UNIVERSITY OF CALIFORNIA, SAN DIEGO

Crosstalk between pancreatic cancer cells and pancreatic cancer-associated fibroblasts through cytokines and a GPCR

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2016

The Thesis of Sarah Esther Chang is approved and is acceptable in quality and form for publication on microfilm and electronically:

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Chair

Dedication

To David, Ruth, and Susanna for their constant love and support

And to all those whose lives have been affected by pancreatic cancer.

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Acknowledgements

I would like to thank my mentor for the last 3 years, Shu Zhou, for teaching me so much and helping me to graduate. Shu has always been there when I needed help or guidance and has encouraged my passion for science. She has always been so patient, kind, helpful, encouraging, and a great friend.

I would like to express my gratitude to Dr. Paul Insel for his mentorship and the opportunity to work in his lab. I am so thankful to have been able to work in the Insel lab and learn so much during my time there.

I would like to thank all the members of the Insel lab for all their help and for their friendship. I would especially like to acknowledge Nakon Aroonsakool for his contribution to the project, his help in the lab, and his encouragement.

I would like to thank Dr. Hedrick and Dr. Tour for agreeing to be a part of my thesis committee.

I would like to acknowledge my friends and family for all their support. I would like to thank Wesley Lau for his constant encouragement. I would like to thank my family who has made all this possible and been there for me every step of the way.

ABSTRACT OF THE THESIS

Crosstalk between pancreatic cancer cells and pancreatic cancer-associated fibroblasts through cytokines and a GPCR

by

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Master of Science in Biology

University of California, San Diego 2016

Professor Paul Insel, Chair

Pancreatic cancer is the third leading cause of cancer death in the United States. Pancreatic ductal adenocarcinoma is the most prevalent type of pancreatic cancer and is characterized by dense fibrosis (termed desmoplasia) in the tumor microenvironment. Cancer associated fibroblasts (CAFs), which are thought to derive from pancreatic stellate cells (PSCs) and fibroblasts (PFs), play

a large part in this desmoplasia. Signaling from cancer cells activates CAFs, which in turn can signal to promote tumorigenesis. Previous work done in the Insel lab had identified GPR68, a proton-sensing G protein-coupled receptor (GPCR), as one of the most highly up-regulated GPCRs in CAFs compared to normal PSCs and PFs. This thesis project identifies TGFβ and TNFα as factors that, increase, respectively, the expression in PSCs of fibrotic markers and GPR68. Other work in this project shows that GPR68 in CAFs regulates the expression and secretion of Interleukin-6 (IL-6). GPR68 signals through a Gs-coupled pathway (via cAMP and protein kinase A [PKA]) to increase IL-6 formation and secretion by CAFs; IL-6 can aid in the development of PDAC. Thus, inhibiting the secretion of IL-6 through GPR68 may be a novel strategy for the treatment of pancreatic cancer.

INTRODUCTION

a. Pancreatic ductal adenocarcinoma

Pancreatic cancer is currently the third leading cause of cancer death in the United States with a 5 year survival rate of <5%[1]. It has the lowest 5 year survival rate of all cancers and is projected to be the second leading cause of cancer death in the US by the year 2020[2,3]. The American Cancer Society predicts that in 2016, 53,070 new cases of pancreatic cancer will occur as well as 41,780 deaths[1]. The main risk factors for pancreatic cancer are smoking, which is thought to contribute to 20-25% of tumors, and family history, which accounts for 5-10% of tumors[4]. Commonly mutated genes in pancreatic cancer include KRAS, BRCA2 and PALB2[5]. Early symptoms of pancreatic cancer include weight loss, nausea, and abdominal pain[6]. These symptoms are nonspecific and often go unrecognized due to a lack of specific diagnostic tools for pancreatic cancer. As a result, about 80% of individuals have locally advanced or metastatic disease upon diagnosis. Only about 20% of individuals with pancreatic cancer are eligible for surgical resection; however, the 5 year survival rate following surgery remains low (15-20%)[7]. For the remaining 80%, gemcitabine-based chemotherapy is used but only typically prolongs survival for an average of 6 months[8]. Low survival rates can be partly attributed to the high chemoresistance of pancreatic cancer cells[9]. One FDA-approved targeted therapy, erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, increases average survival by about two weeks in combination with gemcitabine[10]. The high morbidity rates and lack of early diagnosis show that there is a great need for new diagnostic markers

and novel treatments for pancreatic cancer.

The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) which makes up about 95% of all cases[1]. PDAC begins to develop through noninvasive precursor lesions, the most common form being pancreatic intraepithelial neoplasias (PanINs) in the head of the pancreas[11]. These lesions in the pancreatic duct are about 5mm in diameter and have been found to be largely driven by activating mutations in K-ras[12]. As inactivating mutations in tumor suppressors such as p16, p53 and SMAD4 follow, the lesions progress through three stages, PanIN-1,2,3[11]. In PanIN-1, the nuclei of epithelial cells are still basally oriented and uniform. In PanIN-2, the cells begin to lose their polarity and atypia, loss of structure, begins to increase to become PanIN-3 and eventually invasive PDAC[13.14]. PDAC tumors are large masses that can invade and obstruct the pancreatic duct[15]. PDAC is characterized by cancer cells embedded in a dense fibrotic stroma environment known as tumor desmoplasia. Up to 90% of the tumor mass can be made up of the activated stroma, which consists of endothelial cells, activated stellate cells/cancer-associated fibroblasts (CAFs), extracellular matrix (ECM) components, and immune cells[16]. The production of the stroma is driven by signals from cancer cells and include transforming growth factor β (TGFβ), hepatocyte growth factor (HGF), fibroblast growth factors (FGFs), insulin-like growth factor 1 (IGF-1) and EGF[16]. The sonic hedgehog (SHH) pathway has also been shown to promote desmoplasia through the smoothened (SMO) receptor[17]

b. Cancer-associated fibroblasts (CAFs)

CAFs are myofibroblast-like cells[18] that are characterized by an elevated expression of α-smooth muscle actin (αSMA) and enhanced fibrogenic activity. CAFs originate from many different cell types. Studies in breast tissue have shown that CAFs can develop from resident fibroblasts[19]. CAFs can also originate from mesenchymal cells that include endothelial cells, pericytes, stellate cells, pre-adipocytes, and bone marrow-derived cells such as mesenchymal stem cells.[20]

In PDAC tumors, the two major progenitors of CAFs are pancreatic fibroblasts (PFs) and pancreatic stellate cells (PSCs)[21]. PSCs make up approximately 4-7% of the pancreatic cells. In their quiescent phenotype, PSCs express various markers, including desmin and glial fibrillary acidic protein (GFAP), and contain lipid droplets[22]. The activation of PFs and PSCs into CAFs can be promoted by cytokines such as TGFβ and tumor necrosis factor α (TNFα), growth factors such as platelet-derived growth factor (PDGF), oxidative stress, or injury. When PFs and PSCs are activated into CAFs, these cells express high amounts of αSMA[23], cytokines, growth factors, and ECM components such as collagens and fibronectin[24,25]. CAFs also secrete matrix metalloproteinases and their inhibitors, which together can regulate the ECM[26].

CAFs have been shown to promote the proliferation, metastasis, and immune evasion of cancer cells[18]. In studies of PDAC, mice co-injected with PDAC cells and CAFs had increased tumor incidence, size and metastasis compared to mice injected with tumor cells alone[27]. Studies of conditioned media from CAFs have shown that factors secreted from CAFs promote proliferation and migration of PDAC cells while inhibiting apoptosis[28]. ECM components produced by CAFs can increase cancer cell survival and thereby contribute to tumorigenesis[29,30]. The fibrosis produced by CAFs is a major contributor to the chemoresistance of PDAC. Stroma depletion improves drug delivery through a reduction of hypoxia induced by fibrosis[31]. While the desmoplasia in PDAC promotes chemoresistance, CAFs can influence the cancer cells directly: treatment with the secretions of CAFs decreases the sensitivity of PDAC cells to gemcitabine and other types of chemotherapy[27]. CAFs thus play a pivotal role in the development of PDAC and are a potential target for treatment.

c. Cytokines in PDAC

Cytokines play an important role in the development of PDAC and CAFs by enabling the bi-directional communication between the cells. PDAC cells produce cytokines such as tumor necrosis factor-alpha (TNFα), interleukin-6 (IL-6), interleukin-8 (IL-8), and TGFβ[32] to influence other stromal cells in the tumor microenvironment. TNFα is a pro-inflammatory cytokine that regulates acute and chronic inflammation and can induce the secretion of many other cytokines and chemokines[33]. TNFα has been shown to increase the invasiveness of PDAC

cells[34]. Elevated levels of TNFα are found in the blood of PDAC patients and are correlated with advanced disease[35]. IL-6 is another pro-inflammatory cytokine that not only regulates inflammation but also cell proliferation and survival and is involved in many kinds of cancer[36]. In pancreatic cancer, IL-6 plays a crucial role in tumorigenesis through inflammatory signaling and reactive oxygen species[37]. High serum levels of IL-6 and IL-8 of PDAC patients are associated with more advanced disease and poor survival[38]. IL-8, a chemotactic and pro-inflammatory cytokine, is up-regulated in many kinds of cancer[39]. IL-8 promotes invasiveness and angiogenesis in pancreatic cancer[40]. TGFβ is an anti-inflammatory cytokine that inhibits proliferation but this pathway is often disrupted in cancer[41]. However, TGFβ is considered to be a principal activator of PFs and PSCs to become myofiboblast-like CAFs that cause the desmoplasia in PDAC[42]. All of these cytokines as well as others not only help promote the development of PDAC and interact with CAFs and their progenitors but are also being studied as possible prognostic markers for advanced stages of pancreatic cancer.

CAFs secrete their own set of cytokines that can signal to cancer cells and aid in tumorigenesis. These include EGF, PDGF, stromal-cell derived factor (SDF-1), TGFβ, and IL-6[43]. EGF is known to increase cancer cell proliferation, growth, and differentiation[44]. The receptor for EGF (EGFR) is also commonly over-expressed in PDAC cells and is a current target for pancreatic cancer therapy[45]. PDGF also stimulates cell growth and has been shown to play an important role in the invasive ability of PDAC cells[46,47]. SDF-1 (also known as

CXCL12) is a chemokine that is involved in tumor progression, survival, angiogenesis, and metastasis[48]. In pancreatic cancer, SDF-1 interacts with the Hedgehog pathway to increase metastasis[49]. While TGFβ is a driver of desmoplasia in the tumor microenvironment, it can also contribute to tumor growth and metastasis[50]. As noted above, IL-6 is a main driver of tumorigenesis in pancreatic cancer and its serum concentration increases in patients with advanced disease. Cytokines secreted by PDAC cells and CAFs enable a symbiotic relationship whereby cytokine production by PDAC cells activates CAFs which in turn secrete cytokines to promote PDAC tumor progression.

d. G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) are seven transmembrane domain receptors that comprise one of the largest families of proteins in the mammalian genome. The more than 800 GPCRs in the human genome have been classified into five families in vertebrates: rhodopsin, secretin, glutamate, adhesion, and frizzled[51]. There are about 350 non-chemosensory GPCRs (i.e., not visual, taste or odorant receptors), which respond to endogenous signals (peptides, lipids, neurotransmitters, or nucleotides) to regulate processes such as metabolism, reproduction, development, homeostasis, and behavior[52]. GPCRs interact with heterotrimeric G proteins, which are made up of three subunits: α, β, and γ. Gα subunits are divided into four categories based on their signaling pathways: Gs, which activates adenylyl cyclase to produce cAMP and activate downstream effectors, such as protein kinase A (PKA); Gi, which inhibits adenylyl cyclase

and regulates various ion channels[53]; Gq, which acts via the release of calcium stimulated by inositol triphosphate (IP3) and protein kinase C recruited by diacylglycerol (DAG)[54]; and G12/13, which activates RhoGEF, RhoA and downstream effectors[55].

GPCRs are excellent drug targets as they are located on the plasma membrane, making them easily accessible from the extracellular environment. They are also selectively expressed on different cell types and tissues and activated by specific ligands[56]. GPCRs make up approximately 30% of therapeutic drug targets but these drugs target only a small percentage of GPCRs[57]. GPCRs have been shown to be involved in tumor growth and metastasis[58]. Overexpression of receptors, such as the thrombin receptor PAR-1, has been shown to increase tumor cell invasion and metastasis[59]. Other GPCRs are involved in the chronic inflammation often present in cancer[60]. Previous studies in the Insel lab have shown that GPCRs, such as PAR-1, can be involved in a profibrotic phenotype in cardiac fibroblasts[61]. Very few GPCR have been studied in pancreatic cancer. One study showed that stimulation of the GPCR-interacting protein β-arrestin-2 promotes the proliferation and growth of PDAC cells[62]. Another study identified the GPCR somatostatin (SST1) on CAFs and showed that it could be targeted to reduce chemoresistance of PDAC[63].

e. G protein-coupled receptor 68 (GPR68)

Previous work done in the Insel lab defined the differences in expression of GPCRs between normal pancreatic progenitor cells (PSCs/PFs) and CAFs. Use of Taqman GPCR arrays revealed that ~35 GPCRs are at least 2-fold up-regulated in CAFs compared to PFs and PSCs. One of the most highly up-regulated GPCRs was GPR68 (also termed ovarian cancer GPCR 1 [OGR1] as it was first identified in 1996 from an ovarian cancer cell line). GPR68 has 365 amino acids and is expressed in the spleen, testis, small intestine, peripheral blood leukocytes, brain, heart, lung, placenta, and kidney but not normally in the pancreas, thymus, prostate, ovary, colon, liver, or skeletal muscle[64]. GPR68 is one of four proton-sensing GPCRs along with GPR4, GPR65, and GPR132[65]. GPR68 was the only proton-sensing GPCR that was up-regulated in pancreatic CAFs. GPR68 senses protons (H^+) through histidines at the extracellular surface and is fully activated at pH 6.8[66]. GPR68 has been shown to activate Gs and Gq signaling pathways and increase cellular concentrations of cAMP and IP3[67]. G12/13, and Gi signaling have also been associated with GPR68 in studies of breast and prostate cancer[68,69]. 3,5-disubstituted isoxazoles had been shown to activate GPR68 but in a nonspecific manner[70]. More recently, ogerin has been developed as a positive allosteric modulator of GPR68[71]. Psychosine has been identified as an antagonist of GPR68 and related proton-sensing GPCRs[72].

GPR68 is mostly highly expressed in the cerebellum of mice and less so in other areas of the brain[56]. A role for GPR68 has been suggested to regulate

neurogenesis and brain repair in response to acidosis[73]. GPR68 has also been studied in a wide range of other cell types and diseases: osteoclast differentiation and as a regulator of calcium in bone cells in response to acidosis[74,75]; a regulator of pH homeostasis and inflammation (via GPR68 in monocytes) in the intestine[76]; and in contraction of airway smooth muscle cells in response to lower pH[77]. In cancer, GPR68 seems to play a dual role depending on the cell type. It has been identified as a suppressor of invasion and metastasis in prostate and breast cancer[68,69] and in ovarian cancer, overexpression of GPR68 inhibited cell proliferation and migration but increased cell adhesion to the ECM[78]. By contrast, GPR68 is overexpressed in medulloblastomas and may promote cell growth via calcium release[79]. GPR68-deficient mice had less tumorigenesis of melanoma cells, which suggests that GPR68 is a tumor promoter in this case[80]. The current study examines the role of GPR68 in pancreatic cancer and CAFs.

