficult, *Nfkb* becomes increasingly attractive candidate for treatment.

Evidence over the past 5-years has indicated *Pparb* is a feasible target for chemoprevention[12], albeit not without controversy[13]. PPARβ/δ is a ubiquitously expressed ligand-activated transcription factor that controls a number of cellular functions, and involved in several metabolic disorders such as diabetes, obesity, and atherosclerosis[14–16]. It resides in the nucleus where it associated with transcriptional suppressor BCL6[17]. Through ligand activation of PPARβ/δ, BCL6 dissociates from the complex and decreases inflammatory signaling by binding NFκB and *Stat1*[18]. In addition, PPARβ/δ dimerizes with retinoid X receptor (RXR) and directly regulates expression of certain anti-inflammatory genes, such as interleukin-1 receptor antagonist (*Il1ra*). The biological ramifications of complex formation between PPARβ/δ and BCL6 are still

being elucidated, but both appear to be effective repressors of inflammatory markers in cell and animal models[19].

Little is known about the function of *Pparb* and *Bcl6* in the pancreas or their roles in the etiology of PDAC. GW0742, a PPARβ/δ agonist, attenuates inflammatory response in two different pancreatitis mouse models[20]. We sought to determine the role of *Pparb* and *Bcl6* in the human ductal pancreas in regards to inflammation, and the therapeutic potential of GW501516. Our observations show that GW501516-mediated anti-inflammatory signaling via PPARβ/δ is present in two pancreatic cancer cell lines- Mia PaCa-2 and BxPc-3. PPARβ/δ interacts with and suppresses NFκB activity. Several pro-inflammatory markers are inhibited by PPARβ/δ ligands in a BCL6-dependent manner. Conditioned media experiments using RNAi to reduce expression of *Pparb* and *Bcl6* in pancreatic cancer cells implicated both proteins as regulators of inflammatory gene expression in a human macrophage cell line, THP-1 cells, as well as affect macrophage recruitment.

2.3 Materials and methods

Cells and reagents. Human pancreatic cancer cells, Mia PaCa-2 (COX2 negative, CRL-1420) and BxPc-3 (COX2 positive, CRL-1687) were purchased from ATCC (Manassas, VA) and cultured in high glucose DMEM containing 10% FBS. Human embryonic kidney 293 cells were cultured in DMEM containing 10% FBS. THP-1 cells were cultured in RPMI 1640 media supplemented with 10% FBS. All cell media contained 100U penicillin and streptomycin, and cells were cultured in a humidified atmosphere at 37°C containing 5% CO_2. All media components and FBS were purchased from Gibco

BRL/Life Technologies (Carlsbad, CA). GW501516 used as a positive control for PPARβ/δ, and phorbol 12-myristate 13-acetate (PMA), used to differentiate THP-1 cells, was purchased from Sigma Chemical Company. The 2.8kb m*Mcp1* (accession #U12470) promoter fragment cloned into the luciferase reporter vector pGL3-basic (Promega) was provided by Dr. Ronald Evans (Salk Institute for Biological Studies, La Jolla, CA). Transfection control plasmids pRL-TK and pRLCMV were purchased from Promega (Madison, WI). Recombinant hTNFα was purchased from Invitrogen (Carlsbad, CA) and reconstituted in nanopure water. MISSION© PPARβ/δ, BCL6, IL1Ra, and scrambled non-targeting glycerol stocks were purchased from Sigma-Aldrich. High Capacity cDNA Archive Kit and ABI 7300 real-time PCR system were purchased from Applied Biosystems (Foster City, CA). The pPACKH1 packaging plasmids and the pcDNA3.1-*Pparb*-FLAG plasmid were provided by Dr. Curtis Omiecinski (Penn State University). CytoSelect TM 96-well Invasion Assay (basement membrane, fluormetric format) was purchased from Cell Biolabs, Inc. (San Diego, CA) and used according to manufacturer's instructions.

NF-κB1 reporter assays. Mia PaCa-2 cells were seeded at $7.5x10^5$ cells in 10cm tissue culture dishes. Cells were transiently transfected with 9μg pNFκB-luciferase and 1μg pRLCMV using LipofectAMINE (Invitrogen) reagent for 6h and allowed to recover overnight. Cells were challenged with the indicated treatments 24hrs post-transfection, and NFκB-luciferase activity was measured using Dual Luciferase Reporter Assay (Promega) and normalized using control luciferase activity). Cells were transfected with 5μg *Pparb-FLAG* and 4μg p*Nfkb*-luciferase and 1μg pRLCMV.

Mammalian two-hybrid assay. Experiments were performed using the Matchmaker Mammalian-2-Hybrid system (Clontech). Mia MaCa-2 cells were plated in 24-well plates and allowed to recover overnight. Transfection was carried out with LipofectAMINE 2000 reagent according to the protocol of the manufacturer. Each well was transfected with 200ng of pM-PPARβ/δ or empty vector, 200ng of pVP16-empty, pVP16-p50 or pVP16-p65 subunit of NFκB, 100ng each of pFR-luciferase and pRL-TK. Transfected cells were treated with 500nM GW501516 for 6h and assayed for luciferase activity. Relative luciferase activity was corrected using the internal transfection control (pRL-TK) and the Dual Luciferase Kit (Promega).

Isolation of total RNA and quantitative PCR. Total RNA was isolated from Mia PaCa-2 and BxPc-3 cells using Tri-Reagent and the manufacturer's recommended protocol (Sigma). Reverse transcription of 1μg mRNA was done using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Primers for quantitative Polymerase Chain Reaction (PCR) were designed based on published sequences in GenBank and are shown in Table 2.1. The housekeeping gene βactin was used to normalize all the tested genes. The data shown are representative of three independent experiments with triplicate samples.

Table 2.1 List of qPCR primers.

Gene	Forward Primer	Reverse Primer
bactin	AACAAGAGGCCACACAAATAGG	CAGATGTACAGGAATAGCCTCCG
Il1ra	GGGAACTTTGCACCCAACAT	TTGGCAGGTACTCAGCGAATG
Tgfb	AGGTCCTTGCGGAAGTCAATG	CTATTGCTTCAGCTCCACGGA
Sod1	TGCTTCCCCACACCTTCACTGGT	ATGGCGACGAAGGCCGTGTG
Fgf21	CGCTGGCACAGGAACCTGGA	ACCAGAGCCCCGAAAGTCTCCT
Mcp1	GGACGCATTTCCCCAGTACA	CCGAGAACGAGATGTGGACA
Mcp3	ATGAGGTAGAGAAGGGAGGAGCAT	CAAACTGGACAAGGAGATCTGTGC
Tnfa	TGGATGTTCGTCCTCCTCACA	ATCAATCGGCCCGACTATCTC
Il1b	TCCTTAGTCCTCGGCCAAGAC	GTGCCATGGTTTCTTGTGACC
Il6	CCGTCGAGGATGTACCGAATT	GCCACTCACCTCTTCAGAACG
Cox2	CGGTGTTGAGCAGTTTTCTCC	AAGTGCGATTGTACCCGGAC

Mcp1 reporter assay. Mia PaCa-2 cells transiently expressing non-targeting control, BCL6 or PPARβ/δ shRNA were plated in 10cm tissue culture dishes as described. Cells were transiently transfected with 9μg *Mcp1*-luciferase and 1μg pRLCMV for 6h and allowed to recover overnight. Cells were then challenged with the indicated treatments 24h post-transfection and *Mcp1* promoter driven luciferase was assayed and corrected using the internal transfection control pRLCMV.

Differentiation of THP-1 cells with PMA. Differentiation was achieved by resuspending THP-1 cells at a density of 2 x 10^5 cells/mL in serum-free RPMI 1640 media supplemented with 100nM PMA for 24h. Cells were then allowed to recover in media containing 10% FBS for a further 24h before use in experiments.

Lentiviral RNAi. HEK-293 cells were grown to confluency in 10cm tissue culture plates under the conditions described above. The cells were then transiently transfected with 4.6μg of scrambled non-targeting control, or *Pparb*, *Bcl6*, or *Il1ra* shRNA, as well as 2.4μg each of pPACKH1 packaging plasmids, using LipofectAMINE 2000. Cells were transfected for 6h and allowed to recover overnight in normal media. Fresh media was added the following morning, and pseudoviral supernatant was generated for 72h.

Supernatant was then harvested and passed through a 0.4μm filter under sterile conditions. Polybrene (Millipore, Billerica, MA) was then added to a final concentration of 5μg/mL and the pseudoviral supernatant was then added directly to target cells for 6h. Infected cells were allowed to recover overnight following the addition of 6mL complete media and knockdown of target genes was assessed by qPCR 48h post-infection.

Conditioned media experiments. Control or knockdown Mia PaCa-2 cells were plated at a density of 7.5 x 10^5 cells in 10 cm tissue culture plates. Following overnight recovery the cells were challenged with 1ng/mL TNFα with or without 500nM GW501516 for 24h. Conditioned media was collected and centrifuged at 200 x g at 20°C for 10min and any unused media was stored at -80°C. The same volume of normal media containing TNFα with or without 500nM GW501516 was prepared at the start of the experiment and was used as control media. For gene expression assays, undiluted conditioned media from control or knockdown Mia PaCa-2 cells were added directly to THP-1 cells and the cells were incubated for 24h. Conditioned media was removed the following day and total RNA was isolated and reverse transcribed as described above.

Cell migration assay. Experiments were performed using the CytoSelect TM 96-Well Invasion Assay (Basement Membrane, Fluormetric Format) according to the manufacturer's instructions. The basement membrane was allowed to reach room temperature for 30mins, and rehydrated using warm, serum-free DMEM. THP-1 cells were then seeded into each well at a density of 2x10^6 cells/mL in serum-free media. Conditioned media, as well as control media (DMEM containing 10% FBS, along with TNFα with or without GW501516) was added to the feeder tray to act as a

chemoattractant and the entire apparatus was placed in an incubator at 37°C containing 5% CO_2 for 24h. CyQuant® GR dye/lysis buffer solution was added to the invading cells following completion of the assay and the resulting mixture was incubated at room temperature for 20mins. Invading cells were quantified by reading the fluorescence at 480nm/520nm. All measurements were performed in triplicate.

Statistical analysis. Quantitative data are presented as mean±SEM. ANOVA with p-value<0.05 was used to determine whether differences among variables were significant. Normality was checked using Anderson-Darling test, and the general linear model, followed by the Tukey post hoc test to analyze differences between treatments. All data analyses were performed by MiniTAB Ver.14 (MiniTAB, State College, PA) or JMP (SAS Institute, Cary, NC) and data were plotted by Prism 5.01 (GraphPad Software, San Diego, CA).

2.4 Results

§ denotes experiments performed by Jeffrey David Coleman

Over-expression of *Pparb* inhibits *Nfkb*-luciferase activity in Mia PaCa-2 cells. *Nfkb* is a key regulator of inflammation[21] and it is affected by PPARβ/δ via the p65 subunit [19]. To substantiate PPARβ/δ's anti-inflammatory effect in the human pancreatic cancer cells we sought to evaluate its interaction with NFκB. Mia PaCa-2 cells were transfected with an *Nfkb* response element-luciferase along with pcDNA3.1-*Pparb*-FLAG or empty vector. Over-expression of *Pparb* reduced basal *Nfkb*-luciferase activity. Treatment with 1ng/mL tumor necrosis factor α (TNFα) induced *Nfkb* reporter activity almost three-fold in

control, this effect was diminished in cells over-expressing *Pparb*, even in the absence of ligand (Figure 2.1A). This indicates PPARβ/δ associates with and suppresses NFκB activity in human pancreatic cancer cells, suggesting an anti-inflammatory role for PPARβ/δ in the pancreas.

PPARβ/δ physically interacts with p50 subunit of NFκB. Mammalian 2-hybrid assays were performed to assess interaction between PPARβ/δ and NFκB. Mia PaCa-2 cells were transfected with pM-PPARβ/δ or empty vector control along with pVP16-p50, pVP16-p65, or empty control. GW501516 was treated at 500nM and luciferase activity was measured. GW501516 increased Gal4 activity co-expressing PPARβ/δ and the p50 subunit compared to control, but not in cells expressing the p65 subunit (Figure 2.1B). These results are surprising considering the p50 homodimer has been known to increase expression of anti-inflammatory IL10 [40], inhibit IFNβ expression [41], and play a role in M2 macrophage polarization [42].

Activation of PPARβ/δ reduces *Nfkb* activity in Mia PaCa-2 cells. To determine if NFκB activity could be influenced by GW501516-induced interaction with PPARβ/δ in human pancreatic cancer cells, Mia PaCa-2 cells with *Pparb* or *Bcl6* knocked down were transfected with *Nfkb* response element-luciferase and treated with 1ng/mL TNFα in the presence or absence of 500nM GW501516. In control cells, GW501516 reduced TNFα-stimulated *Nfkb* luciferase activity, as well as basal activity slightly though not significant. TNFα-induced reporter activity was significantly increased when *Bcl6* was knocked down, and GW501516 slightly lowered this effect. GW501516 treatment did not alter basal activity. Both basal and TNFα-stimulated *Nfkb* activity were unaffected by

GW501516 treatment when *Pparb* was knocked down (Figure 2.1C). These results indicate the observed reduction in *Nfkb* activity by GW501516 may require both PPARβ/δ and its associated tumor suppressor BCL6.

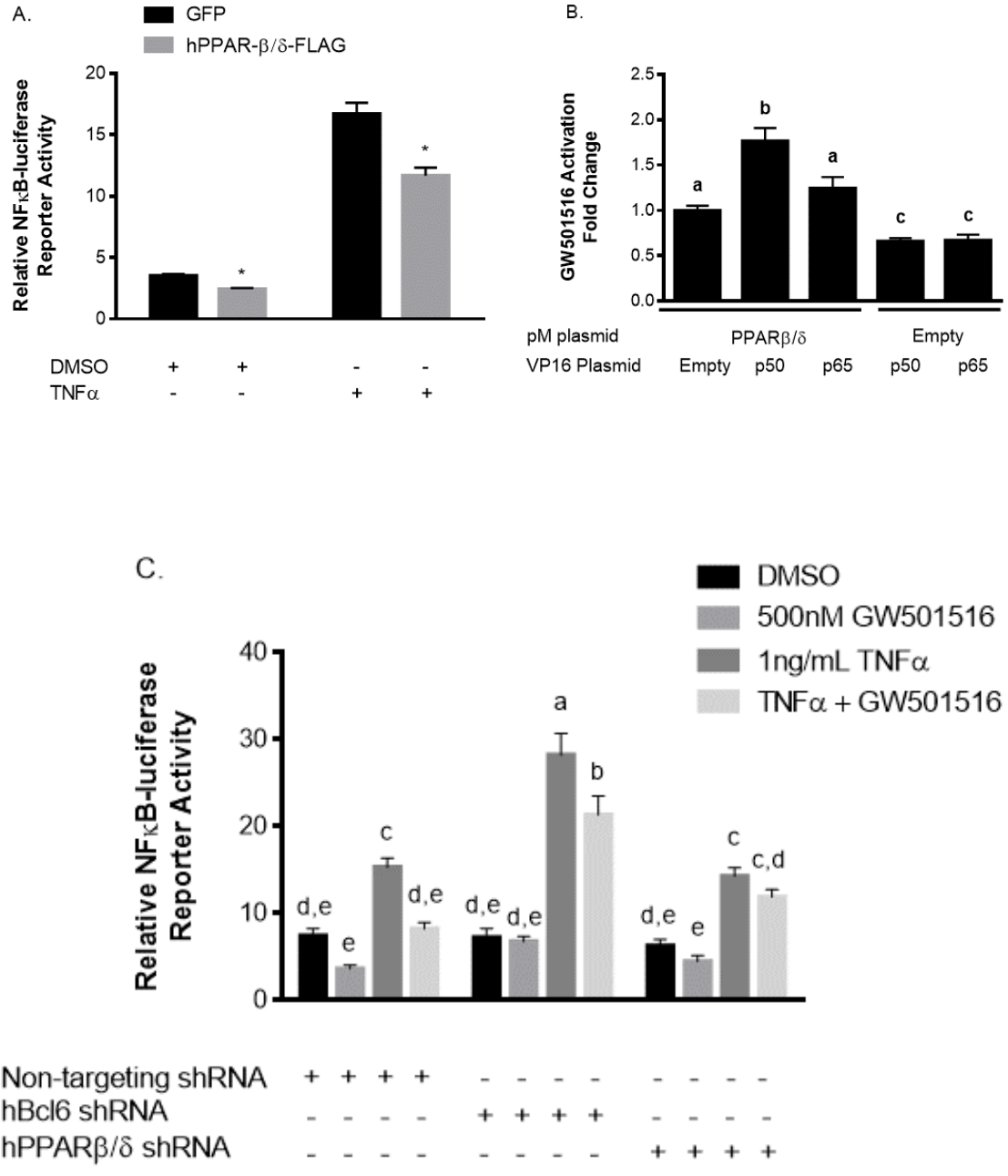


Figure 2.1 Effects of PPARβ/δ expression and activation on NFκB1 interaction and activity. **§**A. PPARβ/δ expression decreases basal and stimulated NFκB activity. Mia PaCa-2 cells were seeded at $7.5x10^5$/mL in 10cm culture dish and transfected with 9μg NFκB1-luciferase/1μg pRLCMV with or without pcDNA3.1-*Pparb*-FLAG for 24h before challenge with TNFα or DMSO control. Luciferase activity was assayed and corrected for transfection efficiency. **P<0.05* **§**B. PPARβ/δ interacts with the p50 subunit of NFκB. Mia PaCa-2 cells were seeded in 24-well plates and transfected (200ng) with pM-PPARβ/δ or empty vector, and either pVP16-p50, pVP16-p65 or pVP16-empty as indicated. Cells were treated with either DMSO or 500nM GW501516 24h post-transfection and pFR luciferase (100ng) activity was assayed and corrected for transfection efficiency (pRLTK; 100ng). Different letters indicate a statistical difference at *P<0.05* using Tukey's multicomparison test. **§**C. BCL6 also plays a role in suppressing NFκB. Mia PaCa-2 cells were seeded at $7.5x10^5$/mL in 10cm dish, infected with 4.6μg of siRNAs targeted against PPARβ/δ or BCL6, 2.4μg of pPACKH1 packaging plasmid for 6h, and were transfected with NFκB1-luciferase. Cells were then challenged with the indicated treatments for 24h and luciferase activity was assayed and corrected for transfection efficiency. Different letters indicate a statistical difference at *P<0.05* using Tukey's multicomparison test.

<u>GW501516 induces expression of anti-inflammatory genes in PPARβ/δ-dependent manner</u>. Activation of PPARβ/δ affects anti-inflammatory genes in many cell lines[21–23]. Mia PaCa-2 cells with *Pparb* or *Bcl6* knocked down were treated with 500nM GW501516 or DMSO control, and mRNA expression was assessed via qPCR. GW501516 increased expression of interleukin-1 receptor agonist (*Il1ra*), transforming growth factor β (*Tgfb*), superoxide dismutase-1 (*Sod1*), and fibroblast growth factor 21 (*Fgf21*) in control and cells with *Bcl6* knocked down. When PPARβ/δ was knocked down, GW501516 treatment did not increase expression of these target genes (Figure 2.2). These results indicate the anti-inflammatory effects of GW501516 are mediated in part by the direct induction by PPARβ/δ.

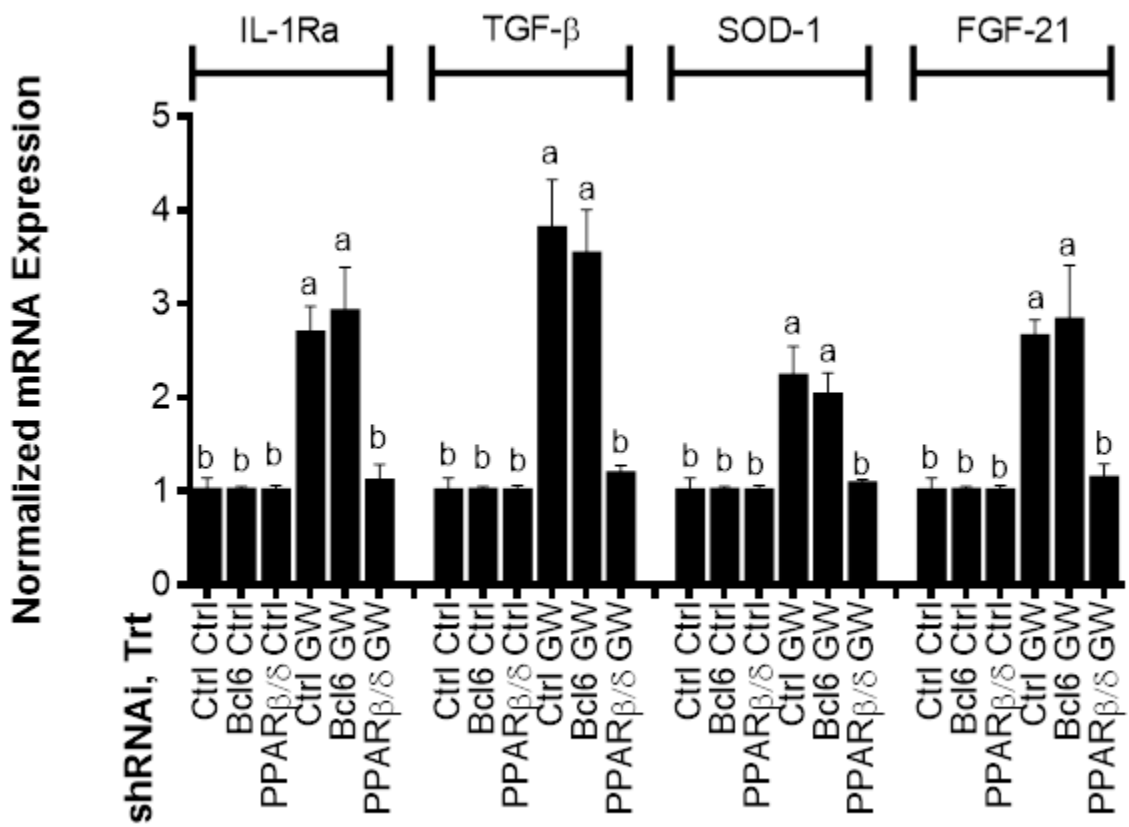


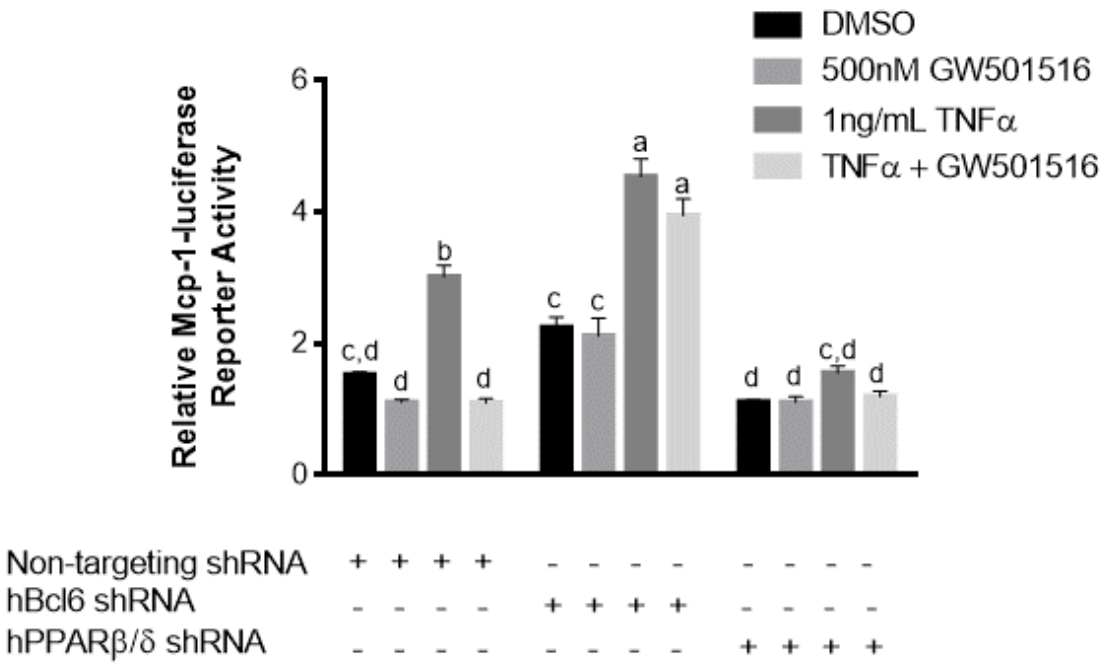
Figure 2.2 GW501516 activated PPARβ/δ exerts anti-inflammatory effects by increasing expression of target genes. Mia PaCa-2 cells were seeded $7.5x10^5$/mL in 10cm dish and transiently infected with 4.6μg of lentiviral-mediated shRNAs targeted against PPARβ/δ or BCL6 for 6h, and 2.4μg of pPACKH1 packaging plasmid. Cells were then treated with 500nM GW501516 or vehicle for 24h. Gene expression was determined by qPCR and expressed as fold induction following normalization to βactin. Different letters indicate a statistical difference at *P<0.05* using ANOVA and Tukey's post hoc multicomparison test.

