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The CXCR2-dependent role of cancer-associated fibroblasts in pancreatic ductal adenocarcinoma

Mohammad Awaji
University of Nebraska Medical Center

THE CXCR2-DEPENDENT ROLE OF CANCER-ASSOCIATED FIBROBLASTS IN PANCREATIC DUCTAL ADENOCARCINOMA

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Mohammad Awaji

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Supervisory Committee:

Surinder K Batra, Ph.D.

Joyce Solheim, Ph.D.

Benjamin J. Swanson, M.D., Ph.D.

Sushil Kumar, Ph.D.

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Mohammad Awaji, Ph.D.

University of Nebraska Medical Center, 2019

Supervisor: Rakesh K. Singh, Ph.D.

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, the fourth leading cause of cancer-related deaths in the USA with over 40,000 deaths per year. Unlike other major cancer types, the progress in dealing with PDAC is plodding, attributed mainly to the asymptomatic nature of the disease, the late diagnosis and the ineffectiveness of current therapies. A better understanding of the biology of the disease could permit the discovery of novel diagnostic and therapeutic tools. With that in mind, we present this dissertation that investigates the tumor-stromal interaction underlined by genetic alterations and inflammation. PDAC develop as a consequence of the accumulation of genetic mutations like Kras. Oncogenic Kras is known to propagate inflammatory signals such as CXCR2. PDAC is known for the prominent desmoplasia that enables therapy resistance and tumor dissemination, which is mainly mediated through cancer-associated fibroblasts (CAFs). Little is known about the connection between oncogenic Kras, CXCR2 signaling and CAFs. In this study, we show that CAFs can produce CXCR2 ligands and can respond to CXCR2 signaling. We indicated that through paracrine factors such as CXCL8 and FGF-2, CAFs support the survival of the aggressive PDAC cells and enable means for progression. We demonstrate that oncogenic Kras is associated with a subset of CAFs with a prominent secretory function mediated through CXCR2 signaling. Lastly, we exhibit a differential role of CXCR2 in PDAC that was dependent on genetic mutations, which may indicate a temporal context of CXCR2 roles in PDAC. Together, CAFs, as well as CXCR2, could still be worthy targets in PDAC in the

right context. Further studies that investigate the progression and timely roles of CAFs and CXCR2 are warranted.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
CALLA	Common acute lymphoblastic leukemia antigen
CD10	Cluster of differentiation 10
CSC	Cancer stem cell
Ctgf	Connective tissue growth factor
Cxcr2$^{-/-}$	CXCR2 knockout
DAB	3, 3'-Diaminobenzidine
DAMP	Damage-associated molecular patterns
DAPI	4'6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPC4	Deleted in pancreatic cancer 4
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELR	Glutamic acid-Leucine-Arginine
EMEM	Eagle's Minimum Essential Medium

EMT	Epithelial-mesenchymal transition
EtBr	Ethidium bromide
FAP	Fibroblast-activation protein α
FBS	Fetal bovine serum
FSP-1	Fibroblast-specific protein 1
GEMM	Genetically engineered mouse model
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
G-MDSC	Granulocytic-MDSC
GPR77	G protein-coupled receptor 77
H&E	Hematoxylin and eosin
HGF	Hepatocyte growth factor
HH	Hedgehog pathway
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HPβCD	Hydroxypropyl-β-cyclodextrin
IF	Immunofluorescence
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IL-	Interleukin

iNOS	Nitric oxide synthase
IPMN	Intra-ductal papillary mucinous neoplasm
KC	Pdx1-cre;LSL-$Kras^{(G12D)}$
KPC	LSL-$^{KrasG12D/+}$; LSL-$Trp53^{R172H/+;}$ Pdx1-Cre
KRAS	Kirsten rat sarcoma viral oncogene homolog
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MCN	Mucinous cystic neoplasm
MDSC	Myeloid-derived suppressor cells
M-MDSC	Monocytic-MDSC
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PanIN	Pancreatic intra-epithelial neoplasia
PBS	Phosphate buffered saline
PC	Pancreatic cancers
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma

PDGF Platelet-derived growth factor

PDGFR-β Platelet-derived growth factor-receptor-β

PPIA Peptidylprolyl Isomerase A

PSC Pancreatic stellate cells

qRT-PCR Quantitative reverse transcription polymerase chain reaction

RIPA Radioimmunoprecipitation assay buffer

ROS Reactive oxygen species

RPL13A Ribosomal protein large 13 A

RPMI Roswell park memorial institute medium

SDS Sodium dodecyl sulfate

SEM Standard error of the mean

SHH Sonic hedgehog

STAT1 Signal transducer and activator of transcription 1

TAM Tumor-associated macrophage

Tgfbr2 Transforming growth factor beta receptor type II

TGF-β Transforming growth factor beta

TNFα Tumor necrosis alpha

Treg T regulatory lymphocytes

TTBS Tween 20 tris-buffered saline

VEGF Vascular endothelial growth factor

αSMA α-smooth muscle actin

THIS THESIS IS DEDICATED TO MY BELOVED

PARENTS

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CHAPTER I: INTRODUCTION

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SIGNIFICANCE OF THE STUDY

Pancreatic cancers (PC), particularly pancreatic ductal adenocarcinoma (PDAC), have a very poor prognosis that often leads to deaths. The major challenge in dealing with the disease is that it is presented at a very advanced stage often when PDAC has already disseminated to other organs. PDAC is also characterized by high recurrence rates and frequent resistance to conventional therapies. Finding screening and diagnostic tools that can detect the disease early when it is still manageable, and developing new therapeutic approaches to deal with advanced stages PDAC are two cornerstones in successfully dealing with this malignancy. This starts by understanding the dense and complex tumor microenvironment of the disease, determining the major players in tumor initiation and progression, and identifying the crucial checkpoints that determine the tumor's fate. Cancer-associated fibroblasts (CAFs) present with abundance in PDAC. They are known to excessively produce extracellular matrix (ECM) proteins as well as many paracrine factors. By interacting with malignant cells and other cells in the tumor microenvironment, CAFs have been implicated in tumor growth, immunosuppression, therapy resistance, and invasion. This study presents an effort to understand the role of CAFs in PDAC and their role in promoting tumor progression, dissemination, or lack thereof via their paracrine interactions. The finding from this study will help in adding to the current knowledge of the molecular and histological features of PDAC, thus, aids in classifying and stratifying PDAC, which will allow more precise new targeted therapy approaches.

OVERVIEW OF THE PANCREAS: ANATOMY AND PHYSIOLOGY

Anatomy and function:

The pancreas is a retroperitoneal organ of both gastrointestinal and endocrine systems. It is located in the posterior portion of the upper abdomen behind the stomach. Pancreas divides into the head (right), body (middle) and tail (left). It weighs around 100 g and extends to 14-25 cm long. Exocrine pancreas, ~95% of organ mass, secrets digestive enzymes to the duodenum through the pancreatic duct at the hepatopancreatic ampulla, through which the common bile duct from the liver and gallbladder also enters the duodenum. The valve of the sphincter of Oddi, not only regulates the flow of bile and pancreatic juice into the duodenum but also prevents the reflux of intestinal contents into the pancreatic duct. Endocrine pancreas, comprised of islets, secretes insulin, glucagon, somatostatin, and pancreatic polypeptide into the blood. The junction of the head and body is referred to as the neck. The neck is thinner than the adjacent portions of the head and body of the pancreas. Posterior to the neck, run major blood vessels such as the superior mesenteric artery, superior mesenteric-portal vein, inferior vena cava, and aorta, which limits the option for a wide surgical margin during pancreatectomy (Horan 2009, Longnecker, Gorelick et al. 2018).

Histology:

The two distinct functions of the pancreas are modulated by two discrete histologic components. The endocrine pancreas that secrets hormones, including insulin, into the bloodstream is defined as the spherical or ellipsoid structures

known as pancreatic islets (islets of Langerhans; dispersed throughout the exocrine pancreas). The islets contain alpha (α) cells, beta (β) cells, gamma (γ) cells, delta (δ) cells, epsilon (ε) cells, and pancreatic polypeptide cells. On the other hand, a network of tubules composed of acinar and duct cells that synthesize, secrete, and carry digestive enzymes into the intestine makes up the exocrine pancreas. Acinar cells contain zymogen granules, the storage compartment for pancreatic digestive enzymes. Acinar cells arrange in clusters, like grapes, at the ends of a branching duct system. Duct, composed of epithelial cells, make up the branching ductal system that collects acinar juices to the gastrointestinal system via the pancreatic duct (Longnecker, Gorelick et al. 2018).

PANCREATIC CANCER

Epidemiology

When compared to other major human malignancies, PC has a lower incidence; yet, the disease remains one of the deadliest types (Lowenfels and Maisonneuve 2006). PC currently ranks as the fourth leading cause of cancer-related death in the United States of America. PC is expected to take over as the second leading cause of cancer-related deaths by 2030 (Siegel, Miller et al. 2018). For the past few years, the American Cancer Society estimated an average of over 50,000 new cases and over 40,000 deaths of PC in both sexes combined. PC is more common in older people and slightly has a higher incidence in males. The overall five-year survival rate for PC is 8%. Around 20% of PC patients present with localized tumors for which surgical resection can improve the survival rate to 32%. Nonetheless, most of the PC cases present clinically at distant metastasis stage with only a three-percent survival rate (Siegel, Miller et al. 2018). The overall five-year survival for all cancers combined has improved from 49% in 1977 to 69% in 2014. In comparison, the survival rate for PC only improved from 3% to 9% (Siegel, Miller et al. 2018). This poor improvement in patient survival can explain why the PC is expected to become the second leading cause of cancer-related deaths after another decade. Furthermore, the disease has a high rate for recurrence, even for those who undergo surgical resection (Network 2016), as well as a high chance of developing resistance to conventional therapy (Hidalgo 2010). The challenges that remain as obstacles in properly improving PC survival rates

include the late presentation and the lack of early detection tools, and high rates of distant metastasis, recurrence, and therapy resistance.

Risk factors

There is not yet any described definitive cause for the occurrence of PC. Many factors have been identified as risk factors for PC, including smoking, obesity, physical inactivity, genetic predisposition, diabetes, and certain diets (Lowenfels and Maisonneuve 2006, Raimondi, Maisonneuve et al. 2009).

Based on descriptive epidemiology, factors like ages, gender or geographical location can impact the rate of PC occurrence. PC is common in older people. The median age of diagnosis of PC is 72 years. Only about less than 10% of patients develop PC are under 50 (Raimondi, Maisonneuve et al. 2009). The American Cancer Society reports the comparatively highest number of deaths 10,594 (males) and 9,076 (females) in the age group of 60-79 years in comparison to other age groups (Siegel, Miller et al. 2018). Moreover, PC presents with higher occurrence in males than in females (Lowenfels and Maisonneuve 2006, Raimondi, Maisonneuve et al. 2009). Race as well can play a role. African-Americans have a higher tendency to get PC than white-Americans. Even so, countries and locations closer to the equator such as Egypt and Zimbabwe have lower rates of PC than northern countries such as Finland and Iceland (Raimondi, Maisonneuve et al. 2009).

Environmental factors such as smoking and specific diets may increase the risk of PC. Exposure to tobacco smoke is associated with about 25% of PC cases

(Lowenfels and Maisonneuve 2006, Raimondi, Maisonneuve et al. 2009). Cigarettes-smokers have around 75% higher risk of PC than non-smokers, and this increased risk persists for at least ten years after smoking cessation (Ilic and Ilic 2016). Furthermore, increased body-mass-index, both general and abdominal obesity, and increased caloric consumption have been linked to the risk of PC (Lowenfels and Maisonneuve 2006, Aune, Greenwood et al. 2011).

Certain people have a predisposition for PC. Around 10% of PC cases are linked to germline mutations. Certain familial syndromes, such as, Peutz-Jeghers syndrome, familial atypical mole-multiple melanoma, cystic fibrosis and Li-Fraumeni syndrome are also linked to increased rate of PC (Raimondi, Maisonneuve et al. 2009). Furthermore, preexisting diseases, such as diabetes and pancreatitis, are linked with increased risk of PC (Lowenfels and Maisonneuve 2006, Raimondi, Maisonneuve et al. 2009).

Histological classification of pancreatic cancer

As the pancreas is divided histologically and functionally into the endocrine and exocrine pancreas, PCs are often classified as such. Endocrine PCs are far less common than exocrine malignancies and often milder. The survival time of patients with endocrine PC is normally two years longer than those diagnosed with exocrine PC (Fesinmeyer 2005). Endocrine tumors are relatively rare, arise in the islet cells and are referred to as islet cell or pancreatic neuroendocrine tumors. Their nomenclature is based on the hormone they overproduce. They are sub-classified into insulinoma, glucagonoma, somatostatinoma or nonfunctional islet cell tumors. The endocrine pancreatic tumors often overproduce normally

occurring substances, such as, insulin and glucagon; exocrine PC tumors are often harsher. Some exocrine malignancies may block pancreatic duct causing jaundice and cachexia (Fesinmeyer 2005). Since the vast majority of PCs are exocrine tumors, we will dedicate the next part to discussing exocrine tumors.

Malignancies of the exocrine pancreas

As described, the exocrine pancreas is mainly made of clusters of acinar cells that secrete digestive enzymes into the branching duct made of ductal epithelial cells (Longnecker, Gorelick et al. 2018). The vast majority of exocrine PCs are ductal adenocarcinomas (Hruban and Fukushima 2007). Less common types of exocrine tumors include cystic tumors that cause a cyst or fluid-filled sac in the pancreas and cancer of the acinar cells. According to World Health Organization and International Agency for Research on Cancer (Bosman, Carneiro et al. 2010), exocrine pancreas malignancies can be classified as follows: PDAC (75% cases), serous cystadenoma, mucinous cystadenocarcinoma, intra-ductal papillary-mucinous carcinoma, and acinar cell carcinoma. PDAC can be further divided into differentiated and poorly differentiated. Other rare forms of exocrine PCs include pancreatoblastoma that affects children and solid pseudopapillary tumors, a rare low-grade neoplasm that mainly affects younger women and has a very good prognosis (Fesinmeyer 2005, Bosman, Carneiro et al. 2010). Because PDAC is the most frequent exocrine tumors with the poorest prognosis, we will mainly focus on discussing PDAC in this dissertation.

PANCREATIC DUCTAL ADENOCARCINOMA (PDAC)

Overview

The cellular phenotype of the PDAC malignant cells is similar to ductal epithelial cells of the exocrine pancreas (Kloppel, Lingenthal et al. 1985, Hruban, Adsay et al. 2001); however, the exact origin is still debated. PDAC may arise from a poorly differentiated ductal cell, a dedifferentiated acinar or islet cell, or a progenitor or stem cell (Li, Lee et al. 2009). PDAC account for 85-90% of all pancreatic neoplasms and is very virulent in nature (Hruban and Fukushima 2007). PDAC can block the pancreatic duct, which results in jaundice and cachexia (Modolell, Guarner et al. 1999, Porta, Fabregat et al. 2005). At the time of diagnosis, 52% of the patients present with a disease that has already metastasized to other organs (Siegel, Miller et al. 2018). PDAC tumors are often firm, but poorly defined in structure with a tendency to invade nearby tissues. Anatomically, 65% of PDAC tumors arise in the head; whereas, around 25% occur in the body and tail. It is common for the tumors in the head to invade the common bile duct or the main pancreatic duct and produce stenosis. In contrast, tumors of the pancreatic body and tail obstruct the main pancreatic duct only (Hruban and Fukushima 2007). Histologically, PDAC is featured with the presence of a dense stromal response known as desmoplasia. Under the microscope, PDAC lesions imitate the appearance of normal pancreatic ducts embedded inside a thick stroma **(Figure 1.1)**. The desmoplastic stroma in PDAC is known to be composed of fibroblasts, stellate cells, endothelial and immune cells (Kloppel, Lingenthal et al. 1985, Hruban, Adsay et al. 2001). The large amount of fibrous stroma explains the

firm consistency of PDAC tumors. PDAC, similar to many other malignancies, does not arise in isolation. The disease progresses over a long period with the contribution of many factors, including, progressive genetic alterations in the malignant cells, as well as contribution from other host cells in the tumor microenvironment. In the next few sections, we will discuss how PDAC progresses, the genetic alterations to allow this progression and the contribution of inflammation and stroma in this disease.

PDAC precursor lesions

The end stage invasive PDAC results from the development of pre-cancerous precursor lesions in the pancreas (Hruban, Wilentz et al. 2000, Hruban, Maitra et al. 2007, Hruban, Brune et al. 2008). Example of these precursor lesions includes intra-ductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN). The most described precursor lesion type is termed pancreatic intra-epithelial neoplasia (PanIN) (Hruban, Maitra et al. 2007). PanINs are microscopic (around ½ cm) neoplastic proliferation in the pancreatic ducts that develop into three histologically distinct stages (PAnIN1-3), before becoming a full-blown invasive PDAC. PanIN1 lesions are composed of columnar epithelial cells with basally oriented nuclei and abundant mucin production and can be either flat (PanIN1A) or papillary (PanIN1B) (Hruban, Brune et al. 2008). PanIN-2 are mostly papillary with some nuclear abnormalities including loss of nuclear polarity, nuclear crowding, and variations in size, hyperchromasia and nuclear pseudostratification (Hruban, Brune et al. 2008). PanIN-3 lesions show the highest form of dysplasia and are architecturally complex with marked cytological abnormalities,

cirbriforming, budding off of epithelial cells and luminal necrosis (Hruban, Brune et al. 2008). Progression from PanIN-1 to PanIN-3 stage occur in stepwise and is accompanied by the onset of various mutations (Hansel, Kern et al. 2003, Hruban, Maitra et al. 2007, Hruban, Brune et al. 2008).

Genetic alterations in PDAC

Molecular and genetic analysis of PDAC indicated that most of the mutations found in the invasive stages are also present in precursor lesions (Feldmann, Beaty et al. 2007). Such a thing suggests that these lesions occur in conjunction with the accumulation of genetic mutations.

The signature genetic events of PDAC lesions include mutations of Kras, CDKN2A, TP53, BRCA2, and Smad4/DPC4, among many others (Kern, Schutte et al. 1995, Hansel, Kern et al. 2003, Löhr, Klöppel et al. 2005, Hruban and Adsay 2009). With the progression of PanINs to higher grades, the number of genetic alteration increase. Activating mutations in the Kras oncogene are detected very early in tumor progression (Feldmann, Beaty et al. 2007). Other notable events include mutations of CDKN2A, TP53, and SMAD4 that happen as a result of the loss of heterozygosity at chromosome 9q, 17p and 18q respectively (Siegel and Massagué 2003, Maitra and Hruban 2008). As the focus of this thesis is to elucidate the role of the CAFs in conjunction with the paracrine signaling in PDAC development and progression, we will only discuss the details of Kras and SMAD4 mutations.

Activating Kras mutations (will be referred to henceforth as oncogenic Kras), are among the earliest genetic events and are found in nearly 95% of PDAC cases (Feldmann, Beaty et al. 2007). Kras is a member of the RAS family, small GTPases with 21-30kDa in size. These RAS proteins reverse between a GTP-bound on-state and GDP-bound off-state. Kras is the only RAS protein that has been reported to mutate in PDAC. A point mutation in this protein results in constitutive activation of RAS leading to persistent downstream signaling. The predominant version occurs at position G12; but activating mutations at other positions have been identified as well (Löhr, Klöppel et al. 2005, Feldmann, Beaty et al. 2007). Oncogenic Kras allows the malignant cells in PDAC to attain more survival, proliferation, cytoskeletal remodeling and motility (Bryant, Mancias et al. 2014). Oncogenic Kras is associated with increase tumor-supporting inflammatory response such as CXCR2 signaling (Ling, Kang et al. 2012, Baumgart, Chen et al. 2014, Purohit, Varney et al. 2016). During the course of this thesis, we will discuss how oncogenic Kras and CXCR2 can play a role in CAFs orientation and function in PDAC.

Smad4, also known as DPC4 (deleted in pancreatic cancer 4), is a member of Smad proteins that mediate signal transduction for a variety of pathways in which transforming growth factor beta (TGF-β) pathway is the most relevant (Blobe, Schiemann et al. 2000, Massagué 2012). TGF-β, a multifunctional cytokine, often found in in the extracellular matrix and is produced by macrophages, lymphocytes, fibroblasts, epithelial cells, and platelets. TGF-β is vital in prenatal and postnatal development, organ maintenance and homeostasis,

and wound healing (Blobe, Schiemann et al. 2000, Massagué 2012). Intact TGF-β/Smad4 signaling works as a tumor suppressor by blocking cell cycle progression, inducing apoptosis of epithelial cells, and maintaining genomic integrity and tissue hemostasis (Liu, Pouponnot et al. 1997, Massagué 2008, Ahmed, Bradshaw et al. 2017). Smad4 inactivation results in ligand accumulation that signal in tumor cells (in a Smad-independent manner) as well as in stromal cells (Zhang 2009). Loss of Smad4 activates non-smad TGF-β pathways including Erk MAPK and JNK/p38 MAPK pathways that play an essential role in epithelial-mesenchymal transition (EMT). Besides, TGF-β mediates EMT, cytoskeletal organization and motility via Rho-like GTPases, RhoA and Rac (Masamune, Kikuta et al. 2003, Massagué 2008). In fibroblasts, TGF-β is known to induce activation and EMC deposition Furthermore, sustained TGF-β inhibits the synthesis of Matrix Metalloproteinases (MMPs), thus, inhibiting degradation of newly synthesized ECM (Shek, Fmj et al. 2002). In PDAC, elevated TGF-β levels are found in both plasma and tumor tissues. The role of TGF-β in regulating EMT and tumor stiffness could explain how high TGF-β expression and the loss of Smad4 correlate with metastasis and poor survival in PDAC (Tascilar, Skinner et al. 2001, Tang, Katuri et al. 2005, Blackford, Serrano et al. 2009, Singh, Srinivasan et al. 2012, Xia, Wu et al. 2014).

Tumor microenvironment of PDAC

Mutations associated with malignancies often enable tumor cells to have sustained signals for growth; however, these events are not enough to maintain the tumor's overall autonomous survival. Several kinds of normal host cells are recruited and oriented by malignant cells to support tumor growth and progression

(Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). One of the hallmarks of PDAC is the presence of dense desmoplasia in the tumor microenvironment of this disease. Desmoplasia in PDAC account for 80-90% of the overall tumor mass and can be defined as the exuberant proliferation of stromal cells, abundant production of ECM with increased collagen deposition (Chu, Kimmelman et al. 2007, Kleeff, Beckhove et al. 2007). This desmoplastic reaction has been implicated in resistance to chemotherapy and radiotherapy and can be attributed to the hypovascularity often observed in PDAC. The stromal compartment in PDAC is composed of multiple cell types including fibroblasts, immune cells, endothelial cells, and ECM proteins. It has been demonstrated that cancer stroma plays an active and dynamic role in tumor growth, invasion, and metastasis (McAllister and Weinberg 2010).

Inflammation in PDAC

It has been established that both innate and adaptive immune cells present at sites of many tumors including PDAC (Chu, Kimmelman et al. 2007, Kleeff, Beckhove et al. 2007). There is more evidence now that support the notion of the tumor supporting-inflammation in regard to the presence of immune cells at the tumor site. Inflammation can contribute to cancer progression by supplying several molecules to enable sustained tumor growth, prevent tumor eradication, facilitate angiogenesis, and promote invasion and metastasis (DeNardo and Coussens 2007, Grivennikov, Greten et al. 2010, Qian and Pollard 2010).

Paracrine factors derived from malignant cells and stromal cells orchestrate the recruitment and the orientation of the different components of the tumor microenvironment to facilitate tumor progression (Pietras and Östman 2010, Matsuo, Takeyama et al. 2012). CXCR2, a chemokine receptor, axis plays a key role during PDAC initiation and progression. CXCR2 and its ligands are linked to increased tumor cell proliferation, pro-tumor immunosuppression and resistance to therapy (Chan, Hsu et al. 2016, Purohit, Varney et al. 2016, Steele, Karim et al. 2016).

In this dissertation, we discuss the contribution of paracrine interaction between malignant cells and CAFs in tumor progression or lack thereof.

CXCR2 SIGNALING

Overview

Chemotactic cytokines, i.e., chemokines, are small secreted molecules that are either homeostatic or inflammatory. They are known to play roles in multiple cellular processes such as leukocyte migration, embryogenesis, angiogenesis, and hematopoiesis. Homeostatic chemokines have a constitutive expression in specific cell types and tissues to maintain tissue homeostasis and development. In contrast, inflammatory chemokines are inducible and up-regulated by inflammatory stimuli (Vandercappellen, Van Damme et al. 2008). Structurally, based on the position of the conserved N-terminal cysteine residues, they are classified into four families: C, CC, CXC, and CX_3C. Chemokine receptors are members of the seven-transmembrane G-protein coupled receptor family. CXCR2 is the receptor for a group of inflammatory and angiogenic chemokines referred to as Glutamic acid-Leucine-Arginine $(ELR)^+$ CXC chemokines that include CXCL1-3, 5-7, and 8 that also interacts with CXCR1(Strieter, Burdick et al. 2006, Lazennec and Richmond 2010). Several studies have reported that CXCR2 and its ligands play an important role in regulating tumor growth, angiogenesis, and metastasis in PDAC and many other tumors (Strieter, Burdick et al. 2006, Singh, Sadanandam et al. 2007, Wang, Wu et al. 2013, Purohit, Varney et al. 2016). To understand the role of CXCR2 in PDAC, we will first discuss the role of CXCR2 during physiological inflammation.

CXCR2 signaling during inflammation

CXCR2 is known to mediate many processes during inflammation. CXCR2 is mainly expressed on several cell types such as neutrophils (Bajrami, Zhu et al. 2016), monocytes (Moser, Barella et al. 1993, Patel, Charlton et al. 2001, Murdoch 2004), mast cells (Maltby, Khazaie et al. 2009, Wynn and Barron 2010), and endothelial cells (Li, Cheng et al. 2011). Ligation of CXCR2 to its chemokines induces calcium release, activates Ras/MAPK and PI3K signaling cascades, and results in many immune responses including directed neutrophil migration (Wu, Wang et al. 2011). Neutrophils represent the largest component of the innate immune system. Neutrophils homeostasis is maintained by balancing their release from bone marrow and their clearance from circulation. CXCL12, a chemokine that works through CXCR4, has an antagonistic effect to CXCR2. CXCR4 activity enhances hematopoietic cells retention in the bone marrow. Loss or decreased activity of CXCR4 results in mobilization of neutrophil to the blood (Eash, Greenbaum et al. 2010). CXCR2 signaling mediates neutrophil migration from blood circulation to the inflamed tissue. During an inflammatory response, neutrophils are one of the first responders. As the first line of host defense against infection, neutrophils travel to sites of infection to then control the bacterial burden; however, prolonged and excessive neutrophil infiltration can cause tissue damage. Recent reports described that that neutrophil recruitment during inflammation occurs in two phases. The early phase is mediated by short-lived signals, whereas the amplification phase is mediated through leukotriene-B4 and CXCR2 chemokines (Grivennikov, Greten et al. 2010, de Oliveira, Rosowski et al. 2016). Tissue remodeling is a crucial step to maintain the structural and functional

integrity of the inflamed tissue. Following the inflammation clearance, many processes occur including re-epithelialization, neovascularization (angiogenesis) and scarring of the collapsed tissue (Devalaraja, Nanney et al. 2000, Midwood, Williams et al. 2004). Angiogenesis, the formation of new capillary blood vessels, is essential to provide a supply of nutrients and oxygen to the newly generated tissues. CXCR2 signaling has a key role in the process of angiogenesis. Endothelial cells, which form the inner lining of blood vessels, constitutively express CXCR2 and respond to chemokine stimulation. CXCL8 was reported to directly enhances endothelial cell proliferation, survival, and their expression of metalloproteases; thus, regulating angiogenesis (Strieter, Polverini et al. 1995, Strieter, Burdick et al. 2006, Matsuo, Raimondo et al. 2009).