Based on prior data, I undertook these studies to test the hypothesis that GPR68 plays an important role in the ability of CAFs to promote tumorigenesis in pancreatic cancer and may be a target for future therapies. In my project, I have conducted studies to: 1) elucidate the factor(s) responsible for the up-regulation of GPR68 and fibrotic markers in pancreatic CAFs, 2) analyze the function of GPR68 in CAFs, and 3) determine the signal transduction pathway for GPR68 in CAFs.

MATERIAL AND METHODS

Cell Isolation and Culture

PDAC patient tumors were diced into small pieces (0.3-0.5mm) and embedded in growth factor reduced Matrigel on a 60mm culture dish. Pre-warmed CAF medium was added to immerse the Matrigel and the Matrigel was incubated at 5% CO_2/95%air. CAF media contains high glucose (4.5 g/L) DMEM, 30% FBS, 1ug/ml fetuin, 20ng/ml EGF, 2mM glutamate, 1mM sodium pyruvate, non-essential amino acids, 200IU/ml penicillin, 200µg/ml streptomycin, Fungizone. After ~6 days incubation, explants with CAF outgrowths were harvested by suspending the explant culture in PBS. An equal volume of 0.05% trypsin was incubated with the explant for ~15min at room temperature. After trypsinization, cells were collected by centrifugation at 1000xg for 5 min. Cells were then resuspended and placed on 10 cm plates with CAF media for cell culture. Primary CAFs can be cultured through 12 passages before senescence occurs; thus low passage (P) primary CAFs (P<12) were used. Human pancreatic stellate cells (PSCs) were purchased from Sciencell Research Laboratories (Carlsbad, CA, Cat. #3830), cultured according to the manufacturer's instructions, and used at low passage (P<3). Pancreatic cancer cell lines, AsPC-1, BxPC-3 and MIA PaCa-2, were purchased from American Type Culture Collection (ATCC). AsPC-1 and BxPC-3 were cultured in RPMI-1640 media with 10% FBS, nonessential amino acid, sodium pyruvate and penicillin-streptomycin. MIA PaCa-2 were cultured in high glucose (4.5 g/L) DMEM media with 10% FBS,

nonessential amino acids, sodium pyruvate and penicillin-streptomycin. All cells were culture at 37°C with 95%air/5% CO_2.

Low pH treatment of PSCs

PSCs were grown overnight on 6 well plates at 80,000 cells/well in media at varying pH. The media contained high glucose DMEM, 25 mM HEPES buffer, 2% FBS and 20mM $NaCO_3$. Final pH values were adjusted at room temperature with HCl or NaOH. PSCs were washed and changed to pH media of pH 7.4 and pH 6.8 for further cell culture. The mRNA of PSCs was prepared at 0, 6, 12, 24, 48, and 72 h for qPCR.

Hypoxia treatment of PSCs

Hypoxic chamber HERAcell 150i CO_2 incubator (ThermoFisher Scientific, Waltham, MA) was used at 5% CO_2, 2% O_2, at 37°C. Normoxia was defined as 5% CO_2, 21% O_2 and 37°C. PSCs were grown overnight on 6-well plates at 80,000 cells/ well. The next day PSCs were washed twice and then placed in fresh media. Hypoxia/normoxia treatment of PSCs was continued for 96 h. Cells were monitored daily for viability by microscopy. The mRNA of PSCs was prepared at 0, 6, 12, 24, 48 and 72 h for qPCR.

Co-culture of PSCs with PDACs

PSCs were grown overnight in a 6-well plate at 50,000 cells/well. PDAC cell lines, AsPC-1, BxPC-3 and MIA PaCa-2 were grown overnight in 24mm 6-well

transwell permeable supports (0.4 µm pore size) (Corning, Corning, NY) at 80,000 cells/ well. The transwells were then placed on top of PSCs and co-cultured in high glucose (4.5g/L) DMEM media with penicillin-streptomycin and media without FBS. Control cells did not have transwells placed on top. PSCs were assessed (qPCR from purified mRNA) at 0, 6, 12, 24, 48 and 72 h. Healthy and viability of cells was confirmed by microscopy throughout all time points.

TGFβ and TNFα treatment of PSCs

PSCs were grown overnight on a 6-well plate at 80,000 cells/well. The next day PSCs were washed twice with high glucose DMEM media with penicillin-streptomycin without FBS. Human recombinant TGFβ (ProSpec, East Brunswick, NJ) and recombinant human TNFα (InvivoGen, San Diego, CA) were dissolved in the same media (final concentration, 50 ng/m). PSCs were treated with TGFβ or TNFα and incubated at 37°C for 2 days. The mRNA of PSCs was prepared at 0, 6, 12, 24, 48 and 72 h for qPCR. Healthy and viability of cells was confirmed by microscopy throughout all time points.

Real-time qRT-PCR

mRNA was collected from samples and isolated using an RNeasy kit with DNase treatment (Qiagen, Valencia, CA). Samples were converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Irvine, CA) according to the manufacturer's protocol. All cDNA samples were used at a concentration of 10 ng/µl. 5 µl of SYBR green (Quanta Biosciences, Gaithersburg, MD), 3 µl of RNase free water,

1 µl of cDNA, and 1 µl of primer (2 µM) were combined for a final volume of 10 µl qRT-PCR amplification on a DNA Engine Opticon 2 system. The PCR program was as follows: preheat at 95°C for 10 min, denature at 95 °C for 15 sec, primer annealing at 55°C for 20 sec and transcription elongation at 72 °C for 40 sec with a total of 40 cycles. Primers were designed using Primer Premier 6 software (PREMIER Biosoft, Palo Alto CA). The primer sequences are 18S-F: 5'-GCCGTTCTTAGTTGGTGGAG-3'; 18S-R: 5'-TCACAGACCTGTTATTGCTCAA-3'; GPR68-F: 5'-CCTTCCGCTTCCACCAGTT-3'; GPR68-R: 5'-TCGTCCTCGATGACCTCCT-3'; ACTA2-F: 5'-TTACTACTGCTGAGCGTGAGAT-3'; ACTA2-R: 5'-CATGATGCTGTTGTAGGTGGTT-3'; COL1A1-F: 5'-TCGGAGGAGAGTCAGGAAGG-3'; COL1A1-R: 5'-CAGCAACACAGTTACACAAGGA-3'; IL6-F: 5'-ACAGCCACTCACCTCTTCAG-3'; IL6-R: 5'-GCAAGTCTCCTCATTGAATCCA-3; COL1A2-F: 5'-ATGCCTAGCAACATGCCAATC-3'; COL1A2-R: 5'-AAAGTTCCCACCGAGACCAG-3'; COL3A1-F: 5'-GCTGGCTACTTCTCGCTCT-3'; COL3A1-R: 5'-TCTCTATCCGCATAGGACTGAC-3'; CTGF-F: 5'-ACATTAGTACACAGCACCAGAA-3'; CTGF-R: 5'-GCTACAGGCAGGTCAGTGA-3'; POSTN-F: 5'-ACGGTGCGATTCACATATTCC-3'; POSTN-R: 5'-TAATGTCCAGTCTCCAGGTTGT-3'; PAI1-F: 5'-GGCTGACTTCACGAGTCTTTC-3'; PAI1-R: 5'-GCGGGCTGAGACTATGACA-3'; PDGFRA-F: 5'-GCGACAGAACTTCAGCATTGT-3'; PDGFRA-R: 5'-TGGATGGATTGAGTGGTCTCTT-3'; PDGFRB-F: 5'-

GGAATGAGGTGGTCAACTTCG-3'; PDGFRB-R: 5'-GGATGGAGCGGATGTGGTAA-3'. Gene expression levels were quantified as ΔCt using 18S as reference gene. Comparison of expression between different treatment groups was quantified as fold change = $2^{-(\Delta\Delta Ct)}$.

Transfection and siRNA Knockdown

PSCs were transfected with an empty pLX304 vector and a pLX304 vector containing GPR68 (DNASU Plasmid Repository, Tempe, AZ). Approximately one million PSCs were seeded on a 10 cm plate in 12 mL of media. 12 µg of plasmid DNA was mixed with 48 µl of Fugene reagent (Promega, Madison, WI) and Opti-MEM media (ThermoFisher Scientific, Waltham, MA) to a total volume of 600 µl. Cells were incubated with plasmid mixture for 24 h. For GPR68 knockdown siGENOME non-targeting control siRNA and siGENOME human GPR68 siRNA (GE Dharmacon, Lafayette, CO) were transfected into CAFs using Lipofectamine RNAiMAX reagent (Invitrogen). 80,000 CAFs/well were seeded onto 6-well plates. 250 µl opti-MEM media that contained 7.5 µL lipofectamine reagent and 25 pmol siRNA was added into each well. Cells were incubated for 72 h.

Growth Curve

CAFs with control or GPR68 siRNA knockdown were seeded at 50,000 cells/ well in 6-well plates. The next day, media was changed to either pH 7.4 or 6.8 media and cell culture was continued for 96 h. Cells were counted using a hemocytometer at 24 h intervals from 0 to 96 h.

Collagen Synthesis Assay

CAFs with control or GPR68 siRNA knockdown were seeded at 30,000 cells/ well in 6-well plates. The next day, media was changed to either pH 7.4 or 6.8 media and [^{3}H]proline was added (1 µCi/mL). After incubation for 48 h, cells were washed once with 2 mL of cold PBS, then lysed with 300 µl of 0.5 N NaOH and incubated for 5 min at 37^o before collecting into tubes. Wells were washed with 300 µl of 0.5 N HCl which was added to the tubes. TCA was added to a final concentration of 20% and the tubes were incubated at 4^{o}C overnight prior to centrifugation at 10,000 rpm for 10 min. The pellet was washed 3 times with 1mL 5% TCA with 5 min centrifugation in between washes. The pellet was then dissolved in 200 µl of 0.2 N NaOH and neutralized with 200 µl of 0.2 N HCl. pH paper was used to confirm the samples were at neutral pH. 200 µl of collagenase solution was added to each sample. Collagenase solution contains 50 mM Tris-HCl, 5mM $CaCl_2$, 0.02% sodium azide, 2.5 mM N-ethylmaleimide, and 10 mg/mL collagenase II. Samples were incubated with collagenase at 37^{o}C for 1 h. TCA was added to a final concentration of 10% TCA. Samples were incubated at 4^{o}C for 1 hr and centrifuged at 10,000 rpm for 10 min. The supernatant was removed into 5 mL scintillation fluid and counted on liquid scintillation counter.

Western Blotting

Cells were cultured in 6-well plates and cell lysates were collected by scraping wells in the presence of 60µL RIPA buffer (Cell Signaling Technology, Danvers, MA) which contained protease inhibitors and phosphatase inhibitors. Cell lysates

were homogenized by sonication and protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (ThermoFisher). Proteins were separated by SDS/PAGE in 4~12% polyacrylamide gels (Invitrogen). Gels were transferred to PVDF membranes using an iBLOT transfer machine (Invitrogen). Membranes were blocked using 5% BSA (w/v) in PBS Tween-20 (PBST) for 2 h, then incubated overnight at 4°C with primary antibodies that were diluted in 1% BSA (w/v) in PBST. The next day, membranes were washed 3 times in PBST for 10 min and incubated with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. The membranes were washed 3 times in PBST and then visualized by Lumigen ECL Ultra (Lumigen, Southfield, MI). Densitometry quantification of protein expression was analyzed by Image J software (U.S. NIH, Bethesda, IL). Antibodies used were GPR68: H-75 (Santa Cruz Biotechnology, Dallas, TX), phospho-CREB (Ser133): 87G3 (Cell Signaling Technology, Danvers, MA), CREB-1: D-12 (Santa Cruz Biotechnology).

IL-6 ELISA Assay

CAF conditioned media were collected from 6-well plates and filtered to remove debris. IL-6 protein level in the conditioned media was detected using an IL-6 ELISA kit (ThermoFisher). 50 µl of standard or media sample was added to 50 µl of biotinylated antibody reagent in a 96-well plate. The plate was incubated for 2 h at room temperature and washed; 100 µl of streptavidin-HRP solution was added and the plate was incubated for 30 min and washed. The process was

repeated with addition of 100 µl of TMB substrate. Finally, 100 µl of stop solution was added to each well and absorbance was measured at 450 nm.

PKA Inhibitors

CAF cells were incubated overnight at 20,000 cells/ well in a 24-well plate. The next day, the media was changed to pH 7.4 or pH 6.8. H-89 (InvivoGen) or PKI:14-22 (Life technologies) were diluted in pH 6.8 media (10 µM, final concentration) and added to CAFs for 6 h prior to mRNA purification and qPCR.

cAMP Assay

CAFs transfected with control siRNA or GPR68 siRNA were plated on a 96-well plate (10,000 cells/well) and incubated overnight. cAMP levels were measured by HitHunter cAMP assay (DiscoverX, Fremont, CA). Cell culture media was changed to 30 µl of a series of pH media (pH 6.4- 7.4) and incubated for 30 min. Then 15 µl of 3mM isobutylmethylxanthine (IBMX, a cyclic nucleotide phosphodieserase inhibitor, 1 mM final concentration) was added to each well for anadditional 10 min incubation. 15 µl of cAMP antibody solution and 60 µl of cAMP detection solution were added to each well. The plate was incubated for 1 hr at room temperature (RT) in the dark. Then 60 µl of cAMP solution A was added and the plate was incubated for 3 h at RT in the dark. Chemiluminescence signal was then measured by a DTX800 multimode plate reader (Beckman Coulter, Carlsbad, CA).

Immunofluorescence staining

CAFs and CAFs with GPR68 knockdown were grown overnight on 12mm round coverslips (Corning, Corning, NY) in 24-well plates (20,000 cells/ well). The next day wells were washed twice with PBS and then incubated for 10 min with 2% paraformaldehyde/ PBS, followed by washing with 10mM glycine (pH7.4) in PBS for 5 min. Cells were permeabilized in 0.1% TritonX/PBS for 10 mins at RT. After washing with PBS/Tween 20 (0.1% Tween), coverslips were blocked in 1% BSA/ PBS/0.05% Tween for 20 min at RT. Primary antibodies were diluted in 1%BSA/PBS/0.05% Tween 20 with 1:100 ratio for GPR68 antibody (H-75, Santa Cruz Biotechnology) and 1:1000 ratio for IL-6 antibody (ab9324, Abcam, Cambridge, MA). Coverslips were incubated with primary antibodies for 48 h at 4°C. After being washed three times with 1%BSA/PBS/0.05% Tween, coverslips were incubated with diluted secondary antibodies at RT for 1 h. Coverslips were also incubated with DAPI as a stain for nuclei. Images were obtained using a Zeiss AxioObserver D1 microscope equipped with an LD A-Plan 20X/0.35 Ph1 objective.

Data Analysis and Statistics

All statistical analysis was done using GraphPad Prism 7. Error bars represent mean±SD. **p<0.01, ***p<0.001, ****p<0.0001.