GW501516 inhibits expression of TNFα-induced pro-inflammatory genes in BCL6-dependent manner. The anti-inflammatory role of PPARβ/δ is mediated in part by BCL6[16,24], and involves several pro-inflammatory markers[17,22,24]. Mia PaCa-2 cells expressing shRNA targeting *Pparb* or *Bcl6* along with monocyte chemoattractant protein-

1 (*Mcp1*) promoter-luciferase, were treated with 1ng/mL TNFα to induce an inflammatory response. *Mcp1* promoter-luciferase activity was increased in control cells, and treatment with GW501516 reduced TNFα-induced reporter activity to basal levels. GW501516 reduced basal levels as well, however, not to a significant degree. TNFα-induced *Mcp1* promoter-luciferase activity was significantly higher when *Bcl6* was knocked down, and GW501516 had no effect. When *Pparb* was knocked down, TNFα-stimulated *Mcp1* promoter-luciferase activity was reduced to approximately basal level with GW501516 having no effect (Figure 2.3A). Reduction in BCL6 appears to increase TNF-induced activity of pro-inflammatory *Mcp1*, whereas its release (via reduction in PPARβ/δ) decreases reporter activity.

To see if this effect was observed at the mRNA level, qPCR was performed on *Mcp1* and other known BCL6 target genes in cells under the same conditions. GW501516 activation reduced mRNA in *Mcp3*, *Tnfa*, and interleukin-1β (*Il1b*) in control infected cells, with minor effect on *Mcp1*. This affect was not present when *Bcl6* was knocked down, and mRNA levels were increased with or without GW501516. When *Pparb* was knocked down, mRNA levels were reduced despite TNFα treatment (Figure 2.3B). Taken together, these results confirm the anti-inflammatory effect of GW501516 is mediated in part by BCL6.

A.


DMSO
500nM GW501516
1ng/mL TNFα
TNFα + GW501516
Relative Mcp-1-luciferase Reporter Activity
Non-targeting shRNA
hBcl6 shRNA
hPPARβ/δ shRNA

B.

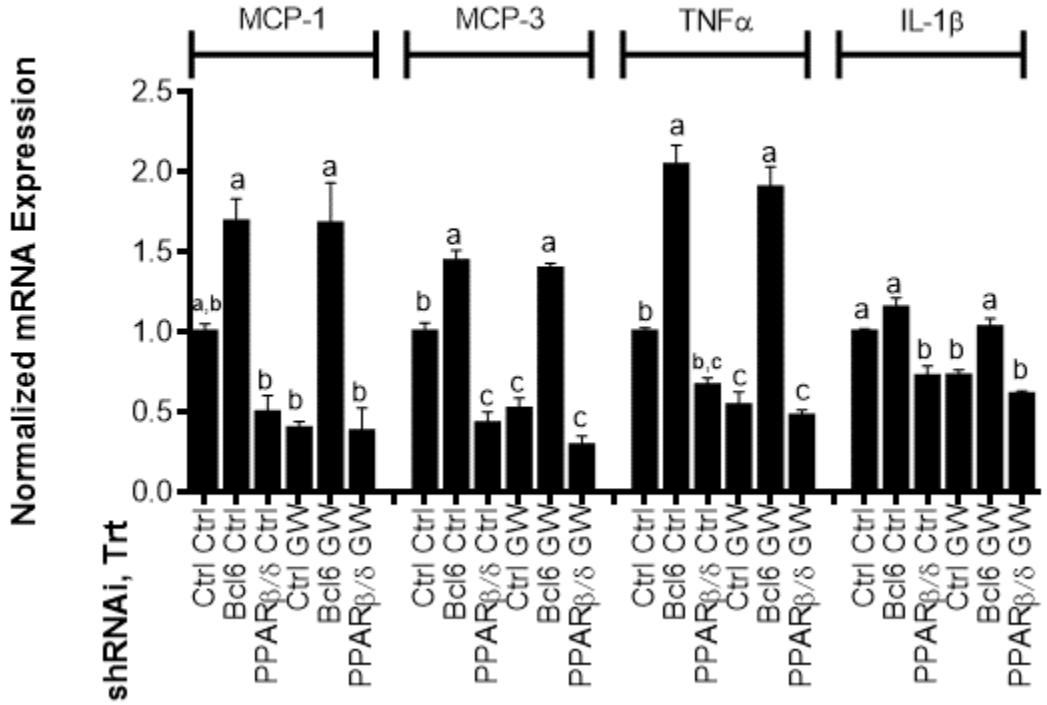

MCP-1
MCP-3
TNFα
IL-1β
Normalized mRNA Expression
shRNAi, Trt
Ctrl Ctrl
Bcl6 Ctrl
PPARβ/δ Ctrl
Ctrl GW
Bcl6 GW
PPARβ/δ GW

Figure 2.3 The transcriptional repressor BCL6 contributes to the anti-inflammatory actions of GW501516 by suppressing target gene expression. **§**A. Effects of *Pparb* and *Bcl6* knock-down on TNFα-induced *Mcp1* promoter driven luciferase. Mia PaCa-2 cells transiently expressing 4.6μg of the indicated shRNAs for 6h and were transfected with 9μg of pGL3-*Mcp1* promoter luciferase and treated with vehicle, 500nM GW501516 or 1ng/mL TNFα with or without GW501516 for 24h before luciferase activity was assayed. B. Repression of TNFα-induced pro-inflammatory target genes by BCL6. Mia PaCa-2 cells expressing the indicated shRNAs were treated with TNFα with or without GW501516 for 24h. Gene expression was determined by qPCR and expressed as fold induction following normalization to βactin. Different letters indicate a statistical difference at *P<0.05* using ANOVA and Tukey's post hoc multicomparison test.

GW501516 is anti-inflammatory in *Cox2* positive human pancreatic cancer cells. Since Mia PaCa-2 cells are reported to not express prostaglandin-endoperoxide synthase 2 (*Cox2*) or interleukin 6 (*Il6*), it is important to substantiate these results in a *Cox2* positive cell line such as the pancreatic cancer cells, BxPc-3. *Cox2* plays a substantial role in angiogenesis by the metabolism of arachidonic acid to factors that contribute to angiogenesis signaling propagation [43]. This cell line was transiently infected with *Pparb*, *Bcl6*, or scrambled control shRNA and treated with TNFα with or without GW501516. GW501516 reduced the TNF-induced expression of the *Pparb* target *Il6* [25] though not significant, and there was no change in *Cox2* mRNA. Knock down of *Bcl6* significantly increased inflammatory response (*Il6* and *Cox2* expression) and was not significantly affected by PPARβ/δ activation. *Pparb* knock down resulted in lower expression of *Il6* mRNA despite GW501516 (Figure 2.4). These results substantiate in BxPc-3 cells, that GW501516 exerts its affects via release of BCL6 from the PPARβ/δ complex. Additionally, in our view, the therapeutic potential of this pathway is not negatively altered by the absence of this mechanism in Mia PaCa-2 cells.

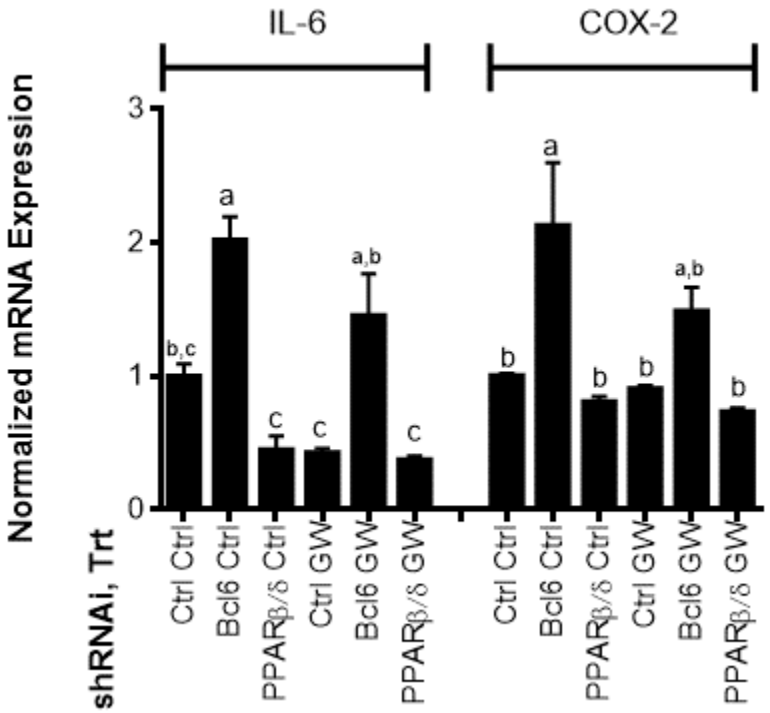


Figure 2.4 **§** Effects of *Pparb* and *Bcl6* knockdown in *Cox2* positive human pancreatic cancer cells. BxPc-3 cells transiently expressing 4.6μg of the indicated shRNAs were challenged with TNFα with or without 500nM GW501516 for 24h. RNA was extracted by standard tri-reagent protocol. Gene expression was determined by qPCR and expressed as fold induction following normalization to βactin. Different letters indicate a statistical difference at $P<0.05$ using ANOVA and Tukey's post hoc multicomparison test.

GW501516 affects crosstalk between human pancreatic cancer cells and THP-1 macrophages. Tumor associated macrophages secrete molecules that aid in the immunosuppressive microenvironment allowing malignancy and cancer progression by promoting angiogenesis and metastasis[26]. To determine if conditioned media from Mia PaCa-2 cells could influence gene expression in macrophages, Mia PaCa-2 cells were treated with 1ng/mL of TNFα with or without 500nM of GW501516 after RNAi knockdown of *Pparb*, *Bcl6*, *Il1ra*, and scrambled control for 48h. Conditioned media was

added for 24h to THP-1 cells differentiated to macrophages. Accumulation of mRNA was examined via qPCR. Media in absence of cells and media from cells not infected with the pseudovirus were also used as controls along with media conditioned by cells infected with scrambled non-targeting shRNA. Our results show GW501516-induced reduction of pro-inflammatory markers within the THP-1 cells including *Mcp1*, *Mcp3*, *Tnfa*, *Il1b*, and *Il6*, but not *Cox2* in cells treated with control media and media from the scrambled control shRNA group (Figure 2.5). Conditioned media taken from BCL6-knock down cells treated with TNFα increased expression of inflammatory markers in THP-1 cells, this inflammatory response was decreased upon treating Mia PaCa-2 cells with GW501516. When *Pparb* was knocked down in Mia PaCa-2 cells, TNFα and IL6 production in condition-media treated THP-1 cells was repressed (Figure 2.5C&E). *Il1ra* knock downs in Mia PaCa-2 did not alter gene expression in THP-1 cells compared to control. *Mcp1* and *Mcp3* were repressed in THP-1 macrophages following conditioned media from GW501516-treated *Il1ra* knock downs suggesting IL1Ra does not play a significant role in the crosstalk between pancreatic cancer cells and macrophages.

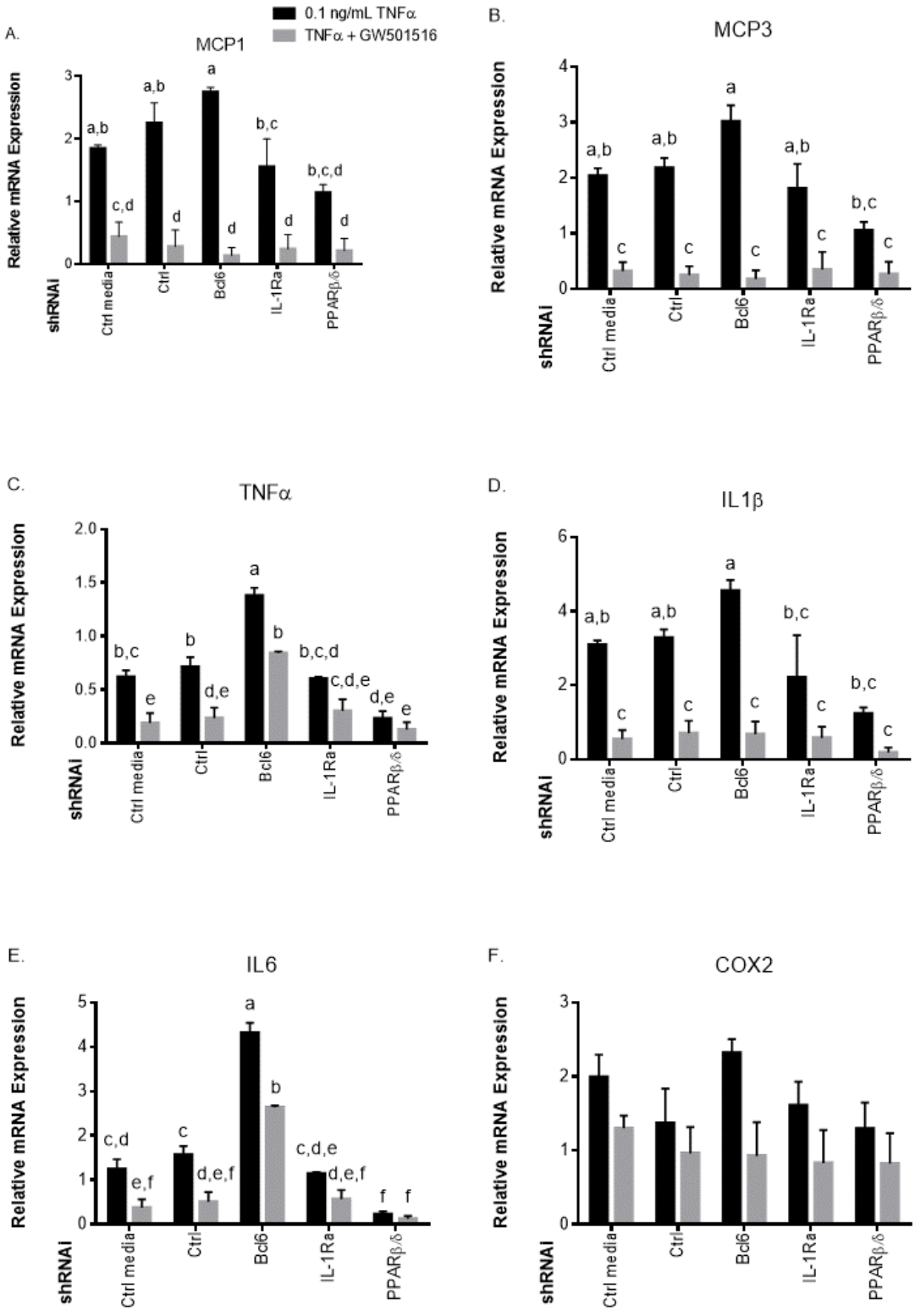

A.
MCP1
0.1 ng/mL TNFα
TNFα + GW501516
Relative mRNA Expression
shRNAI
Ctrl media
Ctrl
Bcl6
IL-1Ra
PPARβ/δ
B.
MCP3
C.
TNFα
D.
IL1β
E.
IL6
F.
COX2

Figure 2.5 Conditioned media from Mia PaCa-2 cells influences gene expression in differentiated THP-1 cells. Mia PaCa-2 cells were infected with 4.6μg of the indicated shRNAs for 6h, recovered overnight, and treated with 1ng/mL of TNFα with or without 500nM GW501516 to condition the media for 48h. Equal aliquots of conditioned media or control media (media placed in a 10cm dish without cells) were then added to PMA-differentiated THP-1 cells. Gene expression of the pro-inflammatory *Mcp1* (A), MCP3 (B), TNFα (C), IL1β (D), IL6 (E) and COX2 (F) was determined by qPCR and expressed as fold induction following normalization to βactin. Different letters indicate a statistical difference at $P<0.05$ using Tukey's multicomparison test.

GW501516 reduces THP-1 cell invasion in BCL6-dependent manner. The BCL6 dissociation from PPARβ/δ by GW501516 activation suppresses production of genes involved in macrophages chemotaxis, therefore it was necessary to see if media conditioned by pancreatic cancer cells under the same conditions would influence macrophage migration across a basement membrane. THP-1 cells were seeded into the upper chamber of an invasion plate in serum-free media, and conditioned media from Mia PaCa-2 cells expressing the indicated shRNAs treated with TNFα with or without GW501516 was used as the chemoattractant. GW501516-treated Mia PaCa-2 conditioned media reduced the amount of invading THP-1 cells, this was not seen when *Pparb* was knocked down in the pancreatic cell line. Interestingly, TNFα induced invasion was increased 70% when *Bcl6* was knocked down in Mia PaCa-2. THP-1 migration was decreased 30% when *Pparb* was knocked down in pancreatic cancer cells regardless of TNFα or GW501516 (Figure 2.6). BCL6 plays an integral part in the anti-inflammatory effects of GW501516, and seems to be the case for its anti-migratory effects as well. Though, PPARβ/δ is clearly anti-inflammatory when activated by GW501516, it appears that when in the inactive state, PPARβ/δ holds association with BCL6 preventing it from exerting its effects.

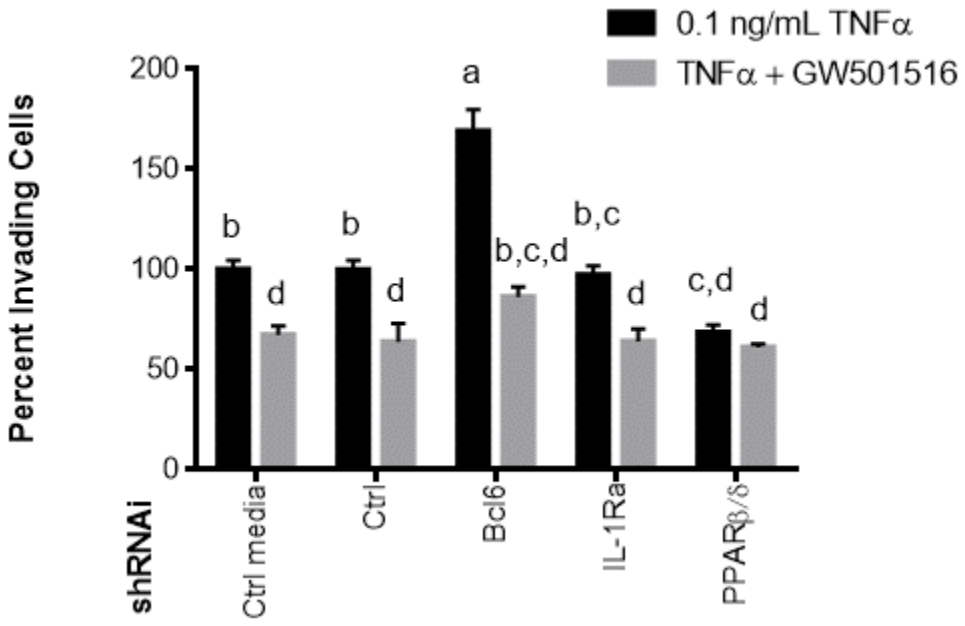


Figure 2.6 **§** Mia PaCa-2 GW501516-conditioned media reduces the percentage of invading THP-1 cells across a basement membrane. Media was conditioned by Mia PaCa-2 cells expressing the indicated shRNAs and treated with TNFα with or without 500nM GW501516 for 48h. Conditioned media was used as a chemoattractant for THP-1 cells. THP-1 monocytes were allowed to migrate across the membrane for 24h and relative invasion was quantified using the CytoSelect 96-well cell invasion assay with fluorometric readings at 480nm/520nm. Different letters indicate a statistical difference at $P<0.05$ using Tukey's multicomparison test.

2.5 Discussion

There is a growing amount of evidence[12] in support of the health benefits of PPARβ/δ agonists in particular in regard to protecting cells from inflammation. Several studies have shown inflammation to be correlated with pancreatic cancer progression[3,21,27–29]. This study provides further evidence that GW501516-activated PPARβ/δ reduces TNFα-induced *Nfkb* activity and inflammatory signaling via direct

induction of anti-inflammatory genes, as well as indirect inhibition of pro-inflammatory genes by BCL6 dissociation in human pancreatic cancer cells. Additionally, GW501516 treatment of Mia PaCa-2 cells affected gene expression in macrophages in manner consistent with decreased invasive potential. This may indicate PPARβ/δ agonists affect crosstalk between cancer cells and tumor-associated macrophages. This is imperative because neoplastic response to therapy is not solely based on the genomic anomalies the cancer cell contains, but also the numerous factors within the tumor microenvironment. Macrophages are key regulators of the tumor microenvironment, angiogenesis, and metastasis[30]. Re-polarization of these immune cells, in response to extracellular cues may provide an effective therapeutic option, although many intracellular signals that control polarization are still being elucidated. Whether macrophage re-polarization or reduction in macrophage population would be a more effective strategy based on tissue type and disease progression is being investigated [31]. Macrophage deletion in breast[32], and Ewing's sarcoma animal models have resulted in decrease tumor incidence[33].

Pparb over-expression significantly reduced basal and TNFα-induced *Nfkb* reporter activity. According to our findings this process is likely mediated, in part, by direct interaction with the p50 subunit. However, previous studies have found the p50 homodimer to be anti-inflammatory by increasing expression of *Il10* and the M2 polarization while decreasing inflammatory *Ifnb* [40-42]. Not only does our data contradict these findings, but the p50 subunit in our mammalian two-hybrid assay had very modest induction compared to p65 and empty controls despite still being significant. Perhaps the presence of a false positive, or some other type of artifact is to explain for this.

GW501516 reduced *Nfkb* activity in control cells, but not when *Pparb* or *Bcl6* were knocked down, indicating the potential repressive mechanism is dependent on both proteins. The p65 subunit of NFκB is constitutively active in human pancreatic cancer cells[34], and is implicated in the progression of pancreatic tumorigenesis[35]. Inhibition of NFκB via PPARβ/δ has already been established in endothelial cells and cardiomyocytes[36], but GW501516-mediated inhibition in the pancreas occurred directly or indirectly via ERK1/2 map kinase phosphorylation as in adipocytes[25] has yet to be determined. TNFα-induced *Nfkb* activity was significantly increased when *Bcl6* was knocked down indicating BCL6 does participate in regulation of *Nfkb* activity. GW501516 may provide a useful therapeutic strategy by attenuation of *Nfkb* by both PPARβ/δ and BCL6.

GW501516 also increased mRNA expression of anti-inflammatory markers- *Il1ra, Tgfb, Sod1,* and *Fgf21*. IL1Ra, an *Il1b* antagonist[37], and *Tgfb* are both PPARβ/δ targets that contribute to the inhibition of IL1β-induced cell migration. *Sod1* was also induced, a gene involved in oxidative stress and contains a functional peroxisome proliferator response element (*PPRE*) in its 5'-flanking region[22]. *Fgf21* is regulated by PPAR-dependent pathways in mice, and protects pancreatic acini from damage via inflammation[38]. We demonstrate here the anti-inflammatory properties of GW501516 in human pancreatic cancer is in part through activation of PPARβ/δ and that the reduction in inflammatory response is in part through direct induction of anti-inflammatory genes by PPARβ/δ.

Unlike PPAR β/δ, the closely related subtypes PPARα and PPARγ lack affinity for BCL6. Since dissociation of BCL6 from the PPARβ/δ complex is largely responsible for the anti-inflammatory properties of PPARβ/δ ligands in mouse macrophages[24], and in animal models[16], this is a unique mode of action available to this receptor. Our data shows this BCL6 pathway is active in the human pancreas and is dependent on activation of PPARβ/δ. Conversely, PPARβ/δ in the inactive state sequesters BCL6, preventing its anti-inflammatory properties. This may indicate increased expression of PPARβ/δ, despite being anti-inflammatory when activated, may play a pro-inflammatory role in the unliganded state as well.