CXCR2 expression and signaling in PDAC

More compelling evidence for the adverse role of CXCR2 signaling in PDAC is now available. Both PDAC and the normal pancreas express CXCR2, but PDAC is more responsive. Oncogenic Kras in PDAC occurs very early, often as soon as the inception of the PanINs (Feldmann, Beaty et al. 2007). The upregulation of CXCR2 signaling in PDAC has been reported to be directly linked to oncogenic Kras (Purohit, Varney et al. 2016). The genetically engineered mouse model (GEMM; Pdx1-cre;LSL-*Kras*$^{(G12D)}$ known as KC mouse model) of PDAC exhibited a progressive increase in the expression of CXCR2 and its ligands in the malignant ductal cells (Purohit, Varney et al. 2016). Oncogenic KRAS-CXCR2 axis created a feed-forward loop that contributed to tumor progression by supporting tumor cell growth. Furthermore, CXCR2 chemokines have been linked with increasing

migration potentials of malignant cells as well as facilitating resistance to chemotherapy by the induction of cancer stem cells (CSCs) (Chan, Hsu et al. 2016, Purohit, Varney et al. 2016).

CXCR2 signaling can additionally aid in the tumor progression, not only by promoting autonomous aggression characteristic in malignant cells but also through other cells in the tumor microenvironment (Strieter, Burdick et al. 2006, Highfill, Cui et al. 2014). We have discussed the role of CXCR2 signaling during inflammation depicted as facilitating migration of innate immune cells and inducing angiogenesis. Such features can be utilized adversely by malignant tumors by enabling recruitment of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) and facilitating tumor invasion through stimulating angiogenesis (Highfill, Cui et al. 2014, Ramachandran, Condamine et al. 2016).

MDSCs, a heterogeneous population of immature myeloid cells, induce immunosuppression by causing defective T cell function (Fujimura, Mahnke et al. 2010). They classify into granulocytic-MDSCs (G-MDSCs; mouse: CD11b+Ly6G+; human: CD11b+ CD15+) and monocytic (M-MDSCs; mouse: CD11b+Ly6C+; human: CD11b+ CD14+) (Ostrand-Rosenberg and Sinha 2009, Goedegebuure, B. Mitchem et al. 2011). In cancer patients, MDSCs have marked a systemic expansion in the spleen, lymph nodes, and blood circulation, as well as at the tumor sites (Gabrilovich and Nagaraj 2009). These MDSCs can cause immunosuppression at the tumor site by the production of nitric oxide synthase (iNOS) and arginase 1 that suppress the proliferation and activation of T cells (Nagaraj and Gabrilovich 2008, Gabrilovich and Nagaraj 2009, Fujimura, Mahnke

et al. 2010, Goedegebuure, B. Mitchem et al. 2011). MDSCs can also suppress T cells function in antigen-specific fashion via reactive oxygen species (ROS) (Nagaraj and Gabrilovich 2008). Moreover, MDSCs promote the recruitment of T regulatory lymphocytes (Tregs) to the tumor sites and blocking the entry of effector T cells (Nagaraj and Gabrilovich 2008, Gabrilovich and Nagaraj 2009, Fujimura, Mahnke et al. 2010, Goedegebuure, B. Mitchem et al. 2011).

CXCR2 axis has been implicated in the recruitment and expansion of MDSCs. A report demonstrated that MDSCs reduction in tumor site was accompanied with reduced CXCL5 protein expression (Weiss, Back et al. 2009). Another report also indicated that CXCL1, 2 and 5 are responsible for the G-MDSCs recruitment to the primary tumors (Toh, Wang et al. 2011). Furthermore, colon cancer model exhibited high levels of CXCR2 ligands and the loss of CXCR2 diminished the G-MDSCs infiltration (Katoh, Wang et al. 2013). In PDAC, MDSCs were shown to be present as early as the PanIN lesions and found further increased infiltration in PDAC (Clark, Hingorani et al. 2007). Using GEMM of PDAC with both oncogenic Kras and p53 mutations, inhibiting CXCR2 reduced MDSCs recruitment and improved response to immunotherapy (Steele, Karim et al. 2016). Kumar et al. demonstrated that CAFs aid in recruiting MDSCs by producing chemokines including CXCL1, 2 and 5 (Kumar, Donthireddy et al. 2017).

CXCR2 axis has been as well adversely implicated in enhancing angiogenesis in many cancer types including lung, melanoma, and pancreas (Matsuo, Ochi et al. 2009, Matsuo, Raimondo et al. 2009, Singh, Varney et al. 2009). The adverse role of CXCR2 in cancer has made it a hot target for inhibition.

Multiple attempts to study the effect of CXCR2 inhibition have been completed. It was reported that inhibiting CXCR2 suppressed inflammation-driven tumorigenesis in skin and intestine cancer models (Jamieson, Clarke et al. 2012). In addition, using small interfering RNA, inhibiting CXCR1 and CXCR2 reduced melanoma tumor growth and invasion (Singh, Sadanandam et al. 2010). For PDAC, the pelleted supernatants from PDAC cell lines were injected into rat corneal micropocket model, where blocking CXCR2 exhibited reduced angiogenesis compared to supernatant injection alone (Wente, Keane et al. 2006). Using *Kras+Tgfbr2*^{KO} mice with conditional pancreas epithelium-TGF-β receptor type II (Tgfbr2) knockout and Kras activation, inhibition of CXCR2 disrupted the tumor-stromal interactions and improved mice survival by lowering the expression of connective tissue growth factor (Ctgf) that promotes fibrosis and tumor progression (Ijichi 2011). In another PDAC model, Steele et al. examined the effect of CXCR2 inhibition in KPC (*LSL-*$^{KrasG12D/+;}$ *LSL-Trp53*$^{R172H/+;}$ *Pdx1-Cre*) that carries both pancreas-specific p53 mutation and oncogenic Kras. They concluded that inhibiting CXCR2 suppresses metastasis, augments immunotherapy, and improves survival by reducing MDSCs infiltration (Steele 2016). Furthermore, our laboratory has generated a syngeneic CXCR2 knockout (*Cxcr2*$^{-/-}$) model using KC-derived cells. The CXCR2 stromal ablation caused no change to the tumor size although it halted cancer cell proliferation and increased their apoptosis; it also decreased the recruitment of MDSCs and increased the induction of cytotoxic T lymphocytes re-orienting the tumor's immune status towards an anti-tumor immune response. On the other hand, depletion of CXCR2 increased fibrotic

reaction within the primary tumor increased liver metastasis and increased the abundance of tumor-associated macrophages (TAMs). The increased fibrosis reveals a potential undescribed role of CXCR2 signaling in regulating CAFs in PDAC (Purohit 2015).

CANCER-ASSOCIATED FIBROBLASTS

Overview

PDAC is one of the leading causes of cancer-related deaths in the United States. The late diagnosis, often after the disease has disseminated, and the limited efficacy of the chemotherapy for advanced disease are the major challenges in PDAC. Moreover, resistance to therapy and recurrence are frequent, even for patients diagnosed with localized tumors (Siegel, Miller et al. 2018). PDAC is highlighted with a dense and firm desmoplasia composed of ECM deposition and infiltrating leukocytes, endothelial cells and CAFs (Kleeff, Beckhove et al. 2007). Desmoplasia is implicated in PDAC development, progression, dissemination as well as therapy resistance (Apte, Park et al. 2004, Moir, Mann et al. 2015, Kalluri 2016). Resolving desmoplasia has been attempted through digesting ECM, targeting CAFs or inhibiting desmoplasia-associated pathways (Olive, Jacobetz et al. 2009, Provenzano, Cuevas et al. 2012, Özdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014). Some of these attempts produced accelerated tumor progression and worsened prognosis (Özdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014), which implies that there is more to desmoplasia than we currently know.

CAFs are the major contributor to desmoplasia, and they produce ECM and multiple soluble factors that contribute to tumor progression (Apte and Wilson 2004, Omary, Lugea et al. 2007, Moir, Mann et al. 2015). Although CAFs are often treated as a single entity, they are vastly heterogeneous by origin. There is an

agreement that CAFs have a mesodermal origin, but their molecular definition is still debatable. Currently, CAFs represent cells present in the tumor microenvironment that are not tumor cells, leukocytes, endothelial, or epithelial cells and that carry fibroblastic features such as the expression of fibroblast-specific protein 1 (FSP-1) (Öhlund, Elyada et al. 2014).

In PDAC, pancreatic stellate cells (PSCs) are the most studied CAFs subtype. Stellate cells, referring to their star-like shape, are found in several organs including the kidneys, lungs, intestines, spleen, uterus, and skin; but, they are mainly described in liver and pancreas (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Öhlund, Elyada et al. 2014, Moir, Mann et al. 2015). PSCs are found in the periacinar, perivascular or periductal regions of the exocrine pancreas. In normal conditions, PSCs are usually in the quiescent state with long cytoplasmic extensions and vitamin-A storing fat droplets. PSCs express many markers including intermediate filament proteins desmin, and Glial fibrillary acidic protein (GFAP) that along with Vitamin-A storing droplet can distinguish them from normal fibroblast (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013). PSCs markers also characterize several other cell types such as desmin that is seen in monocytes, GFAP of astrocytes, vimentin that also characterizes leukocytes and endothelial cells, and Nestin of neuroepithelial stem cells (Omary, Lugea et al. 2007). Activation of PSCs occurs as a result of milieu changes such as pancreatic injury or in response to secreted factors such as platelet-derived growth factor (PDGF) and TGF-β (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). When activated, PSCs assume the myofibroblast-like,

phenotype by upregulating α-smooth muscle actin (αSMA) and collagen I, and losing their vitamin A-storing fat droplets in addition to increased nucleus size, prominent ECM production, and increased cell proliferation and migration potentials (Omary, Lugea et al. 2007, Erkan, Adler et al. 2012, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). Additional reports indicated that activated PSCs express fibroblast-activation protein α (FAP) (Bachem, Schünemann et al. 2005, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). Activated PSCs play essential roles in pancreatic repair following injury and acute inflammation via modulating ECM production and tissue remodeling (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013). Following the secession of the pancreatic assault, activated PSCs revert into quiescence or undergo apoptosis. Repeated assaults and chronic pancreatic inflammation cause sustained PSCs activation, which increases the risk of fibrosis and cancer (Omary, Lugea et al. 2007, Apte, Pirola et al. 2015).

Tissue-resident fibroblasts can also contribute to CAFs population (Öhlund, Elyada et al. 2014). A subset of normal fibroblasts was found to express the glycoprotein Thy-1 was able to differentiate into CAFs after treatment with TGF-β. Genetic mutations such as inactivation of p53 and PTEN has been frequently observed in stromal cells and can also turn them into CAFs (Xing, Saidou et al. 2010). Moreover, CAFs can arise by transdifferentiating through EMT or endothelial to mesenchymal transition (Xing, Saidou et al. 2010, Öhlund, Elyada et al. 2014), but more direct sources of CAFs include bone marrow-derived fibrocytes, mesenchymal stem cells, and adipocytes (Xing, Saidou et al. 2010, Öhlund, Elyada et al. 2014). These diverse origins of CAFs can explain the

absence of consensus on a molecular definition. Nonetheless, multiple markers have been widely used to distinguish CAFs including PDGF-receptor-β (PDGFR-β), αSMA and FAP (Bachem, Schünemann et al. 2005, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). These markers are not uniformly expressed in all CAFs (Öhlund, Elyada et al. 2014), which can be due to the presence of CAFs concurrently at multiple differentiation stages or because of the diverse origins of CAFs. The coexistence of multiple subsets of CAFs could explain the diverse roles and abilities they carry out to promote tumorigenesis and progression and could explain why targeting CAFs using a single marker, such as αSMA, can have an adverse outcome (Özdemir, Pentcheva-Hoang et al. 2014). CAFs have been described to be versatile and to have a wide range of roles in cancer (Omary, Lugea et al. 2007). It is not clear however if all the roles can be carried out by all CAFs or the versatility is due to CAFs diversity. A better understanding of different CAFs subsets could greatly impact our ability to target desmoplasia safely. In this section, we will discuss the functional heterogeneity of CAFs and how the abundance of specific subsets can influence tumor progression or lack thereof.

Role of CAFs in PDAC

PDAC develops as a result of a progressive accumulation of genetic alterations in multiple oncogenes and tumor suppressor genes. Oncogenic Kras occur very early preceding PDAC precursors. The late events of inactivating tumor suppressors such as p53 and Smad4 allow progression to invasive PDAC (Maitra and Hruban 2008, Hidalgo 2010, Vincent, Herman et al. 2011). Although mutations are essential for the malignancy, they do not render them autonomous. Numerous

survival, growth, and invasion cues are obtained through cellular and molecular interactions with other components in the tumor microenvironment. CAFs have been implicated in multiple hallmarks of cancer including sustained proliferative signaling, tumor-promoting inflammation, and invasion and metastasis (Kleeff, Beckhove et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). In some cancers, the accumulation of CAFs and ECM changes were observed prior to tumor formation, which indicates that CAFs recruitment is essential for tumor development and maybe a prerequisite (DeFilippis, Chang et al. 2012, Sasaki, Baba et al. 2014, Ghosh, Vierkant et al. 2017). The most notable adverse contribution of CAFs to the tumor is acting both physically and biochemically to hinder drug delivery and impose resistance. CAFs produce ECM molecules such as collagen, fibronectin, and hyaluronan (Bachem, Schünemann et al. 2005, Nikitovic, Tzardi et al. 2015). The increased deposition of such molecules physically impairs drug delivery to the tumor (Jacobetz, Chan et al. 2012, Provenzano and Hingorani 2013). Inhibiting the Hedgehog (HH) pathway, a major promotor of desmoplasia, and using enzymatic digestion of desmoplasia facilitated drug delivery and increased the intratumoral concentration of the chemotherapy agent (Olive, Jacobetz et al. 2009, Provenzano, Cuevas et al. 2012). Moreover, factors secreted by CAFs such as hepatocyte growth factor (HGF), interleukin (IL)-6 and CXCL8 have been implicated in therapy resistance either by activating resistance-associated pathways or inducing stemness in tumor cells (Omary, Lugea et al. 2007, Garrido-Laguna, Uson et al. 2011, Straussman, Morikawa et al. 2012, Chan, Hsu et al. 2016, Su, Chen et al. 2018).

CAFs are also tied to PDAC metastasis. Increased tumor stiffness as a result of increased ECM depositions, can increase tumor cells contractility, thus allowing tumor cell detachment and invasion (Ahmadzadeh, Webster et al. 2017). Biochemically, CAFs can play a role in up-regulating EMT that endows tumor cells with more migratory and invasion potentials. Tumor cells co-cultured with CAFs had a fibroblast-like appearance, increased migration and expressed mesenchymal markers Vimentin, Snail-1 and Zeb (Kikuta, Masamune et al. 2010). One proposed mechanism for CAFs-induced EMT involves TGF-β that is highly produced by myofibroblasts (Shek, Benyon et al. 2002, Shek, Fmj et al. 2002).

CAFs involvement in PDAC also extends to tumor growth, proliferation and nourishment as well as immunosuppression and immune evasion (Apte, Wilson et al. 2013, Moir, Mann et al. 2015, Bynigeri, Jakkampudi et al. 2017). But, is targeting desmoplasia or CAFs a solution for resolving PDAC aggressiveness? There are conflicting reports on usefulness of targeting CAFs. Olive et al. observed increased vascularization, and improved drug delivery as well as decreased αSMA cells and improved the overall survival of the test mice in response to an inhibitor that targets the HH pathway (Olive, Jacobetz et al. 2009). The drug, however, when put into the test in a clinical trial rendered a decreased survival. Ozdemir et al. developed a mice model that is depleted of αSMA cells. This model demonstrated an accelerated PDAC with reduced survival, undifferentiated tumors, increased chemotherapy resistance, stemness, and immunosuppression (Özdemir, Pentcheva-Hoang et al. 2014). Rhim et al. targeted desmoplasia by inhibiting the HH pathway. In this model, PDAC exhibited tumors with undifferentiated histology,

increased vascularity and proliferation, and reduced survival and myofibroblast infiltration (Rhim, Oberstein et al. 2014). Together, these independent experiments demonstrate that inhibiting myofibroblasts results in aggressive PDAC with intense immunosuppression, heightened proliferation, tumor stemness, and therapy resistance.

We have discussed the heterogeneity of CAFs based on their origin; however, it is not clear if they present with functional diversity within the tumor and if their origin impacts their function. Ohlund et al. described a distinct subset of CAFs in PDAC with a secretory function that is different from the typical myofibroblast CAFs (Ohlund, Handly-Santana et al. 2017). These newly described CAFs are characterized with increased secretion of inflammatory mediators, particularly IL-6, and decreased expression of αSMA in addition to their ability to promote tumor cells proliferation (Ohlund, Handly-Santana et al. 2017). Thus, CAFs heterogeneity in PDAC can explain why particularly targeting myofibroblasts can render a more adverse outcome. It is not clear at this point if other functional subsets, other than myofibroblasts and secretory CAFs, present. In the next sections, we will discuss the contexts by which myofibroblasts or secretory CAFs develop and their impact on the tumor outcome.

Myofibroblast CAFs

Overview

For long, CAFs and myofibroblasts were considered synonymous in the context of cancer and often used interchangeably. We know now that is not

accurate. Besides cancer, myofibroblasts are often described in the context of wound healing, in which quiescent fibrotic cells get activated to undertake tissue repair and remodeling. Cancers are often referred to as “wounds that do not heal” (Dvorak 1986). Looking into the wound healing process can provide insights into the dynamics of CAFs activity in cancer.

Wound healing

Tissue injury causes plasma leakage from local blood vessels. Shortly after, extravasated plasma initiates wound sealing by forming a clot of fibrin, fibronectin, and platelets to trap the blood inside. The sealant clot acts as a provisional scaffold for the migration of inflammatory cells recruited through factors secreted from the damaged tissue cells as well as the platelets. Inflammatory cells clear debris, infectious agents and degrade the clot. Next, activated fibroblasts form granulation tissue by depositing ECM molecules such as collagen, glycosaminoglycans, and fibronectin. Fibroblasts also enable vascularization by recruiting and modulating endothelial cells. Finally, before they disappear, fibroblasts remodel the granulation tissue allowing few blood vessels and dispersed fibrocytes in the dense collagenous scar that replaced the collapsed tissue (Dvorak 1986, Midwood, Williams et al. 2004). As it appears, wound healing is a very coordinated process. First platelets modulate, provisionally, sealing the wound and recruiting inflammatory cells. Next, neutrophils then macrophages clean the mess before allowing fibroblasts to generate the permanent sealant. Cytokines and chemokines coordinate the timely recruitment and activation of different cells.

Interestingly, ECM deposition and remodeling by fibroblasts occurs after the secession of inflammation (Midwood, Williams et al. 2004). Inflammation during wound healing, in particular neutrophils and macrophages, happens in two phases. Neutrophils are among the first responders, recruited mainly through CXCR1/2, to clear the infectious aggressors (Wu, Wang et al. 2011, de Oliveira, Rosowski et al. 2016). Classically activated macrophages (M1) are known pro-inflammatory cells that ingest and degrade tissue debris, pathogens, retired neutrophils, and ECM scaffold to set the stage for tissue repair. Alternatively activated macrophages (M2; the pro-repair and the anti-inflammatory counterparts of M1) produce cytokines that dampen the inflammation including IL-10 and TGF-β (Wynn and Barron 2010, Xue, Sharma et al. 2015, Wynn and Vannella 2016). The latter is known to activate myofibroblasts and induce ECM deposition and remodeling (Midwood, Williams et al. 2004, Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015), the last step in tissue repair. Fibroblasts in wound healing are mainly described as myofibroblasts that are responsible for ECM deposition and remodeling, but it is not clear if other subsets of fibroblasts present with distinct roles similar to those found in cancer that amplifies inflammation.

The context of myofibroblasts in PDAC

Several secreted mediators, such as PDGF and TGF-β, are considered to have ties to the development of myofibroblasts from quiescent fibrotic cells (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). TGF-β typically signals through the Smad pathway. Smad4, also known as DPC4, which is commonly inactivated in PDAC (Ahmed, Bradshaw et al. 2017). TGF-β,

produced by macrophages, lymphocytes, fibroblasts, epithelial cells, and platelets (Blobe, Schiemann et al. 2000, Massagué 2012), is essential in prenatal and postnatal development, organ maintenance and homeostasis, and wound healing (Blobe, Schiemann et al. 2000, Massagué 2012). Intact TGF-β/Smad4 signaling works as a tumor suppressor (Blobe, Schiemann et al. 2000, Massagué 2008, Massagué 2012); however, Smad4 inactivation results in ligand accumulation that signal in tumor cells through Smad-independent pathways to mediate EMT, cytoskeletal organization and motility (Massagué 2008, Ahmed, Bradshaw et al. 2017). In fibroblasts, TGF-β is known to induce activation and ECM deposition, inhibit MMPs synthesis causing stiffness (Shek, Fmj et al. 2002). In PDAC, elevated TGF-β levels regulates EMT and tumor stiffness, and correlates with metastasis and poor survival (Liu, Pouponnot et al. 1997, Tascilar, Skinner et al. 2001, Tang, Katuri et al. 2005, Massagué 2008, Blackford, Serrano et al. 2009, Zhang 2009, Singh, Srinivasan et al. 2012, Xia, Wu et al. 2014, Ahmed, Bradshaw et al. 2017).

Another molecule that has been linked to myofibroblasts is PDGF. Many reports tie PDGF to fibroblasts activation and ECM synthesis along with TGF-β; however, the effect of PDGF is not the same as TGF-β (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013). Besides enhancing the proliferation of activated fibroblasts, PDGF plays a significant role in blood vessel formation and maintenance (Forsberg, Valyi-Nagy et al. 1993, Crawford, Kasman et al. 2009). PDGF is mainly secreted by activated platelets but can also be produced by other

cells such as macrophages and endothelial cells (Forsberg, Valyi-Nagy et al. 1993, Crawford, Kasman et al. 2009, Hammer, Sizemore et al. 2017).

HH molecules, including sonic (SHH), Indian and desert-HH, are morphogens that play a crucial role in embryologic growth and tissue morphogenesis. SHH is implicated in wound healing and repair (Kayed, Kleeff et al. 2006, Le, Kleinerman et al. 2008). In cancer, SHH is highly implicated in desmoplasia and disrupting HH pathway was shown to reduce myofibroblasts (αSMA+ cells), reduce ECM deposition and enhance angiogenesis and drug delivery (Bailey, Swanson et al. 2008, Bailey, Mohr et al. 2009, Olive, Jacobetz et al. 2009, Tian, Callahan et al. 2009, Smelkinson 2017) (Rhim, Oberstein et al. 2014). Several other molecules have been linked to fibroblasts activation; however, there is not enough evidence to connect them to a certain CAFs subset.

In summary, several molecules, including TGF-β, PDGF and SHH, cooperate to establish and maintain desmoplasia by promoting myofibroblasts-phenotype in CAFs. The abundance of myofibroblasts is associated with ECM synthesis and deposition, tumor stiffness, EMT augmentation, and invasion and metastasis.

Secretory CAFs

Overview

Ohlund et al. identified the presence of two distinct phenotypes of CAFs in PDAC. The typical myofibroblasts (αSMA high) with high ECM synthesis were found adjacent to the tumor cells. The other phenotype that they referred to as

inflammatory CAFs (αSMA low) found at a distance from tumor cells and had a lower ECM expression and a higher expression of inflammatory mediators, in particular, IL-6. Inflammatory CAFs possessed the ability to induce tumor cell proliferation (Ohlund, Handly-Santana et al. 2017).

It is established that CAFs secreted several paracrine factors to modulate both inflammatory and fibrotic processes (Omary, Lugea et al. 2007, Moir, Mann et al. 2015). This, however, was attributed to the plasticity and versatility of CAFs and to their ability to carry out multiple roles at the same time. The notion of specialized CAFs subsets is fairly recent and largely understudied. Nonetheless, several reports have pointed, without directly concluding, towards the ability of CAFs to be secretory in certain contexts. We will first discuss the relationship between inflammation and CAFs.

CAFs and inflammation

Extensive studies of pancreatic inflammation shown that CAFs express several paracrine factors and their receptors, which modulates inflammatory and fibrotic processes. Inflammation and fibroblasts activity are closely linked. In pancreatitis, for instance, damage in pancreatic tissues proceeds a succession of events including interstitial edema, parenchymal cells necrosis, trypsin activation, inflammatory cell infiltration, and lastly the activation and proliferation of PSCs (Omary, Lugea et al. 2007). The activated PSCs are often found in areas rich in cytokines, growth factors, and reactive oxygen species such as near necrotic tissues (Omary, Lugea et al. 2007). The excessive ECM deposition and

remodeling that follows PSCs activation is likely a late step in the tissue repair process similar to that seen in wound healing.

CAFs actively contribute to inflammation by producing several cytokines and chemokines. Besides PDGF and TGF-β that are well recognized in their fibrogenic roles, CAFs secrete several factors including IL-1β, IL-4, IL-6, IL-8, IL-13, vascular endothelial growth factor (VEGF) and many others (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). These factors contribute to cancer progression by providing means for inflammation, immunosuppression, tumor cells proliferation, angiogenesis, and chemotherapy resistance (Apte, Wilson et al. 2013, Moir, Mann et al. 2015).

As we discussed in wound healing, activated fibroblasts only proceed to ECM deposition after the secession of inflammation, which may suggest that inflammation acts as a checkpoint that regulates fibroblasts differentiation into myofibroblasts. This is also similar to the activated PSCs during pancreatitis (Omary, Lugea et al. 2007). It is not clear though if a secretory (or inflammatory) phenotype present during these processes.

The context of secretory CAFs

Ohlund et al. described the secretory (inflammatory) CAFs as they develop when they do not have adjacency to the tumor cells (Ohlund, Handly-Santana et al. 2017). This may implicate far-reaching paracrine factors such as chemokines. There is not enough evidence though to conclude on the exact mechanism by which secretory CAFs develop (Ohlund, Handly-Santana et al. 2017). We will next

discuss a few reports that have indicated secretory functions in CAFs, and we will aim to identify the common denominator that can explain the development of the secretory CAFs.

Chan et al. treated CAFs of breast cancer and PDAC with the maximum-tolerated dose of chemotherapy (Chan, Hsu et al. 2016). The treatment caused CAFs to undergo senescence, activate transcription factors such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the signal transducer and activator of transcription 1 (STAT1), and highly express a group of chemokines that signal through the chemokine receptor CXCR1/2 axis (Chan, Hsu et al. 2016). These secreted factors enhanced tumor cell proliferation and stemness, angiogenesis, recruitment of MDSCs, and rendered larger tumors (Chan, Hsu et al. 2016). Senescence often happens in response to the accumulation of somatic mutations, oxidative stress, telomere dysfunction and shortening, loss of immune surveillance, and chronic inflammation in response to inflammatory mediators such as IL-1β, IL-6, and IL-8 (Campisi and d'Adda di Fagagna 2007, Acosta, O'Loghlen et al. 2008, Kuilman, Michaloglou et al. 2010). Senescence in CAFs has been reported on multiple occasions to impact tumorigenicity and tumor cells behaviors (Collado, Gil et al. 2005, Cichowski and Hahn 2008, Hinds and Pietruska 2017). Senescent fibroblasts promoted proliferation and altered epithelial cell differentiation in breast cancer (Parrinello 2005). Bavic et al. showed that senescent CAFs of prostate cancer promote proliferation of tumor cells through paracrine signaling (Bavik, Coleman et al. 2006). Wang et al. reported that senescent CAFs upregulate CXCL8 and enhance

tumor cells migration and invasion (Wang, Notta et al. 2017). Lastly, the induction of CAFs senescence generates a non-fibrogenic myofibroblast phenotype with lower ECM synthesis (Mellone, Hanley et al. 2016). Although it seems convincing, senescence cannot account for it all. According to Ohlund et al., tumor cells also promoted the proliferation of the secretory phenotype indicating that they are not senescent (Ohlund, Handly-Santana et al. 2017).