RESULTS

Factors that regulate fibrotic maker genes and GPR68 expression in CAFs

CAFs have prominently up-regulated expression of GPR68, cytokines, and fibrotic markers compared to that of precursor PSCs[81]. In order to identify the factor(s) that cause(s) the up-regulation of GPR68 and the transformation into CAFs, I assessed potential factors in the PDAC tumor microenvironment. These factors included mimicking the acidic tumor microenvironment, culturing cells in hypoxia, co-culturing PSCs with PDAC cells and treating PSCs with cytokines produced by PDAC cells (e.g., TGFβ and TNFα). I monitored PSC transformation to CAFs by assessing the mRNA expression of α-smooth muscle actin (αSMA), collagens, and other fibrotic markers.

The PDAC tumor microenvironment has a much lower extracellular pH (6.5-6.9) compared to that of normal cells (7.0-7.2). This difference is attributable to the hypovascular, fibrotic tumor stroma[82]. Thus, I compared gene expression in PSCs cultured in pH 7.4 or 6.8 media. After 48 h incubation, GPR68 expression was 4-fold higher in PSCs cultured at pH 6.8 than at pH 7.4 (Figure 1A). However, a time course study of gene expression is more representative of the effects of low pH. Data from such a study showed that GPR68 expression increased in the first 12 h PSCs are cultured in either pH 7.4 or pH 6.8 but at later times, the level of GPR68 mRNA level decreased in both pH conditions, albeit this decrease was slower in the PSCs grown in pH 6.8 media (Figure 2A).

Moreover, the expression of GPR68 in CAFs grown for 48 h at pH 6.8 was similar to that at 0 h (Figure 2A).

The results for GPR68 contrasted with those obtained at pH 6.8 for the expression of αSMA, collagen 1 and IL-6 (Figure 1B-D), all of which were lower after growth of the PSCs for 48h at pH 6.8. These results suggest that low pH has an anti-fibrotic effect on PSCs. Results from a time course study showed that the expression of fibrosis-related genes, αSMA and collagen 1, increased significantly after 24 h growth at pH 7.4 (Figure 2B and 2C). These increases are most likely due to the “spontaneous” transformation of PSCs to myofibroblast-like cells during *in vitro* cell culture[83]. However, the increase in fibrotic activities was blocked at pH 6.8 (Figure 2B and C). IL-6 expression showed inconsistent data in a time course study (Figure 2D) but increased at pH 6.8 after 72 h. Overall, the patterns of gene expression for the entities I assessed were very different with fibrotic markers showing a steady increase of expression at pH 7.4. By contrast GPR68 expression rapidly increased at 6 h and then decreased to basal levels.

Another feature present in the pancreatic tumor microenvironment is hypoxia, which is caused by desmoplasia and plays roles in cancer cell growth and chemoresistance[84]. PSCs were cultured in hypoxia (2% oxygen) and compared to PSCs cultured in normal (21%) oxygen for 96 h. After 48 h, GPR68 expression was lower in hypoxia than normoxia (Figure 3A). By contrast, after 48

h, hypoxia increased the expression of αSMA and slightly decreased expression of collagen 1 without changing IL-6 expression (Figures 3B-3D).

A time course study showed that the expression of GPR68 mRNA decreased in both normoxic and hypoxic conditions but more rapidly in hypoxia (Figure 4D). αSMA expression slightly increased in hypoxia condition after 48 h incubation (Figure 4B), but overall hypoxia had no significant effect on collagen I and IL-6 expression (Figure 4C and D). Again, the pattern of change in fibrotic markers exprsesion differed from that of GPR68. The fibrotic markers showed a slight decrease in expression followed by an increase that was exaggerated by hypoxic conditions. GPR68 decreased in expression in both conditions while hypoxia seemed to promote the decay in expression of the receptor mRNA.

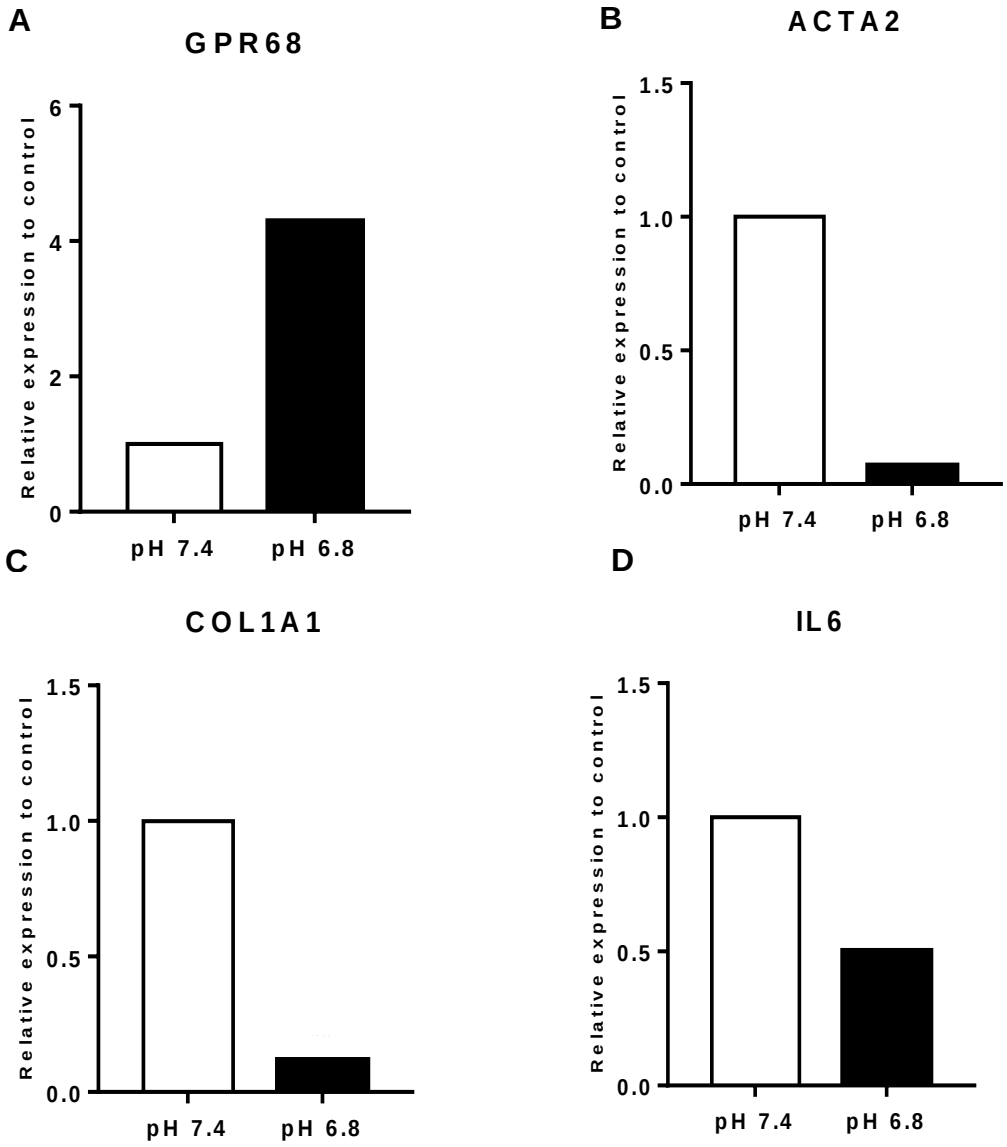

Figure 1: Gene expression determined by qRT-PCR of PSCs cultured in pH 7.4 or pH 6.8 media for 48 h. Expression of GPR68 (A), ACTA2 (B), COL1A1 (C), and IL-6 (D) of PSCs incubated at pH 6.8 was normalized to that of pH 7.4 media. Relative expression $=2^{(\Delta CtpH7.4-\Delta CtpH6.8)}$.The ΔCt values were measured in triplicate and presented as mean, n=1.

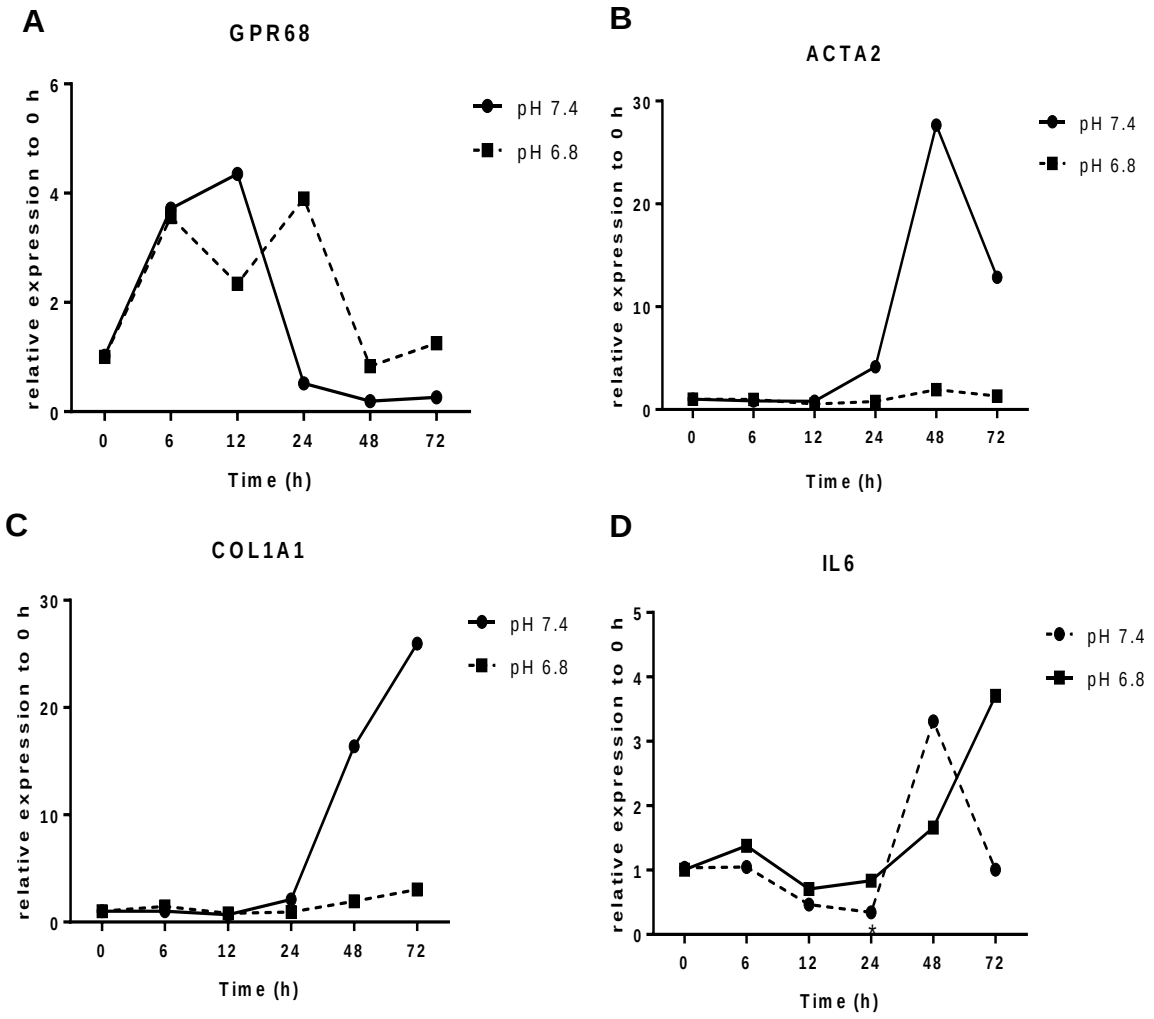

Figure 2: Gene expression determined by qRT-PCR of PSCs cultured for up to 72 h in media at pH 7.4 or pH 6.8. Expression of GPR68 (A), ACTA2 (B), COL1A1 (C), and IL-6 (D) of PSCs was normalized to that at 0 h. Note different scales for each gene. ΔCt values represent one experiment with three replicates presented as mean, n=1.

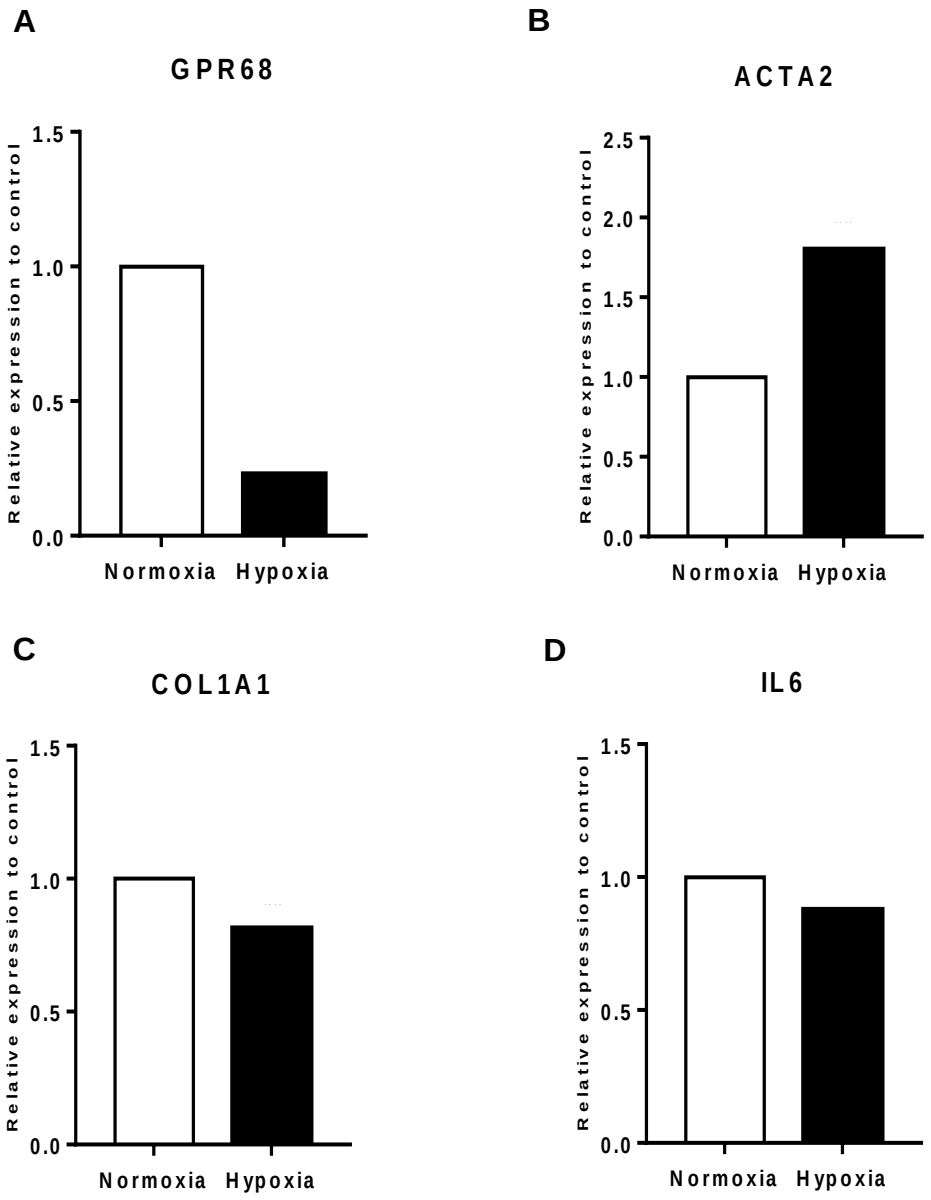

Figure 3: Gene expression determined by qRT-PCR of PSCs cultured at normoxia (21% oxygen) or hypoxia (2% oxygen) for 48 h. Expression of GPR68 (A), ACTA2 (B), COL1A1 (C), and IL-6 (D) of PSCs cultured in hypoxia was normalized to that from growth in normoxia. Relative expression $=2^{(\Delta Ctnormoxia-\Delta Cthypoxia)}$. The ΔCt values were measured in triplicate and are presented as mean, n=1.