Inhibition of *Vegf* reduced macrophage infiltration and cancer growth in an orthotopic mouse model of pancreatic cancer[39]. Conditioned media from TNFα-treated Mia PaCa-2 cells effected gene expression in THP-1 cells, inducing pro-inflammatory gene expression including *Vegf*. Conditioned media from cells treated with GW501516 significantly lowered mRNA expression of pro-inflammatory markers in macrophages. Utilizing RNAi in this study has shown PPARβ/δ and BCL6 may play a role in this pancreatic cancer-macrophage crosstalk. GW501516 reduced macrophage infiltration by 30% in control cells, and a reduction in BCL6 protein showed a 70% increase in invasion clarifying the importance of this transcriptional suppressor in macrophage migration. More importantly, however, was the reduction in PPARβ/δ protein lowered invading macrophages compared to control. Further experimentation is required to fully elucidate the role PPARβ/δ and BCL6 play in the exocrine pancreas, but this data is in support of

the anti-inflammatory properties of this pathway and potential for ameliorating cancer:macrophage crosstalk in the human pancreas.

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Chapter 3

Role of Peroxisome proliferator-activated receptor β/δ and B-Cell Lymphoma 6 in regulation of genes involved in metastasis and migration in pancreatic cancer cells

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3.1 Abstract

PPARβ/δ is a ligand-activated transcription factor that regulates various cellular functions via induction of target genes directly or in concert with its associated transcriptional repressor, BCL6. Matrix remodeling proteinases are frequently over-expressed in pancreatic cancer and are involved with metastasis. The present study tested the hypothesis that PPARβ/δ is expressed in human pancreatic cancer cells and that its activation could regulate *Mmp9,* decreasing cancer cells ability to transverse the basement membrane. In human pancreatic cancer tissue there was significantly higher expression of *Mmp9* and *Pparb,* and lower levels of *Bcl6* mRNA. PPARβ/δ activation reduced the TNFα-induced expression of various genes implicated in metastasis and reduced the invasion through a basement membrane in cell culture models. Through the use of short hairpin RNA inhibitors of *Pparb*, *Bcl6,* and *Mmp9*, it was evident that PPARβ/δ was responsible for the ligand-dependent effects whereas BCL6 dissociation upon GW501516 treatment was ultimately responsible for decreasing *Mmp9* expression and hence invasion activity. These results suggest that PPARβ/δ plays a role in regulating pancreatic cancer cell invasion through regulation of genes via ligand-dependent release

of BCL6 and that activation of the receptor may provide an alternative therapeutic method for controlling migration and metastasis.

3.2 Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths of men and women in the United States. The American Cancer Society estimates for 2013 predicted approximately 45,220 new cases of pancreatic cancer and that 38,460 of those cases would result in death. Lack of identifiable symptoms or biomarkers combined with a 4% five-year survival rate makes pancreatic cancer one of the deadliest malignancies [1]. Although pancreatic cancer is difficult to detect in its early stages, several known risk factors exist, with smoking being the most documented etiologic agent [2]. Several other risk factors include age, diets high in fat [3], excessive alcohol consumption [4], diabetes mellitus [5] and chronic pancreatitis [6]. Common chemotherapeutic treatments have had little success in improving survival rates or restraining the highly metastatic malignancies [7] with the median survival rate of less than six months and surgical resection as the only effective treatment [8]. Prevention strategies and alternative treatments for pancreatic cancer are sorely needed.

Peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. The PPARs consist of three isoforms, PPARα (*Nr1c1*), PPARβ/δ (*Nr1c2*, *Nuc1*, fatty acid-activated receptor (*FAAR*)) and PPARγ (*Nr1c3*). The PPARs effect gene transcription in response to various stimuli, such as fatty acids and their metabolites, xenobiotics and isoform-specific drugs, through a dimerization with retinoid X receptors (RXRs) and subsequent recognition and

binding to peroxisome proliferator-responsive elements (*PPRE*s) within the promoter regions of target genes [9,10]. PPARβ/δ, unlike PPARα or PPARγ which have distinct tissue expression patterns and synthetic ligands, is ubiquitously expressed, often at higher levels than the other isoforms. This receptor regulates fatty acid oxidation and lipid homeostasis [11], cell proliferation and differentiation [12], cell survival [13] and the inflammatory response [14]. The latter response may be via its association with the transcriptional repressor BCL6, which is released upon activation of PPARβ/δ [15]. In the pancreas, PPARβ/δ is expressed in islet cells to a greater extent than either PPARα or PPARγ, and in beta cells where it regulates the inflammatory response [16]. Expression profiling analyses in the mouse demonstrated high PPARβ/δ expression in the cytoplasm of delta cells of the islet of Langerhans, suggesting a potential role for the receptor in the regulation of glucose metabolism [17]. Pancreatic ductal adenocarcinomas are by far the most common of pancreatic malignancies [18], and the role(s) of PPARβ/δ in pancreatic ductal cells is poorly understood.

The matrix metalloproteinases are a family of zinc-dependent proteolytic enzymes that degrade extracellular matrix (ECM) proteins and are well-known regulators of pancreatic cancer cell metastasis and invasion [19, 20]. Matrix metalloproteinase-9 (*Mmp9*, also known as Gelatinase b) in particular is highly expressed in both clinical and experimental models of pancreatic cancer [21]. Furthermore, pancreatic cancer cells display extremely high basal *Mmp9* expression, which is further inducible by phorbol 12-myristate 13-acetate (PMA) [22]. Recently, several studies have linked *Pparb* to *Mmp9*, in *PPARβ/δ* null macrophages, basal *Mmp9* expression is reduced [15], and in vascular smooth

muscle cells (VSMCs) PPARβ/δ activation suppressed the expression of both *Mmp2* and *Mmp9*, with further inhibition on VSMC migration and proliferation [23].

The role(s) of PPARs, particularly PPARβ/δ, in tumorigenesis and cancer cell invasion remains controversial. For example, inhibition of PPARγ suppressed pancreatic cancer cell motility in Capan-1 and Panc-1 cells [24], while its activation in AsPC-1 cells by the specific ligand rosiglitazone increased levels of the tumor suppressor PTEN and decreased levels of phosphorylated AKT [25] and induced caspase-mediated apoptosis in Mia PaCa-2 cells [26]. PPARβ/δ is an APC-regulated target of non-steroidal anti-inflammatory drugs (NSAIDs), suggesting that NSAIDs inhibit tumorigenesis via PPARβ/δ inhibition [27], and genetic disruption of PPARβ/δ contributes to the growth-inhibitory effects of APC [28]. Opposing evidence exists suggesting that PPARβ/δ activation increases [29-31] and decreases cell proliferation [32, 33] in various cell types. Previous evidence, however, establishes a clear link between *Pparb*, *Bcl6* and *Mmp9*, and we sought to elucidate the role(s) of PPARβ/δ activation on potential target genes involved in pancreatic cancer invasion and metastasis. The PPARβ/δ-specific activator GW501516 and shRNAs to decrease expression of *Pparb*, *Bcl6* and *Mmp9* were used in two human pancreatic cancer cell lines, Mia PaCa-2 (*Cox2* negative) and BxPc-3 (*Cox2* positive). The experiments show that ligand-dependent activation of PPARβ/δ causes a BCL6-dependent repression of *Mmp9* and other genes involved in cancer metastasis and decreases indices of cell migration, suggesting that PPARβ/δ agonists may be a beneficial tool in the prevention and treatment of pancreatic cancer.

3.3 Materials and methods

Cells and Reagents. Human pancreatic cancer cells, Mia PaCa-2 (COX2 negative, CRL-1420) and BxPc-3 (COX2 positive, CRL-1687) were purchased from the ATCC (Manassas, VA) and cultured in high-glucose DMEM containing 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere at 37°C containing 5% CO_2. Human Embryonic Kidney 293 cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. All media components and fetal bovine serum (FBS) were purchased from Gibco BRL/Life Technologies (Carlsbad, CA). Ciprofibrate (Cipro), purchased from Sigma Chemical Co. (St Louis, MO), was used as the positive control for PPARα. GW501516 (GW), purchased from Sigma Chemical Co., was used as the positive control for PPARβ/δ. Rosiglitazone (Rosi), purchased from Cayman Chemicals (Ann Arbor, MI), was used as the positive control for PPARγ. Recombinant human TNFα and human MMP9 ELISAs were purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer's instructions. Human pancreatic cancer, chronic pancreatitis and pancreas tissue samples were obtained from Dr. Gerhard Leder (Abt. Allgemein- und Viszeralchirurgie, St. Josef Hospital - Klinikum der Ruhr, University of Bochum, Germany). MISSION® shRNA bacterial glycerol stocks targeted against human PPARβ/δ, BCL6, MMP9, as well as the non-targeting vector, were purchased directly from Sigma-Aldrich. High Capacity cDNA Archive Kit and ABI7300 qPCR System were purchased from Applied Biosystems (Foster City, CA). The pPACKH1 packaging plasmids were kindly provided by Dr. Curtis J. Omiecinski (Penn State University). CytoSelect™ 96-well Cell Invasion Assay (Basement Membrane, Fluorometric Format)

was purchased from Cell Biolabs, Inc. (San Diego, CA) and used according to the manufacturer's instructions.

Isolation of total RNA and qPCR. Total RNA was isolated from Mia PaCa-2 and BxPc-3 cells using Tri-Reagent and the manufacturer's recommended protocol (Sigma). Human pancreatic tissue samples were briefly homogenized in 1mL Tri-Reagent and total RNA was isolated. One μg of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Primers for qPCR were designed based on published sequences in GenBank and are shown in Table 3.1. The housekeeping gene βactin was used to normalize all the tested genes. The data shown are representative of three independent experiments with triplicate samples.

Table 3.1 List of pPCR primers used in this study.

Gene	Forward primer	Reverse primer
Adrp	TGACTGGCAGTGTGGAGAAGA	TGTTAATGCTGCCACTGACCA
bactin	AACAAGAGGCCACACAAATAGG	CAGATGTACAGGAATAGCCTCCG
E-*selectin*	TCCTATTCCAGCCTGCAATGT	AACCCATTGGCTGGATTTGTC
Icam1	ACTCAGCGGTCATGTCTGGAC	GGCATAGCTTGGGCATATTCC
Vcam1	AGTGGTGGCCTCGTGAATG	CACGCTAGGAACCTTGCAGC
Il1b	TCCTTAGTCCTCGGCCAAGAC	GTGCCATGGTTTCTTGTGACC
Mcp1	GGACGCATTTCCCCAGTACA	CCGAGAACGAGATGTGGACA
Mmp9	AGCGGTCCTGGCAGAAATAG	ACGCACGACGTCTTCCAGTAC
Bcl6	GCTCACGGCTCACAACAATG	TCCGGAGTCGAGACATCTTGA
Pparb	AGGCCATTCACCAACTGCTT	ATTGTGGCAGGCAGAGAAGG

Quantification of MMP9 protein by ELISA. MMP9 protein levels were quantified using the human MMP9 ELISA according to the manufacturer's instructions (Invitrogen). Briefly, control Mia PaCa-2 cells or shRNA knock-down cells were plated in 6-well tissue culture plates and treated 1ng/mL TNFα with or without 500nM GW501516 for

24h. At the end of the incubation time, the media was removed and diluted 1:40 in standard diluent buffer. Diluted media samples and MMP9 standards were added to a 96-well microtiter plate containing human MMP9 antibody-coated wells and allowed to incubate at room temperature for 2h. Following the incubation, the media was aspirated and each well washed 5 times with wash buffer. One hundred μL Biotinylated anti-MMP9 (Biotin Conjugate) solution was added to each well and the plate was incubated for 1h at room temperature. Each well was washed a second time with wash buffer, and 100μL of Streptavidin-HRP working solution was added and the plate was allowed to incubate at room temperature for 30mins. After a third wash, 100μL of stabilized chromogen was added to each well and the plate was incubated at room temperature for 30mins in the dark, after which time 100μL of stop solution was added and the absorbance read at 450nm.

Cell migration assay. Either control or knock-down human pancreatic cancer cells were grown to confluence in 10cm tissue culture plates and then pre-treated with TNFα with or without GW501516 for 24h, as above. Cell migration assays were performed using the CytoSelect^{TM} 96-Well Cell Invasion Assay (Basement Membrane, Fluormetric Format) according to the manufacturer's instructions. Briefly, the basement membrane was allowed to reach room temperature for 30mins, and rehydrated using warm, serum-free DMEM. Human pancreatic cancer cells were then seeded into each well at a density of $2x10^6$ cells/mL in serum-free media. Normal cell media (DMEM containing 10% FBS, along with TNFα with or without GW501516) was added to the feeder tray and the entire apparatus was placed in an incubator at 37°C containing 5% CO_2 for 24h. CyQuant® GR

dye/Lysis buffer solution was added to the invading cells following completion of the assay and the resulting mixture was incubated at room temperature for 20mins. Invading cells were quantified by reading the fluorescence at 480nm/520nm. All measurements were performed in triplicate.

Lentiviral shRNA infection. HEK-293 cells were grown to confluency in 10cm tissue culture plates under the conditions described above. The cells were then transiently transfected with 4.6μg of either non-targeting shRNA, or shRNAs targeted against human *Pparb*, *Bcl6*, or *Mmp9*, as well as 2.4μg each of pPACKH1 packaging plasmids, using LipofectAMINE 2000. Cells were transfected for 6h and allowed to recover overnight in normal media. Fresh media was added the following morning, and pseudoviral supernatant was generated for 72h. Supernatant was then harvested and passed through a 0.4μm filter under sterile conditions. Polybrene (Millipore, Billerica, MA) was then added to a final concentration of 5μg per mL and the pseudoviral supernatant was then added directly to target cells for 6h. Infected cells were allowed to recover overnight following the addition of 6mL complete media and knock-down of target genes was assessed by qPCR 48h post-infection.

Statistical Analysis. Quantitative data are presented as mean±SEM. ANOVA with p-value<0.05 was used to determine whether differences among variables were significant. Normality was checked using Anderson-Darling test, and the General linear model, followed by the Tukey post hoc test to analyze differences between treatments. All data analyses were performed by MiniTAB Ver.14 (MiniTAB, State College, PA) or JMP

(SAS Institute, Cary, NC) and data were plotted by Prism 5.01 (GraphPad Software, San Diego, CA).

3.4 Results

§ denotes experiments performed by Jeffrey David Coleman.

Tissue samples from human pancreatic ductal carcinomas show significantly increased levels of *Mmp9* mRNA. It is well known that the matrix metalloproteinases are key regulators of cell proliferation and migration in human pancreatic cancer cells [34] and that MMP9 protein is increased in the pancreatic juice from patients diagnosed with pancreatic ductal adenocarcinomas [35]. Recently, *Mmp9* has been linked to *Pparb* and the transcriptional repressor gene *Bcl6*, in *PPARβ/δ*$^{-/-}$ macrophages, for example, there was lower *Mmp9* expression compared with wild-type cells [15]. Tissue samples from patients diagnosed with chronic pancreatitis or pancreatic cancer were obtained, and we set out to assess the differences in expression of several genes involved in inflammation and metastasis. Indeed, there was a 10-fold increase in *Mmp9* gene expression in ductal carcinomas compared with samples from patients diagnosed with chronic pancreatitis (Figure 3.1). Interestingly, *Pparb* expression was also elevated while mRNA expression of the transcriptional repressor *Bcl6* was almost 3-fold lower in tumor samples compared with those from chronic pancreatitis patients. Despite the low expression of *Bcl6* in ductal carcinomas, however, the relative expression of two BCL6 target genes, *Vcam1* [36] and *Mcp1*, were not significantly elevated in tumor samples. A PPARβ/δ target gene, *Adrp*,

was also not different between tumor, pancreatitis and other pancreatic tissue samples (data not shown).

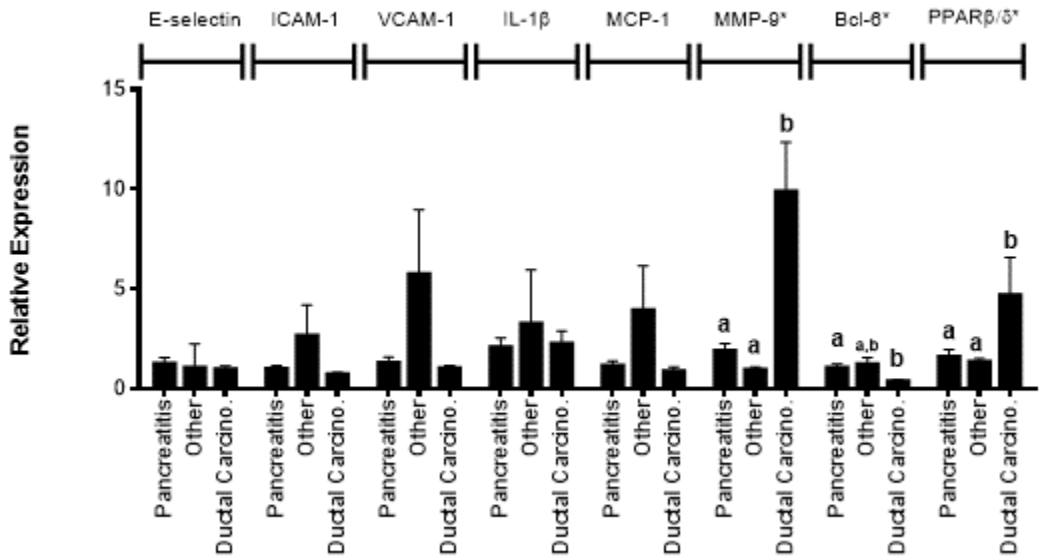

Figure 3.1 **§**Relative mRNA expression in human pancreatic tissues at varying stages of carcinogenesis from chronic pancreatitis to pancreatic cancer. Total mRNA was isolated using standard Tri-reagent protocol and reverse-transcribed. Gene expression was determined using qPCR and expressed as fold induction after normalization to β-actin. **P<0.05*

Regulation of *Mmp9* expression by PPARβ/δ and BCL6 in pancreatic cancer cells.

PPARβ/δ activation negatively influences *Mmp9* gene expression in IL1β-stimulated vascular smooth muscle cells [23]. To study the mechanism of this response and to determine its applicability to another cell type, Mia PaCa-2 cells were transiently infected with non-targeting control, *hBcl6*, *hPparb* or *hMmp9* lentiviral shRNAs (Figure 3.2A). Cells transiently infected with non-targeting control shRNA showed no alterations in

either *Bcl6, Pparb* or *Mmp9* mRNA expression. Mia PaCa-2 cells infected with an shRNA targeted against *Bcl6* showed approximately 50% reduction in *Bcl6* mRNA levels, and cells infected with an shRNA targeting *Pparb* or *Mmp9* showed approximately 70% reduction in corresponding mRNA levels (Figure 3.2A). To determine if gene expression is altered after lentiviral-mediated *Bcl6* or *Pparb* repression, the PPAR target gene *Adrp* was examined upon treatment with three isoform-specific PPAR agonists (ciprofibrate, PPARα, GW501516, PPARβ/δ, rosiglitazone, PPARγ). Control cells and those transiently expressing the indicated shRNAs were treated with either 20μM Ciprofibrate, 500nM GW501516 or 10μM Rosiglitazone. Mia PaCa-2 cells contain functional PPARs as indicated by the ligand-induced expression of *Adrp,* with each treatment resulting in a three-fold increase in transcript levels (Figure 3.2B). Cells expressing *Pparb*-specific shRNA did not induce expression of *Adrp* in response to GW501516 at a concentration that activates only PPARβ/δ [37], while cells expressing *Bcl6*-targeting shRNA retained inducible expression of *Adrp* by all three isoform-specific ligands. Following *Bcl6, Pparb* or *Mmp9* knock-down, cells were treated with 1ng/mL TNFα with or without 500nM GW501516 for 24 hours, and MMP9 protein levels were assessed by ELISA. MMP9 protein levels were significantly elevated in *Bcl6* knock-down Mia PaCa-2 cells following TNFα challenge compared with control cells, while *Pparb* knock-down Mia PaCa-2 cells showed a significant reduction in TNFα-induced MMP9 protein levels (Figure 3.2C), consistent with previous reports in *PPARβ/δ*$^{-/-}$ macrophages. While GW501516 co-treatment significantly suppressed TNFα-induced MMP9 protein levels in control (non-targeting) Mia PaCa-2 cells, this effect was not

observed in either of the *Bcl6* or *Pparb* knock-down cells. Not unexpectedly, lentiviral shRNA targeted against MMP9 significantly reduced both mRNA and protein expression in Mia PaCa-2 cells, and GW501516 activation of PPARβ/δ did not further reduce MMP9 protein levels in these cells.

Mmp9 knock-down reduces Mia PaCa-2 cell invasion. Because *Mmp9* is a key regulator of human pancreatic cancer cell invasion and metastasis, we further examined the effect of lentiviral shRNA-mediated *Mmp9* knock-down on the basal ability of Mia PaCa-2 cells to invade a basement membrane. Mia PaCa-2 cells treated with non-targeting control or *Mmp9*-targeting lentiviral shRNAs were seeded into a 96-well invasion plate and allowed to migrate across a membrane for 24 hours. Using the migration assay described, we found that *Mmp9* knock-down significantly reduced the basal migration of Mia PaCa-2 cells (Figure 3.2D).

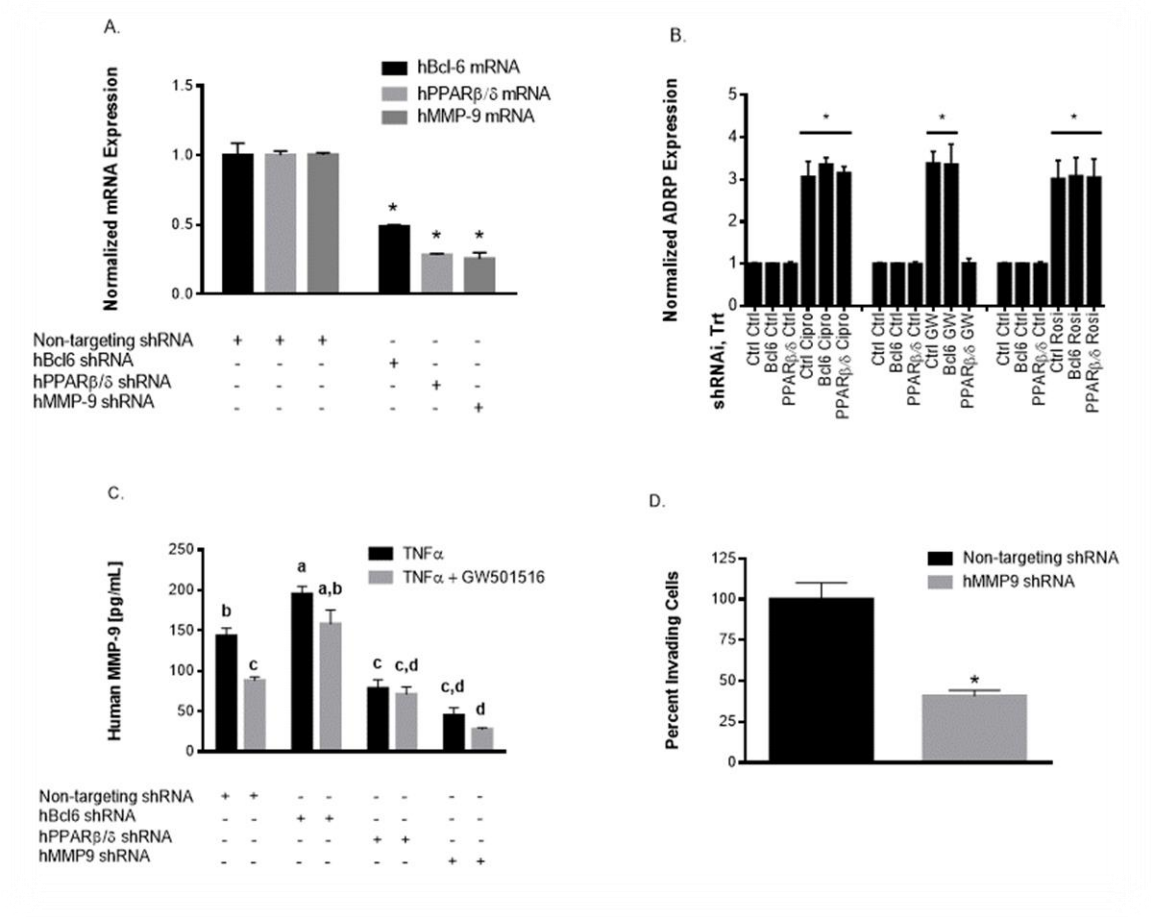

Figure 3.2 Effect of PPARβ/δ activation on MMP9 expression. §A. Mia PaCa-2 cells were transiently infected with non-targeting control, hBCL6, hPPARβ/δ or hMMP9 lentiviral shRNAs for 48h. Total mRNA was isolated and gene-specific knock-down was assessed using qPCR. **P<0.05* §B. Mia PaCa-2 cells contain functional PPARs. Mia PaCa-2 cells were transiently infected with the indicated shRNAs and treated with the indicated PPAR isoform-specific agonists. Induction of the PPAR-target gene ADRP was determined using qPCR. **P<0.05* §C. Mia PaCa-2 cells were stimulated with human TNFα with or without GW501516 for 24h following transient infection with the indicated shRNAs. Human MMP9 protein expression was quantified using MMP9-specific ELISA (Invitrogen). **P<0.05* §D. Mia PaCa-2 cells with reduced MMP9 expression are less invasive than control Mia PaCa-2 cells. Following infection with human MMP9-targeting shRNA, Mia PaCa-2 cells were seeded in 96-well invasion plates (Cell Biolabs, Inc.) and allowed to invade the basement membrane overnight. Relative cell invasion was quantified using the CytoSelect 96-well cell invasion assay with fluorometric readings at 480 nm/520 nm. **P<0.05*

PPARβ/δ activation decreases TNFα-induced expression of pro-inflammatory and cell adhesion genes in human pancreatic cancer cells. PPARβ/δ associates with the transcriptional repressor BCL6 which, upon PPARβ/δ activation, is released and decreases expression of target genes. To determine if the PPARβ/δ/BCL6 pathway is active in human pancreatic cancer cells, we used shRNA knock-down of *Pparb* and *Bcl6* in conjunction with PPARβ/δ-specific activation by GW501516 to analyze the gene expression changes. Mia PaCa-2 cells transiently expressing non-targeting control shRNA, or shRNAs targeted against *Pparb* or *Bcl6*, were stimulated with 1ng/mL TNFα with or without GW501516 for 24 hours. In control Mia PaCa-2 cells, TNFα stimulation induced the robust expression of the cell adhesion molecules *E-selectin*, *Icam1* and *Vcam1*, the pro-inflammatory genes *Il1b* and *Mcp1* and the pro-migratory gene *Mmp9*, while co-treatment with 500nM GW501516 significantly suppressed their expression at the mRNA level (Figure 3.3). Treatment of Mia PaCa-2 cells with a *Bcl6*-targeting shRNA attenuated the GW501516 inhibitory effect on the genes tested, indicating a role for BCL6 in GW501516-mediated repression. Consistent with the findings of Lee *et al*. in *Pparb* $^{-/-}$ RAW264.7 macrophage cells, *Pparb* knock-down Mia PaCa-2 cells displayed significantly lower levels of these genes when challenged with TNFα alone, and PPARβ/δ activation with GW501516 had no further significant repressive effect. Of note is the fact that although *Bcl6* repression resulted in increased MMP9 protein levels in media, it did not concordantly increase the mRNA expression of this gene.