Nonetheless, one common feature of senescent cells is that they activate transcription factors, such as NF-κB and STAT1, that upregulate several paracrine including IL-1β, IL-6, CXCL8, and VEGF (Salminen, Kauppinen et al. 2012, Chan, Hsu et al. 2016, Korc 2016, Lesina, Wormann et al. 2016). NF-κB, in particular, has been under a lot of scrutinies in inflammatory diseases and cancers (Korc 2016, 2017). NF-κB is highly associated with inflammation. Inflammation triggers NF-κB activation, which in turn further amplifies inflammation. Acute inflammation triggered by several factors including cytokines, chemokines, pathogen-associated molecular patterns, and damage-associated molecular patterns (DAMPs) (DiDonato, Mercurio et al. 2012). A recent report by Su et al. indicated that complement components could signal through the G protein-coupled receptor 77 (GPR77) on CAFs of breast and lung cancers to activate NF-κB, which result in upregulation of IL-6 and CXCL8 that promote stemness in tumor cells and cause chemotherapy resistance (Su, Chen et al. 2018). Although the authors could not observe a downregulation in the αSMA or the ECM production, they identified this secretory subset of CAFs using GPR77 and cluster of differentiation 10 (CD10) as surface markers (Su, Chen et al. 2018). CD10 is a small metalloprotease that is

also known as common acute lymphoblastic leukemia antigen (CALLA), where it used as a prognostic marker (Maguer-Satta, Besancon et al. 2011). CD10+ stromal cells have been identified in several cancers including colorectal cancer (Ogawa, Iwaya et al. 2002), breast cancer (Iwaya, Ogawa et al. 2002), gastric cancer (Huang 2005), and PDAC (Ikenaga, Ohuchida et al. 2010). In PDAC, CD10+ CAFs promoted tumor cells growth and invasion and was associated with reduced survival and nodal metastasis (Ikenaga, Ohuchida et al. 2010). It is not clear at this point if CD10 is uniformly expressed in all secretory CAFs or not.

CXCL8, also known as IL-8, is a chemokine that signals via CXCR1/2 axis along with a group of angiogenic chemokines including CXCL1-3 and CXCL5-8, known as ELR^+ chemokines referencing the conserved amino acids motif of Glu-Leu-Arg. ELR^+ chemokines are known chemoattractant of myeloid cells such as neutrophils and MDSCs (Vandercappellen, Van Damme et al. 2008, Zlotnik and Yoshie 2012). For that, CXCR2 axis is often considered pro-tumorigenic in many cancers (Bizzarri, Beccari et al. 2006, Strieter, Burdick et al. 2006). In PDAC CXCR2 axis is involved in MDSCs recruitment, angiogenesis, tumor cells proliferation and migration. Upregulation of CXCR2-axis in PDAC is associated with tumor-supporting inflammation, immunosuppression, angiogenesis and tumor growth. This has made CXCR2 a hot target for PDAC therapy (Vandercappellen, Van Damme et al. 2008, Chao, Furth et al. 2016, Purohit, Varney et al. 2016). The CXCR2 axis adverse role in PDAC was more apparent in line with the oncogenic Kras mutation (Purohit 2015, Purohit, Varney et al. 2016). Purohit et al. generated a syngeneic $Cxcr2^{-/-}$ model using PDAC cells with oncogenic Kras. This stromal

ablation of CXCR2 inhibited tumor growth, reduced immunosuppression by lowering infiltration of MDSCs, reduced angiogenesis, but also increased the fibrotic reaction in the primary tumor and increased metastasis. The increased fibrosis in response to CXCR2 inhibition suggests that CXCR2 axis may play a role in regulating CAFs (Purohit 2015). It is known that CAFs secrete CXCL8 (Omary, Lugea et al. 2007); but, little is known about the role of CXCR2 in CAFs. Few reports have linked CXCR2 to the stromal compartment in PDAC. Inhibiting CXCR2 in the genetically engineered PDAC mouse model that carries oncogenic Kras mutation and TGF-β receptor knockout disrupted the tumor-stromal interactions and improved mice survival (Ijichi, Chytil et al. 2011). Steele et al. used a mouse model with oncogenic Kras and p53 mutations and concluded that CXCR2 is abundant in the stromal regions and that inhibiting CXCR2 suppresses metastasis, and improves survival by reducing MDSCs infiltration, although the author did not elaborate on the effect of CXCR2 inhibition on CAFs (Steele, Karim et al. 2016).

The proposed role of CXCR2 axis in CAFs goes along with what Ohlund et al. reported that the secretory CAFs develop at a distance from tumor cells (Ohlund, Handly-Santana et al. 2017). ELR^+, as well as other chemokines, are considered far-reaching compared to other cytokines such as TGF-β. Chemokines make gradients to recruit target cells from distant locations such as the circulation or the bone marrow, whereas the effect of cytokines is often local. CXCR2 axis is also known to activate NF-κB, and the sustained CXCR2 signaling was even implicated in the induction of senescence (Acosta, O'Loghlen et al. 2008, Acosta,

O'Loghlen et al. 2008, Acosta and Gil 2009, Cavalli, Biavasco et al. 2014, Lesina, Wormann et al. 2016). Overall, we believe that secretory CAFs develop in the context of inflammation. Inflammatory mediators such as ELR^+ chemokines and DAMPs activate inflammatory pathways including NF-κB and STAT1 and render CAFs secretory.

The proposed role secretory CAFs in PDAC

Secretory CAFs produce several paracrine factors including interleukins, chemokines and growth factors such as VEGF (Omary, Lugea et al. 2007, Moir, Mann et al. 2015). The factors with more consensus include IL-6 and CXCL8, among other ELR^+ chemokines. The secreted factors produced by CAFs have been implicated in multiple pro-tumorigenic events including tumor cells proliferation and migration, stemness, immunosuppression, chemotherapy resistance, and invasion; however, some of these events lack consensus. The roles of IL-6 and CXCL8 in cancer is often associated with increased tumor cells proliferation, recruitment of MDSCs, angiogenesis, tumor cells stemness (Singh, Varney et al. 2009, Purohit 2015, Chan, Hsu et al. 2016, Purohit, Varney et al. 2016, Steele, Karim et al. 2016, Su, Chen et al. 2018). Thus, we expect to find out that tumors with abundance in secretory CAFs to be bigger in volume due to proliferation cues and vascularity, immunosuppressive due to MDSCs infiltration, and resistant to chemotherapy with enhanced undifferentiated histology as a result of stemness. On the other hand, the abundance of myofibroblast will likely result in increased stiffness, hypoxia, induction of EMT, infiltration of macrophages and metastasis. Such characteristics observed with stromal CXCR2 deletion.

CONCLUSION

PDAC remains one of the most challenging human malignancies due to its late detection and low effectiveness of current therapies. The characteristic complex tumor microenvironment and the dense desmoplastic reaction in PDAC contribute to tumorigenicity and tumor progression. CAFs represent a major component in PDAC tumor microenvironment and contribute to tumor progression and dissemination. Based on the information available in the literature, we discussed the role of genetic alterations in PDAC development, the impact of inflammation and secreted mediators on tumor progression, the CAFs heterogeneity effect on tumor outcome. We gathered thus far, oncogenic Kras derives tumorigenesis and contribute inflammation by upregulating CXCR2 axis. CXCR2 signaling promotes tumor growth, immunosuppression, and angiogenesis. There are at least two functional entities within the CAFs population. Myofibroblasts, the typical CAFs are characterized by enhanced ECM production and the expression of αSMA; whereas, the secretory CAFs propagates inflammation by secreting mediators such as IL-6 and ELR^+ chemokines. We believe that oncogenic Kras-CXCR2 axis promotes the secretory CAFs and that the abundance of certain CAFs subtype could impact the tumor outcome.

HYPOTHESIS AND SPECIFIC AIMS

PDAC remains one of the most challenging human malignancies due to its late detection and low effectiveness of current therapies. The characteristic complex tumor microenvironment and the dense desmoplastic reaction in PDAC contribute to tumorigenicity and tumor progression. CAFs represent a major component in PDAC tumor microenvironment and contribute to tumor progression and dissemination. Based on the information available in the literature, we discussed the role of genetic alterations in PDAC development, the impact of inflammation and secreted mediators on tumor progression, the CAFs heterogeneity effect on tumor outcome. We gathered thus far, oncogenic Kras derives tumorigenesis and contribute inflammation by upregulating CXCR2 axis. CXCR2 signaling promotes tumor growth, immunosuppression, and angiogenesis. There are at least two functional entities within the CAFs population. Myofibroblasts, the typical CAFs are characterized by enhanced ECM production and the expression of αSMA; whereas, the secretory CAFs propagates inflammation by secreting mediators such as IL-6 and ELR^+ chemokines. We believe that oncogenic Kras-CXCR2 axis promotes the secretory CAFs and that the abundance of certain CAFs subtype could impact the tumor outcome.

Based on that, our ***central hypothesis*** for this project is that: oncogenic Kras-CXCR2 axis modulates the CAFs function and activity and thus impacts PDAC outcome.

Specific Aims

To test our hypothesis, I pursued the following specific aims.

Specific Aim 1: Define the role of oncogenic Kras-CXCR2 axis in CAFs function and activity in PDAC.

Specific Aim 2: Evaluate the CXCR2-dependent role of CAFs in PDAC.

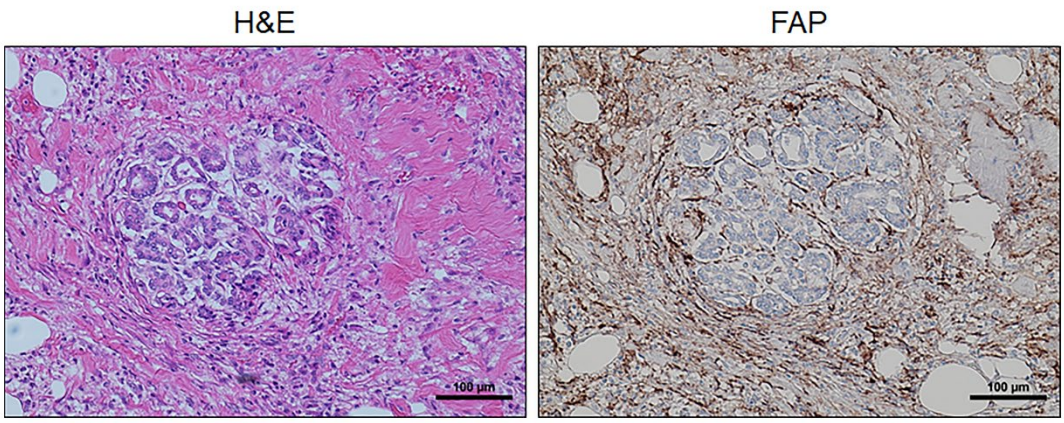

H&E
FAP
100 µm
100 µm

Figure 1.1: Stromal infiltration in human PDAC

Microscopic images that show the extent of CAFs infiltration in human PDAC sections stained with H&E (left) or the CAFs marker FAP (right).

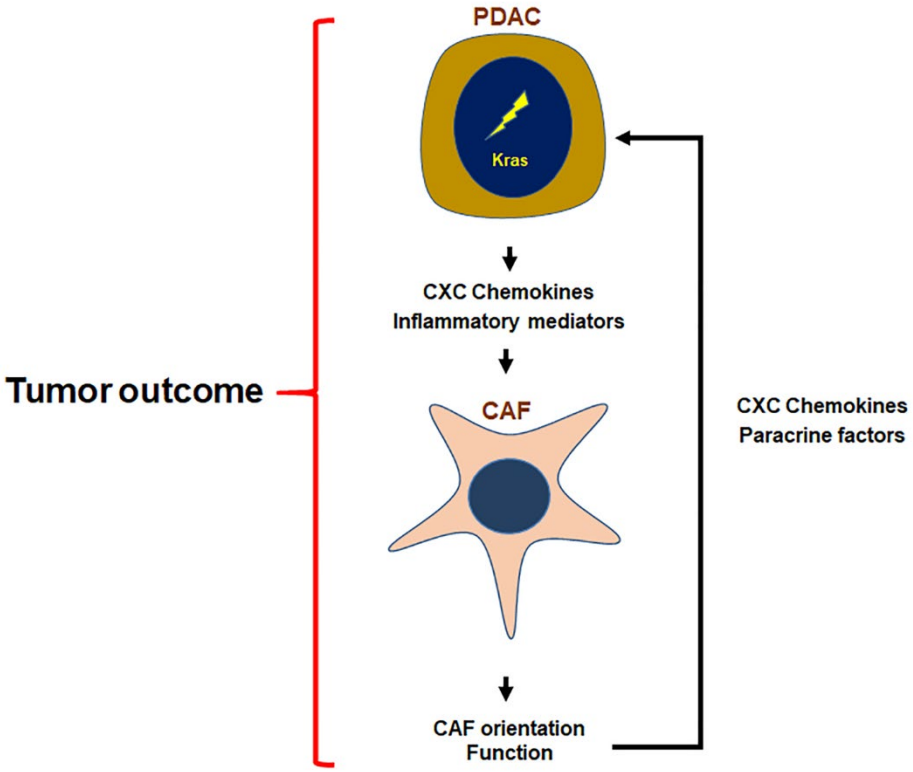

PDAC
Kras
CXC Chemokines
Inflammatory mediators
CAF
CAF orientation
Function
Tumor outcome
CXC Chemokines
Paracrine factors

Figure 1.2: Depiction of the hypothesized CXCR2-dependent role of CAFs in PDAC.

Oncogenic Kras-CXCR2 axis modulates the CAFs function and activity and thus impacts PDAC outcome.

CHAPTER II: MATERIALS AND METHODS

CELL LINE CULTURES

Cell lines and culture conditions

Murine cell lines

PDAC murine cells Panc02 cells and UN-KC-6141 cell line (referred to in this study as KC), and the immortalized mouse pancreatic stellate cells (ImPSC), were a kind gift from Dr. Surinder K. Batra's laboratory at UNMC. Panc02 were maintained in Roswell Park Memorial Institute Medium (RPMI) (HyClone®, GE Life Sciences, UT), and KC along with ImPSC cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (HyClone®, Thermo Scientific, UT). These media were supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, GA), L-glutamine (MediaTech, VA), twofold vitamin solution (MediaTech) and gentamycin (Gibco, Life Technologies, NY).

Human cell lines

CAF cell lines: Immortalized human CAF cell line (10-32 PC Puro, a kind gift from Dr. Surinder K. Batra's laboratory at UNMC) was maintained in RPMI media supplemented with 5% FBS, L-Glutamine, twofold vitamin solution, gentamycin and 5 µg/mL of puromycin Dihydrochloride (Herndon, VA). Normal human fibroblast, BJ cell line, was obtained from ATCC was maintained in Eagle's Minimum Essential Medium (EMEM; Cellgro, Herndon, VA) supplemented with 10% FBS, streptomycin, and penicillin. Immortalized human CAFs (CAF; a kind gift from Dr. Surinder K. Batra's laboratory at UNMC) was derived from the pancreatic tumor tissues. The pancreatic tumor was minced, and fibroblasts were

isolated by differential trypsinization, which was subsequently immortalized using hTERT. It was then maintained in EMEM supplemented with 10% FBS, streptomycin, and penicillin.

HPNE and HPNE-Kras: Immortalized human pancreatic duct-derived cell lines that express exogenous *KRAS*$^{(G12D)}$ (HPNE-Kras) or normally express wildtype Kras (HPNE) (Campbell, Groehler et al. 2007) were maintained in special media consisted of three parts DMEM (HyClone®, Thermo Scientific, UT) and one part in M3:5 growth medium (INCELL, San Antonio, TX) supplemented with 5% FBS, L-glutamine, twofold vitamin solution and gentamycin.

PDAC human cell lines: HPAF was maintained in RPMI 1640 supplemented with 5% FBS, L-glutamine, twofold vitamin solution and gentamycin. HPAF-CD11 were in DMEM supplemented with 10% FBS, L-glutamine, twofold vitamin solution and gentamycin. The previously described CD18/HPAF cell lines that was either transfected with *KRAS*$^{(G12D)}$ knockdown vector (CD18/HPAF-Kras KD) or control vector (CD18/HPAF-scram) (Rachagani, Senapati et al. 2011) were maintained in DMEM supplemented with 5% FBS, L-Glutamine, twofold vitamin solution, Gentamycin and 5 µg/mL of puromycin Dihydrochloride.

Generation of conditioned media

Cells were cultured in their respective complete media for 24h at density of $1x10^5$ cells per well in a six-well plate, then media was removed, cells washed with Hanks's balanced salt solution (HBSS, Cellgro, Herndon, VA) and the media was changed to serum-free media for 24h or 72h.

Generation of ECM from CAFs

We used the methods established by (Mizuguchi, Utoguchi et al. 1997). Briefly, we seeded CAFs cell ($1x10^5$ cells) onto six-well plate and culture them with complete media. After the CAFs cell reach confluence (~24 hours later), we removed the media and washed the cells once with PBS, and then added 1ml of the aqueous solution of 0.02N ammonia to the cells, and incubated them at room temperature for 10 min to lyse the cells. We removed any remaining cellular debris from the culture plate by gentle pipetting and washed the resulting lysate over ten times HBSS.

Co-culture using conditioned media and treatment with exogenous chemokines and inhibitor

Cells were seeded at a density of $1x10^5$ cells/well using six-well plates and maintained in complete media for 24h. Complete media was replaced with serum-free media, diluted conditioned media, or respective treatment and incubated for the respective time.

Co-culture using CAF monolayer or ECM

To generate CAF monolayer, $1x10^6$ cells of CAFs were seeded in the six-well plate and incubated with complete media for 24 hours. Pancreatic cancer cells at a density of $1x10^5$ were then seeded onto the CAFs monolayer or the CAFs ECM and co-cultured in complete media. After a 24-hour incubation, complete media was changed to serum-free media (day 0) and incubated for an additional

72h (day 4). We counted the number of tumor cell and calculated the differences between Day 0 and Day 4.

ANIMAL MODEL AND DETAILS OF *IN VIVO* STUDIES

Study approval

Mice were maintained under specific pathogen-free conditions. All procedures performed were in accordance with institutional guidelines and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC).

Syngeneic mouse models

We utilized two models of a syngeneic immunocompetent mouse model to study the effect of Kras mutation and stromal CXCR2 signaling on the tumor. Two different murine PC cell lines Panc02-GLUC-GFP (wildtype Kras; contains a single nucleotide polymorphism in Kras gene from TAT to TAC at codon 32) (Wang, Zhang et al. 2012) and KRAS-PDAC-GFP (oncogenic Kras) (Purohit, Varney et al. 2016), were inoculated orthotopically in the pancreas of 6-8 weeks old $CXCR2^{+/+}$ (Wildtype) and $CXCR2^{-/-}$ (knockout) mice. Mice were sacrificed after 4-6 weeks as previously described (Purohit 2015). A part of the tumor was fixed in 10% formalin and processed for histological analysis.

REAGENTS AND ANTIBODIES

CXCR2 inhibitors SCH-479833 and SCH-527123, obtained from Schering-Plough Research Institute, and prepared by dissolving in 20% hydroxypropyl-β-cyclodextrin (HPβCD; Acros Chemical St. Louis, MO). Exogenous human CXCL8 and exogenous murine CXCL1 were obtained from (R & D Systems, Minneapolis, MN, USA). A list of all the antibodies used for the present study is available in **Table 2.1**.

GENE EXPRESSION ANALYSIS

RNA isolation

Total RNA was isolated from cells and homogenized tissues using the standard Trizol (Invitrogen, Carlsbad, CA) protocol. Briefly, cells were lysed with 1 ml of Trizol followed by adding 0.2 ml of chloroform and vigorous shaking. After 2-3 min incubation, the mixture was separated by centrifugation at 12,000 g for 15 min. The aqueous phase was then transferred and mixed with 750 µl of isopropanol and incubated in the rotator for 10 min. Tubes were then centrifuged at 12,000 g for 10 min, and the supernatant was discarded. The pellet was washed with 75% ethanol, air dried and eluted in DEPC-treated water.

PCR analysis

Reverse Transcription was performed with 1µg RNA using iScript™ Reverse Transcription Supermix for qRT-PCR (BIO-RAD, Hercules, CA, USA). Regular PCR reactions were performed using Fast Start Taq dNTPack (Roche

Diagnostics, IN, USA). Quantitative real-time PCR reactions were performed using iTaq Universal SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) using the CFX Connect™ Real-Time PCR Detection System (BIO-RAD). Primer sets used for the study are listed in **Table 2.2**. For regular PCR, amplified cDNA was resolved on EtBr containing 2% agarose gels. For real-time PCR mean Ct values of the target genes were normalized to mean Ct values of one or more the housekeeping control genes (Ribosomal protein large 13 A (RPL13A), β Actin, Peptidylprolyl Isomerase A (PPIA) and Hypoxanthine phosphoribosyltransferase 1 (HPRT1)); [-ΔCt = Ct (housekeeping gene) – Ct (target gene)]. The ratio of mRNA expression of target genes versus the housekeeping gene was defined as 2(-ΔCt). Melting curve analysis was performed to check the specificity of the amplified product.

Gene expression microarray

PDAC cell lines HPAF and HPAF-CD11 were cultured alone or on CAF monolayer. Next, nucleic acid was collected for cDNA microarray analysis using a set of two 10K chips (Compugn/Sigma Genosys) that interrogate the full 18+ Compugen Human oligonucleotide at DNA Microarray core facility (UNMC). The library contains 18,861 oligos representing 17,260 unique genes. Raw fluorescent intensity values were collected to determine gene expression levels. Flagged artifacts and negative controls were removed from the series. The data was then normalized, and the channels (Cy3 and Cy5) were background subtracted. The normalized and background subtracted values were log2 transformed. The fold-change was calculated between the Cy3 and Cy5 channels. Emphasis was placed on genes demonstrating greater than 2 fold-change in expression between the two

channels. A list of 169 chemokines and cytokines identified by the KEGG_CHEMOKINE_SIGNALING_PATHWAY and BIOCARTA_CYTOKINE_PATHWAY were identified in the dataset and used for differential expression where indicated. Cluster 3.0 was used to median-center the genes prior to heat map generation in Java TreeView.

PROTEIN ANALYSIS

Protein isolation

The total protein was isolated by lysing cells with RIPA buffer, and the protein concentrations were determined using BCA kit (Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Western blot analysis

Protein samples (40 µg or 25 µg) were electrophoresed on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto Immobilon-p Transfer membrane (Millipore, Billerica, Massachusetts, USA). Membranes were blocked with 3% BSA in PBS for 1 hour at room temperature. Membranes were probed with respective specific primary antibodies (according to dilutions in **Table 2.1**) overnight at 4˚C. Membranes were washed with tween 20 tris-buffered saline (TTBS) buffer, three times and probed with respective secondary antibodies. Following washing with TTBS buffer membranes were visualized using Luminata Forte Western HRP Substrate Kit (Millipore, Billerica, MA).

Enzyme-linked immunosorbent assay (ELISA)

Murine CXCL1,2,5 and 7

Murine cells were seeded at $1x10^5$ density in a six-well plate then treated with respective treatment for the respective time points. The supernatants of cultured cells were collected for ELISA. ELISA assays for mCXCL1, mCXCL2, mCXCL5, and mCXCL7 were performed using a duoset kit (R & D Systems,

Minneapolis, MN, USA) according to the manufacturer's protocol using Bio-Tek plate reader (Winooski, VT). Briefly, plate preparation was done by diluting the capture antibody to the working concentration with PBS and using it to coat the 96-well microplate with 100 µl per well that was then incubated overnight at room temperature. Next day, the coated microplate was washed three times with wash buffer (~400 µl) per well then dried by aspirating the liquid and blotting inverted against paper towels. Plates were then blocked by adding 300 µl of the reagent diluent to each well followed by 1h incubation. After another course of three-time washing, 100 µl of each sample or standard (diluted with sample diluent) was added to the respective well and incubated for 2h at room temperature. Wells were then washed three times with PBS and a 100 µl of the detection antibody working dilution was added per well for 2h followed by three-time washing and adding of the Streptavidin-HRP (100 µl) per well for 20 min in the dark. Lastly, the wells were washed three times then 100 µl of the substrate was added to each well for 20 min in the dark and reaction was stopped by adding 50 µl of the stop solution to each well. Optical density was determined at 450 nm wavelength.

Human CXCL8 and FGF-2

Human cells were seeded according to their respective experiment and supernatants were taken for ELISA analysis.

CXCL8 levels in culture supernatants were determined using an ELISA kit paired antibody purchased from Pierce Inc. (Woburn, MA), according to manufacturer instructions. Briefly, 100 µl of the primary monoclonal antibody

against CXCL8 (2 µg/ml) was coated in Immulon plates in each well. After 1h of incubation at 37oC, the plates were washed and blocked for 1h with blocking buffer (4 % BSA in PBS). After washing the plates four times, 50 µl culture supernatants or standards at different concentrations (recombinant CXCL8 protein, Endogen Inc. Woburn, MA) and 50 µl of biotinylated CXCL8 Ab was added to each well. After 2h of incubation, the plates were washed, and the immunoreactivity determined using the avidin-HRP-TMB detection system (Dako Labs. Denmark). The reactions were stopped by addition of 50 µl of 0.18 N H2SO4 and absorbance determined using an ELISA microtiter plate reader (Bio-Tek Instruments Inc. Winooski, VT) at 450 nm. A curve of the absorbance versus the concentration of CXCL8 in the standard wells was plotted. By comparing the absorbance of the samples to the standard curve, we determined the concentration of CXCL8 in the unknown samples.

For analyzing levels of FGF-2, we used direct ELISA. Samples and different concentrations of recombinant FGF-2 protein (for standard curve) was coated onto ELISA plate overnight. Following washing and blocking non-specific activity, 100 µl of anti-FGF-2 antibody (R & D System, Minneapolis MN) was added into each well. Following two hours of incubation, samples were incubated with biotinylated secondary antibody and immunoreactivity was determined using avidin-HRP-TMB detection system. A curve of the absorbance versus the concentration of FGF-2 in the standard wells was plotted. By comparing the absorbance of the samples to the standard curve, we determined the concentration of FGF-2 in the unknown samples.

Immunofluorescence (IF)

Cells were cultured on four-well chamber slides and treated according to their respective experiment. After ceasing the treatment, cells were washed three times with PBS and fixed using 4% paraformaldehyde, then again washed with PBS for three times. Cells were then blocked with antibody diluent (BD Biosciences) or blocking buffer (PBS with 3% BSA and 0.1% Saponin) for cytoplasmic targets. Cells were probed with the respective antibody (according to **Table 2.1**) at 4˚C overnight. The next day, slides were stained with the respective antibody and counterstained with the nucleic staining 4, 6 diamidino-2-phenylindole (DAPI). Finally, slides were mounted with Vectashield® mounting medium (Vector Laboratories, Burlingame, CA, USA) and observed under a fluorescent microscope.