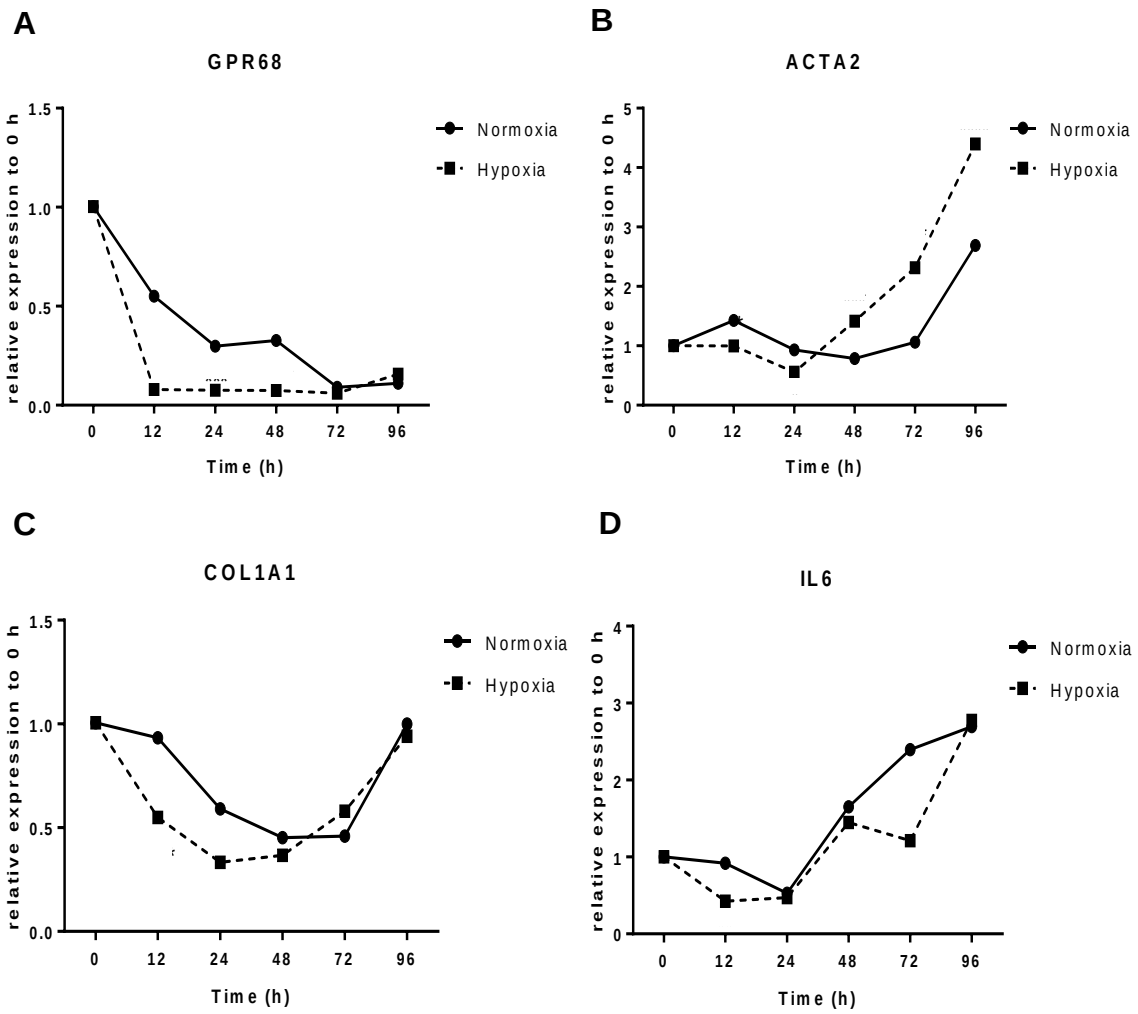

Figure 4: Gene expression determined by qRT-PCR of PSCs cultured in normoxia or hypoxia for up to 72 h. Expression by PSCs of GPR68 (A), ACTA2 (B), COL1A1 (C), and IL-6 (D) was normalized to 0 h. Note different scales for each gene. ΔCt values represent one experiment with three replicates presented as mean, n=1.

In order to assess the effect of PDAC cells on PSCs, a trans-well system was used to co-culture PSCs with three different pancreatic cancer cell lines (ASPC-1, BxPC, MiaPaca). Gene expression was measured for the aforementioned genes as well as other genes involved in fibrosis including COL1A2, COL3A1, connective tissue growth factor (CTGF, also known as CCN2), periostin (POSTN, also known as osteoblast-specific factor OSF-2), plasminogen activator inhibitor-1(PAI-1) and platelet-derived growth factor receptor α and β (PDGFRα/PDGFRβ). CTGF is a secreted protein that promotes fibrosis, migration, and myofibroblast activation and is up-regulated in CAFs[85,86]. POSTN is only expressed in activated CAFs and has been shown to promote proliferation and invasiveness of cancer cells[87]. PAI-1 (also called SERPINE1) promotes expression of collagen and ECM proteins as part of the wound healing response[88]. PAI-1 is highly expressed by PDAC cells and secreted by activated CAFs[89]. PDGFR is a marker of fibroblasts in many kinds of cancer[43]. All three cancer cell lines increased the expression of GPR68 in PSCs after co-culture for 48 h (Figure 5A). Co-culture with ASPC-1 and BxPC increased expression of αSMA (Figure 5B). All PDAC cell lines also increased expression of IL-6, CTGF, POSTN, and PAI-1 (Figure 5D, G, H, I) but co-culture had no significant effects on expression of collagens, PDGFRα, or PDGFRβ (Figure 5C, E, F, J, K).

Data from time course studies showed that co-culture of ASPC-1 cells with PSCs increased GPR68 and IL-6 by 24 h and αSMA by 48 h (Figure 6). GPR68 expression rapidly increased by 6 h in control and co-cultured cells. While in

control cells the expression decreased to basal level by 48 h, co-culture with ASPC-1 seemed to inhibit this decay. αSMA showed a different pattern of expression with steady increases starting at 24 h in control cells and even larger increases in co-cultured cells. The differences in kinetics of gene expression suggest that different mechanisms mediate expression of GPR68 and αSMA. BxPC co-culture showed a similar pattern with both GPR68 and IL-6 expression increasing earlier than αSMA expression (Figure 7). With MiaPaca co-culture, GPR68 and IL-6 expression increased at 24 h but no significant increase was seen in αSMA expression (Figure 8). Overall, these data imply that PDAC cells drive tumorigenesis by secreting factors that can influence the transformation of PSCs to CAFs and by regulating expression of multiple genes, including GPR68. Among the genes I assessed, IL-6 consistently had the largest response, with the highest increase of 55-fold.

In order to identify molecules involved in these gene expression changes, I treated PSCs with two cytokines that PDAC cells are known to produce: TGFβ and TNFα[32]. TGFβ is reportedly involved in the conversion of PSCs into CAFs[23]. Indeed, while TGFβ had little effect on GPR68 expression, 48 h treatment with TGFβ increased αSMA expression 4-fold in PSCs (Figure 9B). COL1A1, CTGF and PDGFRβ expression also increased, consistent with the idea that TGFβ contributes to the transformation of PSCs to CAFs (Figures 9C, G, K and Figure 10).

After 48 h incubation with TNFα, expression of GPR68 in PSCs increased ~15-fold and that of IL6 increased more than 10-fold (Figure 9A, D). In addition, CTGF, PAI1, PDGFRα and PDGFRβ slightly increased in expression in response to treatment with TNFα (Figures 9G, I, J, K). However incubation with TNFα did not significantly increase expression of αSMA and COL1A1, two key fibrotic marker genes (Figure 9B, C). TNFα consistently up-regulated GPR68 expression by PSCs at time points from 6 to 48 h (Figure 11A). A significant increase in IL-6 expression was also observed (Figures 11D).

Together, these data suggest that increased fibrotic activities and increased GPR68 in pancreatic cancer-associated fibroblasts are two independent events regulated by different mechanisms. While TGFβ appears to be important for the conversion of PSCs to myofibroblast-like CAFs, TNFα is a cytokine that up-regulates GPR68 expression. This finding is consistent with previous data showing that TNFα increased GPR68 expression in monocytes[76].

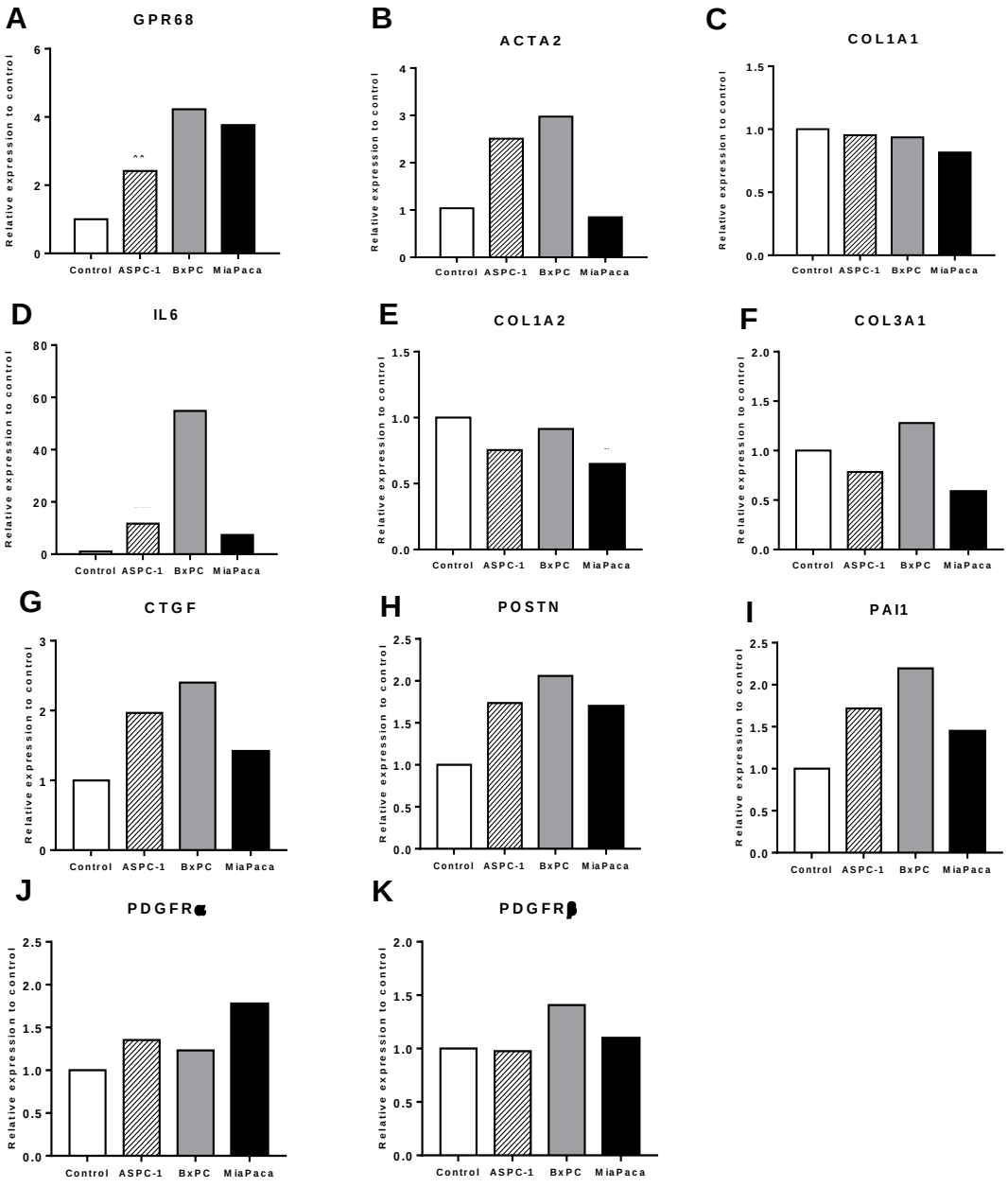

Figure 5: Gene expression, determined by qRT-PCR, of PSCs co-cultured with PDAC cell lines ASPC-1, BxPC, or MiaPaca for 48 h. Gene expression was normalized to control cells and calculated as relative expression $=2^{(\Delta Ctcontrol-\Delta Ctco\text{-}culture)}$. The ΔCt value was measured in triplicate samples and is presented as mean, n=1.

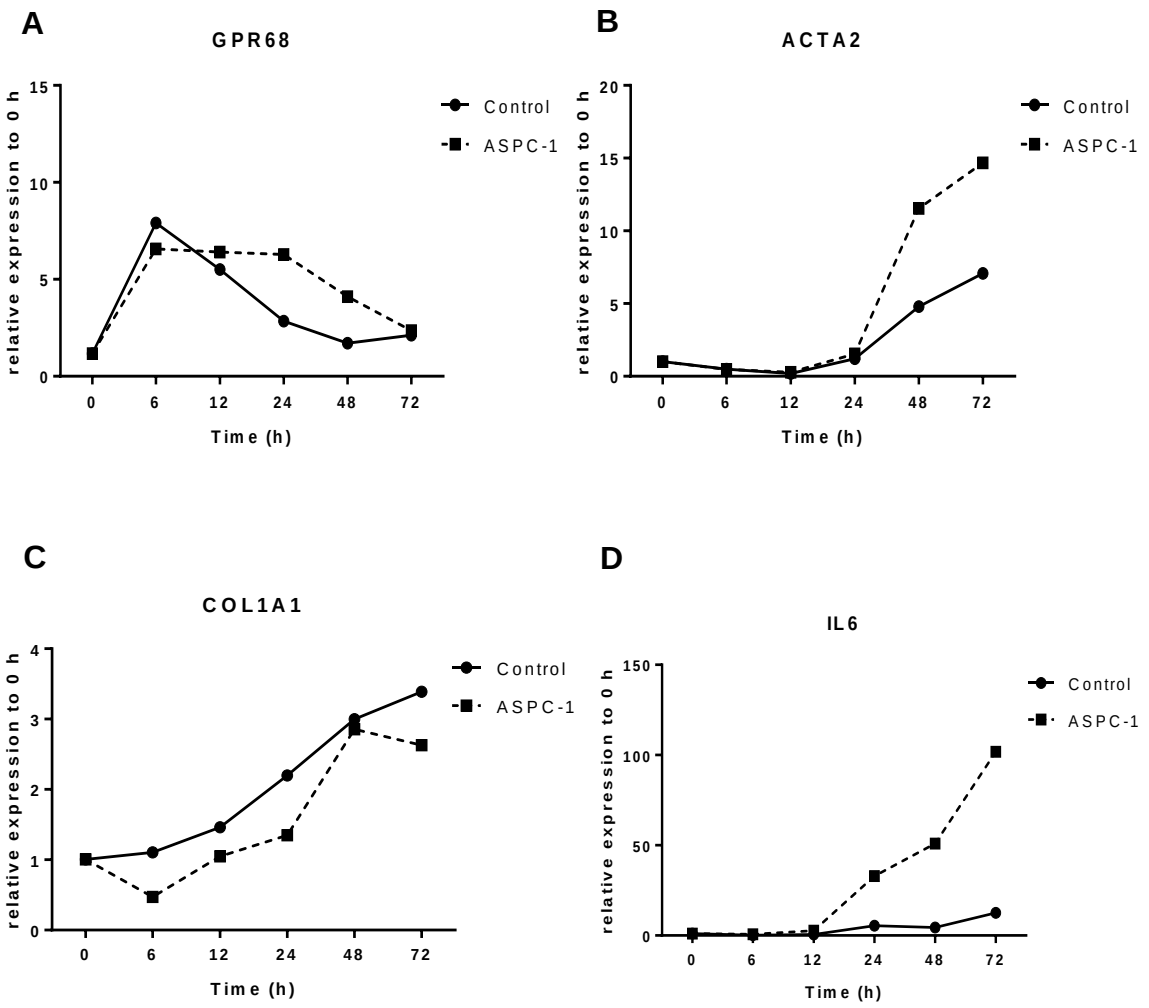

Figure 6: Gene expression determined by qRT-PCR of PSCs co-cultured with PDAC cells line ASPC-1 for up to 72 h. Gene expression was normalized to values at 0 h. Note different scales for each gene. ΔCt values represent one experiment with triplicates presented as mean, n=1.