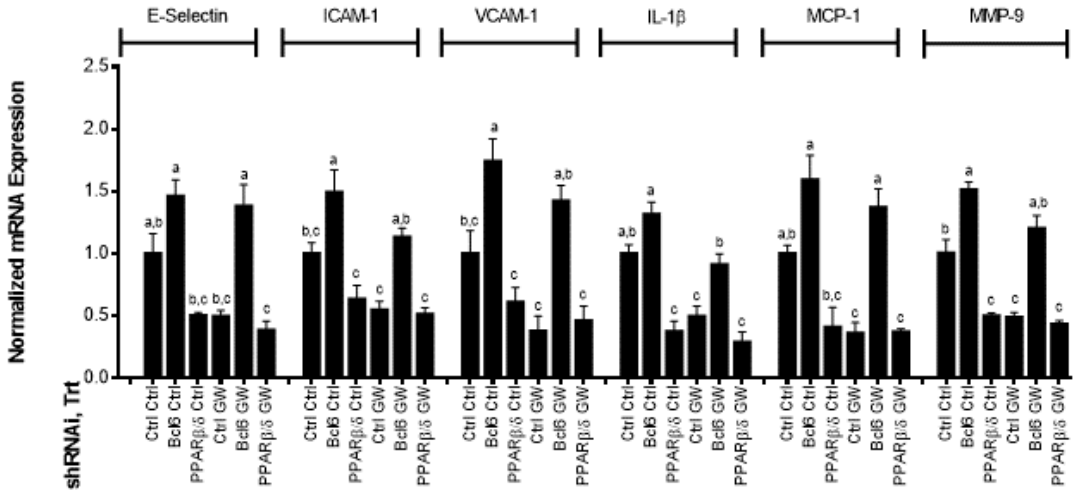

Figure 3.3 Effects of *Pparb* and *Bcl6* knock-down on Mia PaCa-2 gene expression. Mia PaCa-2 cells were transiently infected with non-targeting control, hBCL6 or hPPARβ/δ-specific shRNAs and then stimulated with human TNFα with or without GW501516 for 24h. Total mRNA was isolated and gene expression was determined using qPCR. Data is normalized to βactin and indicated as fold change.

PPARβ/δ activation inhibits human pancreatic cancer cell migration. To examine if PPARβ/δ activation by GW501516 and subsequent repression of pro-inflammatory and pro-migratory genes via BCL6 influenced their ability to invade a basement membrane, Mia PaCa-2 (*Cox2* negative, Figure 3.4A) and BxPc-3 (*Cox2* positive, Figure 3.4B) were treated with non-, *Pparb*- or *Bcl6*-targeting shRNA, and the effects of GW501516 on cell migration were examined. In control cells, GW501516 treatment negatively influenced the ability of either Mia PaCa-2 or BxPc-3 cells to migrate across a membrane (50% reduction). Lentiviral-mediated knock-down of *Bcl6*, however, increased cell migration in both cell lines compared with control cells. GW501516 treatment of *Bcl6* repressed cells did not have an effect on Mia PaCa-2 (Figure 3.4A) but did have an effect on comparable BxPc-3 cells (Figure 3.4B). Interestingly, Mia PaCa-2 and BxPc-3 cells

transiently expressing an shRNA targeted against PPARβ/δ showed significantly reduced cell migration compared with control cells with or without GW501516 treatment. These results suggested that the transcriptional repressor BCL6 mediates the anti-migratory actions of GW501516 in human pancreatic cancer cells, but does so in a PPARβ/δ-dependent manner.

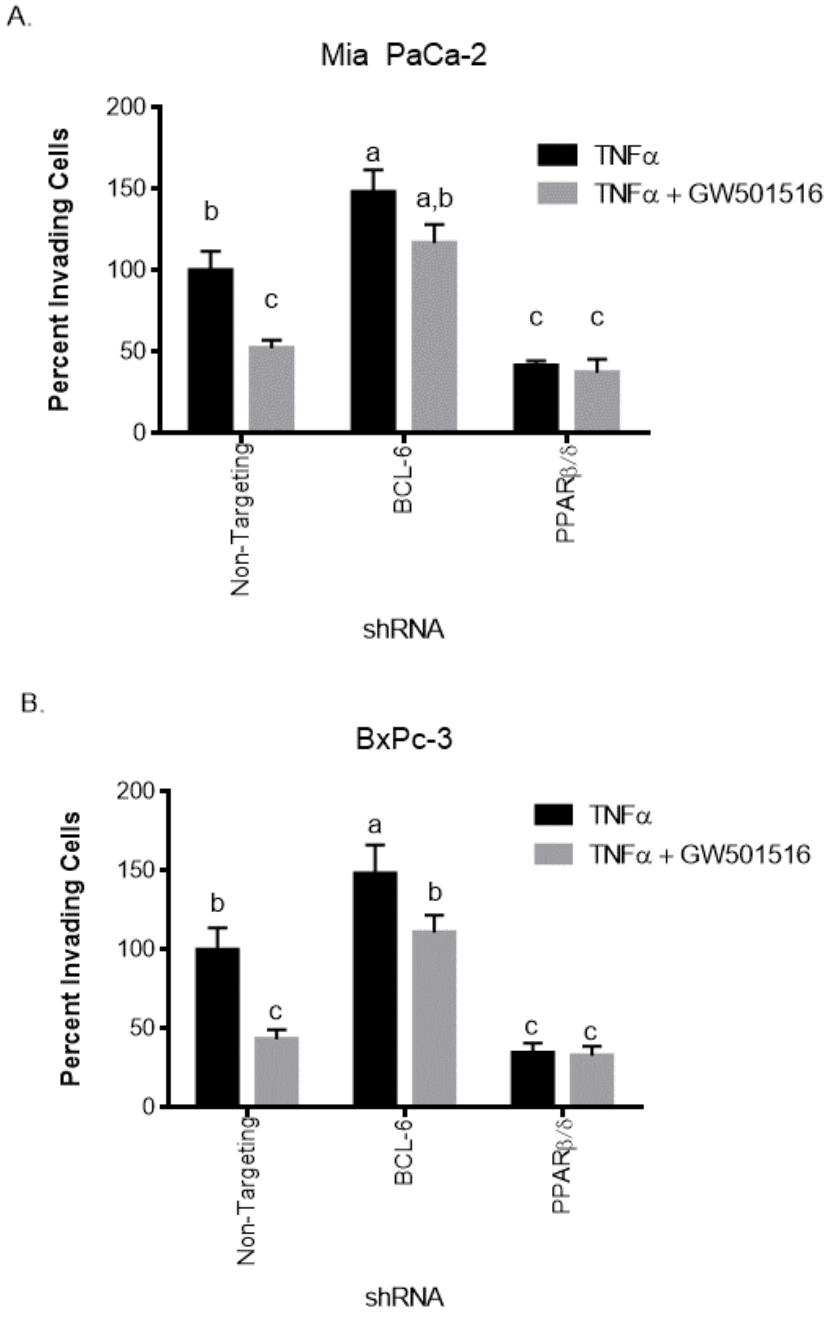

Figure 3.4 GW501516 treatment reduces TNFα-stimulated Mia PaCa-2 and BxPc-3 cell invasion through a basement membrane. Human pancreatic cancer cells were transiently infected with the indicated shRNAs and stimulated with human TNFα with or without GW501516. Cells were allowed to invade a basement membrane overnight, and relative invasion was quantified using the CytoSelect 96-well cell invasion assay with fluorometric readings at 480 nm/520 nm. GW501516 treatment inhibits the invasion of

the COX2 negative pancreatic cancer cell line Mia PaCa-2 **§**(A) and the COX2 positive pancreatic cancer cell line BxPc-3 **§**(B) through a basement membrane.

3.5 Discussion

The PPAR nuclear receptors are regulators of inflammation and proliferation in human pancreatic cells [16, 38, 39]. Although several studies implicate PPARγ activation in inhibition of pancreatic cancer cell growth, little is known about the role of PPARβ/δ, save for its role in suppressing inflammation via BCL6 [16]. Generally, the role of PPARβ/δ in cancer cell growth and tumorigenesis remains controversial. In colorectal cancer cells, non-steroidal anti-inflammatory drugs inhibit tumorigenesis through inhibition of *Pparb* [27], and PPARβ/δ promotes intestinal carcinogenesis [40]. Studies in the *Pparb*-null mouse, however, show that activation of PPARβ/δ induces terminal differentiation [41], and PPARβ/δ-specific ligands inhibit the growth of keratinocytes *in vivo* [42, 43] and *in vitro* [32]. Furthermore, PPARβ/δ activation is linked to inhibition of IL1β-stimulated proliferation and migration of vascular smooth muscle cells [23] via regulation of *Il1ra* and *Tgfb* and negative regulation of *Mmp9*. Our results show that PPARβ/δ activation by GW501516 suppresses expression of *Mmp9* in human pancreatic cancer cells via BCL6, with further inhibition on the ability of two cell lines, Mia PaCa-2 and BxPc-3, to invade a basement membrane.

Consistent with previous work [35], analysis of the expression levels of several genes in both ductal carcinomas and chronic pancreatitis showed elevated levels of the matrix-remodeling gene *Mmp9*. Several studies link increased MMP9 levels to increased invasiveness and metastasis [44, 45]. MMP9 is a critical player in the early stages of tumor

invasion by degrading basement membrane type IV collagen [46], considered to be a crucial step in tumor cell invasion [47]. MMP9 also participates in the degradation of the various components of the ECM [48]. Inhibition of *Mmp* activity by orally bioavailable matrix metalloproteinase inhibitors has shown promise in decreasing tumor metastasis in clinical trials [46]. In the cell lines BxPc-3 and Mia PaCa-2, treatment with the neurotransmitter Norepinephrine increased cell invasiveness via augmented *Mmp2*, *Mmp9*, and *Vegf* [49], while treatment with the β-blocker propranolol inhibited these effects. Clearly the regulation of *Mmp* activity is important in controlling, and possibly treating, pancreatic cancer. The association between *Mmp9*, *Pparb* and *Bcl6* was established by Lee *et al.* [15], demonstrating that *Mmp9* expression in *PPARβ/δ*$^{-/-}$ macrophages is repressed compared to wild type. Activation of the receptor significantly decreased the expression of pro-inflammatory markers, suggesting that BCL6 released from the PPARβ/δ complex plays a role in the regulation of *Mmp9*. A similar result was obtained in VSMCs [23].

In the present studies, *Pparb* mRNA were increased in ductal carcinomas, while *Bcl6* expression was decreased. In colorectal cancer tissue samples, PPARβ/δ expression increased during multistage carcinogenesis and were tightly associated with a highly malignant morphology [50]. It is possible that PPARβ/δ plays a role in human pancreatic cells, but whether PPARβ/δ contributes to pancreatic cancer cell metastasis or if its over-expression is the result of some altered signaling pathway remains unclear. Since the regulatory region of *Pparb* contains several *Ap1* response elements and is controlled by a variety of inflammatory signals [51], the increased expression of this nuclear receptor may be indicative of stress-response and not causally related to the tumor phenotype.

Increased expression of PPARβ/δ in the inactivated state may sequester BCL6, increasing the expression of genes normally controlled by this transcriptional repressor.

Of particular note is the observation that the relative expression of *Bcl6*, a proto-oncogene known to suppress genes involved in cell cycle progression, particularly *Cyclin d1* [52], and inflammation [53], was lower in ductal carcinomas compared with pancreatitis. BCL6 is mutated in several disorders [54-56], and is implicated in cell line immortalization and transformation by overriding cellular senescence downstream of *P53* [57]. Interestingly, DNA-chip hybridization assays identified both *Bcl6* and *Bcl10* as novel candidate genes in pancreatic cancer that were over-expressed in pancreatic cancer cell lines and primary tumor samples [58]. Contrary to ductal cells, BCL6 is absent in pancreatic beta cells, potentially explaining the lack of anti-inflammatory PPARβ/δ signaling in this cell type [16]. Our results suggest that the BCL6 pathway is disrupted as inflamed tissue transforms into tumor, with the possibility that loss of *Bcl6* expression leads to increased cancer invasion via increased *Mmp9* expression.

Activation of PPARβ/δ decreased the expression of MMP9 at the protein level, while knockdown of *Bcl6* increased MMP9 protein production. Activation of PPARβ/δ in *Bcl6* knock-down cells showed no significant effect on reducing MMP9 protein, suggesting a key role for BCL6 in the regulation of *Mmp9*. Knocking down *Pparb* in human pancreatic cancer cells reduced MMP9 protein levels to those comparable to GW501516-treated control cells despite PPARβ/δ activation. It is our hypothesis that *Mmp9* is an indirect PPARβ/δ target gene, and that PPARβ/δ activation releases BCL6 which may then relocate to the *Mmp9* promoter. Further studies, such as chromatin

immunoprecipitation assays, for example, are required to substantiate this point. Our results, however, are in agreement with previous work indicating that low levels of PPARβ/δ result in decreased *Mmp9* expression [15], and we believe that, in the absence of PPARβ/δ, BCL6 is available to repress target genes, either through direct repression on target gene promoters as in the case of *Vcam1* and *E-selectin*, two genes lacking *PPRE*s [36], or through interactions with other cell signaling mediators, such as NFκB [59]. GW0742 has been found to inhibit inflammation by TNFα while not preventing TNFα induced degradation of IκBα and the translocation of NFκB. No decrease in DNA binding activity indicates PPARβ/δ must interfere via a corepressors mechanism at the chromatin level [36].

The relationship between MMP9 and metastasis in pancreatic cancer is well documented. Treatment of Mia PaCa-2 cells with *Mmp9* shRNA reduced protein levels by approximately 50% regardless of GW501516 treatment, with corresponding inhibition on the ability of the cell line to invade a basement membrane. Mia PaCa-2 cells are considered a highly metastatic cell line [60], and our results support the idea that regulating *Mmp9* expression may effectively control human pancreatic cancer cell invasion and metastasis.

PPARβ/δ activation is associated with reduced inflammatory and adhesion cell markers. Our results in Mia PaCa-2 cells show that the PPARβ/δ:BCL6 anti-inflammatory pathway is active and represses *E-selectin, Icam1, Vcam1, Il1b, Mcp1* and *Mmp9*. Repression of *Vcam1*, *E-selectin* [36], and *Mcp1* [15] in particular are dependent upon dissociation of BCL6 from PPARβ/δ and subsequent relocation to the corresponding promoters. Our results demonstrate that the pro-adhesion molecules *E-selectin, Icam1*

and *Vcam1*, the pro-inflammatory *Il1b* and *Mcp1* and the pro-metastasis gene *Mmp9* are BCL6 regulated genes in human pancreatic cancer cells. Treatment with *Bcl6* shRNA attenuated the GW501516-mediated inhibition of TNFα-induced expression of these molecules, while treatment with *Pparb* shRNA reduced their expression at the mRNA level regardless of GW501516 treatment. *E-selectin*, *Icam1* and *Vcam1* are important in pancreatic cancer, where they participate in the detachment of cells from the primary tumor and contribute to cancer spread [61], and their over-expression in pancreatic adenocarcinomas is associated with a stimulation in tumor growth, increased metastatic ability and potentially shorter post-operative survival following tumor resection [62]. IL1β induces *Mmp9* expression in other cell lines [63-65], and enhances the invasiveness of human pancreatic cancer cells [66]. Monocyte chemotactic protein 1 is produced by pancreatic cancer cells in response to TNFα challenge and may contribute to the accumulation of tumor-associated macrophages [67] which influence key events in the tumor invasion process [68]. Taken together, these results suggest that PPARβ/δ activation and subsequent suppression of pro-adhesion and pro-migratory genes via BCL6 might prove useful in the control of pancreatic cancer.

GW501516 treatment also inhibited the TNFα-promoted invasion of a basement membrane by the pancreatic ductal cell lines Mia PaCa-2 and BxPc-3. Pancreatic cancer cells transiently expressing BCL6 shRNA were significantly more invasive, while GW501516 treatment attenuated their invasive potential close to control levels. Conversely, cells transiently expressing PPARβ/δ shRNA were significantly less invasive than control cells, and GW501516 treatment showed no significant effect in further

reducing invasion. We hypothesize that cells expressing lower levels of the transcriptional repressor BCL6 are more invasive owing to a lack of control over pro-migratory gene regulation and increased protein levels of MMP9, while knocking down *Pparb* via shRNA methods allows for a greater population of unassociated BCL6 which is available to repress pro-migratory genes resulting in lower invasion. Although we present here a fairly simplified mechanism, it is possible that more complex signaling cascade is taking place resulting in the inhibition of cell invasion. In VSMCs, repression of *Mmp9* activity was effected in a TGFβ-dependent manner following PPARβ/δ activation [23], and indeed, TGFβ suppresses *Mmp9* expression in monocytes through a prostaglandin E2- and cAMP-dependent mechanism [69]. *Tgfb* is a PPARβ/δ target gene in VSMCs [70], and it is possible that the PPARβ/δ/TGFβ pathway could in fact be active in human pancreatic cancer cells. Our results suggest, however, that BCL6 and PPARβ/δ play critical roles in suppressing pro-migratory gene expression at the mRNA level and in ultimately controlling human pancreatic cancer cell invasion.

Our observations demonstrate that activation of PPARβ/δ by a specific agonist reduces the TNFα-induced mRNA levels of genes known to be involved in the regulation of human pancreatic cancer cell invasion and metastasis, and this negative regulation is also manifested at the protein level. Furthermore, we show that PPARβ/δ activation reduces Mia PaCa-2 and BxPc-3 invasion through a basement membrane, and that the transcriptional repressor BCL6 plays a critical role in the pathway(s) regulating human pancreatic cancer cell invasion. This is not the first time PPAR activators have been shown to negatively influence pancreatic cancer cell invasion, and perhaps further *in vivo*

studies using these mouse-transplantable cell lines could provide more useful insight into the potential therapeutic uses of PPARβ/δ activators in the control and regulation of pancreatic cancer.

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Chapter 4

Sequestration of B-Cell Lymphoma 6 by Peroxisome proliferator-activated receptor β/δ and therapeutic potential in pancreatic cancer

4.1 Abstract

Multiple laboratories contend expression and activation of PPARβ/δ to be inflammatory in colorectal, lung, and breast epithelial cells, while contradictory studies argue PPARβ/δ to be anti-inflammatory, including several studies in our lab regarding the pancreas. PPARβ/δ is usually expressed in lower amounts in cancer compared to normal cells in many tissues. However, the exact mechanism of this pathway in carcinogenesis is poorly understood. Reports from our lab indicate *Pparb* to be highly expressed in tumor tissues of the pancreas compared to non-tumor tissues and BCL6 to be suppressed. RNAi suppression of *Pparb* reduced MMP9 protein and mRNA expression of several pro-inflammatory genes, as well as reduced pancreatic cancer cell invasion across a basement membrane. This led us to hypothesize perhaps *Pparb* expression augments inflammation and cancer progression in the pancreas, specifically by sequestering BCL6, and that treatment with PPARβ/δ-specific ligand GW501516 reverses this sequestration allowing it to exert its anti-inflammatory effects.

We observed an elevated expression of *Pparb* in tumor tissue compared to corresponding adjacent normal tissue in human, and this dysregulation is established approximately nine months into PDAC progression in the Cre-mediated *Kras(*G12D)

mouse model. Basal and TNFα-induced *Mcp1* luciferase activity, an inflammatory chemokine constitutively expressed in several pancreatic cancer cell lines and implicated as a prevalent negative regulator of PDAC, was increased with expression of *Pparb*. Furthermore, *Pparb* expression promoted proliferation in pancreatic cancer cells. Proliferation and *Mcp1* luciferase activity were ameliorated by GW501516. The data presented may indicate a sequestering of BCL6 by PPARβ/δ that occurs during dysregulation of PDAC progression resulting in augmentation of PDAC growth and metastasis, and the therapeutic potential of GW501516 in chemoprevention.

4.2 Introduction

Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) belongs in the nuclear hormone receptor (NR) superfamily of proteins together with the closely related PPARα and PPARγ subtypes [1]. PPARβ/δ is a ligand-activated transcriptional factor that regulates expression of genes involved in lipid metabolism, insulin sensitivity, proliferation, and differentiation [2,3] and is the only PPAR that associates with the transcription suppressor BCL6 [4]. Regulation of gene expression occurs through three main mechanisms: direct ligand-dependent transactivation of PPARβ/δ and retinoid X receptor (RXR) with dimer formation and binding to the peroxisome proliferator response element (*PPRE*) in target genes, through ligand-activated dissociation of BCL6 from the PPARβ/δ complex freeing this transcription regulator to bind to the promoter region of its target genes [5,6], and repression of other transcription factors such as NFkB1 and STAT1 via protein-protein interaction. Together, PPARβ/δ activation affects lipid metabolism and inflammation [4,7] and may be a promising approach in the treatment of metabolic disorders such as obesity,

diabetes, and atherosclerosis [6,8,9]. These two proteins, PPARβ/δ and BCL6, also play a role in cellular senescence [10]. Importantly, metabolic disorders and chronic inflammation are leading causes of development of cancer, including those that arise in the pancreas [11].

Pancreatic cancer (PC) is very aggressive disease, ranking fourth in the leading causes of cancer death in the United States with a 5-year survival rate of 6%. Approximately 29,000 people have been diagnosed each year, and nearly 28,000 will die [12]. Exocrine cancers make up the majority of pancreatic cancer with about 85% classified as adenocarcinoma within the ductal region (pancreatic ductal adenocarcinoma, PDAC). A small minority are neuroendocrine cancers within a the Islets of Langerhans [13]. β cells, within the Islets of Langerhans, contain higher expression of PPARβ/δ compared to the α and γ variants, BCL6 is absent within these cells which may explain the lack of an anti-inflammatory role of PPARβ/δ agonists [14]. Comprehensive gene expression analysis has implicated the PPARβ/δ pathway in development of pancreatic cancer [15] and potential target in treating the disease, although this is not without controversy [16,17].