Immunohistochemistry (IHC)

Sections of 4µm thick from formalin-fixed, paraffin-embedded tissues were deparaffinized with xylene and rehydrated by incubating with decreasing ethanol concentrations. Antigen retrieval was performed using sodium citrate buffer (pH = 6.0) and microwaving for 10 minutes. Endogenous peroxidase was blocked by incubating with 3% hydrogen peroxide in methanol for 30 minutes. After blocking non-specific binding by incubating with serum, slides were probed with primary antibody **(Table 2.1)** overnight at 4ºC. Slides were washed, and the appropriate secondary antibodies were added. Immunoreactivity was detected using the ABC Elite Kit and 3, 3 diaminobenzidine substrate kit (DAB) from (Vector Laboratories,

Burlingame, CA) according to the manufacturer standard protocols. A reddish brown precipitate indicated positive staining. Nuclei were counterstained with hematoxylin. Quantitation was done by counting positive cells in five independent areas at x400.

IN VITRO CELL-BASED ASSAYS

In vitro cell proliferation assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Cells were seeded at appropriate densities in 96-well plates and were allowed to adhere overnight. Cells were washed with HBSS and were incubated with serum-free media alone or with other treatments (conditioned media, CXCR2 inhibitors, exogenous CXCR2 ligands) for 72 hours. Cell viability was determined by MTT assay as previously described (Li et al., 2001). Briefly, 50 µl of the MTT reagent (Thermo Fisher Scientific, Fair Lawn, NJ) was added to each well and incubated for 2-4h. Media and MTT were removed and replaced by 100 µl of dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, Fair Lawn, NJ)

The formula calculated percent inhibition of cell growth: [100 - (A/B) x 100], where ‘A’ and ‘B’ are the absorbance of the treated and Control group, respectively. Percentage of cell growth was calculated by the formula: [(A/B) x 100], where ‘A’ and ‘B’ are the absorbance of treatment and control group respectively.

STATISTICAL ANALYSIS

The statistical analysis was performed using Prism 7 (GraphPad) software. Statistical method and sample size (n; the number of replicates) are indicated in the figure legends. Statistical significance was defined as $p<0.05$. Error bars on figures show standard error of the mean (SEM). Two-tailed Student's t-test, ANOVA and Posthoc comparisons using Mann-Whitney tests with a Bonferroni adjustment were performed when appropriate as indicated in figure legends.

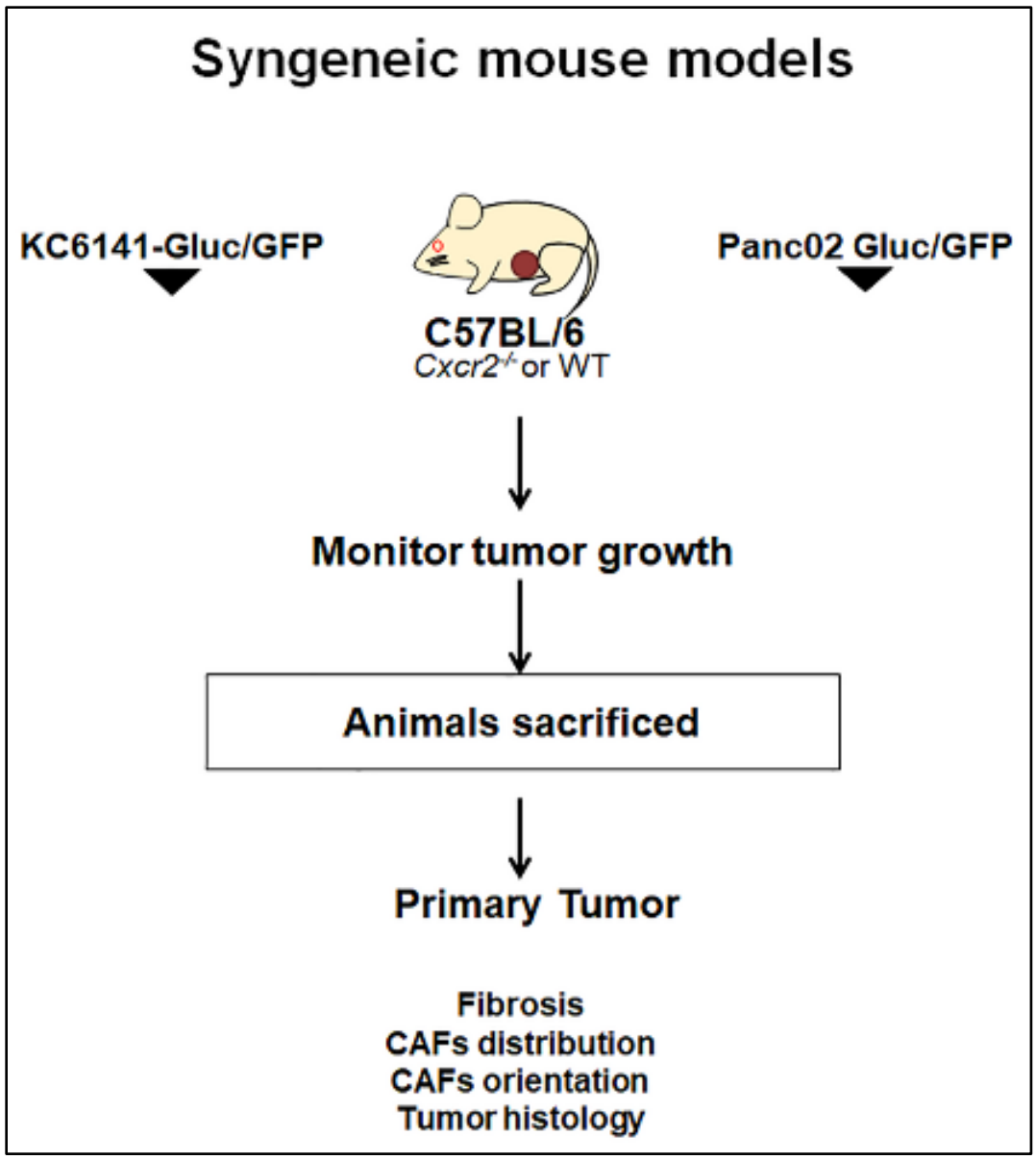

Syngeneic mouse models
KC6141-Gluc/GFP
Panc02 Gluc/GFP
C57BL/6
Cxcr2-/- or WT
Monitor tumor growth
Animals sacrificed
Primary Tumor
Fibrosis
CAFs distribution
CAFs orientation
Tumor histology

Figure 2.1: Mouse models

Syngeneic mouse models: C57BL/6 mice (wildtype or Cxcr2$^{-/-}$) orthotopically transplanted with PDAC murine cells KC (oncogenic Kras) or Panc02 (wildtype Kras).

Table 2. 1: List of antibodies

Species reactivity in this paper	Antibody	Supplier	Catalogue number	Host species	Dilution IHC/IF (WB)
Mouse/Human	CXCR2	Gift from Dr. Strieter		Goat	1:1000
Mouse/Human	αSMA	ThermoFisher	MA5-11547	Mouse	1:250 (1:500)
Mouse/Human	FAP	Abcam	ab207178	Rabbit	1:1000
Mouse/Human	CD10	Abcam	ab951	Mouse	1:50
Mouse/Human	p50	Biolegend	616701	Mouse	1:100
Mouse/Human	HSP70	Santacruz	sc-32239		(1:250)

Table 2. 2: List of primers

Gene	Temp. (ºC)	Species	Sequence
Cxcl1	55˚	Murine	Forward 5'-TCGCTTCTCTGTGCAGCGCT-3' Reverse 5'- GTGGTTGACACTTAGTGGTCT C-3'
Cxcl2	57˚	Murine	Forward 5'-AGTGAACTGCGCTGTCAATG-3' Reverse 5'-TTCAGGGTCAAGGCAAACTT-3'
Cxcl3	68˚	Murine	Forward 5'-GCAAGTCCAGCTGAGCCGGGA-3' Reverse 5'-GACACCGTTGGGATGGATCGCTTT-3'
Cxcl5	68˚	Murine	Forward 5'-ATGGCGCCGCTGGCATTTCT-3' Reverse 5'-CGCAGCTCCGTTGCGGCTAT-3'
Cxcl7	57˚	Murine	Forward 5'-CTCAGACCTTACATCGTCCTGC-3' Reverse 5'-AGCGCAACAAGGATCGTCCTGC-3'
Ccl5	56˚	Murine	Forward 5'-GCTGCTTTGCCTACCTCTCC-3' Reverse 5'-TCGAGTGACAAACACGACTGC-3'
Ccl3	56˚	Murine	Forward 5'-TTCTCTGTACCATGACACTCTGC-3' Reverse 5'-CGTGGAATCTTCCGGCTGTAG-3'
Ccl2	56˚	Murine	Forward 5'-TTAAAAACCTGGATCGGAACCAA-3' Reverse 5'-GCATTAGCTTCAGATTTACGGGT-3'
IL1	56˚	Murine	Forward 5'-GCAACTGTTCCTGAACTCAACT-3' Reverse 5'-ATCTTTTGGGGTCCGTCAACT-3'
IL4	56˚	Murine	Forward 5'-GGTCTCAACCCCCAGCTAGT-3' Reverse 5'-GCCGATGATCTCTCTCAAGTGAT-3'
IL6	55˚	Murine	Forward 5'-CCTCTGGTCTTCTGGAGTACC-3' Reverse 5'-ACTCCTTCTGTGACTCCAGC-3'
IL10	57˚	Murine	Forward 5'-GCTCTTACTGACTGGCATGAG-3' Reverse 5'-CGCAGCTCTAGGAGCATGTG-3'
IL12	57˚	Murine	Forward 5'-TGGGTTTGCCATCGTTTTGCTG-3' Reverse 5'-ACAGGTGAGGTTCACTGTTTCT-3'
IL13	55˚	Murine	Forward 5'-CCTGGCTCTTGCTTGCCTT-3' Reverse 5'-GGTCTTGTGTGATGTTGCTCA-3'
IL17	55˚	Murine	Forward 5'-TTTAACTCCCTTGGCGCAAAA-3' Reverse 5'-CTTTCCCTCCGCATTGACAC-3'
IFN-γ	57˚	Murine	Forward 5'-ATGAACGCTACACACTGCATC-3' Reverse 5'-CCATCCTTTTGCCAGTTCCTC-3'
TNF-α	57˚	Murine	Forward 5'-CCCTCACACTCAGATCATCTTCT-3' Reverse 5'-GCTACGACGTGGGCTACAG-3'
Rpl13a	58˚	Murine	Forward 5'-ACTCTGGAGGAGAAACGGAAGG-3' Reverse 5'-CAGGCATGAGGCAAACAGTC-3'
Actin β	57˚	Murine	Forward 5'-GGCTGTATTCCCCTCCATCG-3' Reverse 5'-CCAGTTGGTAACAATGCCATGT-3'
PPIA	57˚	Murine	Forward 5'-TGTGCCAGGGTGGTGACTTT-3' Reverse 5'-CGTTTGTGTTTGGTCCAGCAT-3'
HPRT	57˚	Murine	Forward 5'-CCTAAGATGAGCGCAAGTTGAA -3' Reverse 5'-CCACAGGACTAGAACACCTGCTAA-3'
Acta2 (αSMA)	56˚	Murine	Forward 5'-CCCAGACATCAGGGAGTAATGG-3' Reverse 5'-TCTATCGGATACTTCAGCGTCA-3'
COL1A1 (Collagen I)	56˚	Murine	Forward 5'-GCCCGAACCCCAAGGAAAAGAAGC-3' Reverse 5'-CTGGGAGGCCTCGGTGGACATTAG-3'
COL4A1 (Collagen IV)	55˚	Murine	Forward 5'-TCCGGGAGAGATTGGTTTCC-3' Reverse 5'-CTGGCCTATAAGCCCTGGT-3'

CHAPTER III: CAFS ENHANCE SURVIVAL AND PROGRESSION OF THE AGGRESSIVE PANCREATIC TUMOR VIA FGF-2 AND CXCL8

This chapter in part is derived from:

Awaji M, Futakuchi, M, Heavican T, Iqbal J, and Singh RK. Cancer-associated fibroblasts enhance survival and progression of the aggressive pancreatic tumor via FGF-2 and CXCL8. *Cancer Microenvironment., 2019 (In press).*

ABSTRACT

PDAC remains one of the most challenging human cancers. Desmoplasia is predominant in this disease exhibiting a strong stromal reaction with an abundance of the CAFs. We aimed in this study to investigate the reciprocal interaction between the tumor cells and the CAFs and its effect on tumor cells survival. We hypothesized that the survival of pancreatic cancer cell with aggressive phenotype is modulated by the interactions between malignant pancreatic tumor cells and surrounding CAFs. To examine this, we utilized co-culture methods where tumor cells with different malignant potentials, HPAF (low) HPAF-CD11 (moderate/high) co-cultured with CAFs. CAFs-conditioned media increased the growth of HPAF-CD11 but not HPAF cells and increased CXCL8 levels highly in HPAF-CD11 and slightly in HPAF. The growth stimulatory effect and elevated CXCL8 level caused by CAFs-conditioned media were diminished by neutralizing the fibroblast growth factor-2 (FGF-2). Also, conditioned media of HPAF-CD11 increased CAFs cell number whereas that of HPAF did not, and these effects were suppressed by neutralizing CXCL8. Furthermore, data from gene expression microarray study exhibited different expression profiles between HPAF and HPAF-CD11 when co-culture with CAFs. A significant increase in CXCL8 and FGF-2 expression was observed with HPAF-CD11/CAFs co-culture and to a lower extent with HPAF/CAFs co-culture. Together, these data demonstrate a paracrine bi-directional interaction between pancreatic tumor cells and the CAFs through CXCL8 and FGF-2 that helps the tumor growth. Future in-depth study of these

pathways will assist in obtaining diagnostic and therapeutic tools for pancreatic ductal adenocarcinoma.

INTRODUCTION

PDAC is the most common type of pancreatic cancers, the fourth leading cause of cancer deaths in the United States (Siegel, Miller et al. 2018). The increased incidence of the disease, as well as the cellular and molecular complexity of the tumor, makes it very challenging to manage. A chance of cure exists for only a minority of the patients, those with locally limited and surgically resectable tumors (Warshaw and Fernandez-del Castillo 1992). At the time of diagnosis, the majority of PDAC patients present at advanced stages beyond surgical resection. Studying the complex cellular and molecular interaction between malignant cells and other cells in the tumor microenvironment can shed more light on how the diseases initiate progresses and spreads.

Desmoplasia is of particular predominance in PDAC exhibiting a strong stromal reaction (Kuniyasu, Abbruzzese et al. 2001, Iacobuzio-Donahue, Ryu et al. 2002, Watanabe, Hasebe et al. 2003). A consistently low ratio of the infiltrating adenocarcinoma component relative to this abundant desmoplastic response is unique to PDAC, in contrast to infiltrating carcinomas in other organ or tissue types (Seymour, Hruban et al. 1994, Kalluri 2016). Typically, these invasive pancreatic tumors are composed of infiltrating adenocarcinoma surrounded by a predominance of dense fibrous (or desmoplastic) stroma (Kloppel, Lingenthal et al. 1985), which itself contains proliferating CAFs, small endothelial-lined vessels, inflammatory cells, and trapped residual atrophic parenchymal components of the organ invaded (Ryu, Jones et al. 2001). CAFs, represent the fibrotic component of the tumor microenvironment, are derived from cells of multiple origins including

tissue resident fibroblasts, bone marrow-derived mesenchymal cells, fibrocytes, and PSCs (Öhlund, Elyada et al. 2014). PSCs, in particular, have gained much attention more than other subsets of CAFs. PSCs, similar to other stellate cells found in other organs such as in liver, kidneys, and lungs, are known to modulate physiological functions by storing vitamin-A at their quiescent state and tissue maintenance and repair at the activated state (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Apte, Pirola et al. 2015, Moir, Mann et al. 2015). In PDAC, activated PSCs have been described to be involved in tumorigenesis, therapy resistance, and metastasis (Xu, Vonlaufen et al. 2010, Lonardo, Frias-Aldeguer et al. 2012, McCarroll, Naim et al. 2014, Moir, Mann et al. 2015, Zambirinis, Levie et al. 2015). Interactions between the malignant cells and surrounding stromal CAFs have been suggested to play a critical role in tumor invasion and progression (Grey, Schor et al. 1989, Camps, Chang et al. 1990). Once tumor cells have spread to different microenvironment, their subsequent growth will depend on the compatibility of the “seed” with the “soil” that they encounter in the microenvironment (Paget 1889, Hart 1982), which depend on the molecular interactions between cancer cells and the stromal cells in the different microenvironment (Chambers, Groom et al. 2002, Fidler, Yano et al. 2002). Invasive cancers do not exist in isolation; rather, they arise from and intimately interact with non-neoplastic host cells (Maehara, Matsumoto et al. 2001, Qian, Mizumoto et al. 2003).

For long, CAFs have been regarded for their role in the formation of desmoplasia, by producing excessive amounts of ECM proteins (Apte and Wilson

2004). Desmoplasia aids in acquiring resistance to current chemotherapy treatments (Olive, Jacobetz et al. 2009, Jacobetz, Chan et al. 2012, Provenzano, Cuevas et al. 2012). Nonetheless, recent literature describes a vast network of CAFs interactions beyond the desmoplasia formation. Through their network of secreted factors, such as cytokine, chemokines and growth factors, CAFs can interact with the multiple components in the tumor microenvironment to modulate tumor progression in different malignancies (De Wever and Mareel 2003, Micke and tman 2004, Cheng, Bhowmick et al. 2005, Paulsson and Micke 2014).

FGF-2 is a member of the FGF family that control multiple cellular processes including proliferation, differentiation, survival, and motility (Basilico and Moscatelli 1992). In the context of cancer, FGF-2 has been shown to promote tumor progression (Polnaszek, Kwabi-Addo et al. 2003). Enhanced FGF-2 protein levels have been shown to correlate with shorter postoperative survival of patients with PDAC (Kleeff, Kothari et al. 2004). Furthermore, FGF-2 was linked to PDAC invasion via its activity in PSCs (Coleman, Chioni et al. 2014).

A member of the CXC chemokine family, CXCL8 signals through CXCR1 and CXCR2 chemokine receptors. These chemokines are known for their role in inflammation by recruiting inflammatory cells and inducing angiogenesis. In malignant tumors, sustained CXCL8 signaling is associated with immunosuppression, angiogenesis, and tumor growth; thus, essential to the progression of PDAC (Saintigny, Massarelli et al. 2012, Liu, Li et al. 2016, Purohit, Varney et al. 2016). There is evidence that CXCL8 and FGF-2 are involved in tumor-stromal interaction (Giri and Ittmann 2001, Coleman, Chioni et al. 2014).

We hypothesized that the aggressive phenotype of PDAC depends on their interaction with CAFs, which involves FGF-2 and CXCL8. To test this hypothesis, we examined the effect of CAFs on pancreatic tumor cells with different malignant potential, HPAF (low) and HPAF-CD11 (moderate/high). HPAF-CD11 is derived from the parent cell line HPAF, where both cells show well-differentiation features and mutations in both *Kras* and *TP53* (Kim, Kern et al. 1989, Egami, Takiyama et al. 1990, Batra, Metzgar et al. 1991, Wang, Knezetic et al. 1996, Ding, Fehsenfeld et al. 2000). We demonstrated that the aggressive phenotype of PDAC has a stronger bi-directional interaction with CAFs through paracrine factors such as FGF-2 and CXCL8.

RESULTS

Aggressive pancreatic tumor cell survived following co-culture with fibroblasts in the absence of serum

We examined the growth of HPAF and HPAF-CD11 cells in the presence or absence of serum-containing media when cultured with or without fibroblasts. Both HPAF and HPAF-CD11 cells showed serum dependency irrespective of their aggressive differential phenotype when cultured in the absence of fibroblasts **(Figure 3.1A and 3.1B)**. Further, the growth of HPAF cells was inhibited following co-culture with fibroblasts (BJ cells monolayer) in the presence of or absence of serum **(Figure 3.1C)** as compared to HPAF cells cultured alone. The level of inhibition of HPAF cells growth following co-culture with fibroblasts was similar to that observed when HPAF cells alone were cultured in the absence of serum **(Figure 3.1A)**. In contrast, we observed an increased survival and growth of HPAF-CD11 cells following co-culture with fibroblasts (BJ cells monolayer). Interestingly the growth of HPAF-CD11 cells was enhanced in following co-culture with fibroblasts in the absence of serum **(Figure 3.1D)**.

Survival of tumor cells is mediated by CAFs conditioned media

To evaluate whether the survival of HPAF-CD11 cells on the fibroblasts monolayer was mediated by the direct contact or paracrine factors, HPAF and HPAF-CD11 cells were incubated with CAF-conditioned media or ECM generated from CAFs, and the increase in cell number was quantitated. No increase in the cell number was observed in HPAF cells in response to CAFs conditioned media, and further inhibition was detected with ECM culture **(Figure**

3.2A). We observed a significant increase in the number of HPAF-CD11 cells following co-culture with both CAFs conditioned media and CAFs ECM (Figure 2B). The increase in HPAF-CD11 cells cultured with CAFs conditioned media was greater than that observed in response to ECM but less than that observed with serum containing media (Figure 3.2B). Next, we examined the dose-dependence of CAFs-conditioned media on the growth of HPAF-CD11 cell (Figure 3.2C) and demonstrated that increasing concentrations of CAFs conditioned media increased the growth of HPAF-CD11 cells. Together, we perceive that CAFs-derived paracrine factors contribute to the survival of the aggressive PDAC cells.

CAFs promote tumor cell survival via FGF-2

To determine the putative growth factors present in CAFs conditioned media, we examined the effect of the neutralizing antibodies of FGF-2 and CXCL8, which have been shown to be involved in the tumor-stromal interaction (Giri and Ittmann 2001, Coleman, Chioni et al. 2014), on the survival of HPAF-CD11 cells. Anti-FGF-2 antibody treatment significantly abrogated the increase of cell number of HPAF-CD11 following culture with CAFs conditioned media (Figure 3.3A), while anti-CXCL8 antibody treatment did not (Figure 3.3B). To confirm that FGF-2 is produced by CAFs, we performed ELISA on the CAF supernatant collected in serum-free media or complete media (Figure 3.3C). These results indicate that FGF-2 but not CXCL8 in the CAFs conditioned media was involved in the survival of HPAF-CD11.

FGF-2 secreted by CAF induces CXCL8 production in tumor cells

CXCL8 and other CXC chemokines that signal through CXCR1/2 axis are known to be expressed in PDAC cells (Le, Shi et al. 2000, Takamori, Oades et al.

2000, Frick, Rubie et al. 2008). Autocrine and paracrine signaling through CXCR1/2 axis plays a vital role in the progression of PDAC by promoting tumor cells growth, angiogenesis, immunosuppression and chemotherapy resistance (Strieter, Burdick et al. 2006, Highfill, Cui et al. 2014, Chan, Hsu et al. 2016, Purohit, Varney et al. 2016). To examine the involvement of FGF-2 in the CXCL8 production by PDAC cells, we determined CXCL8 production in HPAF and HPAF-CD11 cell after treatment with CAFs-conditioned media using ELISA. Our data shows that HPAF-CD11 produce more CXCL8 than their HPAF counterparts by comparing CXCL8 levels produced in serum-free media treatment as well as in response to CAFs-conditioned media treatment **(Figure 3.4A-B)**. By comparing the CXCL8 production in each cell in response to CAFs-conditioned media, we show that CAFs-conditioned media increased CXCL8 level in both HPAF and HPAF-CD11 cells, and neutralizing FGF-2 has lowered the CXCL8 inducing effect of the CAFs-conditioned media **(Figure 3.4C** and **3.4D)**.

Effect of tumor cell conditioned media on the survival of CAFs

Next, we examined the effect of the conditioned media from PDAC cells with different aggressiveness on the survival of CAFs. CAFs were incubated with the conditioned media of HPAF or and HPAF-CD11 for 1, 2, and 3 days, and the increase in cell number was quantitated. We observed a significant increase in the number of CAFs following co-culture with conditioned media of HPAF-CD11 cell at each time point but not with that of HPAF **(Figure 3.5A)**. To determine the putative growth factors present in the conditioned media of HPAF-CD11, we examined the effect of the neutralizing antibodies of CXCL8 **(Figure 3.5B)**. Anti-CXCL8 antibody

treatment significantly abrogated the increase of cell number of CAFs following culture with HPAF-CD11 conditioned media. These results indicate that CXCL8 was involved in the survival of CAFs by the conditioned media of PDAC cells.

Aggressiveness-dependent gene expression of tumor cells when co-cultured with CAFs

Finally, we used gene expression microarray to explore the expressional differences between HPAF and HPAF-CD11 cells upon their co-cultured with CAF. Distinct gene expression profiles were observed for HPAF and HPAF-CD11 when compared alone or in co-culture. More focused look into the expression profile of paracrine factors revealed that CAFs are the major contributor of many cytokines and chemokines **(Figure 3.6A)**. Comparing HPAF/CAF co-culture to HPAF-CD11/CAF co-culture revealed upregulation of motility supporting gene *ELMO1* in HPAF-CD11 co-culture, whereas, mainly cytokines and chemokines were upregulated in the HPAF co-culture **(Figure 3.6A)**. Targeted look into CXCL8 and FGF-2 expression exhibited that CAFs are the leading producer of CXCL8 and that CXCL8 was upregulated in the co-culture condition compared to tumor cells cultured alone for both HPAF and HPAF-CD11 **(Figure 3.6B)**. For FGF-2, only HPAF-CD11 co-culture exhibited significance upregulation of the gene compared to the tumor cells cultured alone **(Figure 3.6B)**. Together, these data demonstrate the versatility of CAFs and their ability to support tumor cells in an aggressive-dependent manner.

DISCUSSION

PDAC, one of the most malignant tumors, is often characterized by an abundant desmoplastic stroma. CAFs, which constitute a primary stromal compartment in PDAC, have been shown to promote the invasive growth of several cancer types such as breast, prostate, and lung (Tuxhorn, Ayala et al. 2002, Micke and tman 2004). CAFs are often only associated with excess extracellular matrix production; thus, their contribution to desmoplasia (Apte and Wilson 2004). Recent studies have addressed the role of CAFs in pancreatic tumor aggressiveness. Non-irradiated CAFs significantly increased the invasive ability of pancreatic cancer cells and the invasiveness was further accelerated when they were co-cultured with irradiated CAFs (Ohuchida, Mizumoto et al. 2004). Nitric Oxide released by CAFs has been shown to lead to the upregulation of IL-1β in pancreatic carcinoma cells, leading to the induction of chemotherapy resistance in these tumor cells (Muerkoster, Wegehenkel et al. 2004). CAFs can produce many paracrine factors including chemokines, cytokines and growth factor, which allow interaction and subsequent modulation of other cells in the tumor microenvironment (Öhlund, Elyada et al. 2014). This secretory role of CAFs remained under-investigated. In the present study, we demonstrated that pancreatic tumors with more aggressive phenotype could interact with CAFs more than non-aggressive cells. These data underscored the importance of the interaction with CAFs in the exertion of the malignant potential of the pancreatic tumor.

FGF-2 is expressed in pancreas cancer (Yamanaka, Friess et al. 1993), as well

as in many other malignant neoplasms (Feng, Wang et al. 1997, Relf, LeJeune et al. 1997). FGFs bind to a family of transmembrane tyrosine kinase receptors (FGFRs 1-4), and FGFR-1 and FGFR-4 are potent receptors for FGF-2 (Ornitz, Xu et al. 1996). A member of the CXC chemokine family, CXCL8, its production has been correlated with tumor growth, immunosuppression, resistance to chemotherapy, angiogenesis, and increased metastatic potential of PDAC (Saintigny, Massarelli et al. 2012, Liu, Li et al. 2016).