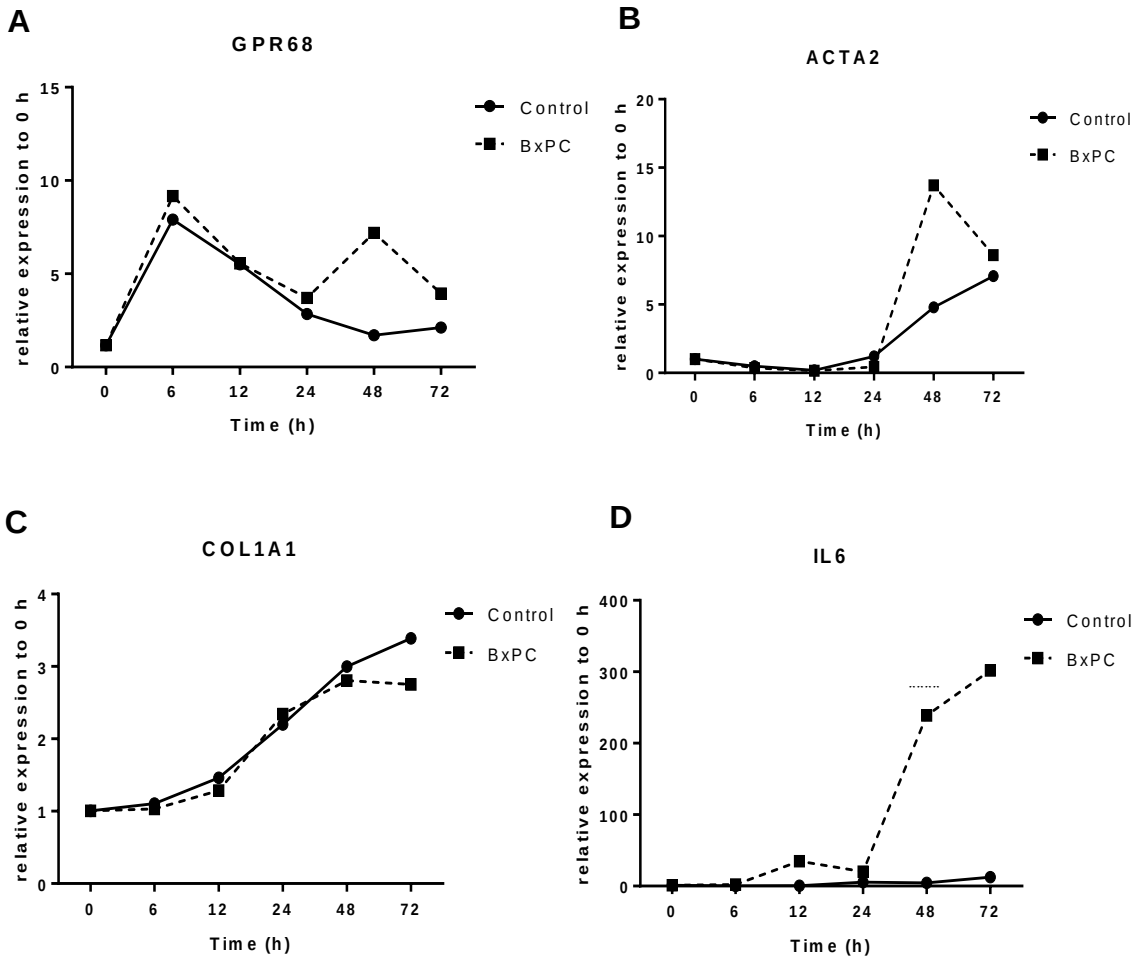

Figure 7: Gene expression determined by qRT-PCR of PSCs co-cultured with PDAC cell line BxPC for 0 to 72 h. Gene expression was normalized to values at 0 hour. Note different scales for each gene. ΔCt values represent one experiment with triplicates presented as mean, n=1.

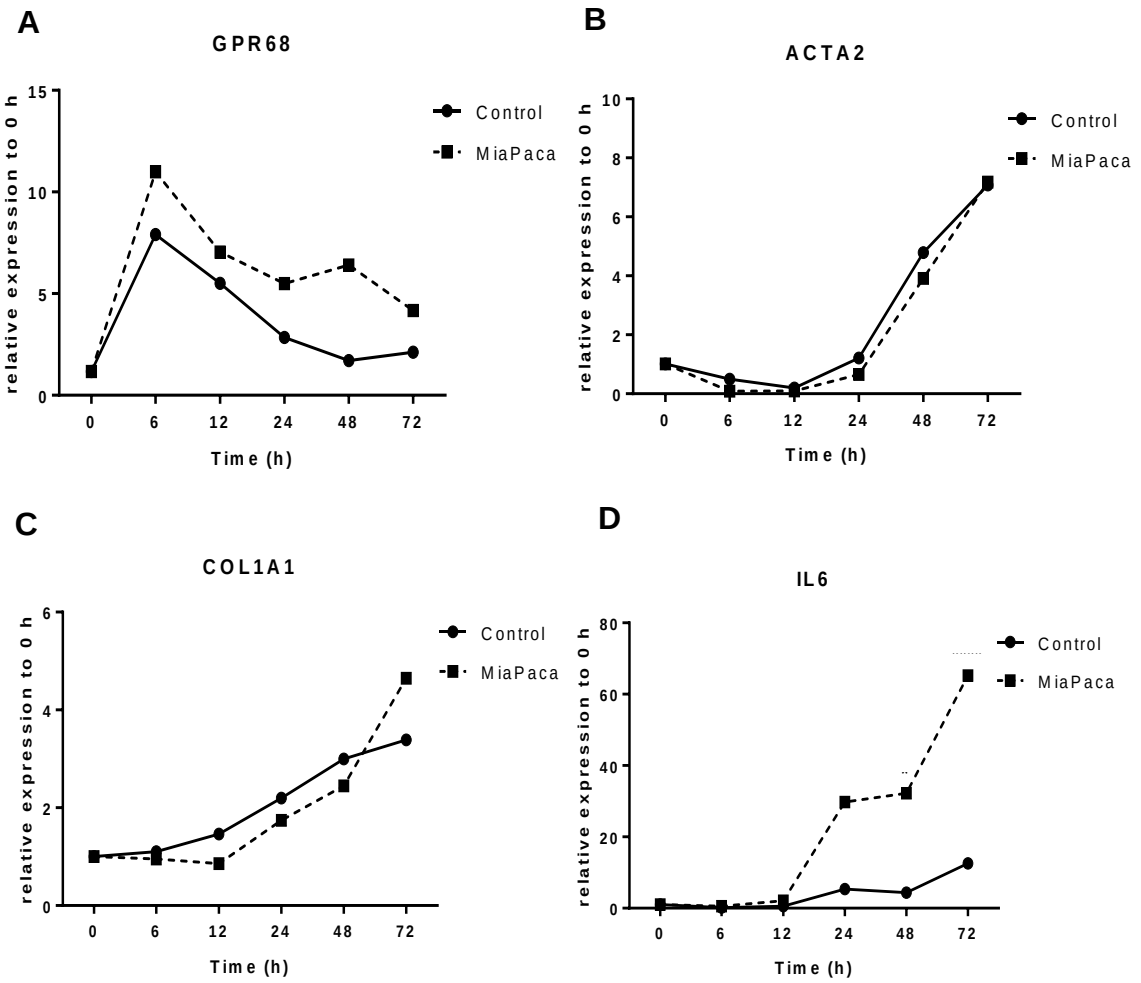

Figure 8: Gene expression determined by qRT-PCR of PSCs co-cultured with PDAC cell line MiaPaca for up to 72 h. Gene expression was normalized to that at 0 h. Note different scales for each gene. ΔCt values represent one experiment with triplicates presented as mean, n=1.

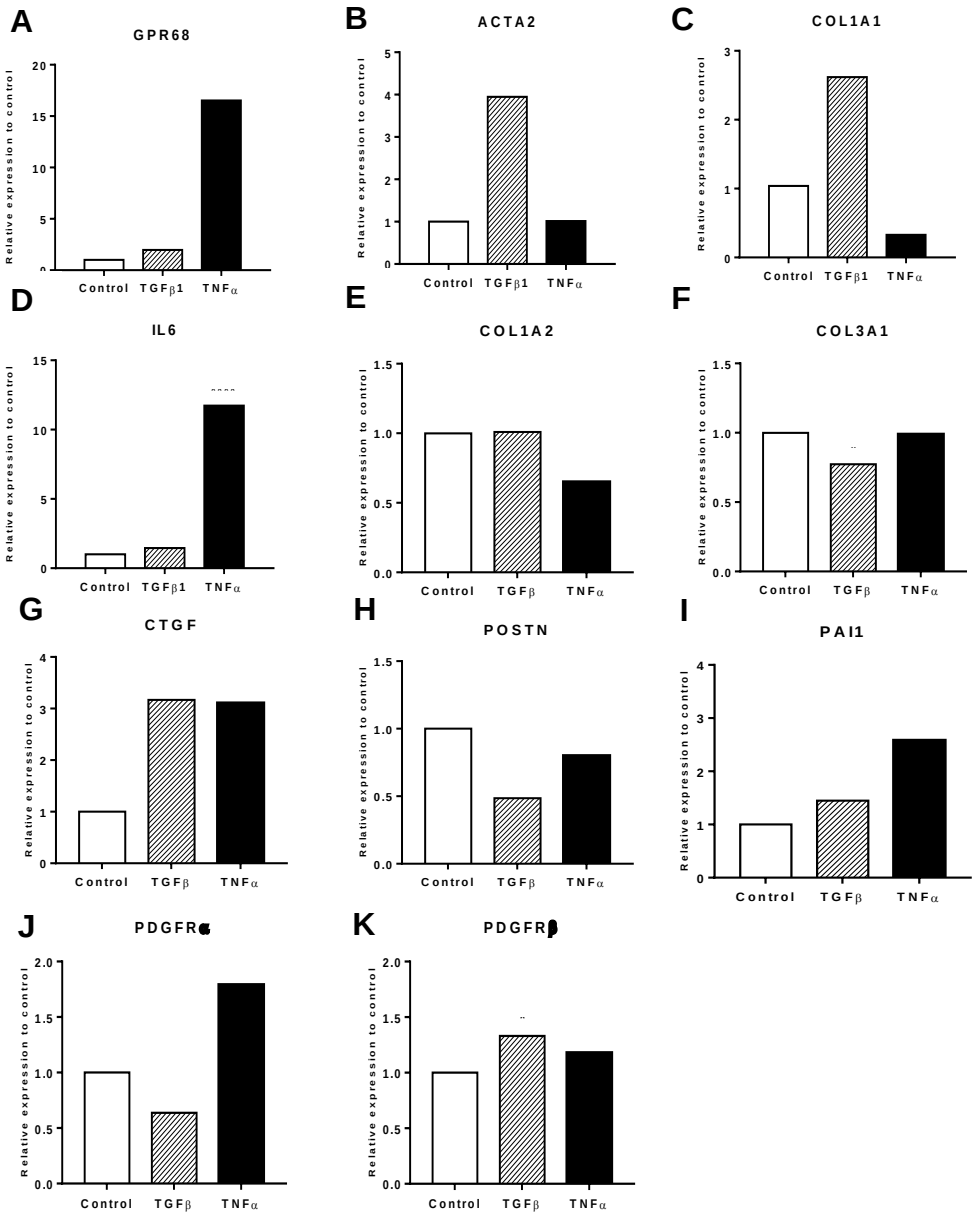

Figure 9: Gene expression determined by qRT-PCR of PSCs incubated with TGFβ or TNFα (50 ng/mL) for 48 h. Gene expression of PSCs treated with TGFβ or TNFα was normalized to control and calculated as relative expression $=2^{(\Delta CtTGF\beta/TNF\alpha-\Delta Ctcontrol)}$. ΔCt values were assayed in triplicate and are presented as mean, n=1.

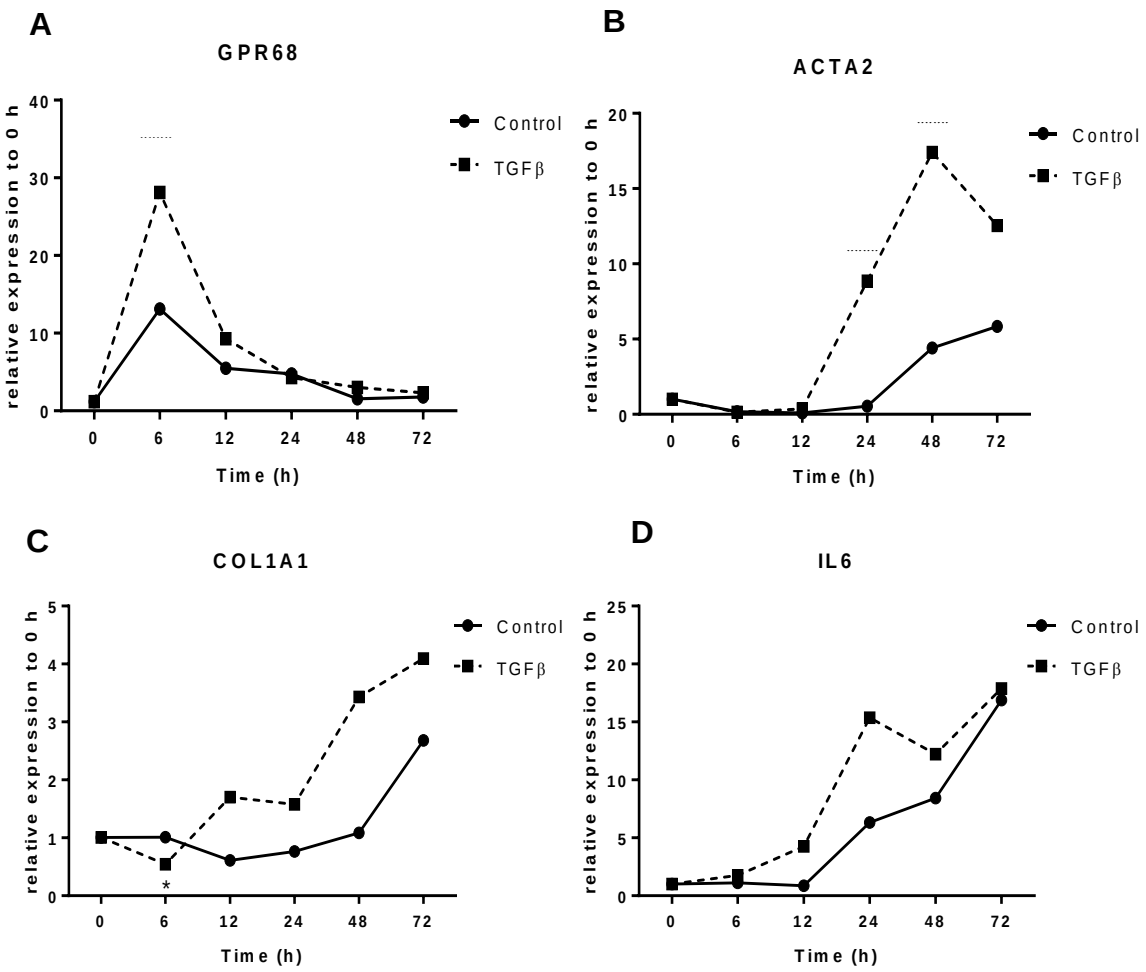

Figure 10: Gene expression, determined by qRT-PCR, of PSCs incubated with TGFβ (50 ng/ml) for up to 72 h. Gene expression was normalized to that at 0 hour. Note different scales for each gene. ΔCt values represent one experiment with triplicates presented as mean, n=1.