The human *Bcl6* gene has three mRNA isoforms. Variant 1 and 2 have distinct transcriptional start sites that encode identical BCL6 proteins (BCL6L). The third isoform lacks exon 7 that encodes the first two zinc finger motifs [18], although the last four such motifs are retained. This short version of BCL6 (BCL6S) is expressed in several human cell lines and tissue, along with the other variants and does not affect DNA binding or nuclear localization, but interaction with its targets including PPARβ/δ are still being elucidated [18]. A single nucleotide polymorphism (SNP) within BCL6 (rs1056932) causes the alternative splicing which produces BCL6S, and has been linked to risk of non-Hodgkin Lymphoma in a population-base case-control study [19].

PPARβ/δ activation via ligands such as GW501516 is associated with decreased inflammatory responses in several cell lines and tissues [20–22], presumably by regulation of genes such as interleukin 1 receptor antagonist (*Il1ra*) via a *PPRE*-dependent mechanism as well as transpression of NFκB activity. Paradoxically, increased expression of PPARβ/δ in its inactive state (without exogenous ligand) increased inflammatory response due to sequestering of BCL6 [23]. Similarly, low doses of doxorubicin increased expression of PPARβ/δ which sequestered BCL6, preventing its anti-senescent effects in cardiomyocites [24]. In this study we examined the PPARβ/δ and BCL6 pathway in pancreatic cancer progression in both humans and in a commonly used mouse model of this disease (*Kras(*G12D) mouse). In addition, the effects of elevated PPARβ/δ protein levels in human pancreatic cancer cell line growth, as well as the interaction between PPARβ/δ relative to that of BCL6, was examined. Taken together, evidence supports the role of over-expression of PPARβ/δ in the etiology of PDAC and points to the potential utility of potent ligands of this nuclear receptor in the treatment of pancreatic cancer.

4.3 Materials and methods

Cells and reagents. Human pancreatic cancer cells, Mia PaCa-2 (COX2 negative, CRL-1420) were purchased from ATCC (Manassas, VA) and cultured in high glucose DMEM containing 10% FBS. Human embryonic kidney 293T cells were cultured in DMEM containing 10% FBS. Cell media contained 100U penicillin and streptomycin, and cells were cultured in a humidified atmosphere at 37°C containing 5% CO_2. All media components and FBS were purchased from Gibco BRL/Life Technologies (Carlsbad, CA). GW501516 used as a positive control for PPARβ/δ was purchased from Tocris

Biosciences (Bristol, UK). The 2.8kb *Mcp1* (accession #U12470) promoter fragment cloned into the luciferase reporter vector pGL3-basic (Promega) was provided by Dr. Ronald Evans (Salk Institute for Biological Studies, La Jolla, CA). High Capacity cDNA Archive Kit and ABI 7300 real-time PCR system were purchased from Applied Biosystems (Foster City, CA). The pCDNA3.1-*Pparb*-FLAG plasmid were provided by Dr. Curtis Omiecinski (Penn State University). The BCL6L-V5 and BCL6S-V5 plasmids used to clone into the pVP16 and pM vector were provided by Dr. Wing Chan (Nebraska Medical Center, Omaha, NE). Antibodies for the ChIP assays were purchased from various sources: anti-IgG (ab2410, Abcam, Cambridge, UK), anti-acetylated Histone H4 (sc-34263, Santa Cruz Biotechnology, Dallas, TX), anti-PPARβ/δ (sc-7197X), anti-BCL6 (5650S, Cell Signaling Technology, Danvers, MA)

Human pancreas tissue. Frozen human pancreas tissue was obtained from the Penn State Hershey Cancer Institute Biorepository, with the characteristics shown in Table 1. Gene expression studies were performed on all tissues, although the data presented herein is based on paired samples (tumor tissue and adjacent normal tissue) in the adenocarcinoma cases. Age, gender or smoking status was not taken into account in these experiments due to the relatively small sample size.

Kras(G12D) mouse model. Mouse pancreatic tumor tissue were provided by Dr. Bogdan Prokopczyk's lab, and were extracted from the progeny of the B6.129-*Kras*^{tm4Tyj} and B6.FVB-Tg (Ipf1-cre) 1Tuv mice. Progeny of these mice contain a *Kras(G12D)* mutation knocked in within its own locus and is transcriptionally silenced by an upstream Lox-Stop-Lox (LSL) cassette. *Pdx1*-driven Cre recombinase excises the LSL cassette resulting in the expression of *Kras(G12D*) in early pancreatic progenitor cells. PanIN

develops and exhibit acinar to ductal metaplasia by 2 months that progress to higher grade PanIN and then PDAC by 8-12 months [25,26]. All mouse models were obtained from the U.S. National Cancer Institute at Frederick Mouse Repository. The whole pancreas was extracted at specified time points for gene expression analysis. Genotyping was performed using primers listed in Table 2 using the Mouse Repository's recommended protocol.

Table 4.1 Human pancreatic tissue samples.

Diagnosis	Samples			Patient's Age			Patient's Gender	
	Tumor	Normal	Matched	Mean	Max	Min	F	M
Adenocarcinoma	27	25	24	69	85	52	12	16
Ampullary Adenocarcinoma	1	1	1	75	75	75	0	1
Carcinoma	1	1	1	54	54	54	0	1
Cystdenoma	0	1	0	82	82	82	0	1
Cysts	0	1	0	79	79	79	0	1
Ductal Adenocarcinoma	20	14	14	66	84	44	9	11
Ductal Papillary Mucinous Carcinoma	2	2	2	67	71	62	1	1
Endocrine Neoplasm	0	4	0	50	67	33	1	3
Endocrine Tumor	3	2	2	57	70	41	2	1
Intraductal Papillary Mucinous Neoplasm	1	1	1	73	73	73	1	0
Islet Cell Tumor	2	3	2	54	81	38	3	0

Metastatic Adenocarcinoma	1	1	1	65	65	65	0	1
Microsystic Adenoma	0	2	0	62	68	55	1	1
Mucinous Adenocarcinoma	1	1	1	65	65	65	1	0
Neuroendocrine Carcinoma	1	1	1	41	41	41	0	1
Papillary Adenocarcinoma	1	1	1	66	66	66	1	0
Total	61	61	51	65	85	33	33	38

Table 4.2 List of genotyping primer sequences.

Forward Primer (Wildtype)	GTc GAc AAG cTc ATG cGG GTG
Forward Primer (Lox)	ccT TTA cAA GcG cAc GcA GAc TGT AGA
Reverse Primer	AGc TAG ccA ccA TGG cTT GAG TAA GTc TGc A
Forward Primer (*Pdx1*)	ctg gac tac atc ttg agt tgc
Reverse Primer (*Pdx1*)	ggt gta cgg tca gta aat ttg

Isolation of total RNA and qPCR. Total RNA was isolated from tissue samples by briefly homogenizing in 1mL Tri-Reagent following the manufacturer's protocol (Sigma, St. Louis, MO). One μg of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Primers for qPCR were designed based on published sequences in GenBank and are shown in Table 3 and 4. The housekeeping gene 18S was used to normalize all the tested genes. The data shown are representative of 122 human samples (61 tumor samples with corresponding adjacent normal tissue), and 65 mouse samples from various time points split into two groups- pre-nine months and post-nine months.

Table 4.3 List of human qPCR primers.

Gene	Forward Primer	Reverse Primer
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Il1ra	GGGAACTTTGCACCCAACAT	TTGGCAGGTACTCAGCGAATG
Tgfb	AGGTCCTTGCGGAAGTCAATG	CTATTGCTTCAGCTCCACGGA
Sod1	TGCTTCCCCACACCTTCACTGGT	ATGGCGACGAAGGCCGTGTG
Fgf21	CGCTGGCACAGGAACCTGGA	ACCAGAGCCCCGAAAGTCTCCT
Mcp1	GGACGCATTTCCCCAGTACA	CCGAGAACGAGATGTGGACA
Tnfα	TGGATGTTCGTCCTCCTCACA	ATCAATCGGCCCGACTATCTC
Cox2	CGGTGTTGAGCAGTTTTCTCC	AAGTGCGATTGTACCCGGAC
Mmp9	ACGCACGACGTCTTCCAGTAC	AGCGGTCCTGGCAGAAATAG
Nfkb	AGGCTATGCAGCTTGCAAAGAG	TGTCACCGCGTAGTCGAAAAG
Esel	TCCTATTCCAGCCTGCAATGT	AACCCATTGGCTGGATTTGTC
Vcam1	AGTGGTGGCCTCGTGAATG	CACGCTAGGAACCTTGCAGC
Hsd11b1	ACAGGAGTCTTCAGGCCAGCTCC	AGCATCTCTGGTCTGAATTCCTCGT
Cbr1	GGGCATCGCCTTCAAGGTTGCT	TCTGTGCACACATCTCGGGTACC
Rbp1	GACAGGCATAGATGACCGCA	CTCACCCTTCTGCACACACT
Tspan8	CCCAGGAGCTATGACAAGCA	TCGCAAAGGCTATCTCCAGG
Bcl6s	CCATGGAGCCTGAGAACCTTG	TGGCTGGCCGGTTGAACTGG
Bcl6l	TCCGGAGTCGAGACATCTTGA	GCTCACGGCTCACAACAATG
Il1a	CGTAAGGCCTCAGCCAGAAG	CTCCCAATCTCCATTCCCAA
Pparb	ATTGTGGCAGGCAGAGAAGG	AGGCCATTCACCAACTGCTT
Adrp	TGACTGGCAGTGTGGAGAAGA	TGTTAATGCTGCCACTGACCA

Table 4.4 List of mouse qPCR primers.

Gene	Forward Primer	Reverse Primer
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Il1ra	ACTCGACATCCGGTTCTGGACAGAAT	ACTCGAGCATTAATGATGTCAGGGATTG
Tgfb	TGGATTCTGCGCTCTCTTCTC	TTTCTGGCCTCTCCGAGTTCT
Sod1	GTGCAGGGAACCATCCACTT	CACAACTGGTTCACCGCTTG
Fgf21	AGATAGTCTCCACGACGCAC	CTCCCAGAGCATCTAGCTTTGT
Mcp1	TTCCACAACCACCTCAAGCA	GCATCACAGTCCGAGTCACACT
Tnfα	CCAACGGCATGGATCTCAA	ACAAGGTACAACCCATCGGCT
Cox2	GTCTGGTGCCTGGTCTGATGA	CACTCTGTTGTGCTCCCGAAG
Mmp9	TCCCCAAAGACCTGAAAACCTC	ACAAGTATGCCTCTGCCAGCTG
Nfkb	CTATGTGTGTGAAGGCCCAT	AGTTGCAAATTTTGACCTGTG
Esel	TGTGAACATGCTGTGATTCCT	GGTGACACCTCATCGTGGAT
Vcam1	CCCTGAATACAAAACGATCGC	CAAGTGAGGGCCATGGAGTC
Hsd11b1	AGCAACCAGAGATAGGCAGC	CCAGGATCGGGAGGAGGTAA
Cbr1	CCGGTCTTGAACCACTCTCC	AAGACATGGCTGCGTGAGAA
Rbp1	TGGACAAACCTGACCCTTCG	GGGAGGATCCATTGTAGCGG
Tspan8	TGCTCTCTATTTCTACCCCCGA	TTTTGCCTGCAGAGATCCGT
Bcl6l	AGACTGTCCACACGGGTGAGA	TGAACTGCGCTCCACAAATG
Il1a	TTTCCACAGCCACGAAGCT	TGATGAAAGGGCTCCCAAGTA
Pparb	GACACTGTGGCAGGCAGAGAA	CCGTTCACCAGCTGTTTCCA
Adrp	AGTGGAAGAGAAGCATCGGCT	TCGATGTGCTCAACACAGTGG

Mammalian two-hybrid assays. HEK293T cells were plated in 96-well plates and allowed to recover overnight. Transfection was carried out with LipofectAMINE 3000 reagent according to the manufacturer's protocol. Each well was transfected with varying concentrations up to 20ng of pM-PPARβ/δ, or empty vector, 20ng of pVP16-empty, or pVP16-BCL6L or BCL6S, 10ng each of pFR-luciferase and pRL-TK. The V5-tagged BCL6L and S were subcloned into pM and pV16 vector's reading frame using restriction enzyme digestion, ligation, and transformation into α-select gold competent cells. Transfected cells were treated with 250nM GW501516 for 6h and assayed for luciferase activity. Relative luciferase activity was corrected using the internal transfection control (pRL-TK) and the Dual Luciferase Kit (Promega, Madison, WI) using the GloMax microplate luminometer (Promega).

CellTiter-Glo luminescent cell viability assays. A 96-well plate was seeded at 2000 cells/well with Mia PaCa-2 cells. Transfection was performed using LipofectAMINE 3000 by manufacturer's protocol with 50ng/well with varying concentration of pcDNA3.1- *Pparb-FLAG,* pcDNA3.1-*Rxra,* and pcDNA3.1-empty control for 6h. Cells were allowed to recover overnight, and treated with 250nM GW501516 for 72h. Viability was assessed using a GloMax microplate luminometer (Promega).

Mcp1 reporter assays. Mia PaCa-2 cells were transfected with varying concentrations of 5ng to 20ng pcDNA3.1-*Pparb-FLAG* and empty control vector in a 96-well plate, as well as 9ng *mMcp1*-luciferase and 1ng pRLCMV for 6h. Cells were then treated with 1ng/mL Tumor Necrosis Factor α (TNFα) with either DMSO or GW501516 for overnight and luciferase assay was measured using dual luciferase kit as described above. Subsequent

transfections were carried out using 20ng of V5-BCL6L with varying amounts (0, 10, 20ng) of pcDNA3.1-*Pparb*-FLAG and empty control vector.

Chromatin immunoprecipitation assays. Mia PaCa-2 cells were grown to confluency in 10 10cm^2 dishes. Cells were treated with GW501516 (5 dishes) or DMSO (5 dishes) for 2h and crosslinked using 1% formaldehyde for 15mins. Crosslinking was quenched with 125mM glycine for 5mins at room temperature. Cells were scraped in Phosphate buffer saline, pelleted, and suspended in 500μL lysis buffer (50mM Hepes-KOH, pH 7.5, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS) containing protease inhibitors on ice. Chromatin was sonicated to shear at a length of about 1kb, size was assessed via 1% agarose gel. Samples were centrifuged at 12,000 x g for 10mins and the supernatant was collected. Samples were diluted to approximately 25μg, input controls were stored at 4°C, and samples were precleared using Goat anti-rabbit IgG magnetic beads (S1432S, New England Biolabs), and incubated by manufacturer's protocols with IgG, acetylated Histone H4, PPARβ/δ, and BCL6 antibodies for DMSO and GW501516 samples overnight at 4°C. Magnetic beads were then incubated at 4°C for 2h. Beads were pelleted and washed 3 times in TE buffer. Samples were pulled through a sucrose cushion via centrifugation, suspended in RIPA buffer, and washed 3 more times in TE buffer. Finally, samples were suspended in elution buffer (1% SDS, 100mM $NaHCO_3$) and crosslinks were reversed at 65°C with proteinase K for 1h. DNA was concentrated using the ChIP DNA Clean and Concentrator kit by Zymo Research (Irvine, CA). Quantitative PCR was run using primers described in Table 4.5.

Table 4.5 List of qPCR primers used in ChIPs.

Gene	Forward Primer	Reverse Primer
Angptl4 negative control	GCCTATAGCCTGCAGCTCAC	CAAGTGGAGAAGGGTACGGA
Angptl4 PPRE	CCTTACTGGATGGGAGGAAAG	CCCAGAGTGACCAGGAAGAC
Angptl4 Trans. start site	ACCGACCTCCCGTTAGCCCC	CGGAGAACCAGCCCTGGGGA
Cd20 control	ACATTTGCCAAGTCCTGACC	GAGGCATGTGCCTTCATTTT
Mcp1 BCL6-binding site	GGAAAAGAAAAAGCAGAGCCACTCCATTCACAC	TTATTGTAAGCCAGGGGGGTGG

Statistical Analysis. Quantitative data are presented as mean±SEM. ANOVA with p-value<0.05 was used to determine whether differences among variables were significant. Normality was checked using Anderson-Darling test, and the General linear model, followed by the Tukey post hoc test to analyze differences between treatments. All data analyses were performed by MiniTAB Ver.14 (MiniTAB, State College, PA) or JMP (SAS Institute, Cary, NC) and data were plotted by Prism 6 (GraphPad Software, San Diego, CA).

4.4 Results

Pparb mRNA higher in tumor tissue than adjacent normal tissue in human pancreas. Previous studies in our lab have shown *Pparb* to be highly expressed in tumor tissue and *Bcl6* to be suppressed [23], corroborated by a previous microarray study in pancreatic tumors [45], and contradictory to several studies in other tissues exhibiting PPARβ/δ to be lower in tumors relative to normal tissue [16]. We acquired 122 human pancreas samples from the Hershey Penn State Cancer Institute Biorepository, 51 of which were tumor samples paired with their corresponding adjacent normal tissue of the same patient. Several PPARβ/δ and BCL6 target genes mRNA abundance was assessed via qPCR.

(Figure 4.1) *Pparb* mRNA was significantly higher in tumor tissue compared to normal tissue. Furthermore, PPARβ/δ target genes *Tgfb* (transforming growth factor β), *Sod1* (superoxide dismutase 1), *Il1ra* (interleukin 1 receptor agonist), and *Fgf21* (fibroblast growth factor 21) mRNA were higher in tumor tissue while *Adrp* (adipose differentiation-related protein) was interestingly not altered. There were no significant difference in the expression of the long or short form of *Bcl6* (BCL6L, BCL6S) between the normal and tumor tissue. However, target genes for BCL6-mediated repression were augmented in the tumor tissue including *Mmp9* (matrix metallopeptidase 9), *Mcp1* (monocyte chemoattractant protein 1), *Esel* (E-selectin) and *Vcam1* (vascular cell adhesion protein 1). *Tnfa* (tumor necrosis factor α), another BCL6 target, was unchanged. Known or proposed prognostic and mechanism markers of PDAC were examined. *Tspan8, Cox2* and *Nfkb* mRNA were increased in tumors versus normal tissue, although *Hsd11b1, Cbr1* and *Rbp1* were not affected.

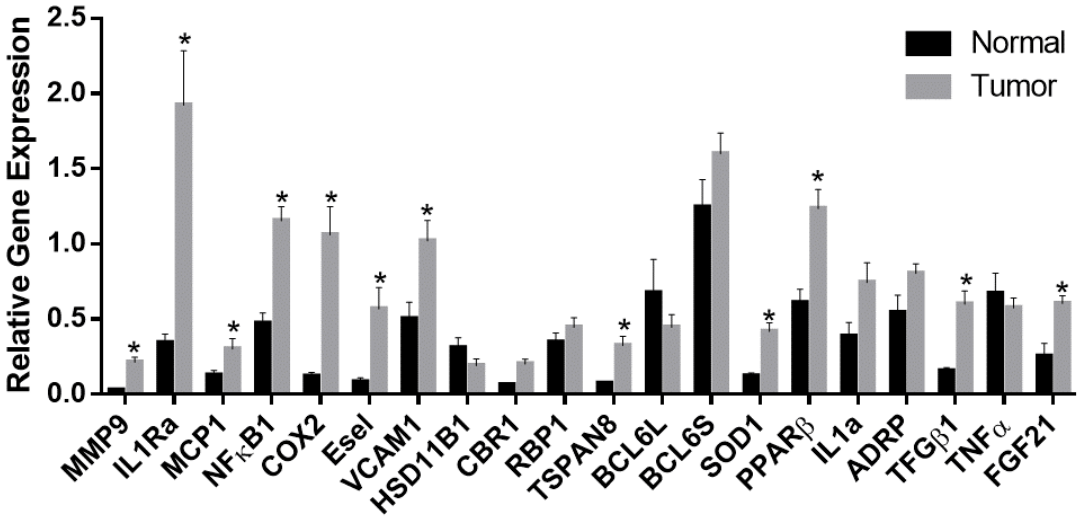

Figure 4.1 PPARβ/δ, BCL6, and their targets' mRNA expression in normal and tumor human pancreatic tissue. Tissue samples were sectioned in liquid nitrogen, homogenized in tri-reagent, and total RNA was isolated using standard Tri-reagent protocol and reverse-transcribed using the High Capacity cDNA Reverse Transcription kit. Gene expression was determined using qPCR and expressed as fold induction after normalization with 18S. *$P<0.05$*

Pparb mRNA abundance elevated in time-dependent manner in *Kras(G12D)* mouse model. To substantiate these findings we decided to observe PPARβ/δ expression over time during the course of PDAC progression. The *Kras(G12D) Pdx1*-Cre mouse model recapitulates the entire spectrum of pancreatic intraepithelial neoplasia (PanIN) observed in humans [25]. Negative ($Kras^{G12D-/-}$) and heterozygous ($Kras^{G12D+/-}$) mice were sacrificed at an age range of 6 to 20 months. It is assumed that homozygous ($Kras^{G12D+/+}$) mice died *in utero*. Whole pancreas tissue were extracted and DNA was isolated for genotyping and RNA was reverse transcribed and assessed via qPCR. (Figure 4.2)

There was no significant difference in any of the mRNA examined between the negative and heterozygous mice sacrificed within the range of 6 to 9 months. (Figure 4.2, top panel) However, after approximately 9 months, mRNA expression looked similar to what was observed in the human tumor samples. (Figure 4.2, bottom panel) PPARβ/δ expression was higher in heterozygous mice compared to the negative mice. PPARβ/δ target genes *Tgfb, Sod1, Fgf21*, and *Il1ra* exhibited elevated transcript levels. *Adrp* was also unchanged like in the human tumor samples. *Bcl6* mRNA was unchanged while its targets, *Mmp9, Mcp1*, *Vcam1*, and *Tnfa* were elevated in heterozygous mice. We have observed an elevation in *Pparb* in 3 independent tumor models of the pancreas.

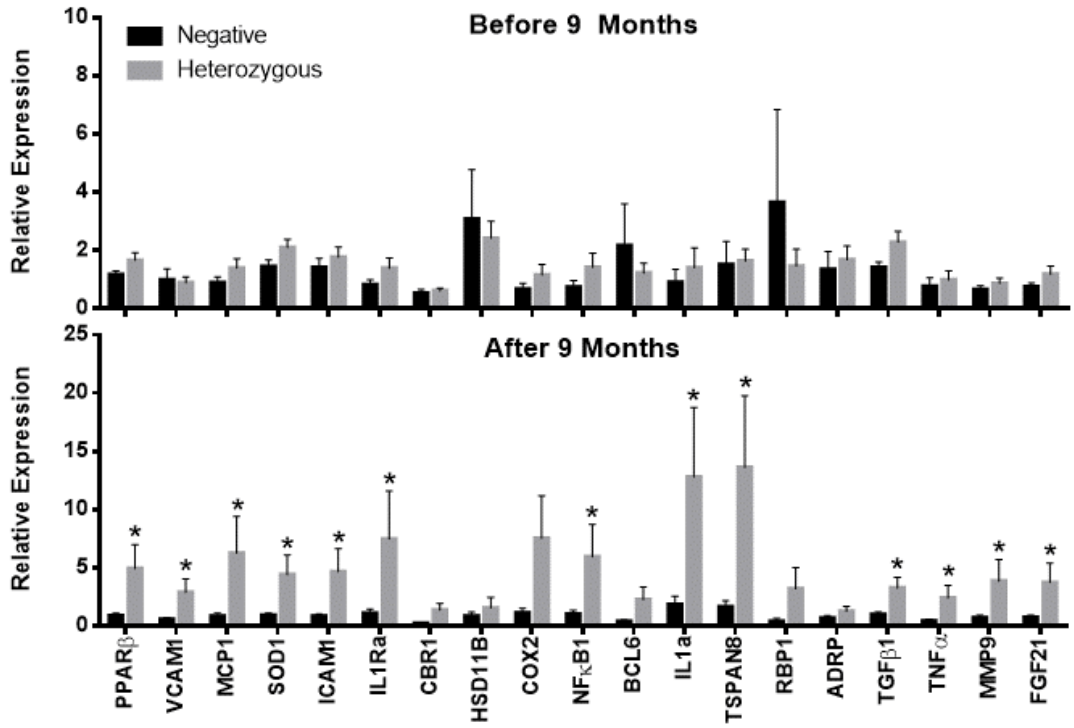

Figure 4.2 PPARβ/δ, BCL6, and their targets' mRNA expression in the Cre-mediated *Kras*^{G12D} mouse model and divided into two groups, pre- and post- nine months. Tissue was received from Penn State Hershey Biorepository, from Dr. Bogdan Prokopczyk's lab. Samples were sectioned in liquid nitrogen, homogenized in tri-reagent, and total RNA was isolated using standard tri-reagent protocol and reverse-transcribed using the High Capacity cDNA Reverse Transcription kit. Gene expression was determined using qPCR and expressed as fold induction after normalization with 18S. *$P<0.05$*

Pparb expression increases Mia PaCa-2 cell growth. Next, we sought to determine if increasing levels of *Pparb* expression affected cancer cell growth in a cell culture model. Mia PaCa-2 cells transfected with pcDNA3.1-*Pparb-FLAG* and pcDNA3.1-*Rxr* or empty vector control were treated with GW501516 or DMSO control and allowed to grow for 72h (Figure 4.3). There was a 2-fold increase in cell growth when *Pparb* and *RXRα* at 20ng plasmid and a 3.5-fold increase at 50ng plasmid (Figure 4.3A). This effect was reversed by the addition of GW501516 in cells transfected with 20-50ng plasmid. Similar

trends were observed when *Pparb* was transfected without the addition of *RXRα* (Figure 4.3B), albeit to a lesser extent.