In the current study, we demonstrate a bi-directional interaction between tumor cells and CAFs that creates a feedforward loop to promote the survival of the tumor cells in PDAC. The said interaction was more obvious with HPAF-CD11 cells that acquire more PDAC aggressive features. Culturing tumor cells on top of CAFs monolayer proved that the interaction between malignant cells and CAFs could promote survival and growth of PDAC cells. Nonetheless, culturing tumor cells in CAFs-derived conditioned media demonstrated that the survival stimulation effect of CAFs on malignant cells is mediated through paracrine factors rather than direct interaction. The use of neutralizing antibodies demonstrated that FGF-2 is the putative factor that stimulates malignant cells survival; whereas, it is clear that FGF-2 was present in CAFs-conditioned media, the difference in the expression of the appropriate FGF-2 receptors is possibly responsible for the difference between HPAF and HPAF-CD11. Moreover, CXCL8 has been shown to enhance endothelial cell proliferation and to regulate angiogenesis (Li, Dubey et al. 2003, Waugh and Wilson 2008). In this study, we show that CAFs-conditioned media increased CXCL8 production by HPAF-CD11. Subsequently, CXCL8 may induce

angiogenesis necessary for further tumor progression. On the other hand, HPAF cells that carry less aggressive potential produce less CXCL8. Therefore, the CXCL8 level induced by CAFs-conditioned media may be one of the determinants for malignant potential. Recently, CXCL8 has been shown to be produced by prostatic epithelial cells of benign prostatic hyperplasia which consists of slow but progressive growth of both epithelial and stromal cell and can act as a paracrine inducer of FGF-2 production by prostatic stromal cells *in vitro* (Giri and Ittmann 2001). In our study, conditioned media of the more aggressive PDAC cells, HPAF-CD11, stimulated and maintained the survival of CAFs through the secretion of CXCL8. Therefore, pancreatic tumor cell-derived CXCL8, released as a consequence of FGF-2 stimulation, may act on CAFs to stimulate further FGF-2 production. On the other hand, we have shown that CAFs derived FGF-2 can serve as a paracrine inducer of CXCL8 production by pancreatic tumor cells.

Looking into the differential gene expression profiles of HPAF and HPAF-CD11 upon their co-culture with CAFs can reveal the extent of CAFs contribution to tumor progression. CAFs appear to have a high baseline of several cytokines and chemokines including CXCL8. An interesting observation is that HPAF-CD11 cell/CAFs co-culture upregulates *ELMO1* gene that has been associated with motility (Grimsley, Kinchen et al. 2003, Sanui 2003). If we put this together with the ability of CXCL8 to induce angiogenesis, we can assume that CAFs can contribute to tumor cells spread to other organs.

In conclusion, interactions between pancreatic tumor cells and CAFs promote the survival of tumor cells with aggressive potentials and promote CXCL8

production. CXCL8, released as a consequence of FGF-2 stimulation, act on CAFs to stimulate further FGF-2 production. Thus, such bi-directional interactions between pancreatic tumor cells and CAFs help the tumor growth in different microenvironments, which leads to the pancreatic tumor progression and spread.

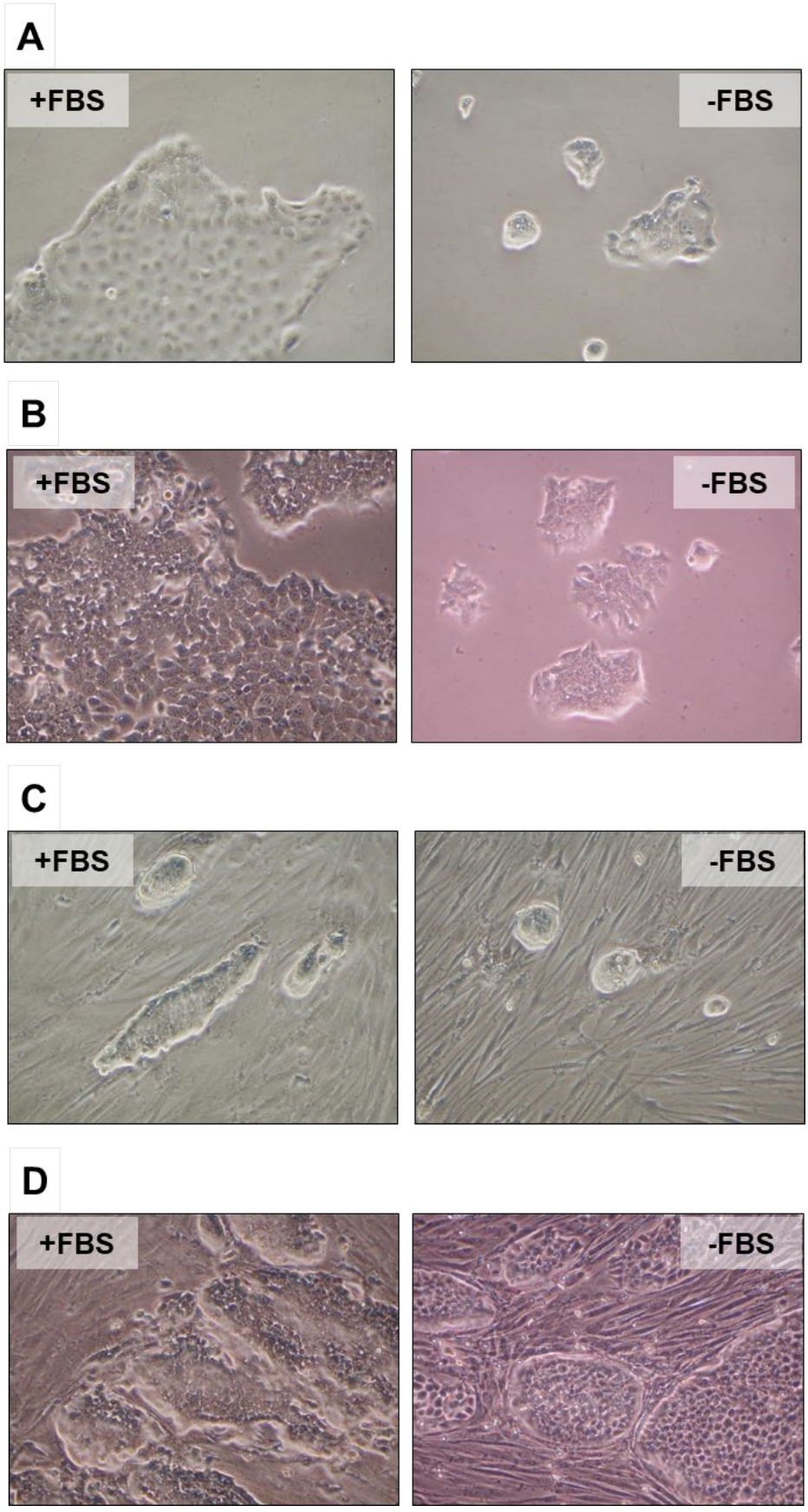

A
+FBS
-FBS
B
+FBS
-FBS
C
+FBS
-FBS
D
+FBS
-FBS

Figure 3.1: Fibroblasts promote the survival of aggressive pancreatic tumor cell

(**A**) Light microscope images of HPAF cells cultured in complete media or serum-free media. (**B**) Light microscope images of HPAF-CD11 cells cultured in complete media or serum-free media. (**C**) Light microscope images of HPAF cells cultured on top of fibroblasts (BJ) monolayer with complete media, or serum-free media. (**D**) Light microscope images of HPAF-CD11 cells cultured on top of fibroblasts (BJ) monolayer with complete media, or serum-free media.

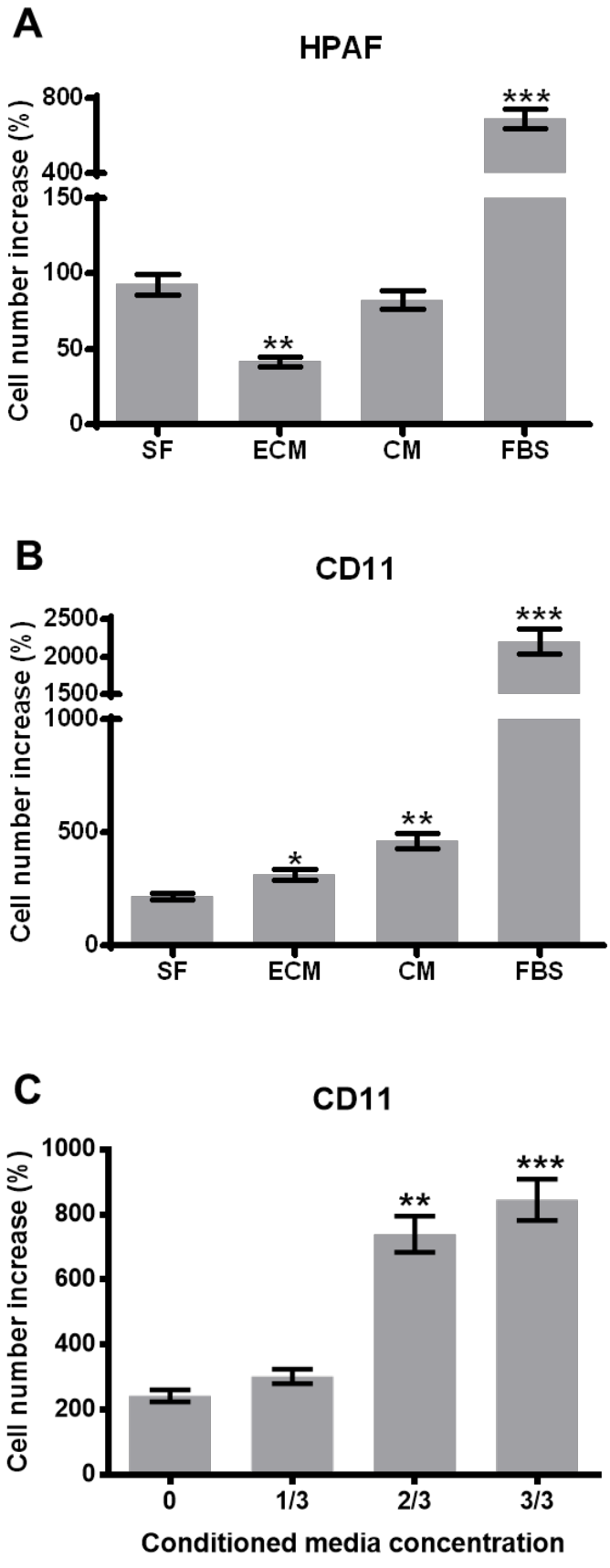
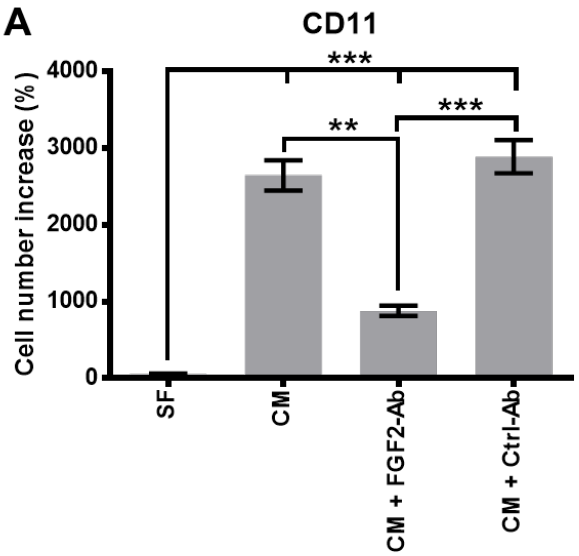
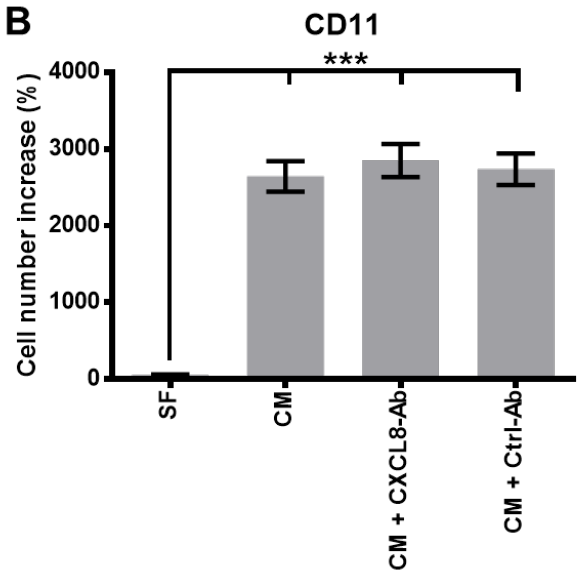

A
HPAF
Cell number increase (%)
800
400
150
100
50
0
***
**
SF
ECM
CM
FBS
B
CD11
Cell number increase (%)
2500
2000
1500
1000
500
0
***
*
**
SF
ECM
CM
FBS
C
CD11
Cell number increase (%)
1000
800
600
400
200
0
**
***
0
1/3
2/3
3/3
Conditioned media concentration

Figure 3.2: CAFs promotes the survival of tumor cells through paracrine contact

(**A** and **B**) Survival of HPAF cells (**A**) and HPAF-CD11 (**B**) following their culture with CAFs-ECM, CAFs-conditioned media, or complete media as compared to serum-free media. (**C**) Survival of HPAF-CD11 cells in increasing amounts of CAFs- conditioned media to serum-free media. (n=3), student's t-test. *p<0.05, **p<0.01, ***p<0.001


A
CD11
***
**
***
Cell number increase (%)
4000
3000
2000
1000
0
SF
CM
CM + FGF2-Ab
CM + Ctrl-Ab


B
CD11
***
Cell number increase (%)
4000
3000
2000
1000
0
SF
CM
CM + CXCL8-Ab
CM + Ctrl-Ab

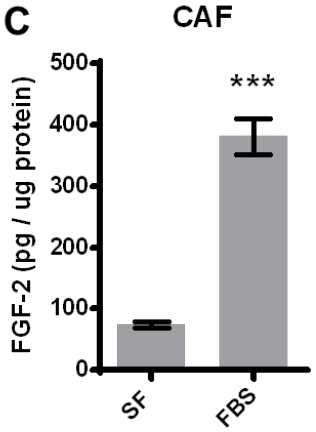

C
CAF
***
FGF-2 (pg / ug protein)
500
400
300
200
100
0
SF
FBS

Figure 3.3: Effects of CAFs conditioned media on the survival of HPAF-CD11 is mediated by FGF-2

(**A**) Number increase of HPAF-CD11 in response to CAFs-conditioned media +/- FGF-2 neutralizing antibody as compared to serum-free media. (**B**) Number increase of HPAF-CD11 in response to CAFs-conditioned media +/- CXCL8 neutralizing antibody as compared to serum-free media. (**C**) FGF-2 concentrations, evaluated by ELISA, in the supernatant of CAF incubated in serum-free media or complete media. (n=3), student's t-test. *p<0.05, **p<0.01, ***p<0.001

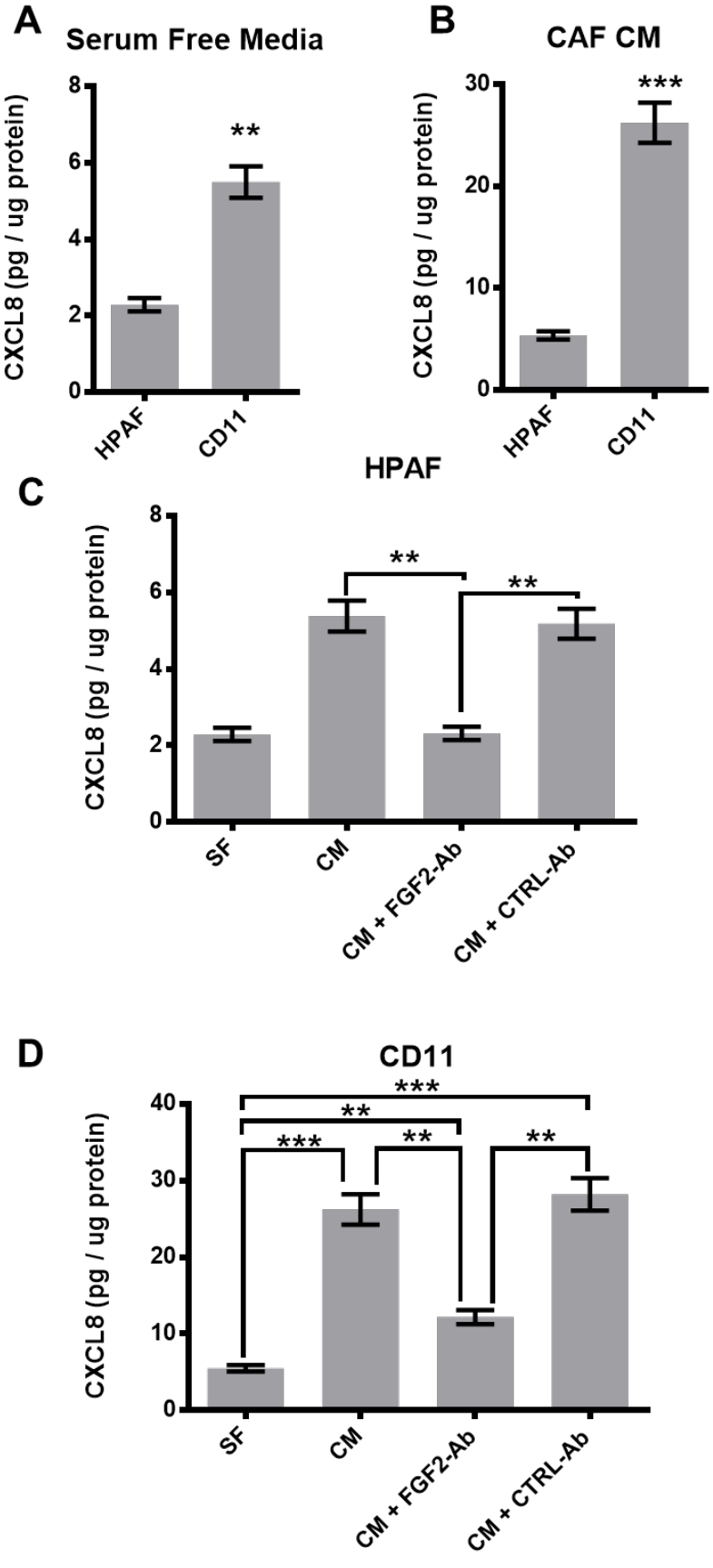
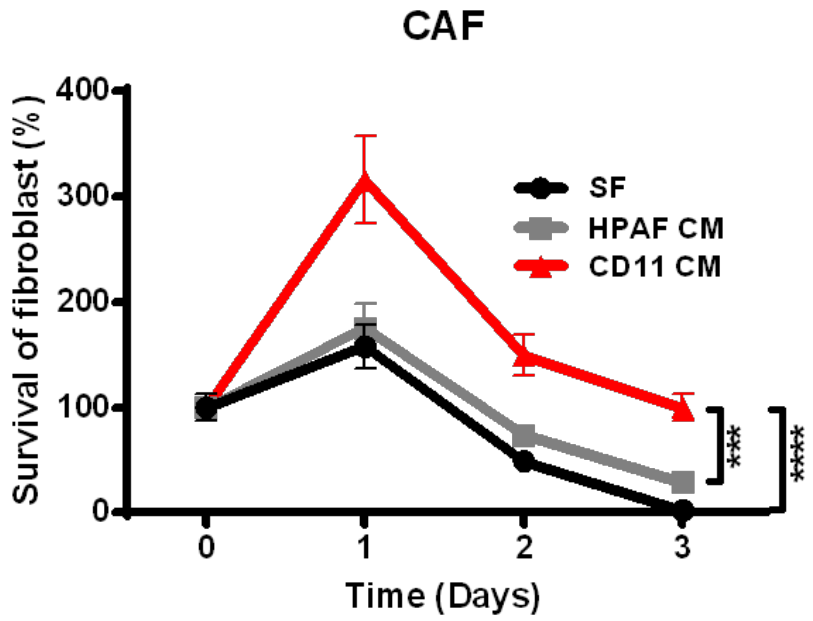

A
Serum Free Media
CXCL8 (pg / ug protein)
8
6
4
2
0
**
HPAF
CD11
B
CAF CM
CXCL8 (pg / ug protein)
30
20
10
0
***
HPAF
CD11
C
HPAF
CXCL8 (pg / ug protein)
8
6
4
2
0
**
**
SF
CM
CM + FGF2-Ab
CM + CTRL-Ab
D
CD11
CXCL8 (pg / ug protein)
40
30
20
10
0
***
**
***
**
**
SF
CM
CM + FGF2-Ab
CM + CTRL-Ab

Figure 3.4: FGF-2 in the CAFs conditioned media promotes the CXCL8 production of pancreatic tumor cells

(**A**) CXCL8 concentration, evaluated by ELISA, in the supernatant of HPAF cells and **(B)** HPAF-CD11 after treatment with serum-free media or CAFs-conditioned media. (**C**) CXCL8 concentration, evaluated by ELISA, in the supernatant of HPAF cells after treatment with CAFs-conditioned media +/- FGF-2 neutralizing. (**D**) CXCL8 concentration, evaluated by ELISA, in the supernatant of HPAF-CD11 cells after treatment with CAFs-conditioned media +/- FGF-2 neutralizing. (n=3), student's t-test. *p<0.05, **p<0.01, ***p<0.001

A


CAF
Survival of fibroblast (%)
400
300
200
100
0
0
1
2
3
Time (Days)
SF
HPAF CM
CD11 CM
***
****

B

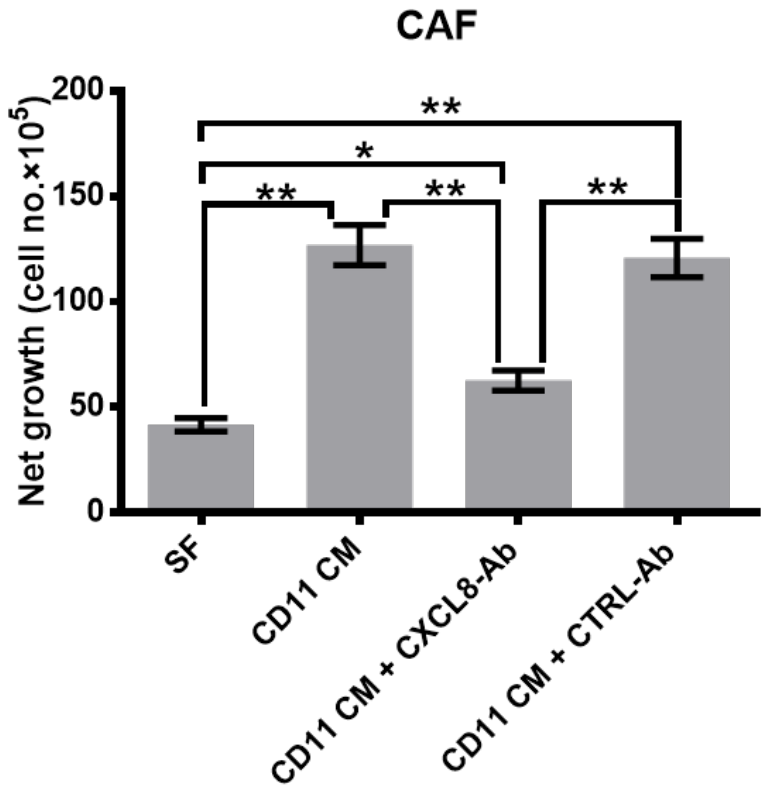

CAF
Net growth (cell no.×10^5)
200
150
100
50
0
**
*
**
**
**
SF
CD11 CM
CD11 CM + CXCL8-Ab
CD11 CM + CTRL-Ab

Figure 3.5: Effect of tumor cell conditioned media on the survival of CAFs

(**A**) Survival, increase in cell number, of CAFs incubated with the conditioned media of HPAF or and HPAF-CD11 for 1, 2, and 3 days, and the increase in cell number was quantitated. (**B**) Net growth, determined by the increase in cell number, of CAFs in HPAF-CD11 conditioned media +/- CXCL8 neutralizing antibody. (*n*=3), student's *t*-test. **p*<0.05, ***p*<0.01, ****p*<0.001

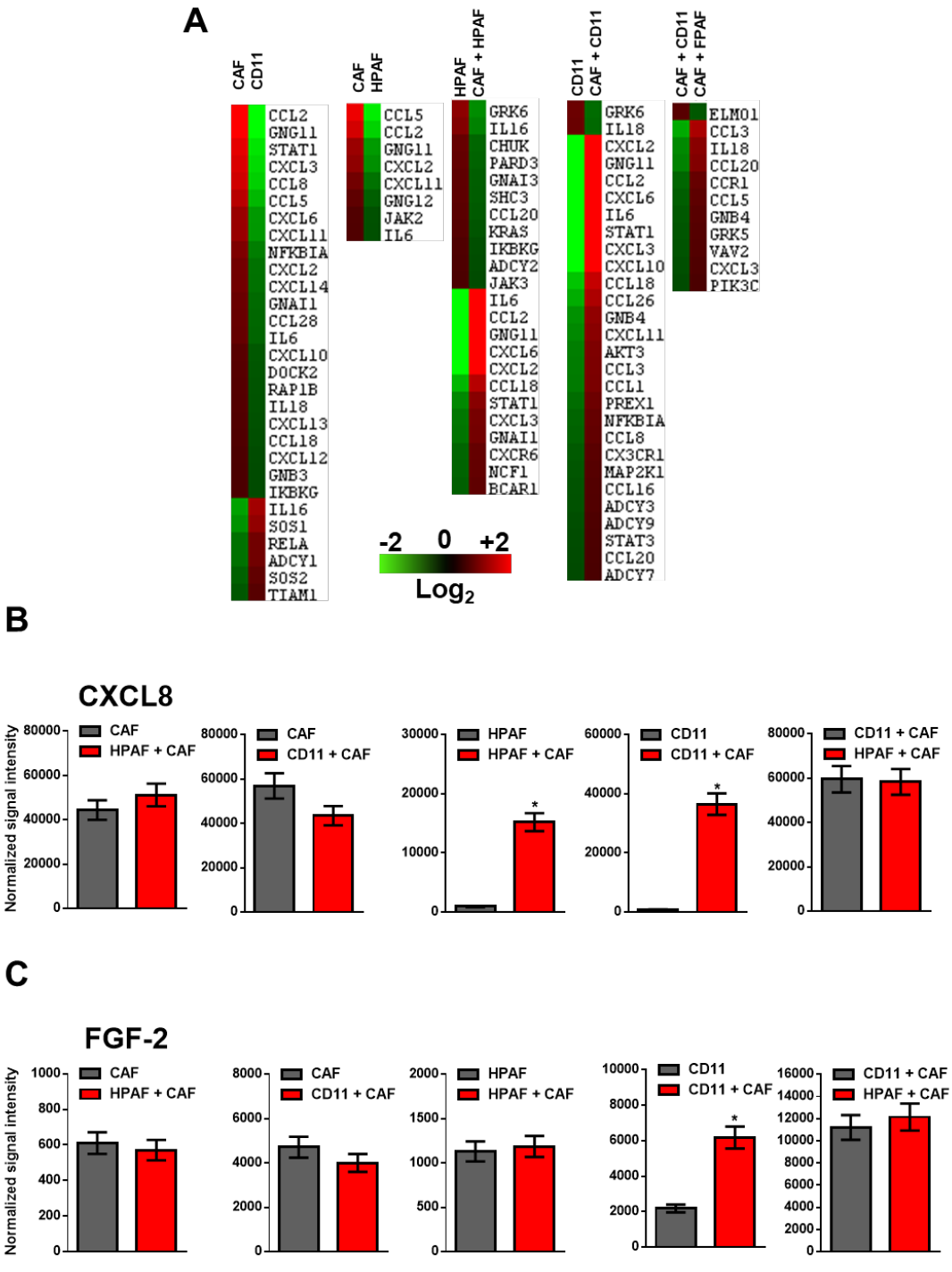

A
CAF
CD11
CCL2
GNG11
STAT1
CXCL3
CCL8
CCL5
CXCL6
CXCL11
NFKBIA
CXCL2
CXCL14
GNAI1
CCL28
IL6
CXCL10
DOCK2
RAP1B
IL18
CXCL13
CCL18
CXCL12
GNB3
IKBKG
IL16
SOS1
RELA
ADCY1
SOS2
TIAM1
CAF
HPAF
CCL5
CCL2
GNG11
CXCL2
CXCL11
GNG12
JAK2
IL6
HPAF
CAF + HPAF
GRK6
IL16
CHUK
PARD3
GNAI3
SHC3
CCL20
KRAS
IKBKG
ADCY2
JAK3
IL6
CCL2
GNG11
CXCL6
CXCL2
CCL18
STAT1
CXCL3
GNAI1
CXCR6
NCF1
BCAR1
CD11
CAF + CD11
GRK6
IL18
CXCL2
GNG11
CCL2
CXCL6
IL6
STAT1
CXCL3
CXCL10
CCL18
CCL26
GNB4
CXCL11
AKT3
CCL3
CCL1
PREX1
NFKBIA
CCL8
CX3CR1
MAP2K1
CCL16
ADCY3
ADCY9
STAT3
CCL20
ADCY7
CAF + CD11
CAF + FPAF
ELMO1
CCL3
IL18
CCL20
CCR1
CCL5
GNB4
GRK5
VAV2
CXCL3
PIK3C
-2
0
+2
Log2
B
CXCL8
Normalized signal intensity
CAF
HPAF + CAF
CAF
CD11 + CAF
HPAF
HPAF + CAF
CD11
CD11 + CAF
CD11 + CAF
HPAF + CAF
C
FGF-2
Normalized signal intensity
CAF
HPAF + CAF
CAF
CD11 + CAF
HPAF
HPAF + CAF
CD11
CD11 + CAF
CD11 + CAF
HPAF + CAF

Figure 3.6: Differential gene expression of tumor cells cultured with CAFs

(**A**) Heat map of gene expression of HPAF and HPAF-CD11 cultured alone or with CAFs determined using gene expression microarray. Heat maps show chemokines and cytokine with > 2-fold increase. (**B**) Expression of CXCL8, represented as normalized signal intensity, in HPAF and HPAF-CD11 cultured alone or with CAFs determined using gene expression microarray. (**C**) Expression of FGF-2, represented as normalized signal intensity, in HPAF and HPAF-CD11 cultured alone or with CAFs determined using gene expression microarray.