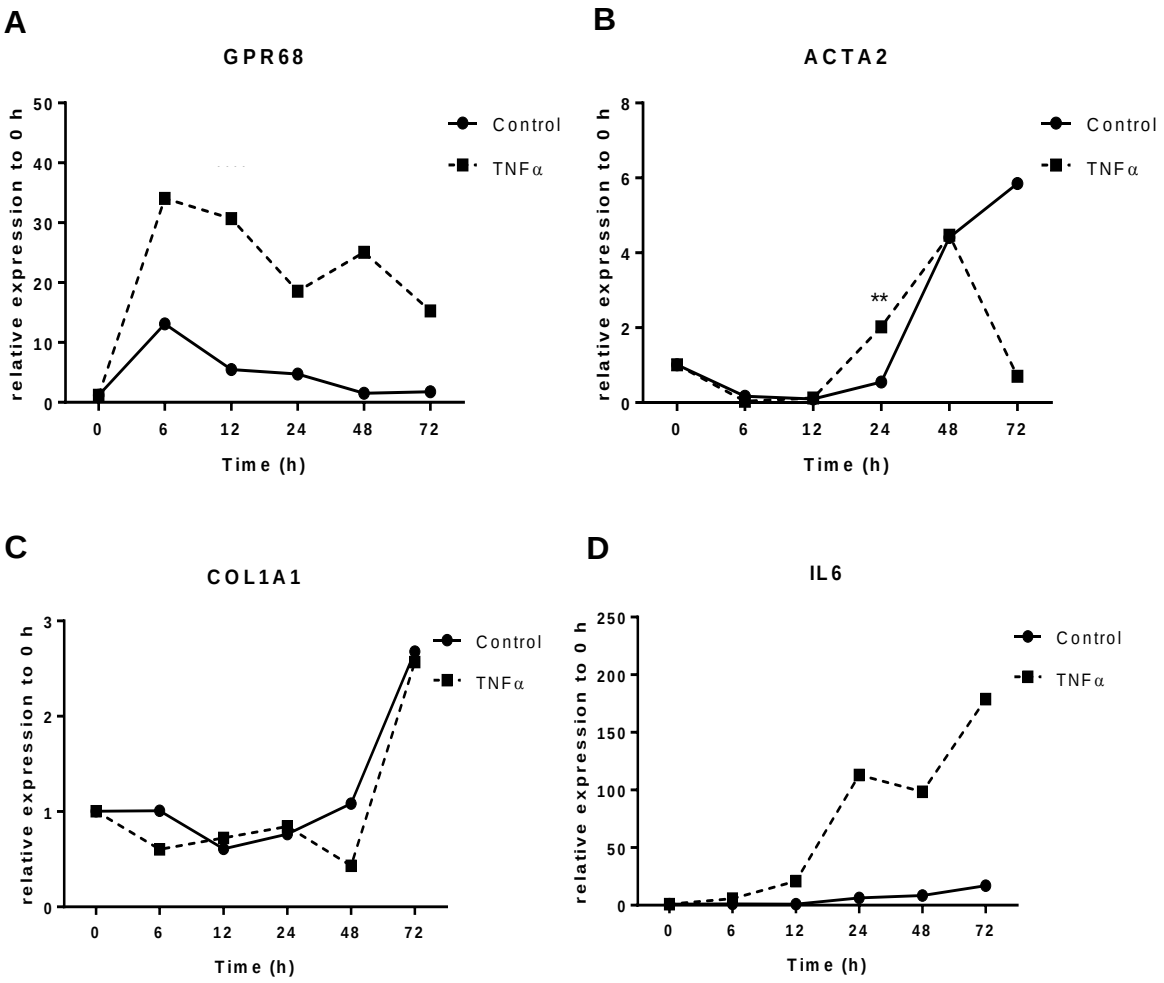

Figure 11: Gene expression determined by qRT-PCR of PSCs treated with TNFα (50 ng/ml) for up to 72 h. Gene expression was normalized to values at 0 h. Note different scales for each gene. ΔCt values represent one experiment with triplicates presented as mean, n=1.

GPR68 regulates the expression and secretion of IL-6 in CAFs

As shown in Figure 11 (Figures 11 A and 11D), I found that TNFα treatment of PSCs increased expression of both GPR68 and IL-6 but that and up-regulation of GPR68 occurred 6 hours earlier than that of IL-6. This led me to hypothesize that GPR68 regulates IL-6 expression in pancreatic CAFs. To test this hypothesis, I used both gain-of-function and loss-of-function experiments to assess the effect of GPR68 on IL-6 expression. GPR68 cDNA was transfected into PSCs for 24 h, resulting in increased levels of GPR68 mRNA and GPR68 protein (Figure 12). This increase in GPR68 expression was associated with an increase in IL-6 expression compared to PSCs transfected with a control vector (Figure 12). siRNA knockdown of GPR68 in CAFs decreased GPR68 mRNA and protein levels and significantly decreased IL-6 mRNA (Figure 13) and IL-6 protein (assessed by immunofluorescences, (Figure 14).

To assess the impact of GPR68 on cell proliferation, CAFs with and without GPR68 knockdown were cultured at pH 7.4 or pH 6.8 for 96 h. I observed no difference in proliferation within the first 24 h. After 48 h more CAFs were detected at pH 7.4 than at pH 6.8 but no significant difference in proliferation was observed between CAFs subjected or not to GPR68 knockdown (Figure 15). These data imply that GPR68 does not affect proliferation of CAFs.

A collagen synthesis assay was used to assess the impact of GPR68 on the production of collagen by CAFs. CAFs with and without GPR68 knockdown

and grown for 48 h at pH 7.4 or 6.8 were assayed. No significant difference was observed in CAFs with or without GPR68 knockdown (Figure 16). GPR68 thus appears not play a direct role in the ECM production of CAFs.

To further evaluate regulation of IL6 by GPR68, CAFs that were or were not subjected to GPR68 knockdown were cultured at pH 7.4 and pH 6.8 for 6 h. In the control CAFs, activation of GPR68 with pH 6.8 increased IL-6 expression ~10-fold, whereas in the CAFs with GPR68 knockdown, IL-6 was increased only 5-fold by incubation at pH 6.8 (Figure 17). Consistent with those results, CAFs released more IL-6 at pH 6.8 into the extracellular media and the amount of IL-6 in the media was less from CAFs in which GPR68 was knocked down by siRNA (Figure 18). These results showed that GPR68 positively regulates the production and secretion of IL-6 from CAFs.

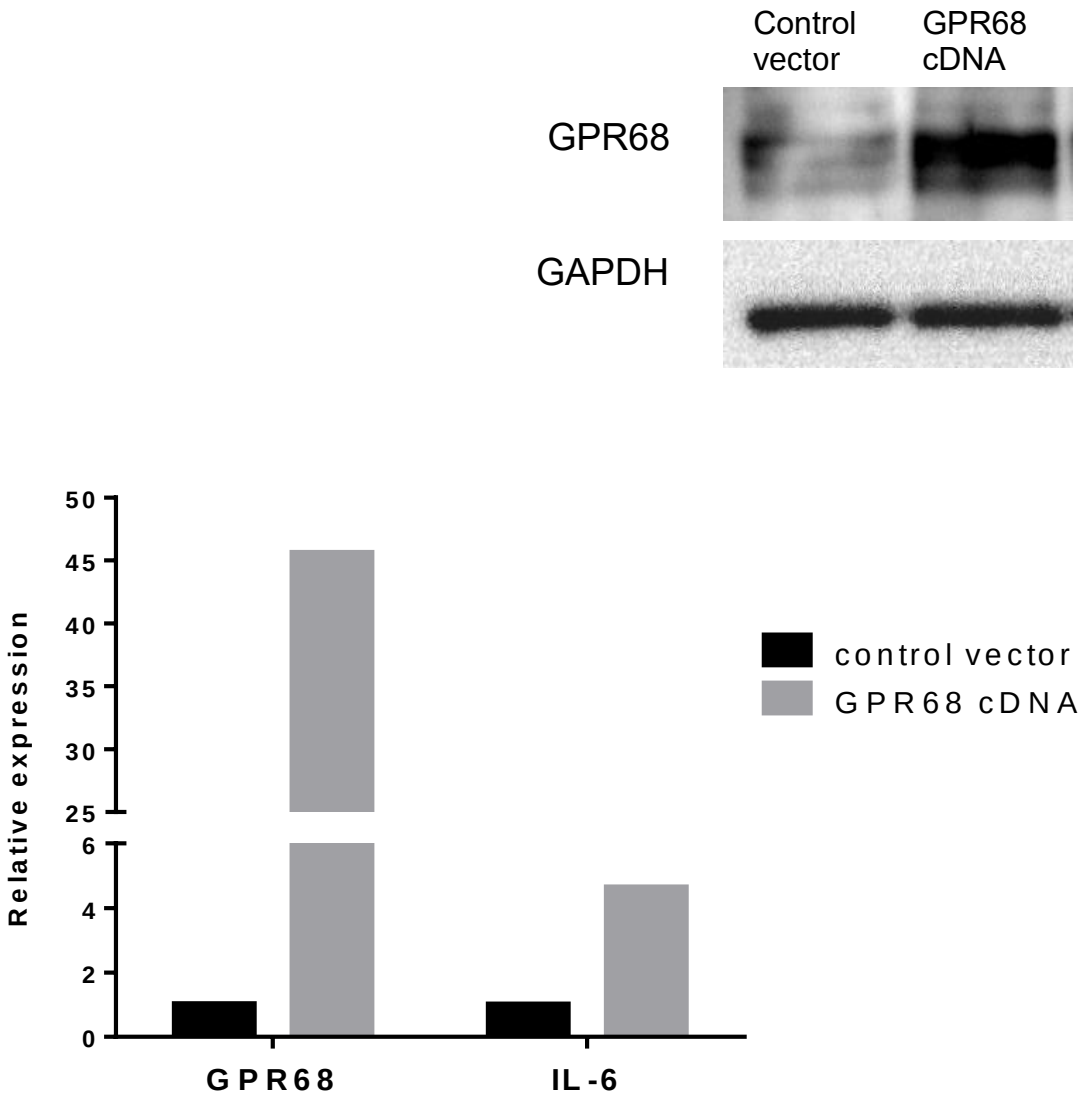

Figure 12: Increasing expression of GPR68 in PSCs by plasmid transfection increases IL-6 expression. Gene expression (by qPCR) of GPR68 and IL6 in PSCs transfected with GPR68 cDNA was normalized to control vector. Relative expression $=2^{(\Delta Ctcontrol-\Delta CtGPR68)}$. The ΔCt values were assayed in triplicate and shown as mean, n=1. The inset shows the increase in GPR68 protein expression in PSCs transfected with the GPCR68 construct.

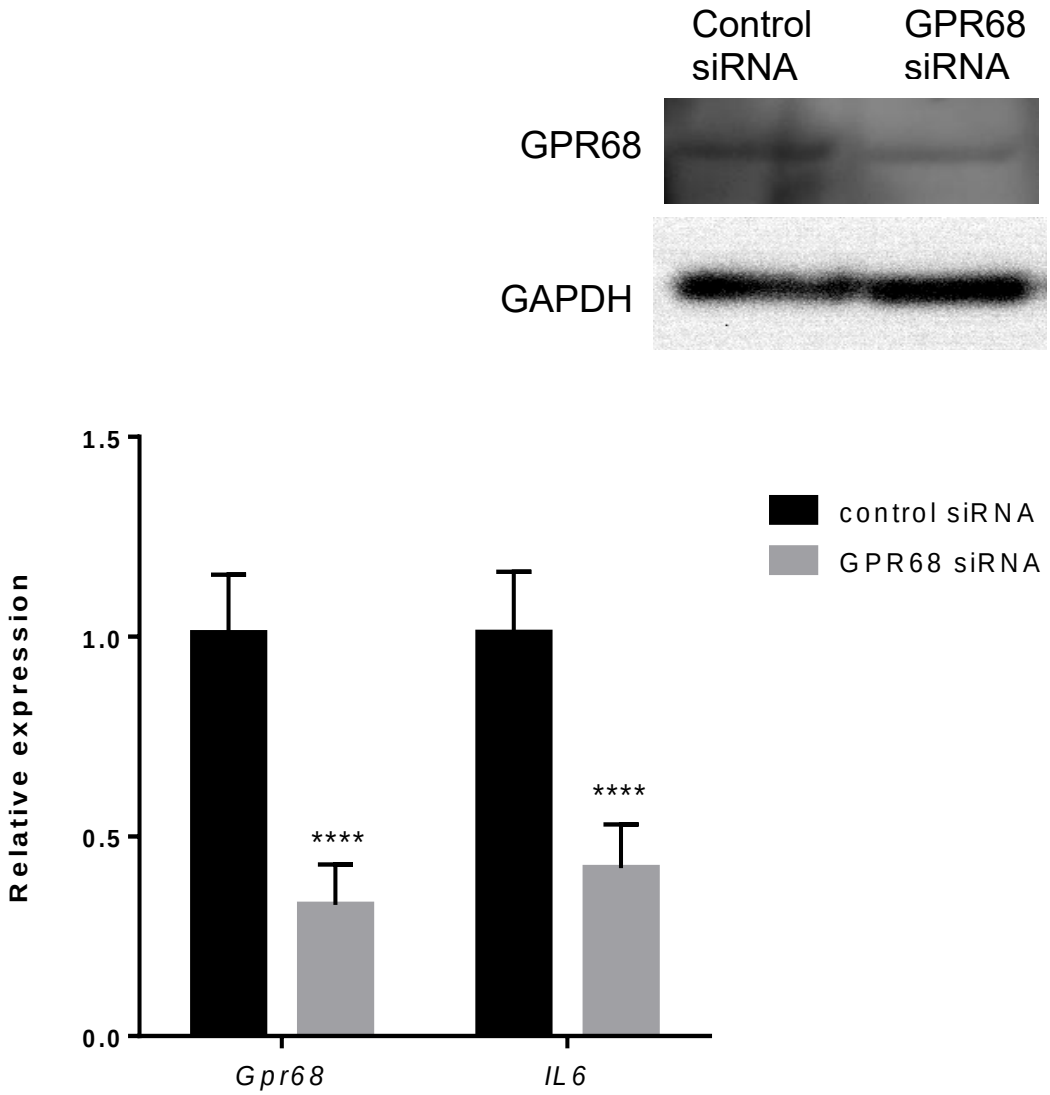

Figure 13: siRNA knockdown of GPR68 in CAFs decreases IL-6 expression. Gene expression (by qPCR) of GPR68 and IL6 in CAFs transfected with GPR68 siRNA was normalized to control siRNA. Relative expression $=2^{(\Delta Ctcontrol-\Delta CtGPR68)}$. ΔCt values were measured in triplicate and shown as mean±SD for n=3. Unpaired t-test, *p<0.05, **p<0.01, ***p<0.001. The inset shows the decrease in GPR68 protein expression in CAFs transfected with the GPCR68 siRNA construct.