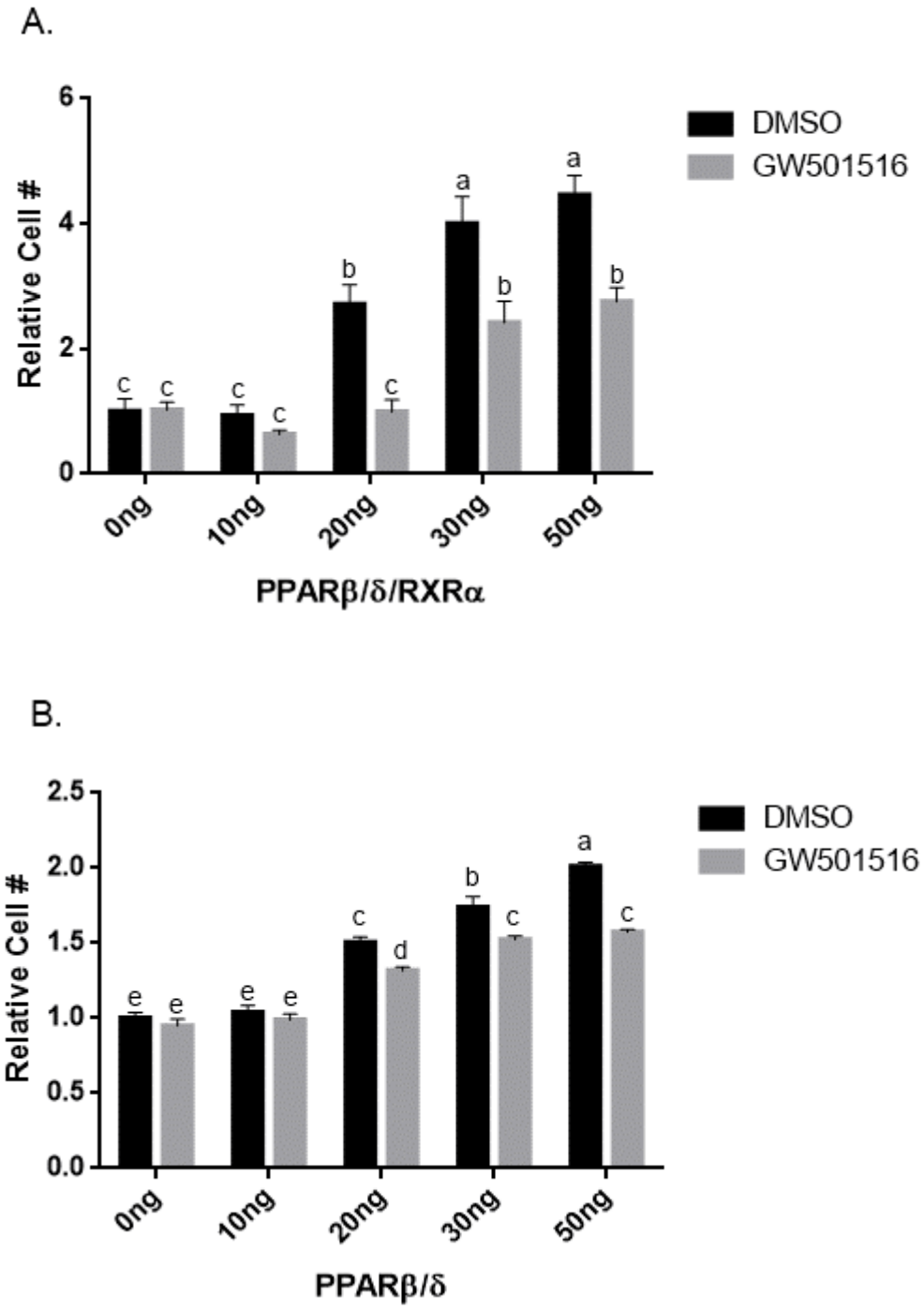

Figure 4.3 PPARβ/δ expression increases Mia PaCa-2 cell growth and is ameliorated by GW501516. Mia PaCa-2 cells were seeded in 96-well plates at $2x10^3$ per well, treated with 250nM GW501516 or DMSO control, and allowed to grow for approximately 72h. Plates were analyzed for luminescence using a GloMax microplate luminometer set at 750nm. Data are relative to control (Empty transfection, no GW). Different letters indicate a statistical difference at $P<0.05$ using Tukey's multicomparison test.

Mcp1 luciferase activity increases with *Pparb* expression and is abrogated by GW501516. Next, we sought to determine if increasing expression of *Pparb* affected a known BCL6 target and prognostic marker of pancreatic cancer, *Mcp1*. Mia PaCa-2 cells transfected with higher levels of *Pparb* showed increased *Mcp1* luciferase activity in both presence and absence of TNFα stimulation (Figure 4.4). The TNFα-induced *Mcp1* luciferase activity was decreased by GW501516 regardless of the amount of *Pparb* transfected. Furthermore, the inclusion of BCL6, transfected at 20ng, showed almost complete inhibition of TNFα-induced *Mcp1* activity, the exception being when PPARβ/δ was transfected at 20ng, again an effect reduced by GW501516.

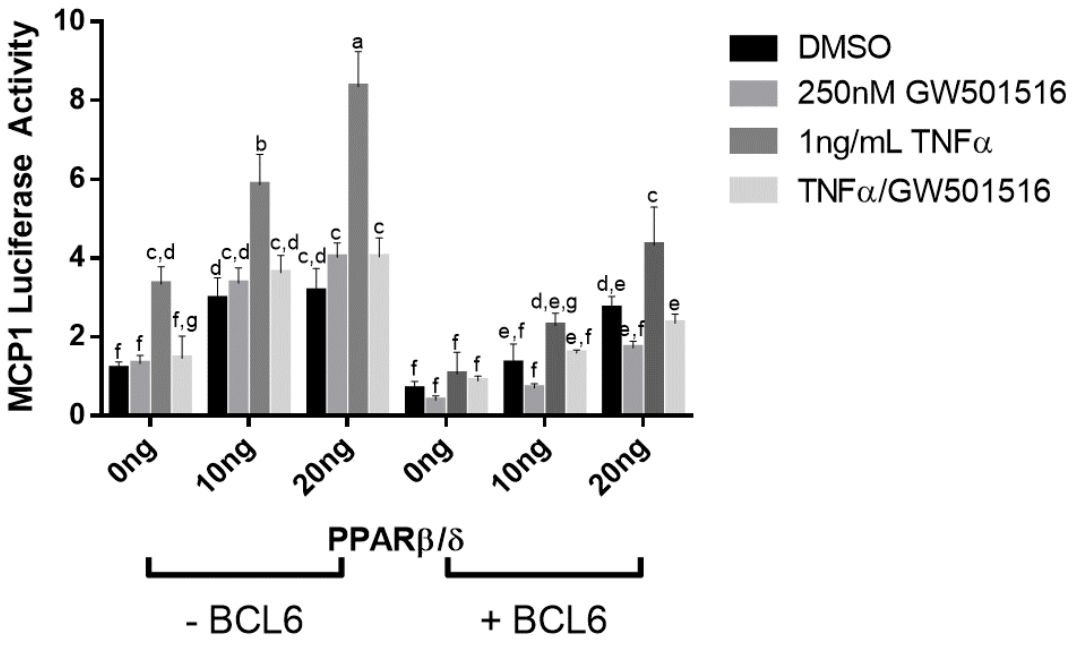

Figure 4.4 *Mcp1* luciferase activity in Mia PaCa-2 cells transfected with *Pparb*. Mia PaCa-2 cells were seeded at $2.5x10^4$ per well in a 96-well plate, and transfected with pcDNA3.1- *Pparb* -FLAG and a 9ng of luciferase containing the *Mcp1* promoter. Cells were treated with 1ng/mL TNFα, 250nM GW501516, or both. Luciferase activity was measured and corrected using 1ng of pRLCMV (renilla) values for transfection efficiency. Different letters indicate a statistical difference at $P<0.05$ using Tukey's multicomparison test.

Short transcript of BCL6 does not alter PPARβ/δ-Gal4 activity. A mammalian two-hybrid assay was carried out in HEK293T cells to determine if the short transcript of BCL6 lacking the first two zinc finger motifs altered its interaction with PPARβ/δ [18]. As shown in Figure 4.5, BCL6S does not differentially activate luciferase expression than the BCL6L variant. GW501516 was treated overnight at 250nM to assess differential dissociation between BCL6L and BCL6S from PPARβ/δ. Indeed, luciferase activity was decreased when GW501516 was treated signifying separation of the activation domain from the Gal4 DNA binding domain. When increasing concentrations of BCL6 was

transfected, there was an increase in luciferase activity suggesting PPARβ/δ and BCL6 were interacting.

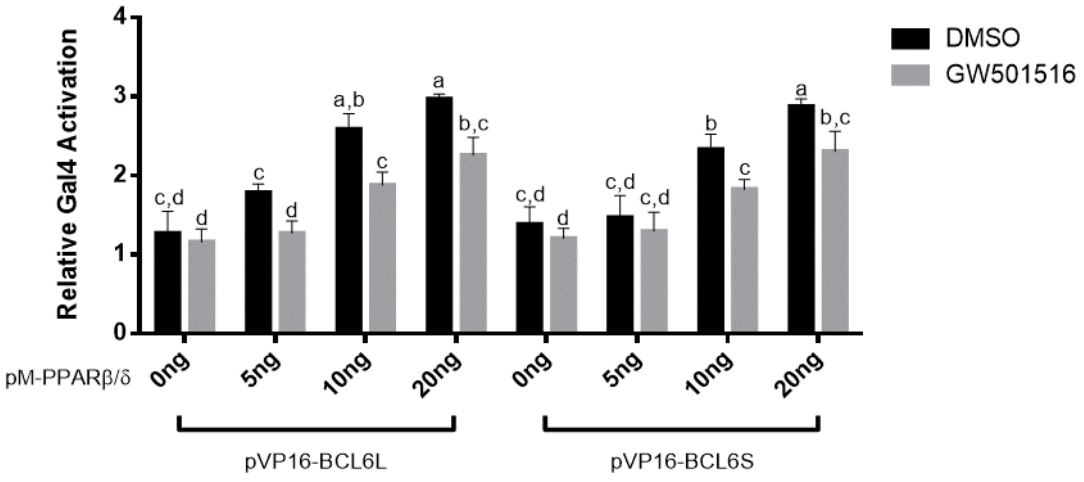

Figure 4.5 Mammalian two-hybrid assay of PPARβ/δ interaction with BCL6 variants. HEK293T cells were seeded in a 96-well plate at $4x10^5$ per well, transfected with pM-PPARβ/δ or empty vector, and 20ng of either pVP16-BCL6L, BCL6S, or empty vector. Cells were treated with DMSO or 250nM GW501516 for 24h. Luciferase activity was measured using a GloMax luminometer and corrected for transfection efficiency. Different letters indicate statistical significance at *P<0.05* using Tukey's multicomparison test.

Chromatin immunoprecipitation of BCL6. ChIPs were performed to elucidate the mechanism of PPARβ/δ's sequestration of BCL6, and its effects on interaction with their targets. *Angptl4* (angiopoietin like-4) and *Mcp1* were used as prototypical PPARβ/δ and BCL6 target genes, respectively. Quantitative PCR data for each immunoprecipitation was normalized to the input DNA controls. PPARβ/δ's association with the *Angptl4 PPRE* was increased upon treatment with GW0742 (Figure 4.6, left panel). BCL6 was present at the *PPRE* and its association was slightly, albeit not significantly, reduced by

GW0742 treatment. As expected, the Histone H4 induced-acetylation at the *Angptl4* transcription start site was increased by PPARβ/δ ligand (Figure 4.6 middle panel). The BCL6 binding site within *Mcp1* showed a high basal association with BCL6 (right panel). Unexpectedly, GW0742 treatment increased PPARβ/δ association while decreasing BCL6 presence, in contrast to the *Mcp1* reporter data presented above. This may indicate a masking of the antibody epitope on BCL6 upon ligand treatment.

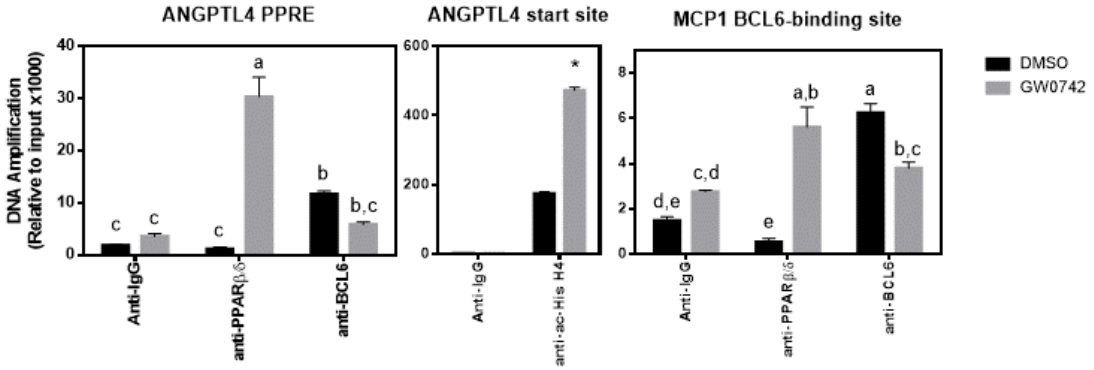

Figure 4.6 Chromatin immunoprecipitation assay of PPARβ/δ and BCL6 binding to elements of ANGPTL4 and *Mcp1*, respectively. Mia PaCa-2 cells were seeded in 10cm dishes (per treatment) to confluency and treated with 250nM GW501516 or DMSO control. Cells were crosslinked using 1% formaldehyde for 10mins and stopped using 1.25M glycine. Cells were harvested by scraping in PBS, and lysed using lysis buffer with protease inhibitors. Samples were sonicated using a Branson Sonifier 250, pelleted by centrifugation, and supernatant was collected. 50ng of DNA were immunoprecipitated at 4°C using antibodies described previously (overnight for primary, 2h for secondary) after preclearing against resin. Crosslinks were reversed in 65°C water bath overnight. Samples were washed using TE buffer. Samples were centrifuged in a sucrose cushion and RIPA buffer before final elution. A DNA concentrator by Zymo was utilized before qPCR analysis. Data are representative of three independent experiments for PPARβ/δ and two for BCL6. Data was normalized to the input control. Different letters indicate statistical significance at $P<0.05$ using Tukey's multicomparison test.

4.5 Discussion

PPARβ/δ remains controversial in regards to its role in cancer, especially in the colon, although very little is known about the function of this receptor in other tissues. In colorectal cells, non-steroidal anti-inflammatory drugs (NSAIDS) prevent PPARβ/δ from binding to its response elements and decrease tumorigenesis [27]. *Pparb* and *Cox2* mRNA are increased in colorectal cancer cells [28]. PPARβ/δ expression and activity are elevated by mutation-induced activation of *Kras* in rat intestinal epithelial cells and activation required *Cox2*-induced production of prostacyclin [29]. This *Cox2* activity is modulated by PPARβ/δ, a positive feedback loop, that may play an important role in cholangiocarcinoma[30]. However, adenomatous polyposis coli $(APC)^{min/+}$ mice, containing a point mutation in the *Apc* gene, grew polyps at a 5-fold increase upon GW501516 treatment compared to control [31]. Knockout of *Pparb* in $Apc^{min/+}$ mice decreased intestinal adenoma [32]. A *Pparb*-null colon cancer cell line developed in HCT116 cells grew slower than the wild-type and when inoculated in nude mice formed tumors at a higher frequency [33].

There are several reports that contradict these findings of a pro-cancer role for PPARβ/δ. GW501516 and GW0742, PPARβ/δ-specific ligands, did not exacerbate growth of colorectal cancer and liver cells and *Cox2* was not elevated in mice with intact APC [34]. In several studies utilizing the Apc^{min} mice, polyp formation appeared to be enhanced in absence of PPARβ/δ [35,36], and treatment with GW0742 had no effect on tumorigenesis with and without PPARβ/δ [37]. *Pparb* has been implicated in anti-inflammatory signaling in several *in vitro* [23,38] and *in vivo* [39,40] models, and is commonly expressed in lower abundance in tumor tissue compared to normal tissue [16]. PPARβ/δ

promoted keratinocyte terminal differentiation and inhibited cell proliferation [39], and also induced apoptosis [41]. There is a growing collection of evidence supported by a higher level of reproducibility in favor of PPARβ/δ being anti-inflammatory and a terminal differentiator [16]. Surprisingly, the role of PPARβ/δ in the pancreas is lacking. Previous studies in our lab have implicated PPARβ/δ as being therapeutic in pancreatic adenocarcinoma [23]. Treating the human pancreatic cancer cell line Mia PaCa-2 with a specific ligand or upon RNAi knockdown of *Pparb* there was decreased expression of *Mmp9* and several inflammatory genes. Importantly, there was also a decreased invasion of pancreatic cancer cells across a basement membrane upon either knockdown of *Pparb* or activation of this nuclear receptor [23].These seemingly dichotomous observations are hypothesized to be due to release of BCL6 from the PPARβ/δ complex, thereby allowing for interaction and decreased expression of its target genes, including *Mmp9* and *Mcp1*. This was confirmed by reducing expression of *Bcl6* in Mia PaCa-2 cells with concomitant decrease in GW501516's effects on *Mmp9* and *Vcam1* expression as well as cell invasion, *in vitro*[23].

We demonstrate in human and mouse pancreatic tumor models that *Pparb* mRNA expression was not only significantly elevated in tumor tissue compared to normal tissue, but expression increased in concert with PDAC progression. Importantly, *Bcl6* expression remained unchanged, thus allowing for a suspected increase in bound and decrease in free, functional BCL6. *Mmp9* and other BCL6 target genes and markers of PDAC was also elevated in these tumor tissue, consistent with our observations [23]. Several PPARβ/δ target genes were elevated in tumor tissue including *Tgfb*1, *Il1ra*, *Sod1*, and *Fgf21*. In both human and mouse pancreas tissues, BCL6 inflammatory target genes were increased

in tumor tissue such as *Vcam1*, *Mcp1*, *Tnfa*, *E-selectin*, and *Icam1*. As expected, *Nfkb*, *Cox2*, and *Tspan8* genes involved in inflammation and metastasis, respectively, increased in both human pancreatic tumors and in pancreas of nine month old *Kras(G12D) Pdx1*-cre mice. Taken together, this is in support of our hypothesis that elevated expression of PPARβ/δ leads to a sequestering effect on the tumor suppressor BCL6, preventing its anti-inflammatory role.

The *in vivo* gene expression patterns were further validated by several *in vitro*, molecular studies to better understand the implications of PPARβ/δ-BCL6 complex formation. Forced expression of PPARβ/δ in a pancreatic cancer cell model, Mia PaCa-2, augmented proliferation over 72 hours. The inclusion of RXRα, a dimer partner involved in PPARβ/δ function, exacerbated proliferation to a greater extent compared to just PPARβ/δ alone. GW501516 reduced proliferation, and in the presence of RXRα was able to reduce this effect by almost half. Although RXRα is an obligatory partner of the PPARs for their ability to regulate target genes via a *PPRE*-dependent pathway, it is not known whether RXRα modulates the interaction between PPARβ/δ. Forced over-expression of *Pparb* increased *Mcp1* activity, a gene suppressed by BCL6 with and without stimulation by TNFα. Again, GW501516 reversed this effect to control levels. This is of particular relevance since *Mcp1* is a potent recruiter of inflammatory cells to the pancreas and is important in the progression of PDAC in humans [14,42].

As previously described, the presence of a SNP results in an exonic splice variant in *Bcl6* that deletes the first two zinc finger motifs [18]. As far as we know, there are no molecular data to indicate a functional difference between the full length BCL6 and its shorter variant, however the shorter variant may be linked to non-Hodgkin lymphoma [19].

A mammalian two hybrid assay using both variants fused to a *pVP16* activation domain and the full length *Pparb* fused to a *pM Gal4* construct were transfected into HEK293T cells. Increasing levels of the *Pparb-Gal4* construct led to increasing luciferase activity, the signal was effectively reduced by treatment with GW501516 indicating a dissociation between *Pparb* and *Bcl6* constructs. However, there did not appear to be a difference between the long and the short construct. Further experimentation is required to fully elucidate the mechanistic difference, if any, between BCL6 variants and PPARβ/δ, and their targets. However, at least in the relatively small set of human tissue studied herein, there was no difference in expression of either variant in tumors versus normal pancreatic tumors.

Chromatin immunoprecipitation was carried out in Mia PaCa-2 cells to investigate the interplay between PPARβ/δ and BCL6 and their interactions with their respective consensus sequences while treated with GW0742 or DMSO. High levels of acetylated Histone H4 was detected at the transcriptional start site of *Angptl4*, a PPARβ/δ target, while under treatment with GW0742 indicative of activation of the transcriptional machinery, and PPARβ/δ was detected at higher levels at its response element as well. Unexpectedly, the GW0742 group showed higher levels of PPARβ/δ and significantly lower levels of BCL6 at the BCL6-binding site on *Mcp1*. There are a few possibilities that may serve as viable explanations for this: 1) due to structural alterations by recruitment of corepressors perhaps the anti-BCL6 was unable to identify the epitope, 2) a high turnover rate may result in the degradation of the tumor suppressor resulting in low signal, 3) the quality of antibody for our purposes may be poor and an antibody that recognizes a different epitope may be required. Further experimentation, antibody and

time-point optimizations are required to better understand the PPARβ/δ modulation of BCL6.

These data are in support of a sequestering of BCL6 by PPARβ/δ that occurs when PPARβ/δ becomes upregulated during PDAC progression and that cancer growth becomes compounded by this effect. This mechanism has been reported previously in foam cells and cardiomyocytes [24,43], and have been found to be pro-inflammatory. Studies in our lab may confirm not only the presence of this mechanism in the pancreas, but that it may play a significant role in carcinogenesis. These findings provide insight into the controversial nature of PPARβ/δ in cancer, and furthermore, the therapeutic potential of GW501516 in the pancreas. Therapeutic targeting of a single protein pathway has proven largely ineffective and challenges of overcoming the stromal barrier in chemotherapy drug delivery [44] makes treatment of PDAC very difficult. The PPARβ/δ/BCL6 pathway may provide an early prognostic marker for PDAC development as well as an effective candidate in a multi-targeting drug therapy.

4.6 References

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Chapter 5

Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) and B-Cell Lymphoma 6 (BCL6) genetic polymorphisms and association with altered gene expression in human pancreatic cancer

5.1 Abstract

The nuclear receptor Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) and the transcriptional repressor B-cell lymphoma 6 (BCL6) regulate genes involved in inflammation, metabolism and cancer in pancreas. Single nucleotide polymorphisms (SNPs) have been identified within PPARβ/δ and BCL6 that are associated with metabolic disease and cancer. Previous studies have indicated a relationship between non-coding SNPs in *Pparb* rs6902123, rs2076167, and rs1053049 on glucose uptake in skeletal muscle and rs2267668 with effectiveness of lifestyle intervention on aerobic fitness, insulin sensitivity, and body composition. A putative exonic splicing polymorphism in the *Bcl6* gene (rs1056932) affects the risk of non-Hodgkin lymphoma. It has long been established the link between glucose metabolism and metabolic rate with occurrence of pancreatic cancer, and these PPARβ/δ/BCL6 polymorphisms have not yet been studied in relation to pancreatic cancer. Utilizing TaqMan genotyping assays, high resolution melting, along with real-time gene expression these polymorphisms in human pancreatitis and pancreatic cancer tissue were examined. Although there were no differences in SNP frequency between individuals with pancreatitis versus pancreatic cancer, expression of *Bcl6* and the *Pparb* target gene matrix metallopeptidase 9 (*Mmp9*) mRNA were significantly different in these tissues. *Pparb* SNPs rs1053049 and

rs9658056 were associated with altered *Mmp9* mRNA while *Pparb* rs2076167 and *Bcl6* rs1056932 associated with *Bcl6* mRNA levels. Here, we show a correlation between *Pparb* SNPs and differential mRNA expression of inflammatory and macrophage invasion genes that may indicate a connection between these polymorphisms and pancreatic cancer.