CHAPTER IV: CXCR2 SIGNALING ACTIVATES NF-KB AND PROMOTES SECRETORY CAFS

ABSTRACT

Due to its late detection and low success of current therapies, PDAC remains one of the most challenging malignancies. Desmoplasia and tumor-supporting inflammation are hallmarks of PDAC. In addition to the autonomous aggressiveness feature of PC malignant cells, host tumor microenvironment contribute greatly to tumor progression and spread. CAFs, a major component of the tumor microenvironment in PDAC, are implicated in facilitating therapy resistance and metastasis. Recent reports emphasized the concurrence of multiple subtypes of CAFs that carry out different roles. CXCR2 is a chemokine receptor that is known for its role during inflammation and its adverse role in PDAC. Oncogenic Kras upregulates CXCR2 and its ligands and; thus, contribute to tumor proliferation, immunosuppression, therapy resistance by stemness induction. The deletion of CXCR2 in a PDAC syngeneic mouse model, render fibrosis revealing a potential undescribed role of CXCR2 in regulating CAFs. We hypothesize that CXCR2 regulates CAFs function in PDAC and contribute to CAFs heterogeneity. Using co-culture methods, gene and protein expression methods, we demonstrated that PDAC tumor cells with oncogenic Kras express more CXCR2 ligands. CXCR2 ligands derived from PDAC cells inhibited CAFs growth, decreased the expression of the myofibroblasts-associated markers including αSMA and collagen I, and increased the expression of immunosuppressive cytokines and tumor-promoting chemokines including IL-4, IL-10, IL-13 and CXCL7 through the activation of NFκB. Together, we demonstrate that sustained

signaling through CXCR2 activates NFκB and induces a secretory phenotype of CAFs in PDAC that upregulates pro-tumor factors.

INTRODUCTION

Pancreatic cancer, one of deadliest human cancers, is the fourth leading cause of cancer-related deaths in the USA (Siegel, Miller et al. 2018). The lack of both early detection tools and viable treatment options made pancreatic cancer a very stubborn and one of the deadliest human malignancies (Warshaw and Fernandez-del Castillo 1992, Hidalgo 2010). Making progress in dealing with the disease starts by understanding the complex cellular and molecular interactions and identifying the checkpoints that are crucial for the disease initiation, progression and spread. PDAC is the most common and most aggressive subtype of pancreatic cancers (Fesinmeyer 2005, Bosman, Carneiro et al. 2010). PDAC develops progressively as a result of accumulating genetic and epigenetic alterations (Hruban and Fukushima 2007, Hruban and Adsay 2009). Oncogenic Kras often develops very early before the inception of the full-blown disease. Premalignant lesions, known as PanIN, develop with the Kras mutation and not until other mutations such as the p53 and the Smad4 inactivation, the disease transforms into a blunt PDAC (Hruban and Fukushima 2007). Oncogenic Kras is tightly linked to inflammatory signals that contribute to tumor growth and immunosuppression; thus, enabling disease progression (Grivennikov, Greten et al. 2010, Baumgart, Chen et al. 2014, Hamada, Masamune et al. 2014). The disease is additionally characterized by a dense and complex desmoplastic tumor microenvironment composed of ECM deposition, fibrotic cells, endothelial cells, and immune cells (Chu, Kimmelman et al. 2007, Kleeff, Beckhove et al. 2007). Inflammatory signals such as cytokines and chemokines secreted by the malignant

cells and other components of the tumor microenvironment contribute to the tumorigenicity and progression of PDAC (Grivennikov, Greten et al. 2010, Baumgart, Chen et al. 2014, Hamada, Masamune et al. 2014). One of the major inflammatory signals in PDAC is CXCR2 that has strong ties to the prominent oncogenic Kras (Baumgart, Chen et al. 2014, Purohit, Varney et al. 2016).

CAFs represent a major component of PDAC tumor microenvironment (Chu, Kimmelman et al. 2007, Kleeff, Beckhove et al. 2007). CAFs are fibrotic cells of multiple origins including PSCs, resident fibroblasts, bone marrow-derived mesenchymal stem cells, fibrocytes and others (Öhlund, Elyada et al. 2014). In response to external cues, quiescent fibrotic cells get activated and when this activation occurs in the context of cancer, they become CAFs (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). CAFs have been associated with supporting tumor in all the stages from initiation to spread (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). Myofibroblasts, activated fibrotic cells with extensive ECM synthesis, have long been used as a synonym for CAFs in the context of cancer (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). More recent reports indicate the coexistence of multiple functional subsets of CAFs (Ohlund, Handly-Santana et al. 2017, Su, Chen et al. 2018). The typical myofibroblasts are characterized by upregulation of αSMA and ECM molecules such as collagen I (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). The other subset referred to as inflammatory or secretory CAFs develops through paracrine signaling and tend to have low expression of αSMA and high expression of inflammatory mediators

(Ohlund, Handly-Santana et al. 2017). This functional heterogeneity could explain the versatility of CAFs and the failure of targeting desmoplasia to resolve PDAC (Özdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014).

Chemokines are small secreted molecules that are renowned for their role in inflammation (Ransohoff 2009, Griffith, Sokol et al. 2014, Roy, Evans et al. 2014, Hughes and Nibbs 2018). Based on the position of the conserved N-terminal cysteine residues, they are classified into four families: C, CC, CXC, and CX3C. Chemokine receptors are G-protein coupled receptor molecules (Ransohoff 2009, Griffith, Sokol et al. 2014, Roy, Evans et al. 2014, Hughes and Nibbs 2018). CXCR2 is the receptor for multiple chemokines that include a group of angiogenic chemokines referred to as ELR^+ CXC chemokines that include CXCL1-3, 5-7, and 8 that also interacts with CXCR1 (Bizzarri, Beccari et al. 2006, Strieter, Burdick et al. 2006). CXCR2 signaling helps in recruiting granulocytes to the site of inflammation and also enable angiogenesis (Bizzarri, Beccari et al. 2006, Strieter, Burdick et al. 2006, Bajrami, Zhu et al. 2016, Zhang, Guo et al. 2018). These features in the context of cancer can be considered adverse. The chemoattracting ability of CXCR2 signaling allows recruitment of immunosuppressive cells such as neutrophils and MDSCs (Highfill, Cui et al. 2014, Kumar, Donthireddy et al. 2017), and the angiogenic feature of CXCR2 enables easy access for tumor growth and spread (Waugh and Wilson 2008, Matsuo, Raimondo et al. 2009, Singh, Varney et al. 2009). Furthermore, CXCR2 signaling can directly contribute to tumor growth by promoting malignant cells proliferation (Waugh and Wilson 2008, Purohit, Varney et al. 2016). Our group has demonstrated the presence of an association

between the occurrence of oncogenic Kras and the progressive upregulation of CXCR2 axis in PDAC (Purohit 2015, Purohit, Varney et al. 2016). In-vitro inhibition of CXCR2 was able to reduce tumor cells proliferation, migration, and anchorage (Purohit 2015, Purohit, Varney et al. 2016). Several attempts to assess if CXCR2 inhibition can be utilized as a target for PDAC treatment. Inhibiting CXCR2 disrupted tumor to stromal interaction and improved the survival in *Kras*$^{+}$*Tgfbr2*^{KO} PDAC mouse model (Ijichi, Chytil et al. 2011). In KPC (*LSL-*$^{KrasG12D/+;}$ *LSL-*$^{Trp53R172H/+;}$ *Pdx1-Cre*) mice, the global CXCR2 inhibition suppressed metastasis, but the loss of CXCR2 in epithelial cells only was not able to inhibit metastasis (Steele, Karim et al. 2016). Our group generated a syngeneic mouse model with a global CXCR2 knockout (*Cxcr2*$^{-/-}$) transplanted with cells from *Pdx1-Cre; Kras*^{G12D} (KC) mouse. In this model, CXCR2 deletion played tumoritoxic and tumoristatic roles by halting the proliferation of tumor cells and enhancing their apoptosis, suppressing angiogenesis, and inducing an anti-tumor immune response. Nonetheless, the deletion of CXCR2 also increased the induction of fibrosis and increased metastasis (Purohit 2015). Taken together, we believe that CXCR2 signaling in the CAFs inhibits the myofibroblast phenotype and induces a phenotype with a secretory function. In the present study, we demonstrate that CXCR2 signaling interaction in CAFs causes them to assume a phenotype that is characterized with lower expression of αSMA and ECM proteins, and higher expression of pro-tumorigenic secreted factors including immunosuppressive cytokines and tumor-supporting chemokines. We demonstrate that this phenotype is mediated through activating NF-κB transcription factor activity.

RESULTS

Kras-dependent paracrine inter-talk between CAFs and PDAC cells

In the beginning, we sought to find out if there is a paracrine inter-talk between tumor cells and CAFs. We utilized unidirectional coculture techniques for this purpose. The proliferation of PDAC cells was measured following treatment with conditioned media collected from the CAFs ImPSC (murine) or 10-32 PC Puro (human). Relative to the treatment with serum-free media, ImPSC promoted the growth of tumor cells with activated oncogenic Kras (KC) while inhibiting cells with wildtype Kras (Panc02) **(Figure 4.1A)**. Similarly, the conditioned media of human CAFs cell line 10-32 PC Puro enhanced the growth of CD18/HPAF-Scr that possesses oncogenic Kras and has a control vector while inhibited the growth of BxPC3 cells (with wildtype Kras) as well as CD18/HPAF-Kras kd (with oncogenic Kras knockdown) **(Figure 4.1B)**. Next, we decided to investigate if the Kras-dependent differential response present also in CAFs treated with PDAC cells conditioned media. Conditioned media of PDAC cells carrying oncogenic Kras mutation (KC) inhibited the growth of ImPSC cells contrary to wildtype cells (Panc02) that enhanced CAFs growth **(Figure 4.1C)**; and similarly, BxPC3 cells and CD18/HPAF-Kras kd cells conditioned media promoted the growth of 10-32 PC Puro CAFs, when the CD18/HPAF-Scr has inhibited their growth **(Figure 4.1D)**.

To confirm this observation, we utilized immortalized human pancreatic duct-derived cell lines that express exogenous *Kras*$^{(G12D)}$ (HPNE-Kras) or normally

express wildtype Kras (HPNE). The conditioned media of 10-32 PC Puro cells enhanced the growth of HPNE-Kras; whereas, HPNE-Kras conditioned media inhibited the growth of 10-32 PC Puro cells while HPNE conditioned media promoted it **(Figure 4.2)**. Together, the observations we described here indicate the presence of Kras-dependent response orchestrated through paracrine factors secreted by tumor cells and stromal cells, which could be involved in tumor progression.

PDAC cells paracrine factors promote phenotype alterations in CAFs

When activated, fibrotic cells assume the myofibroblast phenotype that exhibits increased ECM synthesis and is characterized by the expression of αSMA as a marker. In cancer, myofibroblast was used synonymously to CAFs for many years (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). We decided next to investigate if the PDAC cells-derived paracrine factors have any effect on the expression of myofibroblasts markers. ImPSC cells were treated with conditioned media of KC cells that carry oncogenic Kras mutation, then the expression of αSMA, Collagen I, and Collagen IV was examined. KC conditioned media downregulated the mRNA expression of αSMA (*Acta2*) and the ECM proteins Collagen I (*Col1A1*) and Collagen IV (*Col4A1*) **(Figure 4.3A)**. Protein levels of αSMA determined by immunofluorescence and western blot showed that conditioned media of KC cells had decreased the expression of αSMA in ImPSC **(Figure 4.3B)**. We gather from this that oncogenic Kras promotes the secretion of paracrine factors that can alter CAFs away from their typical myofibroblast phenotype.

PDAC cells paracrine factors induce secretion of pro-tumor cytokines and chemokines in CAFs

Ohlund et al. described the presence of a CAFs phenotype with a higher secretory function and a lower fibrotic function that arises through paracrine signaling (Ohlund, Handly-Santana et al. 2017). To determine if the Kras promoted CAFs phenotype alterations, we assessed the expression of multiple cytokines and chemokines. We analyzed the mRNA levels of multiple cytokines and chemokines in ImPSC cells treated with conditioned media of KC cells in comparison to serum-free media control. The analysis of mRNA levels indicated several changes in the expression of multiple cytokines **(Figure 4.4A)**. Notably, *IL6* expression was downregulated contrary to expectations **(Figure 4.4B)**. Nonetheless, the most significant changes observed as upregulation of cytokines that are considered pro-tumorigenic by promoting immunosuppressive conditions in cancer (Matsuo, Takeyama et al. 2012, Suzuki, Leland et al. 2015) including *IL4*, *IL10,* and *IL13* **(Figure 4.4C)**. Additionally, CXCR2 chemokines are known to play an adverse role in PDAC mainly by promoting tumorigenesis, immunosuppression and angiogenesis (Strieter, Burdick et al. 2006, Highfill, Cui et al. 2014, Purohit, Varney et al. 2016). Analysis of CXCR2 chemokines revealed enhanced mRNA expression of *CXCL2* and *CXCL7* in response to the KC conditioned media treatment **(Figure 4.5A-B)**. Analysis of the levels of chemokines in the supernatant of ImPSC cells showed that CAFs express baseline high levels of CXCL1 and CXCL5, moderate levels of CXCL2 and lower levels of CXCL7. Treating CAFs with conditioned media of KC cells did not increase CXCL1 or CXCL5 but slightly increased CXCL2 and significantly increased CXCL7 **(Figure 4.5C)**. Taken

together, we demonstrate that the activation of oncogenic Kras in PDAC promotes the secretion of paracrine factors that contribute to modulating CAFs by altering them into a phenotype with more secretory function and lower fibrogenic features.

CXCR2 signaling in CAFs promotes the secretory phenotype in CAFs

The paracrine factors CXCL1-3 and CXCL5-8 signal through CXCR2 chemokine receptor (Bizzarri, Beccari et al. 2006, Strieter, Burdick et al. 2006). Our group has previously demonstrated the presence of a link between the activation of oncogenic Kras and the upregulation of CXCR2 axis in PDAC (Purohit, Varney et al. 2016). The stromal ablation of CXCR2 increased the fibrotic reaction in the syngeneic KC mouse model of PDAC suggesting a role of CXCR2 signaling in regulating the fibrotic component in PDAC (Purohit 2015). Thus, we decided to investigate if CXCR2 signaling is involved in the CAFs phenotype alterations. First, we confirmed that our CAF cell lines express CXCR2 using immunofluorescence **(Figure 4.6A)**; then we used ELISA to measure CXCR2 ligands concentrations in the conditioned media of PDAC cells. KC conditioned media expressed more CXCL1, CXCL5 and CXCL7 than Panc02 **(Figure 4.6B)**, and CD18/HPAF-Scr produced more CXCL8 than both BxPC3 **(Figure 4.6C)**. We treated ImPSC cells with conditioned media of KC cells in the presence or absence of CXCR2 pharmacological inhibitors. Blocking CXCR2 reduced the inhibitory effect of KC conditioned media and increased the growth stimulatory effect of Panc02 **(Figure 4.7A)**. Recombinant CXCL1 exhibited a dose-dependent inhibition in the growth of ImPSC cells similar to growth inhibition induced by treating 10-32 PC Puro cells with a recombinant CXCL8 that was reduced with

CXCR2 inhibitor **(Figure 4.7B)**. Lastly, ImPSC cells treated with recombinant CXCL1 downregulated *Acta2* (SMA) and *Col1A1* (Collagen I) and upregulated *IL4, IL13* **(Figure 4.7C)** and *CXCL7* **(Figure 4.7D)**. Hereby, we provided evidence that signaling through CXCR2 is involved in altering CAFs towards the secretory phenotype.

CXCR2 signaling in CAFs activates NF-κB

NF-κB is a transcription factor that has strong ties to both inflammation and cancer (DiDonato, Mercurio et al. 2012). Furthermore, oncogenic Kras and CXCR2 have been reported to contribute to tumor progression through NF-κB (Richmond 2002, Ling, Kang et al. 2012, Walana, Wang et al. 2018). Lastly, a new report described that NF-κB activation gives rise to a secretory subset of CAFs in breast and lung cancers that express GPR77 and CD10, which could be new markers for the secretory CAFs (Su, Chen et al. 2018). So, to investigate the involvement of the CXCR2 signaling in CAFs in NF-κB activation and CD10 expression. ImPSC cells treated with KC conditioned media and 10-32 PC Puro cells treated with CXCL8 showed increase NF-κB nuclear translocation, an indication of increased NF-κB activity. Blocking CXCR2 was able to reduce this NF-κB activity **(Figure 4.8A)**. CD10 expression in ImPSC cells was not changed in response to KC conditioned media; whereas, 10-32 PC puro cells had low expression of CD10 that was slightly increased after CXCL8 treatment **(Figure 4.8B)**. Collectively, we demonstrate that CXCR2 signaling in CAFs of PDAC causes activation of NF-κB that causes CAFs to assume a secretory phenotype.

DISCUSSION

PDAC, one of the most malignant tumors, is characterized by both tumor-supporting inflammation and abundant desmoplastic reaction (Chu, Kimmelman et al. 2007, Kleeff, Beckhove et al. 2007, Grivennikov, Greten et al. 2010, Baumgart, Chen et al. 2014, Hamada, Masamune et al. 2014). Oncogenic Kras that occur early during PDAC progression is associated with inflammatory signals such as CXCR2 (Baumgart, Chen et al. 2014, Purohit, Varney et al. 2016). CXCR2 signaling in many cancer, including PDAC, has been proven adverse by contributing to tumor growth, immunosuppression, angiogenesis and chemotherapy resistance (Waugh and Wilson 2008, Matsuo, Raimondo et al. 2009, Singh, Varney et al. 2009, Highfill, Cui et al. 2014, Chan, Hsu et al. 2016, Purohit, Varney et al. 2016, Kumar, Donthireddy et al. 2017, Su, Chen et al. 2018). CAFs are the major contributors to desmoplasia in many cancers including PDAC (Apte, Wilson et al. 2013, Gore and Korc 2014, Moir, Mann et al. 2015, McCarthy, El-Ashry et al. 2018). Desmoplasia adds to tumorigenicity by supporting therapy hindrance and resistance, and metastasis (Olive, Jacobetz et al. 2009, Pandol, Edderkaoui et al. 2009, Bynigeri, Jakkampudi et al. 2017). CAFs for long have been regarded as myofibroblasts characterized by expression of αSMA and ECM proteins (Apte, Wilson et al. 2013, Moir, Mann et al. 2015). Although CAFs have been known to secrete several paracrine factors, including CXCR2 ligands (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Chan, Hsu et al. 2016), it is only recently that this secretory function gained enough attention. Recent reports described distinct CAFs subsets with a specialized secretory function (Ohlund,

Handly-Santana et al. 2017, Su, Chen et al. 2018). Several studies suggested the presence of a relationship between CXCR2 signaling and stromal activity (Ijichi, Chytil et al. 2011, Steele, Karim et al. 2016). Our group has demonstrated that stromal ablation of CXCR2 enhances fibrosis (Purohit 2015). The secretory CAFs phenotype was described to develop when there is no adjacency to the tumor cells (Ohlund, Handly-Santana et al. 2017), which suggests the involvement of far-reaching paracrine molecules such as CXCR2 ligands. The purpose of the present study was to determine the role of oncogenic Kras-CXCR2 axis in CAFs activity and function in PDAC.

Our data indicate a growth stimulatory effect of CAFs-derived paracrine factor on PDAC cells with oncogenic Kras but not PDAC cells with wildtype Kras. On the other hand, PDAC cells-derived paracrine factors stimulated CAFs growth when the cells had wildtype Kras and inhibited CAFs growth when the cells had oncogenic Kras mutation. This Kras-dependent response was shown to extend beyond tumor cells proliferation to alter the expression of myofibroblast CAFs-associated proteins, including αSMA and ECM synthesis and to upregulates pro-tumor cytokines and chemokines. By investigating the putative mechanisms by which these CAFs phenotype alterations occurred, we determined that CXCR2 signaling in CAFs is involved. Blocking CXCR2 ameliorated the inhibitory effect of the oncogenic Kras tumor cells paracrine factors and further enhanced the stimulatory effect of wildtype cells. Furthermore, recombinant CXCR2 ligands treatment altered the expressions profile in CAFs to reduce myofibroblasts markers and to upregulate pro-tumor paracrine factors. Lastly, we determined that

the CXCR2-induced secretory function in CAFs is mediated through NF-κB activation. We also confirmed that the secretory phenotype express CD10, a marker that has been recently linked to secretory CAFs in breast and lung cancers (Su, Chen et al. 2018).

It is known that CAFs population is heterogeneous by cell origins (Öhlund, Elyada et al. 2014); however, functional heterogeneity is rather a recent concept. Myofibroblast is the typical CAFs phenotype; but, there is more attention now to subsets of CAFs with a specialized secretory function (Ohlund, Handly-Santana et al. 2017, Su, Chen et al. 2018). Albeit, the notion of secretory roles of CAFs is not new. It is known that CAFs express many receptors and secrete many paracrine factors; however, this was mainly attributed to CAFs versatility rather than specialized functional subsets (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). Looking into literature, we can find some reports that have described what it seems to be an abundant secretory function in CAFs. Chemotherapy treatment of CAFs of breast cancer and PDAC induced senescence and activated inflammatory transcription factors including NF-κB and STAT1, which resulted in upregulation of CXCR2 ligands that induced chemotherapy resistance by promoting cancer stemness (Chan, Hsu et al. 2016). Senescent CAFs have been reported to assume secretory functions. For example, senescent CAFs were reported to produce inflammatory mediators such as CXCL8 and IL-6, which contributed to tumor growth, tumor cells migration, and epithelial differentiation alteration (Parrinello 2005, Lawrenson, Grun et al. 2010, Wang, Notta et al. 2017). Both oncogenic Kras and CXCR2 have links to senescence,

which is mediated through activation of inflammatory transcription factors such as NF-κB (Acosta, O'Loghlen et al. 2008, Acosta, O'Loghlen et al. 2008, Vizioli, Santos et al. 2014, Lesina, Wormann et al. 2016).

Markers such αSMA, FAP, and PDGFR have been utilized to identify CAFs (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). Nonetheless, targeting CAFs using αSMA depletion rendered an adverse outcome by accelerating tumor progression as a result of enhanced therapy resistance and immunosuppression (Özdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014). Based on our current knowledge, we believe that the adverse outcome was a consequence of increasing the abundance of the secretory CAFs. Taken together, it is clear that the markers that have been heavily utilized are likely not uniformly expressed on CAFs but it is not clear if this because of CAFs origin or functional heterogeneity. We are warranted to dive into the CAFs biology and attain better markers that allow targeting CAFs safely. There is not much we know about putative markers for the secretory CAFs. In PDAC, the secretory CAFs were described to express FAP and to have a low expression of αSMA (Ohlund, Handly-Santana et al. 2017). The secretory CAFs described in breast, and lung cancer had a similar expression of αSMA, FAP, and PDGFR to the other CAFs; however, they were identified using other surface markers such as GPR77 and CD10 (Su, Chen et al. 2018). CD10, a small metalloprotease, is known as a prognostic marker in hematological malignancies (Maguer-Satta, Besancon et al. 2011). In solid tumors, CD10 is considered a prognostic marker in certain tumors including melanoma, breast, and lung cancers (Shipp, Tarr et al. 1991, Carrel, Zografos et

al. 1993, Chu and Arber 2000, Iwaya, Ogawa et al. 2002). Stromal cells that express CD10 have been identified in several cancers including colorectal cancer, breast cancer, gastric cancer, and PDAC (Iwaya, Ogawa et al. 2002, Ogawa, Iwaya et al. 2002, Huang 2005, Ikenaga, Ohuchida et al. 2010). In PDAC, $CD10^+$ CAFs promoted tumor cells growth and was associated with reduced survival and nodal metastasis (Ikenaga, Ohuchida et al. 2010). It is not clear; however, if all the $CD10^+$ CAFs carry out a secretory function or if all the secretory CAFs express CD10.

In conclusion, we report in this study that the sustained CXCR2 signaling in CAFs of PDAC promotes activation of NF-kB and produces a secretory phenotype characterized by the production of pro-tumor paracrine mediator, low fibrogenic potentials, and expression of CD10. These findings add to our current knowledge about CAFs heterogeneity. Further studies are needed to fully characterize CAFs and determine if the abundance of specific subsets of CAFs is associated with certain cancer outcomes.