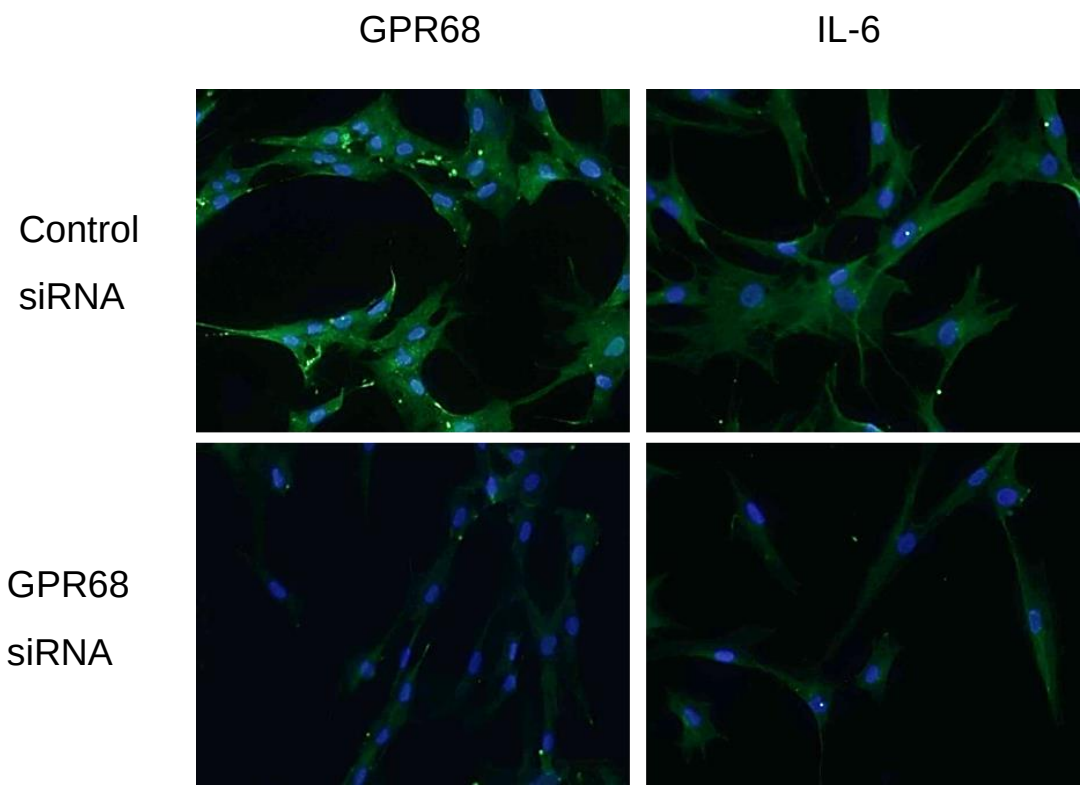

Figure 14: Immunofluorescence staining of GPR68 and IL6 in CAFs and CAFs with GPR68 knockdown. Blue (DAPI) and Green (GPR68/IL6).

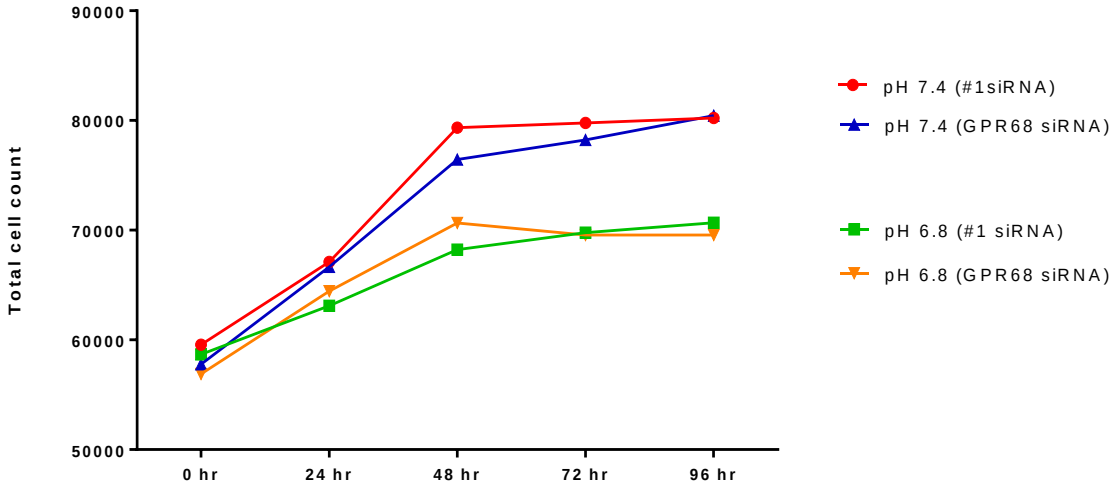

Figure 15: Cell growth curve (over 96 h) of CAFs and CAFs with GPR68 knockdown in pH 7.4 and pH 6.8 media, n=2.

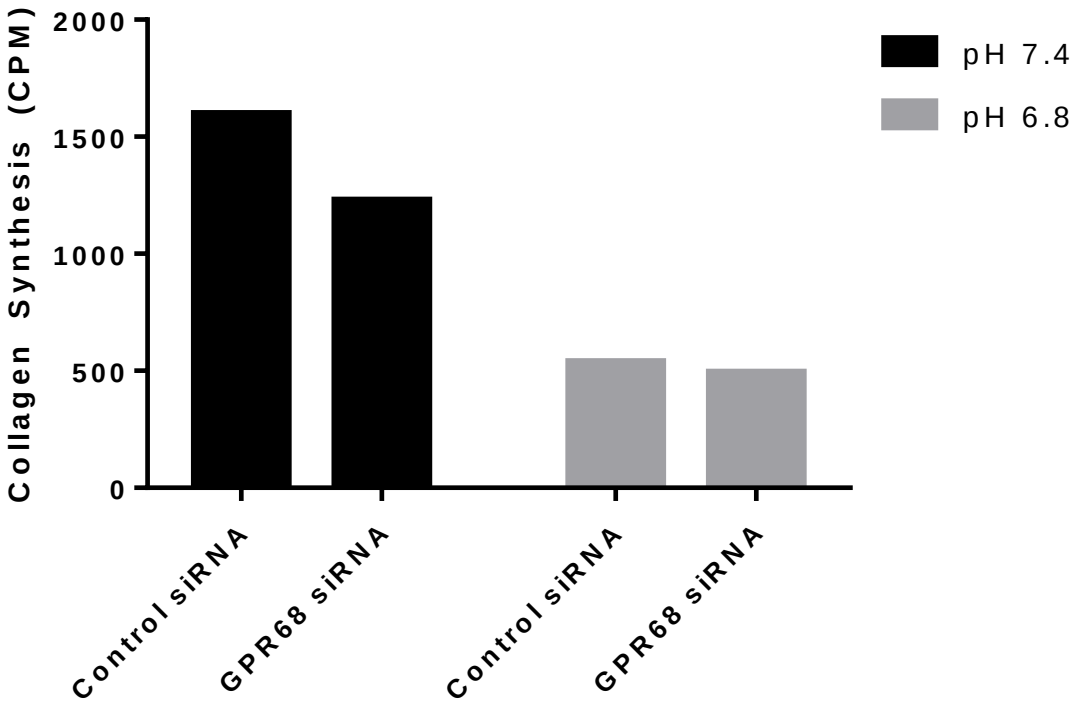

Figure 16: Collagen synthesis in CAFs subjected or not to GPR68 knockdown and cultured in pH 7.4 or pH 6.8 media, n=1.

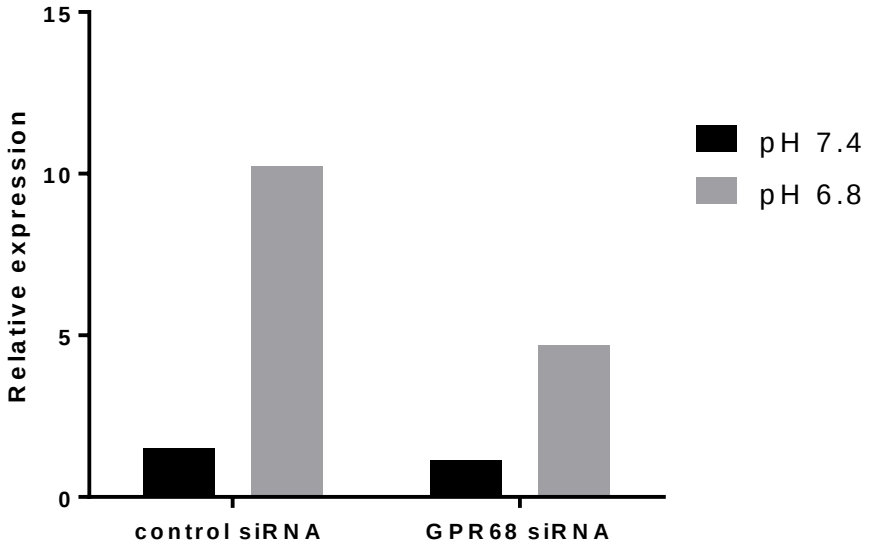

Figure 17: qPCR analysis of IL6 mRNA expression in CAFs and CAFs with GPR68 knockdown in both pH 7.4 and pH 6.8 media. Expression of IL6 mRNA was normalized to that of control siRNA at pH 7.4. The ΔCt value was measured in triplicate samples and are shown as mean, n=1.

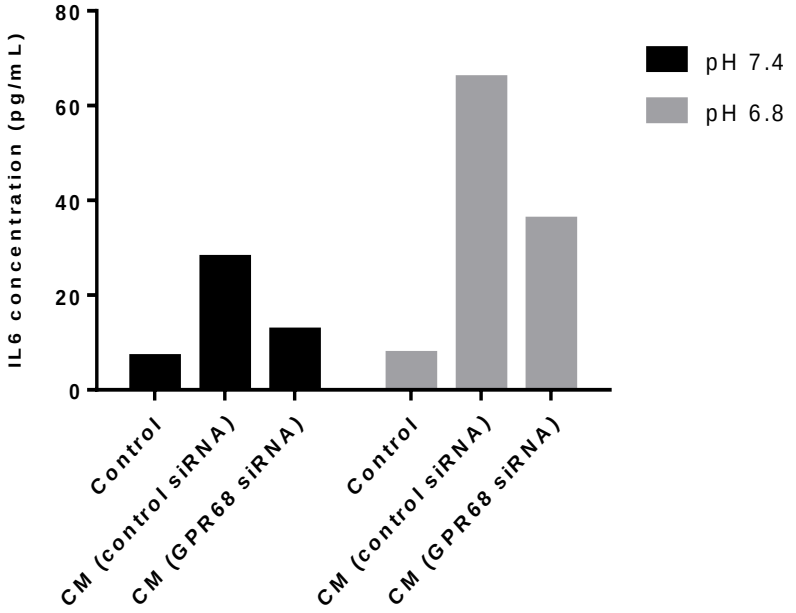

Figure 18: ELISA analysis of IL6 protein secreted in pH 7.4 and pH 6.8 media by CAFs and CAFs with GPR68 knockdown. Each data point was measured in triplicate and data are shown as mean, n=1.

GPR68 signals through Gαs linked signaling pathway

I sought to begin to assess the signaling pathway through which GPR68 increases the production and secretion of IL-6. Assay of cAMP revealed that CAFs treated with a dose-dependent lowering of pH (pH 7.4-6.4) had increased amounts of cAMP; siRNA knockdown of GPR68 in CAFs abrogated the low pH induced increase in cAMP (Figure 19). These data imply indicate that GPR68 is able to increase the activity of the heterotrimeric GTP binding protein, Gαs, which activates adenylyl cyclase to increase cellular cAMP concentrations. The increase in cAMP, in turn, activates PKA to phosphorylate and activate the transcription factor CREB. Upon activation, phosphorylated CREB translocates to the nucleus and alters gene transcription. I found that treatment of CAFs with specific PKA inhibitors H89 and PKI both abolish the low pH-induced IL-6 up-regulation in CAF even though treatment of CAFs with H89 and PKI did not affect GPR68 expression in CAFs (Figure 20), thus implying a role for PKA in the low-pH-promoted increase in IL-6 expression..

I also assayed Ser^{131} phosphorylation of CREB (the PKA site of phosphorylation). CAFs cultured at lower pH had increased CREB- Ser^{131} phosphorylation of CREB (Figure 21), results that support the idea that GPR68 regulates IL-6 expression through the Gs/cAMP/PKA signaling pathway.

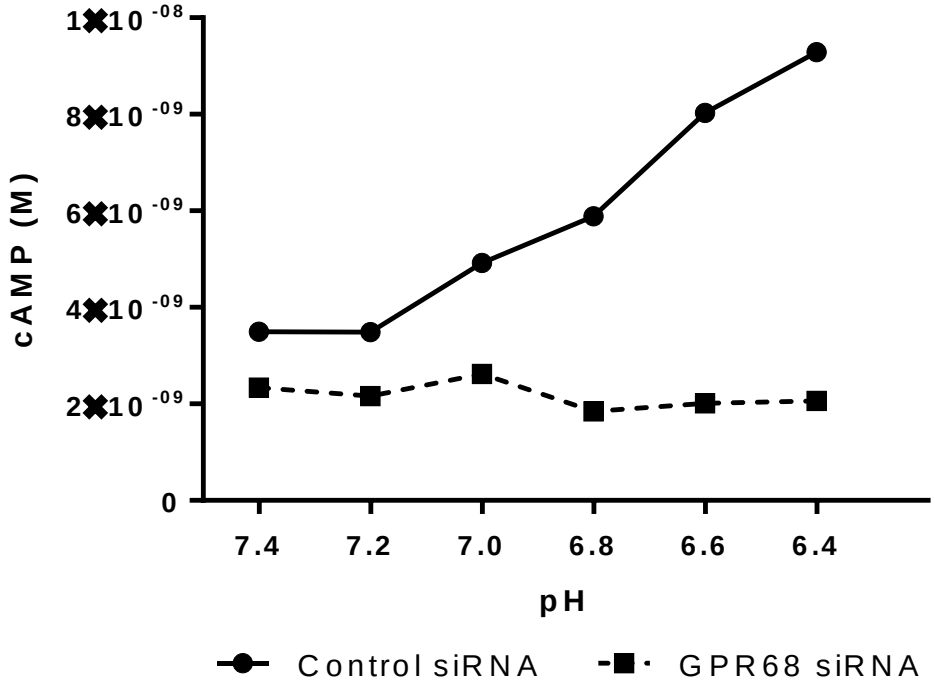

Figure 19: cAMP assay of CAFs treated with pH media shows an increase in cAMP with a lowering of pH. siRNA knockdown of GPR68 in CAFs decreases cAMP production. Each data point was measured in triplicate and data are shown as mean, n=1.