5.2 Introduction

Single nucleotide polymorphisms (SNPs) have been identified in *Pparb* and *Bcl6* that have been associated with higher incidence of metabolic disease and cancer. Previous studies have already established PPARβ/δ as a potential target in metabolic disorders such as diabetes [1–3], atherosclerosis [4–6], and obesity [7–9], and emerging as a potential option in chemoprevention [10]. PPARβ/δ regulates proliferation in certain cell types in part by direct transcriptional events as well as through association with the transcriptional suppressor, BCL6 [11]. Non-coding SNPs in *Pparb*: rs6902123, rs2076167, rs2267668, and rs1053049 are associated with insulin sensitivity and glucose uptake in skeletal muscle [12], and rs2267668 on effectiveness of lifestyle intervention on aerobic fitness, insulin sensitivity, and body composition [13,14]. Several other *Pparb* SNPs have been associated with plasma lipid levels, Body Mass Index, and the risk for diabetes and coronary heart disease [15]. Furthermore a SNP in *Bcl6*, rs1056932, located in exon 6 provides an alternative binding site for a splicing enhancer [16]. This polymorphism is responsible for the transcription of a shorter variant of *Bcl6* lacking the first two zinc finger motifs [17], and has been associated with higher risk of non-Hodgkin lymphoma [16].

We have demonstrated in previous studies that PPARβ/δ and BCL6 pathway is altered in pancreatic tumors and that these transcription factors control cell cycle, inflammation and metastasis in cell culture models. Utilizing high resolution melting (HRM) we sought to identify mutations within the coding region of PPARβ/δ that could help explain the alterations in gene expression associated with PDAC. Also, known polymorphisms in both PPARβ/δ and BCL6 were examined to determine if they could add further proof that this signaling pathway is important in the etiology of pancreatic cancer in humans.

5.3 Materials and methods

Cells, tissue samples, and reagents. The human pancreatic ductal cancer cell line Mia PaCa-2 (COX-2 negative, CRL-1420), were purchased from the ATCC (Manassas, VA). Human pancreatic cancer and chronic pancreatitis samples were obtained from Dr. Gerhard Leder, (Abt. Allgemein- und Viszeralchirurgie, St. Josef Hospital - Klinikum der Ruhr, University of Bochum, Germany). High Capacity cDNA Archive Kit and ABI7300 Real-time PCR System were purchased from Applied Biosystems (Foster City, CA). Human pancreatic tumor tissue with adjacent normal tissue was obtained from the Penn State Hershey Cancer Institute Biorepository, as previously described. (Table 4.1)

Isolation of total RNA, RT-PCR, and qPCR. Total RNA was isolated from human pancreatic tissue samples which were briefly homogenized in 1mL Tri-Reagent (Sigma, St. Louis MO) and total RNA was isolated following the manufacturer's protocol. One μg of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit

(Applied Biosystems, Foster City, CA). Primers for qPCR were designed based on published sequences in GenBank and are shown in Table 5.1.

Table 5.1 List of qPCR primers used in this study.

Gene	Forward primer	Reverse primer
bactin	AACAAGAGGCCACACAAATAGG	CAGATGTACAGGAATAGCCTCCG
Esel	TCCTATTCCAGCCTGCAATGT	AACCCATTGGCTGGATTTGTC
Icam1	ACTCAGCGGTCATGTCTGGAC	GGCATAGCTTGGGCATATTCC
Vcam1	AGTGGTGGCCTCGTGAATG	CACGCTAGGAACCTTGCAGC
*Il1*β	TCCTTAGTCCTCGGCCAAGAC	GTGCCATGGTTTCTTGTGACC
Mcp1	GGACGCATTTCCCCAGTACA	CCGAGAACGAGATGTGGACA
Mmp9	AGCGGTCCTGGCAGAAATAG	ACGCACGACGTCTTCCAGTAC
Bcl6	GCTCACGGCTCACAACAATG	TCCGGAGTCGAGACATCTTGA
Pparb	AGGCCATTCACCAACTGCTT	ATTGTGGCAGGCAGAGAAGG

DNA extraction. Tissue samples were homogenized in 0.1M Tris-HCl/0.05M EDTA DNA extraction buffer with 1.25% SDS. Buffer was supplemented with 0.002mg/mL RNase. Proteinase K was added to the samples and incubated at 55°C for 3h. DNA was extracted using a standard Phenol/Chloroform protocol. Samples went through two 70% ethanol wash steps and a 100% wash step and centrifuged at full speed for 10mins. Samples were resuspended in TE buffer. Samples were quantitated using a Spectrophotometer 1000 (Nanodrop, Wilmington, DE).

Primer design. NCBI SNP web search was used to identify flanking sequences around the polymorphic site, sequences were copied to the Primer Design software provided by Idaho Technologies. The SNP was identified and the software yielded optimal primer sequences (Table 5.2) based on parameters (60°C annealing temperatures yielding products 100-250bp) recommended by the manufacturer (Idaho Technologies, Salt Lake

City, UT). Primer sequences were created at the Genomics Core Facility at Penn State University.

Table 5.2 Primer sequences used for amplifying PPARβ/δ.

Amplicon	Forward Primer	Reverse Primer
Exon 4 197bp	tatgactgggcctgcaggtgt	tgtcccctgccagtcctctc
Exon 5 231bp	ctgcccctccatcgtgtgtc	accatgtgaccacagcctcag
Exon 6 202bp	ctggtggcctttcctcacct	ccgtgagttgccctggtcag
Exon 7 268bp	ctgcctgggctccttgctga	caggccaagcagcagcaaca
Exon 8-1 272bp	gtctcccgaggcctgatctctaac	gcacgccatacttgagaagggt
Exon 8-2 302bp	cccagcttcagcagcctctt	gcccacctgccccactctca
Exon 9 329bp	ctttctgagctccctggcgt	gtgctggccccattggagtc

Polymerase Chain Reaction. Sections of DNA containing the SNP were amplified using dilutions described in Table 5.3 and run at conditions described in Table 5.4 in an MJ Research PTC-200 thermal cycler. Dilutions and cycling were optimized by running amplicons on agarose gel to assess accuracy and strength of amplification.

Table 5.3 Dilutions for PCR reaction.

		Exon 4	Exons 5 and 9	Exon 6, 7, 8-1, and 8-2
Component	Volume	Final Concentration	Final Concentration	Final Concentration
2.5X Lightscanner Master Mix	4µL	1X	1X	1X
10X FWD & REV primers	1µL	0.3µM	**0.25µM**	0.3µM
PCR-grade H_2O	3µL	NA	NA	NA
DMSO	1µL	10%	10%	0%
DNA(15ng/µL)	1µL	15ng/reaction	15ng/reaction	15ng/reaction
Total	10µL			

Table 5.4 PCR cycling conditions using an MJ Research PTC-200 thermal cycler.

		Exons 4, 8-1 & 8-2	Exon 5	Exons 6 & 7	Exon 9
	Time	Temperature	Temperature	Temperature	Temperature
Hold	120 s	95°C	95°C	95°C	95°C
45 cycles	30 s	94°C	94°C	94°C	94°C
	30 s	**68°C**	**66°C**	**72°C**	**70°C**
1 cycle	30 s	94°C	94°C	94°C	94°C
	30 s	28°C	28°C	28°C	28°C

High resolution melt. Amplicons were then transferred to a microplate containing LC Green saturated fluorescent dye (Idaho Technologies), and 25uL of mineral oil. Plates then underwent HRM via a Lightscanner 96 system (Idaho Technologies). Melting began at 70°C and would end at 98°C. Lightscanner analysis software was used to identify SNPs in each reaction analyzing matched hybrids called homoduplexes and mismatched hybrids called heteroduplexes.

TaqMan® single nucleotide polymorphism assays. DNA was isolated from human pancreatic tissue samples which were briefly homogenized in 1mL Tri-Reagent (Sigma, St. Louis MO) and DNA was isolated following the manufacturer's protocol. One hundred ng DNA was amplified using TaqMan SNP. Genotyping was undertaken by using TaqMan assays from Applied Biosystems following the manufacturer's suggestions and analyzed in an ABI 7000 Fast Real-Time PCR System. Allele-specific fluorogenic probes were hybridised to the template in the first step of the 5′ nuclease assay. Then, during the polymerase chain reaction (PCR), the 5′ nuclease activity of the Taq polymerase made it possible for discrimination. In addition, the probes include a 3′ minor

groove binding group that hybridises to single-stranded targets and has greater sequence specificity when compared to the original DNA probes. This reduces nonspecific probe hybridization which leads to low background fluorescence for the 5′ nuclease PCR assay (TaqMan, Applied Biosystems). Cleavage results in the increased emission of a reporter dye. Two unlabeled PCR primers and two allele-specific probes were required for each 5′ nuclease assay. At the 5′ end, each probe is labeled with two reporter dyes. In the present study, VIC and FAM were used as the reporter dyes. The probes and primers used in TaqMan®SNP Genotyping Assays (ABI) were selected according to the information on ABI website (http://www.appliedbiosystems.com). The SNPs selected for examination are shown in Table 5.5. Finally, PCR amplification was performed by using 0.05 μL probes, 3 μL of TaqMan Universal Master Mix, 1 μL DNA, and 1.95 ddH2O in a 6 μL final reaction volume. In addition, thermal cycling conditions for PCR amplification were 95 °C for 10 min, 45 cycles of 95 °C for 10 s and 60 °C for 1 min. Moreover, thermal cycling was undertaken by using Applied Biosystems7000HT Standard Real-Time PCR System, and all 96 well plates were read according to Sequence Detection Systems (SDS) automation controller software v2.3 (ABI).

Table 5.5 PPARβ/δ and BCL6 SNPs. Minor Allele Frequency/Minor Allele Count: Frequency at which the less common allele occurs in a given population/the number of times the allele has been observed.

SNP	Sequence	MAF/MAC
rs2267668 (*Pparb*/Intron)	TAAAATATCT[**A/C/G/T**]AGAGTTGGGG	G=0.161/353
rs6902123 (*Pparb*/Intron)	GGGTCCCTGG[**C/T**]GCTGTCTTGA	C=0.213/467
rs1053049 (*Pparb*/3'UTR)	CCTGCCCCTA[**C/T**]GGGCGCTGCA	C=0.320/700
rs2076167 (*Pparb*/N163N)	CTGGCTCCCCTC[**A/G**]TTTGCAGTCAGC	G=0.309/677

rs9658056 (*Pparb*/5'Promoter)	AAGGTGTGGC[**C/T**]GAAGGAGAGG	T=0.032/69
rs1056932 (*Bcl6*/N387N)	ACAGCCTCAA[**C/T**]CAGAATGCCA	C=0.390/853
rs3172469 (*Bcl6*/Intron)	TTCCCAGTAC[**A/C**]TAAAGCTTTT	C=0.309/676

Statistical analysis. Quantitative data are presented as mean±SEM. ANOVA with p-value <0.05 was used to determine whether differences among variables were significant. Normality was checked using Anderson-Darling test, and the General linear model, followed by the Tukey post hoc test to analyze differences between treatments. All data analyses were performed by MiniTAB Ver.14 (MiniTAB, State College, PA) or JMP (SAS Institute, Cary, NC) and data were plotted by Prism 5.01 (GraphPad Software, San Diego, CA).

5.4 Results

No mutations discovered in *Pparb*. HRM was utilized to scan for mutations in the coding region of *Pparb* in human pancreatic tumor tissue and then compare it to normal pancreatic tissue in effort to identify mutations indicative of pancreatic cancer risk. DNA from 30 paired tissue samples were scanned for mutations as described in Table 5.2. No new mutations were discovered in *Pparb*, however, a non-coding SNP, rs2076167, was identified in preliminary scanning of tumor tissue and validated by sequencing.

No association of SNPs with disease-state. The rs2076167 SNP has been found to be associated with insulin sensitivity and risk of diabetes mellitus [18]. Metabolic disorders are known risk factors for development of pancreatic cancer [19]. We sought to analyze this SNP along with other known non-coding *Pparb* SNPs in pancreatic tissue, perhaps to

identify trends in tumor tissue compared to normal tissue, eluding to a genetic predisposition for pancreatic cancer. However, we didn't have a big enough population to determine any significant associations with non-coding SNPs and pancreatic cancer. Next, we sought to analyze these SNPs and gene function in human pancreatic cancer.

Presence of PPARβ/δ SNPs and MMP9 mRNA expression in pancreatic tissue. DNA from human pancreatitis and pancreatic cancer tissues were genotyped using TaqMan genotyping kits (Table 5.6). There were no differences in frequency in SNPs between pancreatitis and pancreatic cancer. However, *Mmp9*, a BCL6 target gene had significant differences in mRNA abundance based on genotype. *Pparb* SNP, rs1053049 and rs9658056, were associated with altered mRNA expression of *Mmp9*. *Bcl6* SNP rs1056932 was associated with *Bcl6* mRNA levels despite no alteration in target genes: *Mmp9*, *Mcp1*, and *Vcam1*. rs1053049 was also associated with altered expression of *Bcl6*, a transcription factor that binds and inhibits *Mmp9*, rs2076167 showed significant differences in *Bcl6* and *Mmp9* levels as well, *Cox2* and *Il1ra*, genes involved in inflammation, were also differentially expressed.

Table 5.6 Association of SNPs with gene expression in pancreatic tissue. Housekeeping gene βactin was used to normalize all tested genes. Data shown are representative of three independent experiments with triplicate samples.

	rs2076167				rs6902123				rs1053049				rs2267668				rs9658056				rs1056932			
		Mean	SEM	p		Mean	SEM	p		Mean	SEM	p		Mean	SEM	p		Mean	SEM	p		Mean	SEM	p
MMP9	CC	1.06	.	0.08	CT	10.71	4.85	**0.02**	CC	1.35	0.29	**0.05**	AA	4.49	1.42	0.76	CC	4.17	0.99	**0.00**	TT	3.74	0.95	0.74
	CT	7.54	2.21		TT	3.62	0.90		CT	7.91	2.32		AG	5.23	1.88		CT	26.04	.		TC	5.29	1.55	
	TT	2.55	0.72						TT	2.55	0.72										CC	2.79	1.23	
COX2	CC	4.54	.	**0.03**	CT	1.56	0.75	0.99	CC	2.66	1.88	0.20	AA	1.34	0.27	0.16	CC	1.58	0.23	0.86	TT	2.23	0.61	0.11
	CT	1.80	0.34		TT	1.57	0.23		CT	1.86	0.35		AG	2.01	0.38		CT	1.33	.		TC	1.27	0.23	
	TT	1.23	0.26						TT	1.23	0.26										CC	2.41	0.87	
ICAM	CC	1.75	.	0.34	CT	1.25	0.59	0.22	CC	1.14	0.62	0.54	AA	0.91	0.17	0.95	CC	0.92	0.12	0.60	TT	1.02	0.22	0.91
	CT	1.01	0.21		TT	0.84	0.09		CT	1.04	0.22		AG	0.89	0.12		CT	0.52	.		TC	0.88	0.15	
	TT	0.78	0.14						TT	0.78	0.14										CC	0.88	0.36	
Esel	CC	1.35	.	0.41	CT	2.11	1.18	**0.03**	CC	0.88	0.48	0.32	AA	1.20	0.30	0.70	CC	1.14	0.20	0.99	TT	1.06	0.19	0.90
	CT	1.43	0.42		TT	0.96	0.09		CT	1.50	0.45		AG	1.03	0.14		CT	1.15	.		TC	1.12	0.28	
	TT	0.88	0.10						TT	0.88	0.10										CC	1.40	0.27	
BCL6	CC	4.10	.	**0.00**	CT	1.00	0.52	0.60	CC	2.25	1.85	**0.01**	AA	0.66	0.13	0.11	CC	0.84	0.15	0.50	TT	0.58	0.16	**0.00**
	CT	0.96	0.23		TT	0.79	0.14		CT	1.00	0.25		AG	1.14	0.33		CT	0.22	.		TC	0.68	0.13	
	TT	0.54	0.06						TT	0.54	0.06										CC	2.18	0.84	
PPARβ/δ	CC	5.24	.	0.09	CT	5.42	3.99	0.11	CC	2.92	2.33	0.08	AA	2.07	0.98	0.32	CC	2.64	0.78	0.84	TT	1.82	1.06	0.87
	CT	4.31	1.59		TT	2.09	0.52		CT	4.54	1.67		AG	3.66	1.14		CT	1.68	.		TC	2.86	1.02	
	TT	1.05	0.16						TT	1.05	0.16										CC	2.40	1.17	
IL1Ra	CC	6.00	.	**0.03**	CT	2.01	0.60	0.79	CC	3.75	2.26	0.09	AA	1.34	0.26	0.08	CC	1.79	0.36	0.98	TT	1.44	0.42	0.89
	CT	2.33	0.66		TT	1.75	0.41		CT	2.39	0.70		AG	2.65	0.88		CT	1.73	.		TC	1.89	0.45	
	TT	1.12	0.26						TT	1.12	0.26										CC	1.71	1.43	
VCAM1	CC	5.58	.	0.18	CT	1.86	0.88	0.99	CC	3.49	2.10	0.22	AA	1.02	0.23	0.09	CC	1.87	0.68	0.80	TT	1.02	0.18	0.85
	CT	2.93	1.43		TT	1.84	0.77		CT	3.02	1.52		AG	3.43	1.86		CT	0.78	.		TC	2.03	0.92	
	TT	0.74	0.10						TT	0.74	0.10										CC	2.06	1.22	
MCP1	CC	3.83	.	**0.03**	CT	0.50	0.23	0.17	CC	2.39	1.44	0.22	AA	0.85	0.22	0.09	CC	1.09	0.18	0.37	TT	1.31	0.49	0.32
	CT	0.99	0.20		TT	1.17	0.20		CT	1.00	0.22		AG	1.48	0.29		CT	0.08	.		TC	0.90	0.19	
	TT	0.99	0.26						TT	0.99	0.26										CC	1.72	0.76	
PPARα	CC	10.27	.	0.10	CT	9.64	5.01	0.18	CC	5.72	4.55	0.09	AA	4.98	1.50	0.30	CC	5.84	1.24	0.83	TT	6.13	2.72	0.98
	CT	8.47	2.23		TT	5.18	1.09		CT	8.93	2.33		AG	7.63	2.00		CT	7.53	.		TC	5.92	1.54	
	TT	3.47	1.06						TT	3.47	1.06										CC	5.23	2.37	
PPARγ	CC	5.82	.	0.13	CT	2.41	1.94	0.21	CC	3.24	2.58	0.44	AA	1.35	0.52	0.96	CC	1.37	0.38	0.57	TT	0.41	0.13	0.42
	CT	1.37	0.69		TT	1.13	0.27		CT	1.41	0.73		AG	1.31	0.43		CT	0.02	.		TC	1.44	0.48	
	TT	1.08	0.33						TT	1.08	0.33										CC	2.20	1.27	
ADRP	CC	1.41	.	0.31	CT	2.26	1.32	0.24	CC	1.53	0.12	0.31	AA	1.33	0.34	0.54	CC	1.46	0.30	0.95	TT	1.22	0.30	0.86
	CT	1.95	0.60		TT	1.31	0.24		CT	1.97	0.64		AG	1.71	0.53		CT	1.34	.		TC	1.56	0.39	
	TT	1.05	0.18						TT	1.05	0.18										CC	1.18	0.51	
IL1β	CC	8.05	.	0.18	CT	3.52	1.18	0.95	CC	5.03	3.03	0.20	AA	2.15	0.54	0.09	CC	3.39	1.02	0.91	TT	1.64	0.36	0.72
	CT	5.09	2.04		TT	3.35	1.16		CT	5.29	2.16		AG	5.73	2.65		CT	2.68	.		TC	3.81	1.37	
	TT	1.68	0.56						TT	1.68	0.56										CC	3.47	1.61	
CAT	CC	9.14	.	0.99	CT	14.07	6.74	0.54	CC	5.27	3.87	0.87	AA	11.06	3.69	0.77	CC	10.71	2.54	0.66	TT	6.85	2.89	0.77
	CT	10.84	2.63		TT	9.85	2.69		CT	11.43	2.73		AG	9.48	1.69		CT	3.66	.		TC	11.61	3.40	
	TT	10.32	4.22						TT	10.32	4.22										CC	9.62	1.75	
SOD1	CC	6.85	.	0.90	CT	8.16	2.19	0.84	CC	3.95	2.90	0.81	AA	7.63	1.97	0.88	CC	7.59	1.47	0.61	TT	5.40	2.57	0.79
	CT	6.76	1.41		TT	7.34	1.67		CT	7.12	1.45		AG	7.15	1.89		CT	2.91	.		TC	8.06	1.90	
	TT	8.10	2.48						TT	8.10	2.48										CC	7.12	2.06	
THRDX	CC	28.33	.	0.14	CT	8.97	2.41	0.89	CC	16.34	12.00	0.58	AA	9.03	2.14	0.68	CC	9.69	1.65	0.53	TT	7.18	2.59	0.66
	CT	8.18	1.42		TT	9.62	1.88		CT	8.42	1.49		AG	10.44	2.41		CT	3.20	.		TC	9.60	2.04	
	TT	9.71	2.68						TT	9.71	2.68										TT	13.03	5.58	

5.5 Discussion

The prevalence of particular *Pparb* mutations in the pancreas and risk of cancer development is poorly understood. Utilizing High Resolution Melting, SNPs are able to be identified based on the melting curves of amplified regions of DNA. Several coding regions (Table 5.2) of *Pparb* were scanned for mutations in normal and cancerous tissue,

however, no new mutations were discovered. Known non-coding SNP, rs2076167, was identified in tumor tissue and validated by sequencing. Subsequently, several known non-coding SNPs were assessed via TaqMan genotyping kits. Unfortunately, due to the limitations of our population size no significant associations were able to be made between mutation and disease state, and ultimately required us to shift the scope of this study.

Pparb and *Bcl6* polymorphisms and their effects on gene expression in the pancreas and in pancreatic tumors have not yet been reported. However, since the start of this study several recent reports of SNPs in *Pparb* have been associated with metabolic disease state. The *Pparb* SNP, rs2016520, influences the therapeutic effect of repaglinide, an anti-diabetic drug [20]. Furthermore, rs6902123 has been an associated risk factor of type 2 diabetes in middle-aged and elderly Chinese, the C allele was linked to increased fasting glucose and glycated hemoglobin [21], implicated in development of diabetes-related complications. The SNPs rs2267668, rs2016520, and rs1053049 have also been found, individually and in haplotype, to have an effect on athletic performance. The haplotype presence of these polymorphisms were lacking in athletes indicating an unfavorable effect on metabolic demand [22].

Recent studies have also linked several *Pparb* polymorphisms with cancer. In lung cancer patients, CC variant of *Pparb* SNP rs3734254 appeared to show reduced mortality rate compared to the TT variant. This was also true in cancers of the upper aero-digestive tract [23]. In gastric cancer patients the GG variant of rs2076167 was highly present [18]. However, understanding of the metabolic effects of PPARβ/δ polymorphisms in the pancreas is certainly lacking.

The GG variant of the non-coding SNP, rs2076167, in the pancreas was not found in the normal vs. tissue tumor set, however, the pancreatitis and pancreatic tumor set (Table 5.6) showed increased expression of *Mcp1* and *Cox2* compared to the other variants, as well as *Il1ra*. AG variant showed increased *Bcl6* expression and its target *Mmp9*, a gene involved in metastasis. Another non-coding SNP, rs1053049, had higher expression of *Bcl6* and *Mmp9* in the minor allele CC variant. A coding *Bcl6* SNP, rs1056932, the minor CC variant had significantly higher *Bcl6* expression. Gene expression seemed unaffected by disease-state in the normal vs. tumor tissue set.