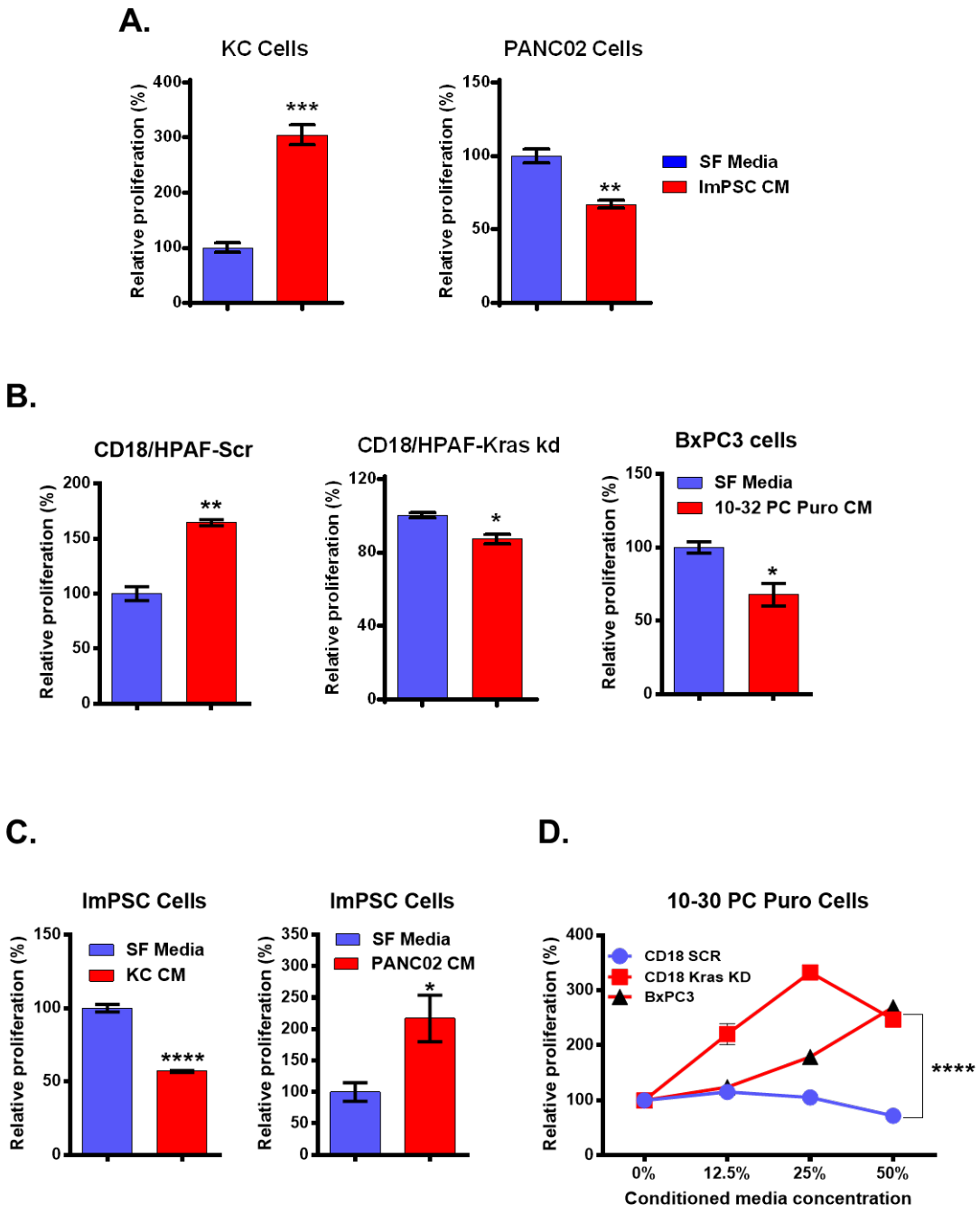
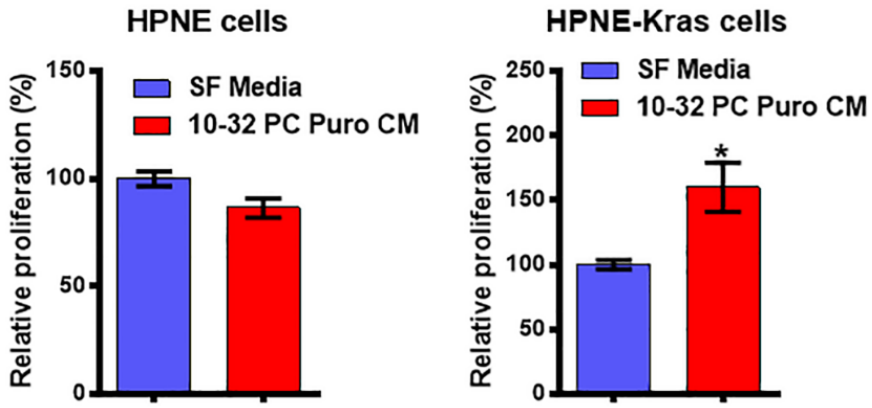

A.
KC Cells
PANC02 Cells
Relative proliferation (%)
400
300
200
100
0
***
150
100
50
0
**
SF Media
ImPSC CM
B.
CD18/HPAF-Scr
CD18/HPAF-Kras kd
BxPC3 cells
Relative proliferation (%)
200
150
100
50
0
**
120
80
40
0
*
150
100
50
0
*
SF Media
10-32 PC Puro CM
C.
ImPSC Cells
ImPSC Cells
Relative proliferation (%)
150
100
50
0
SF Media
KC CM
****
350
300
250
200
150
100
50
0
SF Media
PANC02 CM
*
D.
10-30 PC Puro Cells
Relative proliferation (%)
400
300
200
100
0
CD18 SCR
CD18 Kras KD
BxPC3
****
0%
12.5%
25%
50%
Conditioned media concentration

Figure 4.1: Kras status of PDAC cells determines the growth response in tumor cells and CAFs

(A) The proliferation of murine PDAC cell lines (KC and Panc02) treated with conditioned media of the CAFs cell line (ImPSC) relative to serum-free media treatment. (B) The proliferation of human PDAC cell lines treated with conditioned media of the CAFs cell line (10-32 PC Puro) relative to serum-free media treatment. (C) The proliferation of ImPSC cells treated with conditioned media of PDAC cell lines relative to serum-free media treatment. (D) The proliferation of the human CAFs cell line (10-32 PC Puro) treated with conditioned media of PDAC cell lines relative to serum-free media treatment. Data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test or two-way ANOVA when appropriate. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

A.


HPNE cells
Relative proliferation (%)
150
100
50
0
SF Media
10-32 PC Puro CM
HPNE-Kras cells
Relative proliferation (%)
250
200
150
100
50
0
SF Media
10-32 PC Puro CM
*

B.

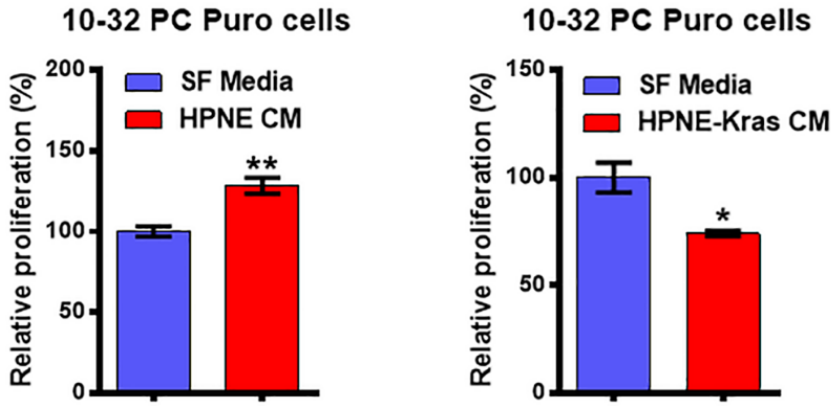

10-32 PC Puro cells
Relative proliferation (%)
200
150
100
50
0
SF Media
HPNE CM
**
10-32 PC Puro cells
Relative proliferation (%)
150
100
50
0
SF Media
HPNE-Kras CM
*

Figure 4.2: Kras-dependent differential proliferation response

(A) The proliferation of immortalized human pancreatic ductal cell lines treated with conditioned media of the CAFs cell line (10-32 PC Puro) relative to serum-free media treatment. (B) The proliferation of the human CAFs cell line (10-32 PC Puro) treated with conditioned media of pancreatic ductal cell lines relative to serum-free media treatment. Data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test. *$p \leq 0.05$, **$p \leq 0.01$.

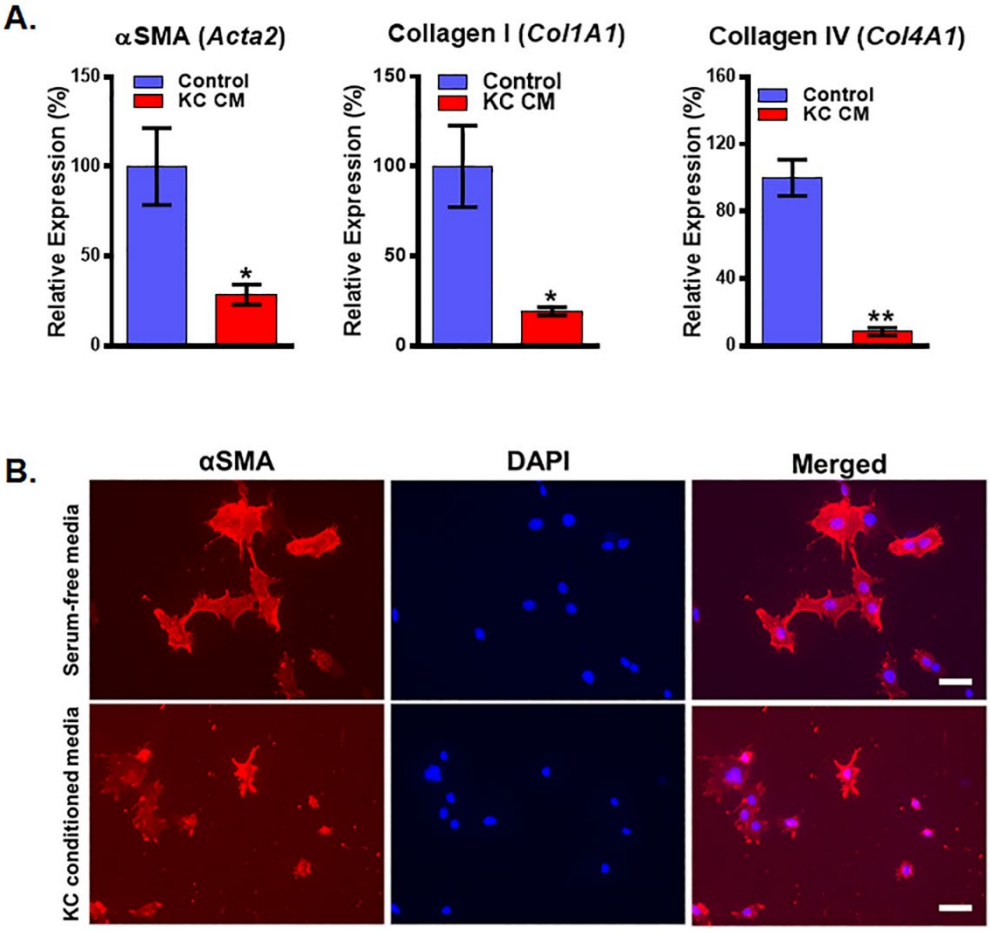

A.
αSMA (Acta2)
Collagen I (Col1A1)
Collagen IV (Col4A1)
Control
KC CM
Relative Expression (%)
150
100
50
0
160
120
80
40
*
**
B.
αSMA
DAPI
Merged
Serum-free media
KC conditioned media

Figure 4.3: Paracrine factors of PDAC cells downregulates myofibroblasts markers

(A) Expression, determined by qPCR, of *Acta2* (αSMA), *Col1A1* (Collagen I) and *Col4A1* (Collagen IV) in ImPSC cells treated with KC conditioned media relative to serum-free media treatment. (B) Representative microscopic image of αSMA expression, determined by IF, in ImPSC cells treated with KC conditioned media or serum-free media. Data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test. *$p \leq 0.05$, **$p \leq 0.01$. Scale bar = 50μm.

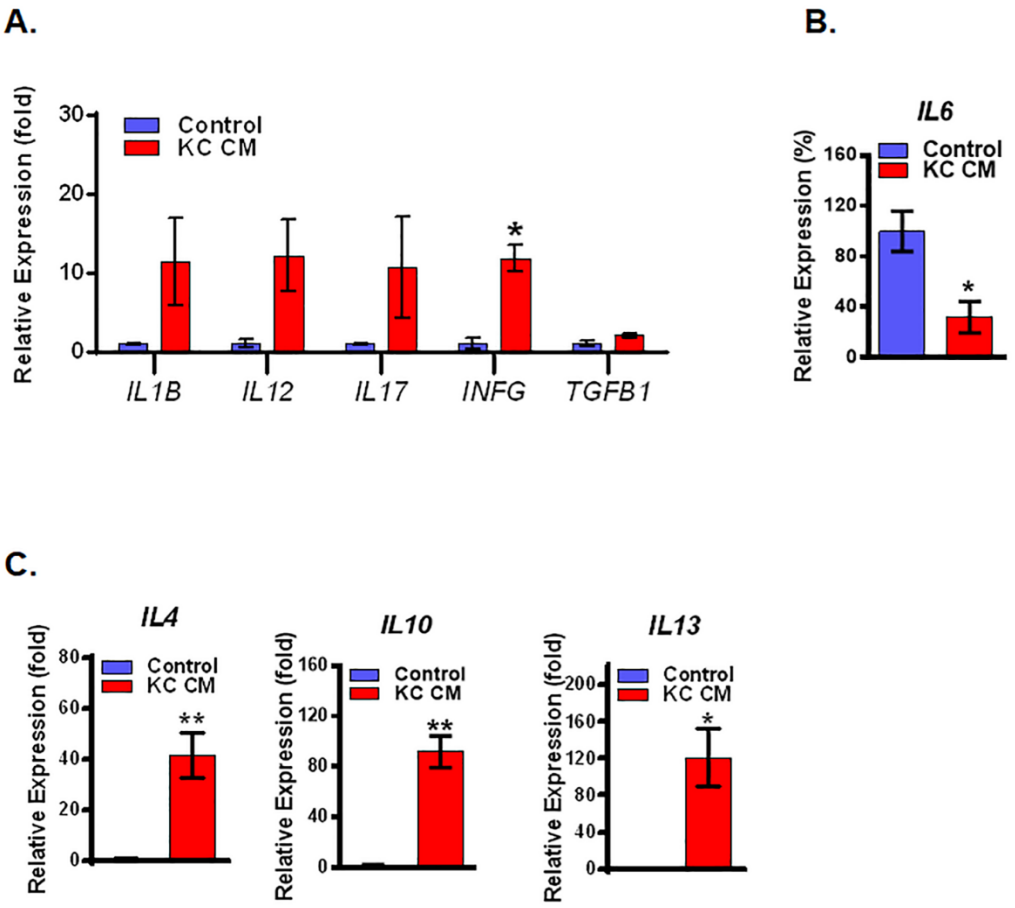

A.
B.
C.
Relative Expression (fold)
Control
KC CM
IL1B
IL12
IL17
INFG
TGFB1
IL6
Relative Expression (%)
IL4
IL10
IL13

Figure 4.4: PDAC cells-derived factors promote the secretion of immunosuppressive cytokines from CAFs

(A) Expression, determined by qPCR, of selected cytokines in ImPSC cells treated with KC conditioned media relative to serum-free media treatment. (B) Expression, determined by qPCR, of *IL6* in ImPSC cells treated with KC conditioned media relative to serum-free media treatment. (C) Expression, determined by qPCR, of *IL4, IL10, and IL13* in ImPSC cells treated with KC conditioned media relative to serum-free media treatment. Data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test. *$p \leq 0.05$, **$p \leq 0.01$.

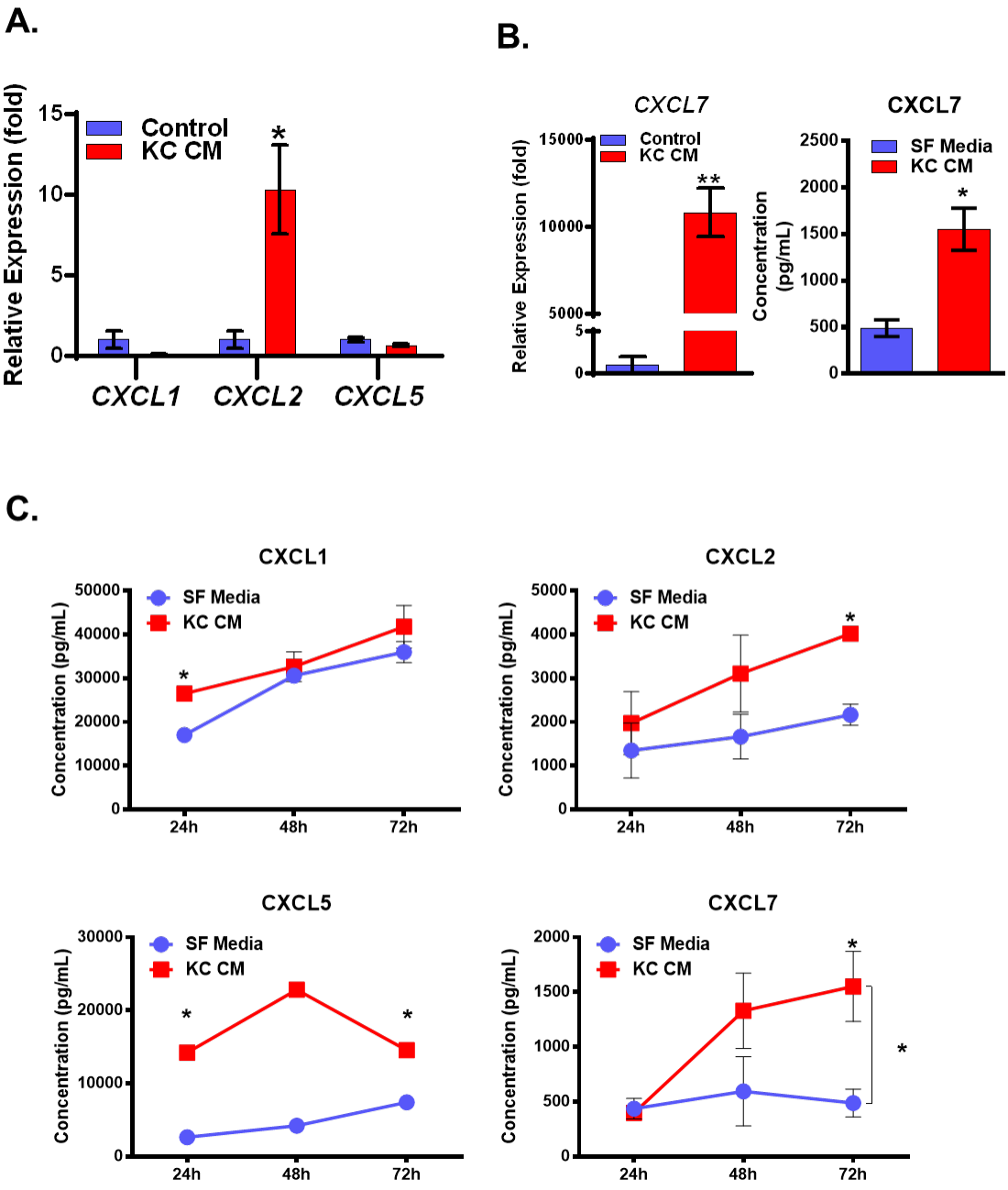

A.
Relative Expression (fold)
15
10
5
0
Control
KC CM
*
CXCL1
CXCL2
CXCL5
B.
CXCL7
Relative Expression (fold)
15000
10000
5000
5
0
Control
KC CM
**
CXCL7
Concentration (pg/mL)
2500
2000
1500
1000
500
0
SF Media
KC CM
*
C.
CXCL1
Concentration (pg/mL)
50000
40000
30000
20000
10000
0
SF Media
KC CM
*
24h
48h
72h
CXCL2
Concentration (pg/mL)
5000
4000
3000
2000
1000
0
SF Media
KC CM
*
24h
48h
72h
CXCL5
Concentration (pg/mL)
30000
20000
10000
0
SF Media
KC CM
*
*
24h
48h
72h
CXCL7
Concentration (pg/mL)
2000
1500
1000
500
0
SF Media
KC CM
*
*
24h
48h
72h

Figure 4.5: PDAC paracrine factors enhances production of CXCR2 ligands

(A) Expression, determined by qPCR, of *CXCL1, CXCL2, and CXCL5* in ImPSC cells treated with KC conditioned media relative to serum-free media treatment. (B) Expression of *CXCL7*, determined by qPCR, and concentration of *CXCL7*, determined by ELISA in ImPSC cells treated with KC conditioned media compared to serum-free media treatment. (C) The concentration of CXCL1, CXCL2, CXCL5, and CXCL7, as determined by ELISA, in the supernatant of ImPSC cells treated with KC conditioned media or serum-free media for 24, 48, or 72h. Data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test or two-way ANOVA when appropriate. *$p \leq 0.05$, **$p \leq 0.01$.

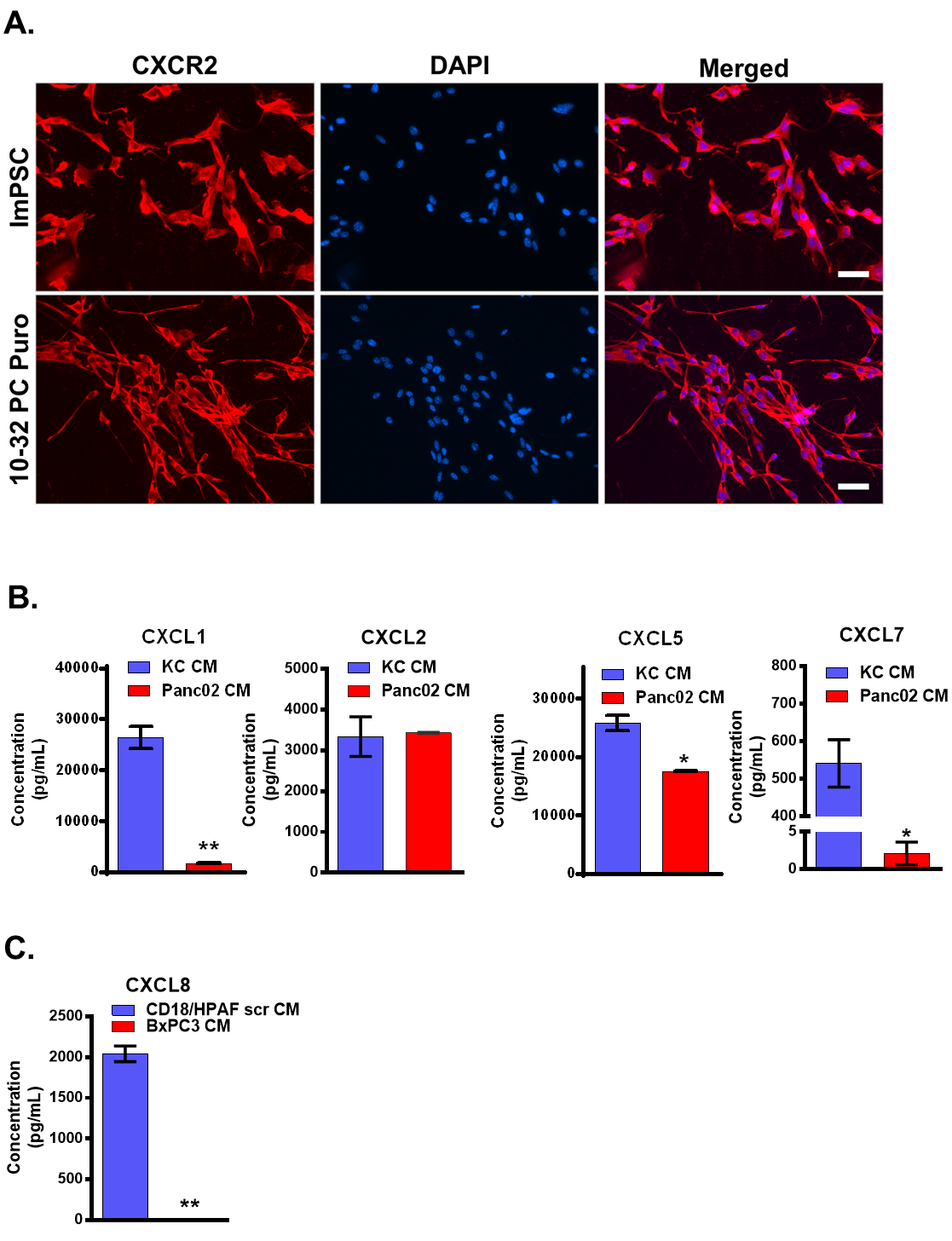

A.
CXCR2
DAPI
Merged
ImPSC
10-32 PC Puro
B.
CXCL1
CXCL2
CXCL5
CXCL7
KC CM
Panc02 CM
Concentration (pg/mL)
40000
30000
20000
10000
0
5000
4000
3000
2000
1000
800
700
600
500
400
5
**
*
C.
CXCL8
CD18/HPAF scr CM
BxPC3 CM
2500
2000
1500
1000
500
0

Figure 4.6: Higher expression of CXCR2 ligands in PDAC cells with oncogenic Kras

(A) Representative microscopic image of CXCR2 expression, determined by IF, in ImPSC and 10-32 PC Puro CAFs. (B) The concentration of CXCL1, CXCL2, CXCL5, and CXCL7, as determined by ELISA, in the conditioned media of KC and Panc02 cells. (C) The concentration of CXCL8, as determined by ELISA, in the conditioned media of CD18/HPAF scr and BxPC3 cells. Data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test. *$p \leq 0.05$, **$p \leq 0.01$. Scale bar = 50μm.