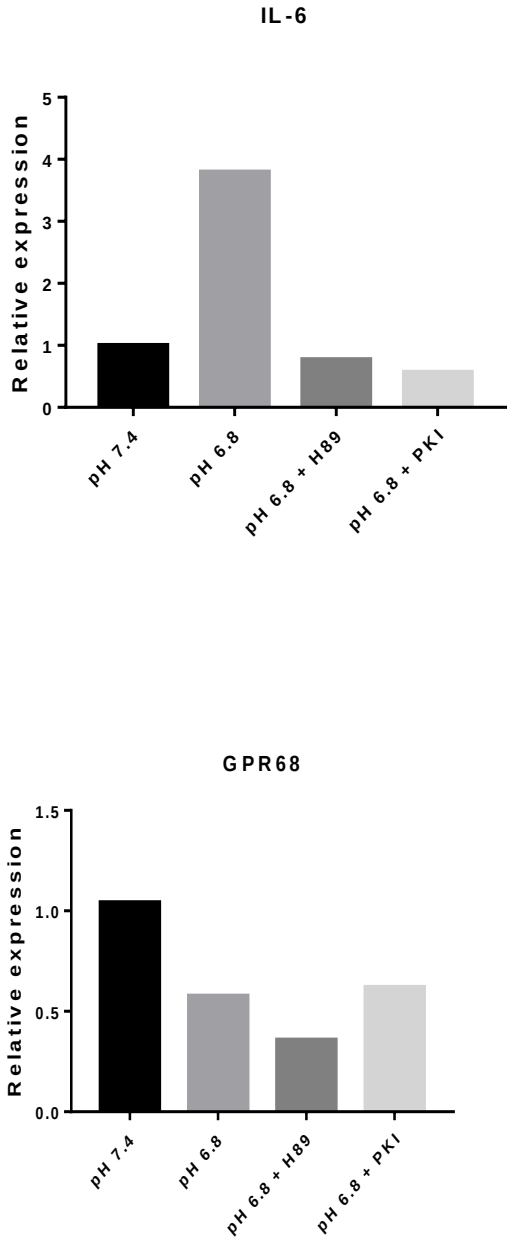

Figure 20: Treatment of CAFs with PKA inhibitors H89 and PKI in pH 6.8 significantly decrease IL-6 expression as determined by qRT-PCR. Relative expression was normalized to pH 7.4 as fold change. Each data point was measured in triplicates and results are presented as mean, n=1.

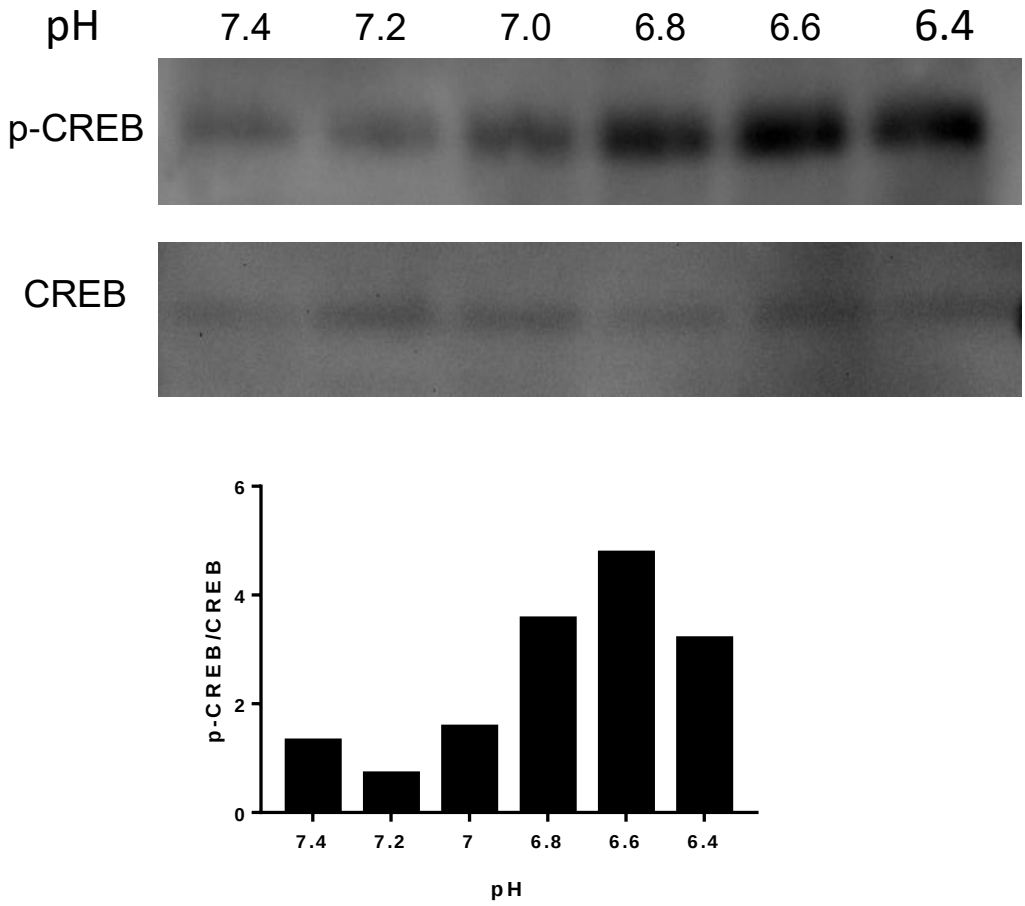

Figure 21: CREB Ser^{131} phosphorylation in CAFs increases as the extracellular pH is decreased from pH 7.4 to 6.4s determined by Western blot. Densitometry quantification calculated as p-CREB/CREB, n=1.

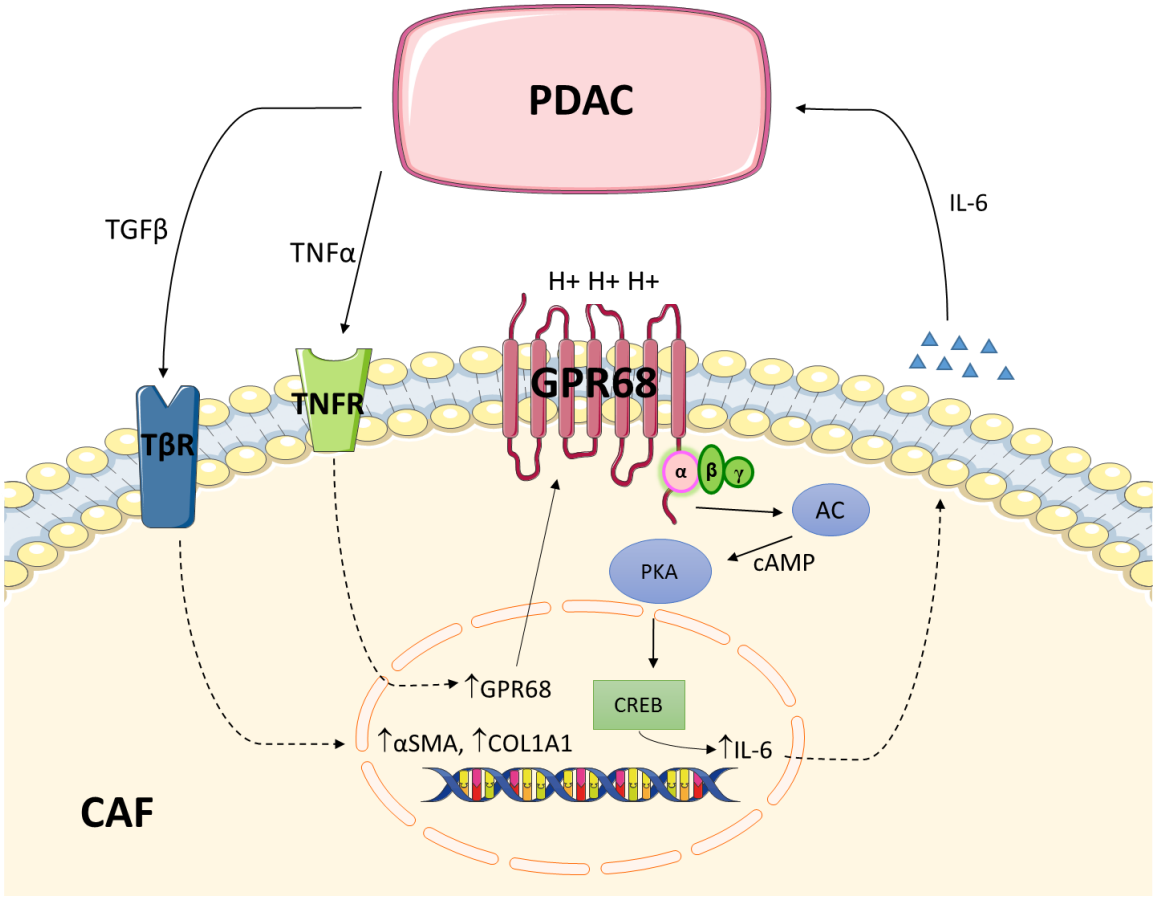

Figure 22: Summary of the crosstalk between PDAC cells and CAFs. PDAC cells secrete TGFβ which increase expression of fibrotic markers. PDAC cells secrete TNFα which up-regulates GPR68. Activation of GPR68 by protons increase cAMP which activates PKA. PKA phosphorylates CREB which leads to the expression and secretion of IL-6.

DISCUSSION

The data in this thesis project revealed a specific pathway and mechanism for the bi-directional interaction between PDAC cells and CAFs (Figure 22). I discovered that PDAC cells signal to PSCs to up-regulate the expression of GPR68 through TNFα; however other factors could also be involved in regulating GPR68 expression. Consistent with previous data[23], treatment of PSCs with TGFβ increased the expression of fibrotic markers, implying that TGFβ is likely a main regulator of the conversion of PSCs to CAFs. Previous *in silico* analysis revealed putative DNA-binding sites for NF-κB and HIF-1α in the GPR68 promotor region[76]. This agrees with the result that TNFα up-regulates GPR68 in PSCs because NF-κB is a downstream transcription factor of TNFα signaling[90].

I tested the effect of low pH and hypoxia on GPR68 expression as a way to see if HIF-1α is another potential transcription factor for GPR68. However, experiments with low pH media and hypoxia showed opposite effects. The spontaneous activation of PSCs into CAFs seemed to be inhibited by the low pH media as the fibrotic markers had lower expression. Although hypoxic cell culture conditions lowered the media pH compared to normoxia (data not shown), hypoxia increased some fibrotic markers but not others. Acidosis and hypoxia are present in the tumor microenvironment but each of these factors alone may not be sufficient to activate PSCs. It will be interesting to evaluate the expression of e GPR68 expression by a combination of hypoxia and in a co-culture system,

perhaps preferably a 3 –D culture system. These two factors might have additive or synergistic effects on regulating GPR68 expression.

In almost every experiment with PSCs, the control cells showed a rapid, short-lived increase in GPR68 expression. Expression decreased over time to return to basal level at around 24-48 h. In the co-culture samples, expression of GPR68 rose at 6 h but continued at high levels past 24 h. Only TNFα-treated samples had a consistently high expression of GPR68. This result agrees with previous studies that showed a role for TNFα in regulating GPR68 expression[76]. My findings seem to demonstrate the instability of GPR68 mRNA in cells and suggest a rapid turnover rate. The pattern of increased expression was different for fibrotic markers, which tended to increase at a delayed time point (~24 h) and remain high through the next 48 h. These distinct kinetic patterns further highlight the idea that the up-regulation of GPR68 and of fibrotic markers are promoted through different mechanisms.

Significant increases of IL-6 expression seemed to follow increasing GPR68 which lead to my hypothesis that GPR68 might regulate IL-6 production in CAFs. Indeed, the correlation between GPR68 and IL-6 was confirmed by overexpression and knockdown experiments. Furthermore, both mRNA and protein levels of IL-6 were increased at pH 6.8 where GPR68 is fully activated. These data show that GPR68 functions in CAFs to positively regulate the expression and secretion of IL-6. GPCRs are involved in many physiological

processes but GPR68 did not seem to be involved in the growth or survival of CAFs nor in CAF fibrotic activities, including the synthesis of collagens. This may mean that GPR68 is not involved in the fibrotic nature of the CAFs themselves but rather contributes to the secretome of CAFs, especially since CAFs secrete many cytokines and growth factors. It will be interesting to investigate which other entities in CAF secretomes are regulated by GPR68. Indeed, a recent study showed that GPR68 regulates IL-8 expression in pancreatic beta cells[91].

Although GPR68 has been discovered to couple to different Gα proteins, I found that GPR68 regulates IL-6 expression through the Gαs signaling pathway. Activation of the receptor by extracellular protons leads to increased production of cAMP which then activates PKA. In turn PKA phosphorylates CREB as the likely mechanism that increases expression of IL-6[92]. It will be important to assess other signaling pathways that might mediate actions of GPR68. One such pathway might involve $G\alpha_q$ coupling since products of such coupling can regulate cAMP formation in other cell types[93].

Many recent studies of pancreatic cancer focus on the significant role of the tumor microenvironment and CAFs. CAFs have been shown to promote tumorigenesis and chemoresistance of PDAC cells, making CAFs an important therapeutic target[28]. Blocking the activation of CAFs can decrease the survival of tumor cells[94]. Based on data in this project, blocking TNFα or TGFβ could be possible therapies for PDAC. However, further research is necessary to confirm

whether these cytokines are the main drivers of CAF activity. There may be other signals secreted by cancer cells that can be elucidated and studied.

The secretion of signals from CAFs helps to drive the development of PDAC[27]. Preventing or decreasing the secretion of cytokines from CAFs is another option for PDAC therapy. GPR68 plays an important role in the cytokine signaling of CAFs and particularly of IL-6. IL-6 has been shown to play a role in the proliferation, invasion, and chemoresistance of PDAC cells[95]. Pancreatic cancer patients are also often characterized by a high amount of IL-6 in their blood serum[96]. Inhibiting the activation of GPR68 and therefore secretion of IL-6, is a novel strategy for pancreatic cancer therapy. There is now a need for the identification of a specific GPR68 antagonist. An important future direction is to screen drugs or antibodies that can block the activity of GPR68 and can decrease the powerful effect that CAFs have on PDAC. Furthermore, *in vivo* studies of pancreatic tumors in both control and GPR68 knockout mice should be used to test the function of GPR68 in PDAC as well as the effects on such tumors of drugs that inhibit GPR68.

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