In conclusion, no new mutations were found and the non-coding *Pparb* SNPs that were assessed appeared to lack any correlation with the pancreatic cancer phenotype. The role of PPARβ/δ and BCL6 in inflammation and modulation of metabolic disorder is well established, the link between inflammation and metabolic disorder with cancer, including that in the pancreas, has also been established. So we decided to shift our analysis towards gene expression with the SNPs to determine if they had any effect on gene function. Despite some significant differential expression in rs2076167 in *Pparb*, these data are largely inconclusive.

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Chapter 6

Conclusion

Ductal adenocarcinoma represents approximately 90% of human pancreatic cancer cases. Accumulating mutations that cause constitutive activation of *Kras* during early stages encompass the vast majority of these cancers, leading to dysregulation of several downstream cell signaling pathways. This dysregulation eventually results in the inactivation of tumor suppressors *Cdkn2a* [1], *Smad4* [2], and *P53* during later stages of the disease[3]. Due to the complexity of the Ras signaling pathways, finding a suitable therapeutic target has been difficult. Single protein targets have been ineffective in chemoprevention [6], and the resiliency of pancreatic cancer cells to chemotherapy such as gemcitabine and 5'fluorouracil have made the search for alternative molecular targets imperative [10]. Aiding in the resiliency of pancreatic cancer is the recruitment of tumor associated macrophages (TAMs), which express higher levels of MMP9 that mediate degradation of the basement membrane and expression of *Vegf* leading to angiogenesis and eventual metastasis [37]. PPARs mediate several key pathways including glucose and lipid homeostasis [5], inhibition of metabolic disease, as well as augmentation of anti-inflammatory signaling [6], and activation/deactivation of macrophages [8,9]. Perhaps induction of this pathway through PPARβ/δ-specific ligands as well as in combination with recent advances in chemotherapeutic regimens such as gemcitabine/FOLFIRINOX and/or nab-Paclitaxel may prove beneficial in improving survival rate of the disease, as well as serve as a potential prognostic marker for early detection.

The PPAR-subtype *Pparb* is ubiquitously expressed at moderate levels in the pancreas compared to other tissues, although its role in cancer has not been studied. Several conflicting reports indicate opposite effects in various human and mouse cancer models, particularly the colon. Intestinal *Pparb* over-expression in transgenic mice showed to enhance susceptibility colon cancer [38]. In support of these findings, HCT116 cells that are *Pparb*-null show reduced tumorigenesis in xenograft models [39]. However, over the years a growing collection of evidence has yielded some common themes such as PPARβ/δ's promotion of terminal differentiation, and inhibition of inflammatory signaling [6]. Data presented in this dissertation are in support of the therapeutic role of PPARβ/δ-specific ligands against inflammation and metastasis of pancreatic cancer cells and for the PPARβ/δ/BCL6 pathway as an effective target against carcinogenesis and progression in the pancreas.

6.1 Summary and perspectives

Studies presented in chapter two of this dissertation substantiated that PPARβ/δ is not only expressed in the pancreas, but its function is anti-inflammatory in pancreatic cancer cell models, Mia PaCa-2 and BxPc-3. In a reporter assay using an *Nfkb PPRE* fused to a luciferase, GW501516-activated PPARβ/δ was sufficient to suppress basal and TNFα-induced *Nfkb* luciferase expression. RNAi knockdown of *Pparb* and *Bcl6* indicated both transcription factors may be required for this repression. Furthermore, over-expression of *Pparb* inhibited basal and TNFα-induced activation of the *Nfkb* luciferase. Previous studies have indicated this inhibition occurs through binding of PPARβ/δ with the p65 subunit in cardiomyocytes [40]. GW501516 treatment induced

several anti-inflammatory genes such as *Tgfb1, Sod1, Il1ra,* and *Fgf21* in a PPARβ/δ-dependent manner as measured by quantitative PCR. GW501516 treatment also suppressed TNFα-induced pro-inflammatory genes such as *Mcp1, Mcp3, Tnfa,* and *Il1b* in a BCL6-dependent manner. Interestingly, *Mcp1*, *Mcp3,* and *Il1b* mRNA expression was reduced when *Pparb* was knocked down. Low doses of doxorubicin, an anthracycline, has been found to increase expression of PPARβ/δ that sequesters BCL6 preventing the tumor suppressor from exerting its suppression of inflammatory genes [41]. Lee *et al*. has also demonstrated in foam cells a sequestering of BCL6 by PPARβ/δ as a function of *PPRE*-independent gene regulation [42]. PPARβ/δ antagonists, ST247 and PT-S58, increased expression of BCL6-target *Mcp1* in mouse bone marrow cells and human monocytes, an effect reversed by PPARβ/δ agonist, L165,041 and GW501516 [43]. We performed a reporter assay using the *Mcp1* promoter fused to a luciferase and substantiated that treatment with GW501516 led to suppression of the luciferase in a BCL6-dependent manner, and RNAi knockdown of *Pparb* also inhibited TNFα-activation. Mia PaCa-2 cells are negative for *Cox2,* which is over-expressed in several malignancies including pancreatic cancer, regulated by *Nfkb,* and contributes to angiogenic signaling [44]. In BxPc-3 cells, positive for *Cox2* and *Il6*, PPARβ/δ activation did not reduce these genes. RNAi knockdown of *Bcl6* increased inflammatory response and knockdown of *Pparb* reduced *Il6* mRNA expression indicating GW501516 regulation is partially through BCL6. Macrophages have been linked to progression of cancer growth by evasion of immune response and promoting metastasis [8–10]. In fact, a recent report has found PPARβ/δ activation to induce an anti-inflammatory macrophage phenotype and inhibit immune-suppressive factors [11]. To investigate macrophage

recruitment in the pancreas, conditioned media experiments were performed to further analyze the crosstalk between pancreatic cancer cells and macrophages. RNAi knockdown of *Pparb*, *Bcl6*, *Il1ra*, and scrambled control all showed a reduction in pro-inflammatory gene expression when treated with GW501516 substantiating in the pancreas that PPARβ/δ ligands relieves inflammatory signaling via crosstalk between cancer cells and infiltrating macrophages. Media conditioned by GW501516-treated Mia PaCa-2 cells attracted fewer THP-1 across a basement membrane compared to TNFα-treated media. Of note, RNAi knockdown of *Pparb* appeared to reduce pro-inflammatory gene expression as well as reduce invading THP-1 cells compared to control. These data suggests PPARβ/δ expression may have an exacerbating effect on inflammation, however, PPARβ/δ-specific ligands such as GW501516 has a clear therapeutic effect by ameliorating inflammatory cell signaling, especially by inhibiting inflammatory macrophage recruitment which has been implicated in sustaining a pro-tumorigenic microenvironment.

In chapter three we went further by investigating if PPARβ/δ plays a role in cell migration and metastasis. Quantitative PCR was performed on human pancreatitis and pancreatic cancer tissue to screen expression of biomarkers of inflammation, migration, and metastasis. MMP9 was expressed 10-fold higher in cancer tissue compared to pancreatitis. *Mmp9* is involved in cancer cell invasion by degradation of the basement membrane type IV collagen [12], and has been implicated in metastasis [13–15]. Substantiating a previous study [45], *Pparb* was found to be over-expressed in tumor tissue compared to pancreatitis, interestingly, *Bcl6* appeared to be suppressed. The reason for this over-expression is unknown. The regulatory region of PPARβ/δ contains several AP1

transcription factor binding sites [16], and a possible explanation for its over-expression may be because of the stress response caused by tumor formation. A link has been established between MMP9 and BCL6 [19], and *Pparb*-null macrophages have significantly lower *Mmp9* expression perhaps because of the abundance of free BCL6 caused by the absence of PPARβ/δ. This may indicate adenocarcinoma of the pancreas has higher expression levels of *Mmp9* because of either an increased expression of PPARβ/δ resulting in a sequestering effect of BCL6 and/or mutational augmentation [17,18]. We further investigated the regulatory role of the PPARβ/δ and BCL6 pathway on *Mmp9* expression in the pancreas. We utilized an ELISA to assess MMP9 protein levels in Mia PaCa-2 cells treated with TNFα with and without GW501516. GW510516 was effective in reducing MMP9 protein levels in control cells. However, RNAi knockdown of *Bcl6* increased MMP9 levels and GW501516 was ineffective. *Pparb* knockdowns reduced MMP9 levels passed control levels suggesting the absence of PPARβ/δ results in higher abundance of free BCL6 which is required for PPARβ/δ regulation of MMP9. The PPARβ/δ pathway regulates MMP9, particular by release of BCL6, not only on the mRNA level but on the protein level as well. This has significant effect on metastasis as shown by RNAi knockdown of *Mmp9* using a cell invasion assay, approximately a three-fold reduction in *Mmp9* mRNA resulted in over 50% reduction in pancreatic cancer cell invasion across an artificial membrane. Quantitative PCR indicated GW501516 treatment led to significant reduction in *Il1b*, *Mcp1*, and *Mmp9* mRNA with slight, but not significant, reduction in *Esel*, *Icam1*, and *Vcam1*. RNAi knockdown of *Bcl6* led to significant increase in *Icam1*, *Vcam1*, and *Mmp9* mRNA, with GW501516 having no effect in reducing these levels. PPARβ/δ knockdowns reduced *Il1b*, *Mcp1*, and *Mmp9*

mRNA. Despite GW510516 having no significant effect in reducing mRNA expression of cell migratory markers *Esel*, *Icam1*, and *Vcam1*, knocking down *Bcl6* clearly shows modulation of the cell migration program by this PPARβ/δ pathway. GW501516 was effective in causing a two-fold reduction in invasion of Mia PaCa-2 and BxPc-3 cells across an artificial basement membrane. The reduction of BCL6 expression led to an increase in invasion with GW501516 only being significantly effective in reducing invasion in BxPc-3 cells. Consistent with previous observations, knockdown of *Pparb* reduced invasive potential of both cell lines more than two-fold from control levels. These data suggest the PPARβ/δ pathway is also effective in reducing metastatic potential through its inhibition of *Mmp9*, and is indicative of a modulatory effect on angiogenic signaling.

In the fourth chapter we sought to explore *PPARβ*/δ and *Bcl6* expression, along with several of their targets and biomarkers of pancreatic carcinogenesis in human tumor tissue including corresponding adjacent normal tissue to compare. As observed in our last tissue set and reported previously, *Pparb* was over-expressed in tumor tissue compared with normal tissue while its anti-inflammatory targets were also elevated: *Tgfb1*, *Sod1*, *Il1ra*, and *Fgf21*. Unlike what was observed in our previous set, *Bcl6* was unchanged. However, BCL6 inflammatory targets were elevated in tumors: *Mmp9*, *Mcp1*, *Esel*, and *Vcam1*. Prognostic and mechanistic markers were also assessed such as *Nfkb*, *Tspan8*, and *Cox2* were elevated, while *Cbr1*, *HSD11B*, *Tnfa*, and *Rbp1* were unaltered. Next, we analyzed a mouse pancreatic tumor model in Cre-mediated expression of *Kras(G12D)* that is known to recapitulate the PanIN spectrum of tumor growth observed in human patients of the disease making it an important tool in early prognostic marker discovery.

We assessed the same genes as previously described above, and no significant differences were discovered up to nine months. Mice that were sacrificed after nine months showed a significant increase in *Pparb* expression, as observed previously there was no change in *Bcl6* or *Adrp*. PPARβ/δ targets *Sod1*, *Il1ra*, *Tgfb1*, and *Fgf21* were all elevated compared to $Kras^{G12D-/-}$ mice. BCL6 targets *Vcam1*, *Mcp1*, *Icam1*, *Mmp9*, and *Tnfa* were elevated as well. Other prognostic markers *Nfkb*, *Tspan8*, and *IL1a* were elevated while no change was observed in *Cox2*, *Cbr1*, *HSD11B*, and *Rbp1*. The data presented so far may suggest an inhibitory effect on *Bcl6* expression, perhaps effected by the over-expression of *Pparb*. Previous studies in cardiomyocytes showed a sequestration effect of BCL6 by PPARβ/δ, an effect that was ameliorated by PPARβ/δ agonists [20,41]. We sought to investigate this sequestration effect in the pancreas. A cell titer glo assay showed Mia PaCa-2 cells over-expressing *Pparb* became significantly more proliferative over a 72 hour timespan, an effect that was reduced with GW501516 treatment. A reporter assay using the *Mcp1* promoter fused to a luciferase was used to determine how elevated expression of *Pparb* affected luciferase expression. The forced expression of *Pparb* increased basal and TNFα-induced luciferase expression by approximately two-fold, and GW501516 treatment reduced this effect to control levels. Luciferase expression was significantly reduced with transfection of 20ng BCL6, however, luciferase expression still elevated with increasing expression levels of *Pparb*. Clearly, *Pparb* levels plays a significant role not only in effecting inflammatory, migratory, and metastatic signaling, but in proliferation of pancreatic cancer cells as well. A SNP in BCL6, rs1056932, creates an exonic splice variant that excludes the first two zinc finger motifs [21], and has been linked to non-Hodgkin lymphoma [22]. We looked at the interaction between

PPARβ/δ and the normal and short splice variants of BCL6 by a mammalian two hybrid assay. The full length PPARβ/δ was fused to the pM (Gal4-DNA binding domain) vector, while the BCL6 variants were fused to a pVP16 (activation domain) vector. No significant differences were discovered between the full and short variant as assessed by luciferase expression, however, dissociation of the proteins were observed while treating with 250nM of GW501516. Indeed, increasing the transfection load of pVP16-BCL6 increased association of the Gal4-DNA binding domain with the activation domain as assessed by luciferase activation. Treating with GW501516 reducing luciferase activation suggesting a dissociation the activation domain of the BCL6 variants from the Gal4-DNA binding domain of the PPARβ/δ. Further experimentation such as pull-down assays are required to fully elucidate the mechanistic detail between these two transcription factors, as well as substantiate the mammalian two hybrid to rule out possible false positives or negatives. Quantitative chromatin immunoprecipitation was performed on the PPARβ/δ and BCL6 protein complex to elucidate the sequestration on the interaction with their target genes. PPARβ/δ was detected in drastically higher abundance at its response element on *Angptl4*, a known PPARβ/δ target, compared to DMSO control. Acetylated *Histone H4* was detected at the transcription start site approximately over two-fold increase indicative of gene activation. Interestingly, PPARβ/δ was detected approximately five-fold increase at the BCL6-binding site on *Mcp1* compared to DMSO. Unexpectedly, BCL6 was significantly higher in DMSO than GW0742 treatment. Perhaps poor antibodies un-optimized for our purposes is the reason for these observations, or protein folding resulting in the antibody to be unable to bind to its target epitope. The BCL6 protein has a very short half-life and instability may be a reason we

were unable to detect the protein at its binding site, the transcription factor may be targeted for ubiquitylation and proteosomal degradation prior to antibody detection. Further optimization and experimentation would be required to fully analyze these mechanisms. These data substantiate an inflammatory and proliferative effect stemming from the up-regulation of *Pparb*. These effects may be a result of a sequestration of BCL6, inhibiting it from exerting its tumor suppressive role. However, these effects are ameliorated well by the treatment of PPARβ/δ agonists, GW501516 and GW0742.

Figure 6.1 illustrates the proposed mechanism by which increased expression of PPARβ/δ, observed over-expressed in pancreatic carcinogenesis, increases binding to tumor suppressor, BCL6. In absence of ligand, PPARβ/δ sequesters BCL6 from exerting its anti-inflammatory and anti-metastatic effects exacerbating carcinogenic progression. Ligand-activation of PPARβ/δ resolves this sequestration, undertaking a direct and indirect anti-inflammatory and anti-metastatic role in the pancreas. The therapeutic potential of GW501516 may be highlighted in two ways: 1) The prevention of the pancreatic cancer switch from pancreatitis by increasing anti-inflammatory gene and decreasing inflammatory gene expression via BCL6, and attenuating macrophage recruitment, a key step in sustaining a pro-tumorigenic microenvironment. 2) Inhibiting the progression of pancreatic cancer by reducing metastatic signaling and attenuating pancreatic cancer cell invasion across the basement membrane. The data presented herein supports GW501516 in chemoprevention in cases of pancreatitis. More so, the reduction in pancreatic cancer progression that may make it an effective addition to chemotherapeutic regimens, described below.

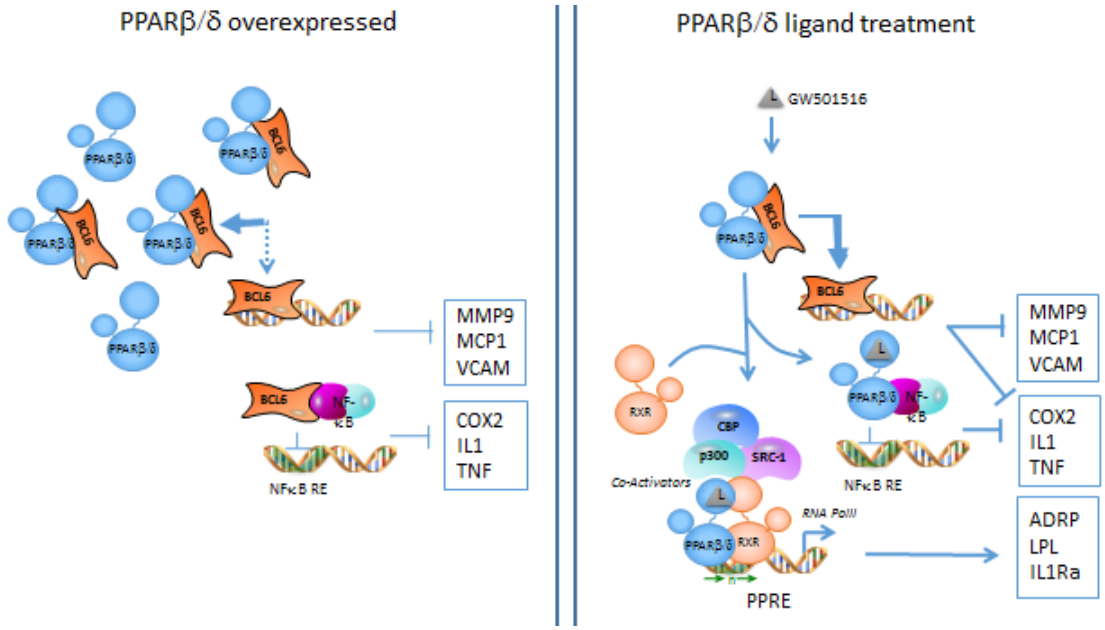

Figure 6.1 Summary illustration of PPARβ/δ over-expression and resulting sequestration of BCL6, and PPARβ/δ-activated release of BCL6 by GW501516 and the subsequent regulation of target genes involved in inflammation and metastasis.

6.2 Future directions

The role PPARβ/δ and BCL6 in regards to cancer in the pancreas remain poorly understood due to lack of *in vivo* data. The Cre-mediated *Kras(G12D)* mouse model was appropriate for studying the PanIN:PDAC progression, despite tumor latency and progression to metastasis being highly variable. *In vivo* studies that investigate the therapeutic effectiveness of GW501516 in chemoprevention of pancreatic carcinogenesis, as well as a chemotherapeutic additive with gemcitabine regimens, in a more consistent tumor model would yield important insight. A mouse xenograft model where a pancreatic cancer cell line (Mia PaCa-2) is injected into the flank of immunocompromised mice may be an appropriate model. Once tumors begin to develop, approximately one to two weeks after inoculation, the mice would be treated with PPARβ/δ-specific ligands with or

without various chemotherapy regimens (gemcitabine in combination with oxilaplatin/cisplatin/capecitabine/FOLFIRINOX/nab-paclitaxel in various timing and dosage [23–26]) to assess tumor development and progression over a four week span. An orthotopic model where the cells may be injected into the pancreas may serve as a better model as this would provide a more proper tumor microenvironment and therefore more of an accurate representation of human tumorigenesis [27]. Further benefits of this study would be the ease of RNAi knockdown of *Pparb* and *Bcl6* to create stable cell lines to transplant and better determine the role of this pathway in tumor development. A downside of this approach would be the lack of an immune response that comes with these mouse models. Tumors may often subvert normal immunosurveillance during its progression aiding in its survival by manipulating the microenvironment, possibly resulting in the reduction of antigen-specific helper and cytotoxic T cells, and promoting expression of pro-inflammatory cytokines. The PPARβ/δ and BCL6 pathway plays a significant role in the immune system through its regulation of T lymphocytes, microglial cells, and astrocytes [5]. Previous studies have demonstrated PPARβ/δ's involvement in reduction of *IFNγ* and *IL17* expression in the brain and spleen, genes implicated in the Th1 inflammatory immune response, as well as reduction of *IL12* and *IL23* expression implicated in Th1 and Th17 polarization, and promoting *IL10* and *IL13* implicated in Th2 anti-inflammatory immune response [28,29].

GW501516 has been extensively studied in regards to the role of PPARβ/δ in lipid metabolism. In obese rhesus monkeys, increasing treatment of GW501516 elevated HDL decreased LDL, VLDL and plasma insulin [34]. In ob/ob mice, GW501516 significantly reduced blood glucose and plasma insulin as well as elevated fatty acid β-

oxidation and increased gene expression involved in fatty acid catabolism [35]. GlaxoSmithKline, creators of the drug, have reached phase III clinical trials with GW501516 before ceasing development. In phase I, randomized, double-blind, placebo-controlled trial of healthy males GW501516 increased HDL [36]. In a phase I/II random crossover trial of males with dyslipidemia GW501516 decreased triglycerides, total cholesterol, and apoB100 levels, while significant increase in VLDLapoB and decreases in LDLapoB production were observed [36]. The ongoing controversy with PPARβ/δ's role in carcinogenesis has discouraged production of the compound. However, pancreatic cancer is a disease of extremely poor prognosis with surgical resection resulting in the loss of up to 95% of the pancreas in patients with localized non-metastasized tumors, and risk of liver failure in patients with metastasized tumors. The potential for colon cancer risk may be considered acceptable for a therapeutic agent of pancreatic cancer. Furthermore, pending on the effectiveness of GW501516/gemcitabine and/or nab-paclitaxel on inhibition of tumor growth and survival rate in the xenograft mice, clinical trials may be performed in patients with metastatic pancreatic adenocarcinoma to assess effectiveness on survival rate, side effects, and measurement of circulating levels of cancer biomarkers such as *Vcam1*, *Mmp9*, and *Mcp1*.

Several leaps have been made in the development of pancreatic cancer models in mice [30–32]. One example of this is the development of a new orthotopic model of invasive pancreatic cancer within an immunocompetent host [33], unlike past immunocompetent models where cells are toxin- or virally-induced and do not mimic human tumorigenesis, this model induces mutations in *Kras* and *p53* (found in 90% and 60% in tumors[1], respectively). Advantages of this model are the ease of orthotopic immunodeficient mice

with the applicability of genetically altered mouse models. Tumors establish consistently within two weeks of implantation, unlike the Cre-mediated *Kras(G12D)* mouse, and progress to advanced stages of the disease by six to eight weeks. Cells are also wild-type and tumor development will eventually result in infiltration into normal parenchyma similar to natural disease progression, unlike the *Kras(G12D)* mouse model where tumors tend to grow throughout the pancreas. This model was derived by subcloning PDAC cells that metastasized to the liver in the *Kras(G12D)* mice [33], and therefore does not recapitulate the full spectrum of PanIN making it a poor candidate for early carcinogenesis studies. The main purpose of this model would be the assessment of PPARβ/δ and BCL6 in tumor development within a natural microenvironment and immune response under treatment by PPARβ/δ-specific ligands in combination with chemotherapeutic regimens. Early prognostic studies would probably best be served by the Cre-mediated *Kras(G12D)* mouse model as described.

6.3 References

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1. **R.W. Smith**, B. Prokopczyk, J.P. Vanden Heuvel, "Sequestration of B-Cell Lymphoma 6 by Peroxisome Proliferator-Activated Recepter β/δ and therapeutic potential in pancreatic cancer." (In preparation)
2. **R. W. Smith**, J. D. Coleman, J.T. Thompson, and J.P. Vanden Heuvel, "Therapeutic potential of GW501516 and the role of Peroxisome proliferator-activated receptor β/δ and B-Cell Lymphoma 6 in inflammatory signaling in human pancreatic cancer cells." (In preparation)
3. J. D. Coleman, J. T. Thompson, **R. W. Smith**, B. Prokopczyk, and J. P. Vanden Heuvel, "Role of Peroxisome proliferator-activated receptor β/δ and B-cell lymphoma-6 in regulation of genes involved in metastasis and migration in pancreatic cancer cells," *PPAR Res.*, vol. 2013, 2013.
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