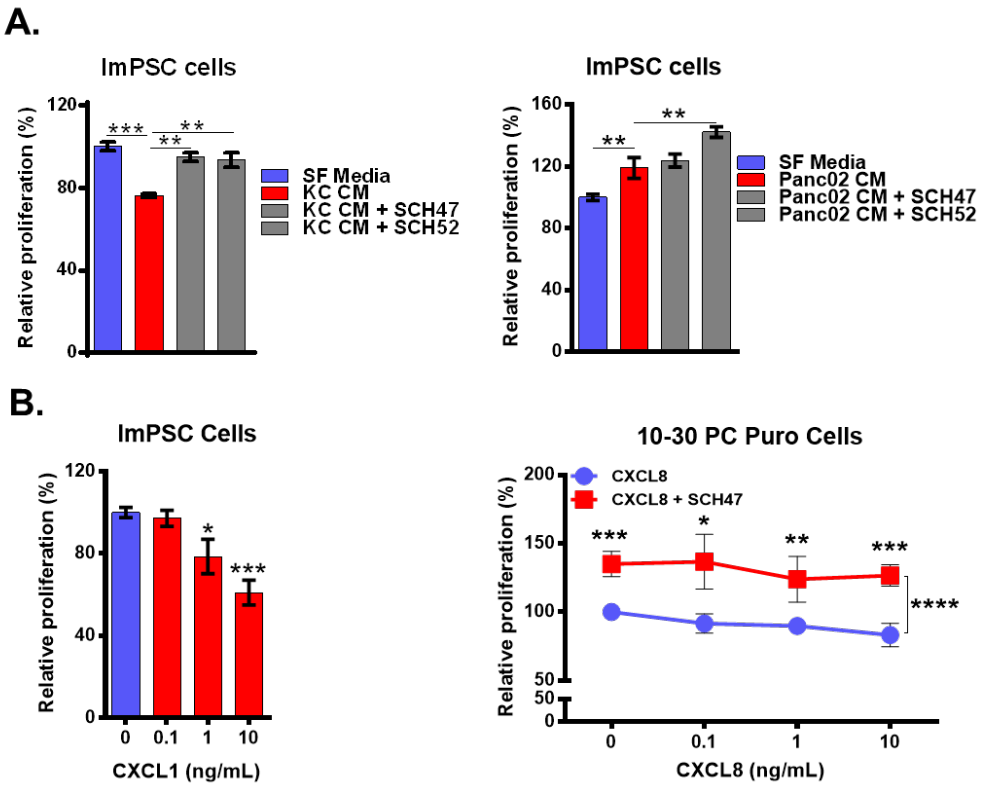

A.
ImPSC cells
Relative proliferation (%)
SF Media
KC CM
KC CM + SCH47
KC CM + SCH52
ImPSC cells
Relative proliferation (%)
SF Media
Panc02 CM
Panc02 CM + SCH47
Panc02 CM + SCH52
B.
ImPSC Cells
Relative proliferation (%)
CXCL1 (ng/mL)
10-30 PC Puro Cells
CXCL8
CXCL8 + SCH47
Relative proliferation (%)
CXCL8 (ng/mL)
C.
αSMA (Acta2)
Collagen I (Col1A1)
IL4
IL13
Control
CXCL1
Relative Expression (%)
Relative Expression (fold)
D.
CXCL7
CXCL7
Control
CXCL1
Relative Expression (fold)
SF Media
CXCL1
Concentration (pg/mL)

Figure 4.7: CXCR2 signaling in CAFs induces a secretory phenotype

(A) The proliferation of ImPSC cells treated with conditioned media of PDAC cell lines ± CXCR2 inhibitors relative to serum-free media treatment. (B) The proliferation of the ImPSC cells treated with increasing concentrations of recombinant CXCL1 and the proliferation of 10-32 PC Puro cells treated with recombinant CXCL8 ± CXCR2 inhibitors relative to serum-free media treatment. Data are presented as mean ± SEM. (C) Expression, determined by qPCR, of *Acta2, Col1A1, IL4* and *IL13* in ImPSC cells treated with recombinant CXCL1 relative to serum-free media treatment. (D) Expression of *CXCL7*, determined by qPCR, and concentration of *CXCL7*, determined by ELISA in ImPSC cells treated with recombinant CXCL1 compared to serum-free media treatment. Statistical analysis was performed using Student's t-test or two-way ANOVA when appropriate. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

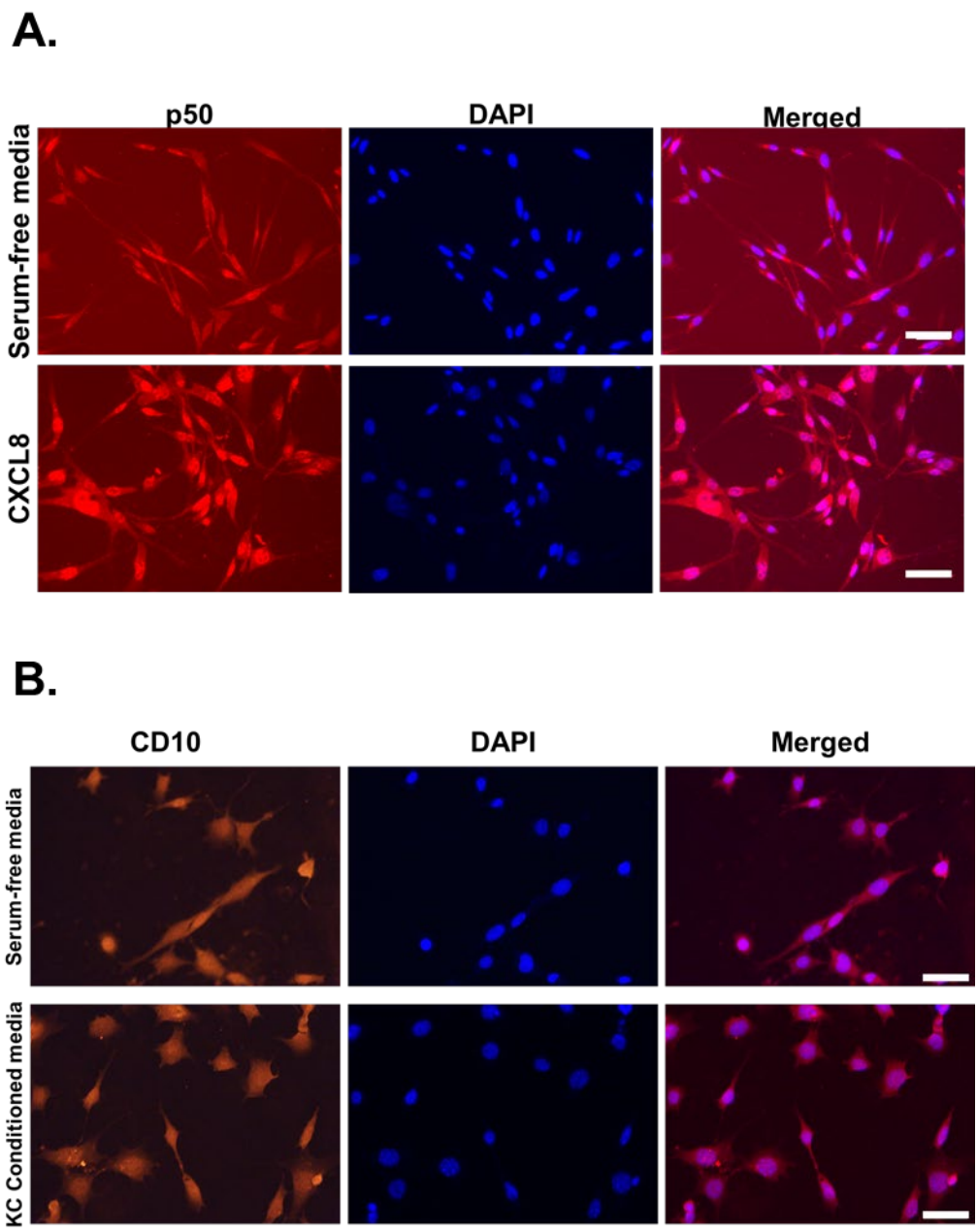

A.
p50
DAPI
Merged
Serum-free media
CXCL8
B.
CD10
DAPI
Merged
Serum-free media
KC Conditioned media

Figure 4.8: CXCR2 signaling induces secretory CAFs via activating NF-kB

(A) Representative immunofluorescence image of p50 in 10-32 PC Puro cells treated with recombinant CXCL8 or serum-free media. (B) Representative immunofluorescence image of CD10 in ImPSC cells treated with KC conditioned media or serum-free media. Scale bar = 50μm.

CHAPTER V: CAFS ORIENTATION IMPACTS TUMOR OUTCOME OF PDAC

ABSTRACT

PDAC is a very ferocious and challenging malignancy with a characteristic of predominant desmoplasia. CAFs, ECM deposition, and infiltrating immune and endothelial cells make up the complex tumor microenvironment of PDAC. CAFs are known for their role in ECM deposition and remodeling, which creates firm tumors that hinders chemotherapy and modulates metastasis. Recently, a specialized subset of CAFs was described to have an association with amplifying inflammation. We aimed to investigate if the abundance of a certain subset of CAFs is associated with certain cancer outcome. To examine this, we utilized syngeneic mouse models of PDAC. Our results demonstrate that oncogenic Kras associated with the abundance of the secretory CAFs and immune infiltration; whereas, myofibroblasts were associated with increased fibrosis. Inhibition of CXCR2 was beneficial in tumors abundant in myofibroblast CAFs. Together, we demonstrate the functional heterogeneity of CAFs in PDAC and that CAFs orientation impacts the tumor outcome.

INTRODUCTION

PDAC is one of the most virulent solid tumors in human due to its frequent spread, late diagnosis, and therapy resistance (Siegel, Miller et al. 2018). Accumulation of genetic mutations, including early events such as oncogenic Kras activation and late events including the inactivation of tumor suppressors such as p53 and Smad4, contribute to tumor initiation and progression (Hansel, Kern et al. 2003, Hruban and Fukushima 2007, Hruban, Maitra et al. 2007, Hruban and Adsay 2009). Nonetheless, more tumor progression cues also produced as a result of cross-talk between tumor cells and other host cells in the tumor microenvironment that include CAFs, immune cells and endothelial cells (Chu, Kimmelman et al. 2007, Kleeff, Beckhove et al. 2007). CAFs population consists of fibrotic cells of different origins including PSCs, tissue-resident fibroblasts, bone marrow-derived mesenchymal cells, and fibrocytes (Öhlund, Elyada et al. 2014). As CAFs, these cells contribute to tumor progression and spread by modulating ECM synthesis and remodeling as well as the secretion of several paracrine factors (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). Recently, more attention has been directed towards CAFs functional heterogeneity. Myofibroblast CAFs are the typical phenotype that is known to produce ECM molecules contributing to tumor stiffness, hypoxia and impairing chemotherapy delivery (Olive, Jacobetz et al. 2009, Apte, Wilson et al. 2013, Moir, Mann et al. 2015). The recently reported secretory CAF phenotype is characterized by a low expression of αSMA and increased secretion of inflammatory and pro-tumor mediators (Ohlund, Handly-Santana et al. 2017, Su, Chen et al. 2018). The CAFs

secreted factors enhance tumor growth, immunosuppression, and chemotherapy resistance by the induction of stemness (Chan, Hsu et al. 2016, Su, Chen et al. 2018).

In the previous chapter, we described how oncogenic Kras and CXCR2 signaling contribute to the secretory CAFs orientation. In this section, we aimed to assess the impact of PDAC mutations and CXCR2 inhibition on CAFs orientation and the overall tumor outcome. To achieve this, we utilized syngeneic PDAC mouse models orthotopically transplanted with KC cells (oncogenic Kras) (Purohit 2015) or Panc02 (contains a single nucleotide polymorphism in Kras gene from TAT to TAC at codon 32 and Smad4 inactivation mutation) (Wang, Zhang et al. 2012). To assess the effect of CXCR2 inhibition, the syngeneic models were performed in mice with *Cxcr2*$^{-/-}$ or CXCR2-wildtype genotypes.

RESULTS

Genetic mutations in PDAC determine CAFs orientation and abundance

We have determined thus far that oncogenic Kras upregulates CXCR2 axis, which in turn controls CAFs orientation and activity. To investigate if the PDAC mutations can exhibit differential CAFs distribution and abundance in-vivo, we utilized syngeneic mouse models transplanted with KC cells that carry oncogenic Kras (Torres, Rachagani et al. 2013) or Panc02 that carry wildtype Kras (Wang, Zhang et al. 2012). Histological assessment of KC tumor exhibited well-differentiated tumors with abundant duct formation, low to moderate infiltration of CAFs, and a high inflammatory infiltration, in particular, polymorph nuclear cells that are likely neutrophils or MDSCs **(Figure 5.1A)**. Panc02 tumors were undifferentiated with no to little duct formation, and a high infiltration of CAFs **(Figure 5.1B)**. To assess CAFs orientation, we used immunohistochemistry technique to stain for αSMA and CD10. In KC tumor, few αSMA^{+} cells were found often between ducts and at the tumor margins; interestingly, CD10 staining was localized to the αSMA areas but also stained much more area where αSMA was negative **(Figure 5.1C)**. This indicates that αSMA^{+} CAFs are also likely $CD10^{+}$, which may represent the myofibroblast population; whereas, $CD10^{+}$ CAFs that are negative for αSMA staining represent secretory CAFs. Lastly, CAFs distribution in Panc02 tumors indicated that both αSMA and CD10 staining were localized to the same areas suggesting that CAFs in Panc02 tumors are likely assumed the myofibroblast phenotype **(Figure 5.1D)**. Together, we show that Kras status of PDAC can affect CAFs phenotypes orientation and abundance.

CXCR2 inhibition renders different impact based on PDAC mutations

Our interest in investigating CAFs of PDAC was as a result to the observation that inhibiting CXCR2 in the syngeneic KC mouse model rendered increased fibrosis revealing a potential role of CXCR2 in regulating CAFs (Purohit 2015). In this section, we aimed to investigate the effect of the stromal CXCR2 deletion in the context of PDAC mutations. We utilized the syngeneic PDAC model of KC and Panc02 that were implanted in *Cxcr2*$^{-/-}$ or wildtype. From our previous study (Purohit 2015), we concluded that CXCR2 deletion caused tumoristatic and tumoritoxic effects by halting proliferation and increasing apoptosis of tumor cells, lowering MDSCs infiltration, and decreasing angiogenesis. Our focus here was to assess the impact of CXCR2 deletion on CAFs and if it is linked to other changes in the context of oncogenic Kras. In KC tumors, deletion of CXCR2 did not exhibit alteration to the differentiation stage of the tumor cells; however, the major observation was the increase of fibrotic reaction **(Figure 5.2A)**. The increased fibrotic reaction in the KC model was associated with increased αSMA^{+} cells that were also localized to the same area as CD10^{+} CAFs suggesting a diminished secretory CAFs repertoire **(Figure 5.2B)**. CXCR2 knockout in Panc02 implanted mice seemed to have a more prominent anti-tumor effect. In addition to increasing the tumor cells apoptosis, the decreased vascularization was more noticeable in these tumors. Moreover, CXCR2 stromal deletion appears to ameliorate the aggressiveness of the tumor cells in Panc02 tumors, which was presented as increased ducts formation indicating a transformation from undifferentiated to poorly-differentiated tumor **(Figure 5.2C)**. Finally, similar to CXCR2-wildtype

tumors, both αSMA and CD10 staining localized to the same areas of the sections, and there was no noticeable change to fibrotic reaction or the abundance of CAFs **(Figure 5.2C)**. Collectively, we demonstrate a mutation-dependent histological difference in PDAC and show that CXCR2 plays a role in both tumors and stromal compartments.

DISCUSSION

Both the tumor-supporting inflammation and the dense desmoplasia are typical characteristics of PDAC (Chu, Kimmelman et al. 2007, Kleeff, Beckhove et al. 2007, Grivennikov, Greten et al. 2010, Baumgart, Chen et al. 2014, Hamada, Masamune et al. 2014). CAFs are now considered a player in both hallmarks by assuming distinct functional phenotypes. Myofibroblasts produce ECM causing tumor stiffness and hypoxia; thus, promoting therapy hindrance and contributing to metastasis (Olive, Jacobetz et al. 2009, Pandol, Edderkaoui et al. 2009, Bynigeri, Jakkampudi et al. 2017). Secretory CAFs secrete paracrine mediators and modulate inflammation, immunosuppression, and chemotherapy resistance (Waugh and Wilson 2008, Matsuo, Raimondo et al. 2009, Singh, Varney et al. 2009, Highfill, Cui et al. 2014, Chan, Hsu et al. 2016, Purohit, Varney et al. 2016, Kumar, Donthireddy et al. 2017, Su, Chen et al. 2018). In this section, we used syngeneic mouse models to assess the involvement of PDAC mutations and the involvement of CXCR2 in tumors' histological features. PDAC tumors with only oncogenic Kras mutation presented well-differentiated histological features, with increased polymorph nuclear cells infiltration and abundance of secretory CAFs. Disrupting stromal CXCR2 signaling, caused an abundance of myofibroblast CAFs, which was associated with the increased fibrotic reaction. On the other hand, the tumors with wildtype Kras and Smad4 inactivating mutation presented undifferentiated tumors with an abundance of CAFs that were mostly myofibroblasts. CXCR2 deletion in these tumors produced a more pronounced

anti-tumor effect by forming poorly-differentiated tumors with decreased angiogenesis.

Targeting CAFs as well as targeting CXCR2 has been attempted before, which exhibited beneficial outcomes in some cases and adverse outcomes in others cases. Targeting CAFs or desmoplasia improved chemotherapy delivery in some instances and accelerated tumor progression in other cases (Olive, Jacobetz et al. 2009, Özdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014). Inhibiting CXCR2 in the context of *Kras* mutation alone, increased the fibrosis and metastasis (Purohit 2015); whereas, CXCR2 inhibition in the context of other mutations, such as *p53* or *Tgfbr2*, rendered beneficial outcomes (Ijichi, Chytil et al. 2011, Steele, Karim et al. 2016). Our data demonstrated that the pronounced anti-tumor effects of CXCR2 deletion were observed in tumors generated using Panc02 cells that carry a wildtype Kras and a Smad4 mutation (Wang, Zhang et al. 2012). Oncogenic Kras mutation occurs early during PDAC pathogenesis; whereas, mutations of the tumor suppressors are often late events (Hruban and Fukushima 2007). This observation could suggest a temporal context for the beneficial or adverse outcomes of CXCR2 inhibition, in which inhibiting CXCR2 in early PDAC could render accelerated disease and inhibiting CXCR2 in late stages could tame the disease.

In conclusion, we report in this study that CXCR2 signaling plays a role in regulating the CAFs of PDAC; thus, affecting the tumor outcome. Further studies are required to characterize CAFs subsets and identify better markers than currently available. Furthermore, there is a need for better understating for the

temporal and mutation context of CXCR2 roles in PDAC. We have witnessed that both targeting CAFs and targeting CXCR2 could have context-dependent outcomes. Both could still be good potential targets for treating PDAC, pending better understanding.

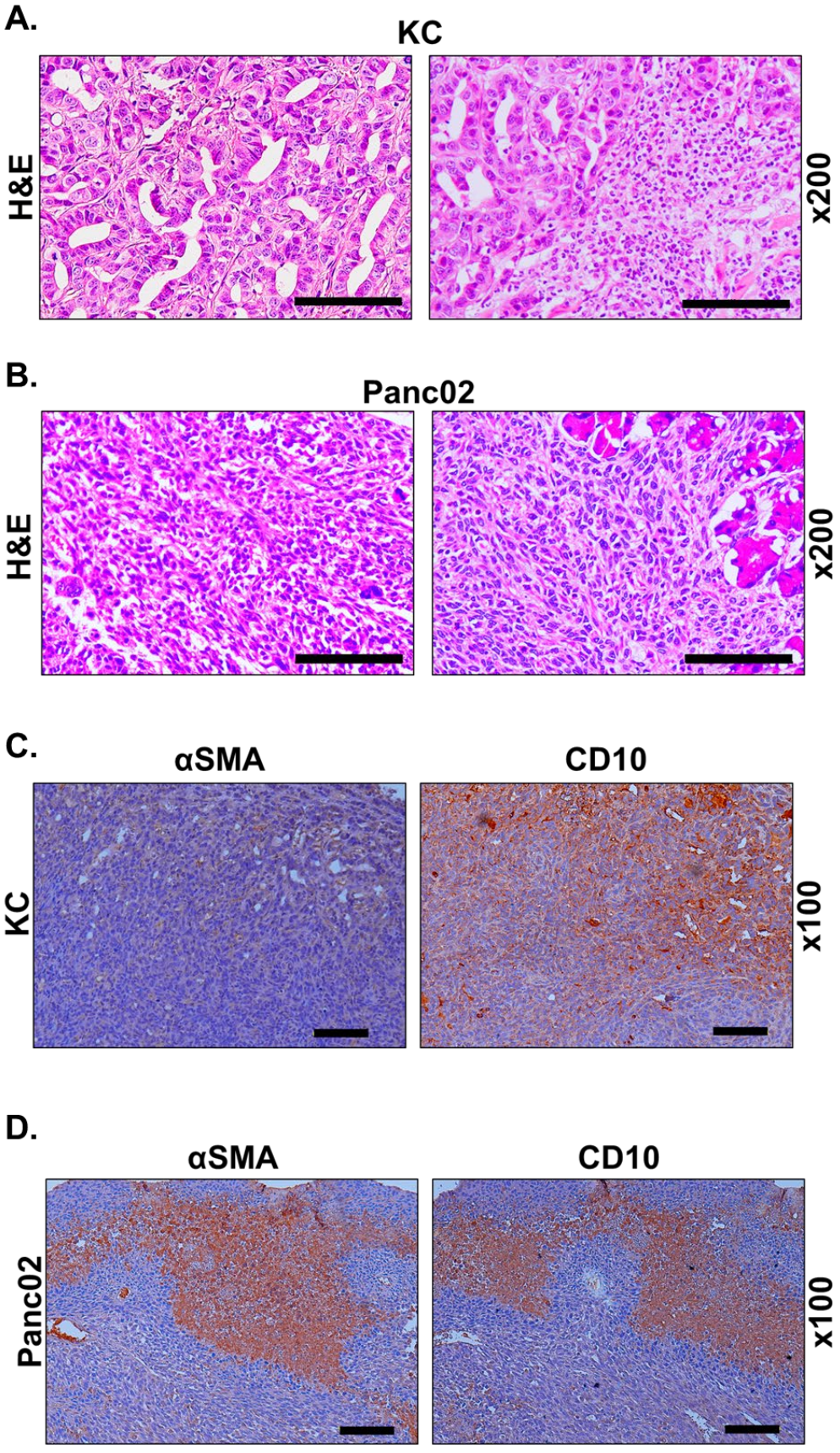

A.
KC
H&E
x200
B.
Panc02
H&E
x200
C.
αSMA
CD10
KC
x100
D.
αSMA
CD10
Panc02
x100

Figure 5.1: Kras-dependent CAFs distribution *in-vivo*

(A) Representative image with H&E staining of tumors derived from KC implanted syngeneic mouse models showing the well-differentiated histology (left) and the abundant polymorph nuclear cells infiltration (right). (B) Representative image with H&E staining of tumors derived from Panc02 implanted syngeneic mouse models showing the undifferentiated histology (left) and the abundant CAFs infiltration (right). (C) Representative immunohistochemistry images of tumors derived from KC implanted syngeneic mouse models showing αSMA staining (left) and CD10 staining (right). (D) Representative immunohistochemistry images of tumors derived from Panc02 implanted syngeneic mouse models showing αSMA staining (left) and CD10 staining (right). Scale bar = 100μm.

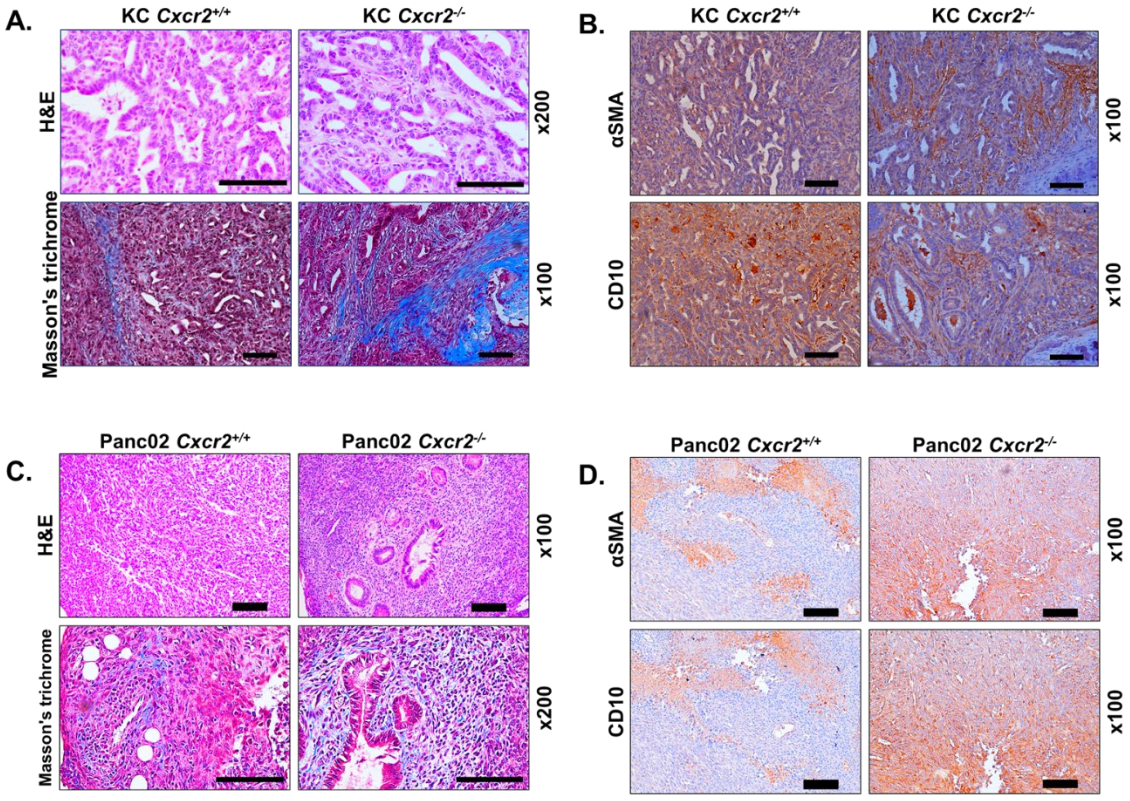

A.
KC Cxcr2+/+
KC Cxcr2-/-
H&E
x200
Masson's trichrome
x100
B.
KC Cxcr2+/+
KC Cxcr2-/-
αSMA
x100
CD10
x100
C.
Panc02 Cxcr2+/+
Panc02 Cxcr2-/-
H&E
x100
Masson's trichrome
x200
D.
Panc02 Cxcr2+/+
Panc02 Cxcr2-/-
αSMA
x100
CD10
x100

Figure 5.2: CXCR2 inhibition affects stromal activity *in-vivo*

(A) Representative immunohistochemistry images with H&E or Masson's trichrome staining of tumors derived from KC implanted syngeneic mouse models. (B) Representative immunohistochemistry images of tumors derived from KC implanted syngeneic mouse models showing αSMA or CD10 staining. (C) Representative immunohistochemistry images with H&E or Masson's trichrome staining of tumors derived from Panc02 implanted syngeneic mouse models. (D) Representative immunohistochemistry images of tumors derived from KC implanted syngeneic mouse models showing αSMA or CD10 staining. Scale bar = 100μm.

CHAPTER VI: MAJOR CONCLUSIONS AND FUTURE DIRECTIONS

INTRODUCTION

Pancreatic cancers, in general, rank the fourth in leading causes of cancer deaths in the United States (Siegel, Miller et al. 2018). PDAC is the most common subtype of pancreatic cancers, and one of the most virulent human cancers. The lack of early screening tools and the asymptomatic nature of the disease cause the late detection that is often at the metastatic stage. A small proportion of PDAC patients are diagnosed with a localized tumor. At such stage, surgical resection may provide a cure for the disease. Patient diagnosed with advanced stages miss the chance for effective therapeutic options. The virulence of PDAC comes from its frequent dissemination, resistance to conventional therapy, and the high recurrence rates even for those patients who underwent surgical resection (Hidalgo 2010). There is an urgent need to develop screening and early detection options as well as to develop effective therapies for advanced PDAC. Understanding the complex tumor-stromal and stromal-stromal interactions, underlined with understanding the driving genetic alterations can aid in the quest for developing both detection tool and therapeutic potentials. Our effort in this study aimed to investigate the relationship between oncogenic Kras mutation, CXCR2 signaling, and CAFs. We also sought to examine the role of CAFs in PDAC in light of CXCR2 signaling CAFs heterogeneity.

The adverse role of CAFs in PDAC as enablers of therapy resistance and metastasis is established (Olive, Jacobetz et al. 2009, Xing, Saidou et al. 2010, Apte, Wilson et al. 2013, Moir, Mann et al. 2015, Su, Chen et al. 2018). CAFs functional heterogeneity has gained momentum recently. Myofibroblast CAFs, the

typical phenotype, develop in response to factors including TGF-β, PDGF and SHH, and contribute to desmoplasia by promoting ECM deposition (Shek, Fmj et al. 2002, Omary, Lugea et al. 2007, Bailey, Swanson et al. 2008). The recently described secretory CAFs are thought to develop in the context of inflammation through paracrine stimulation (Ohlund, Handly-Santana et al. 2017, Su, Chen et al. 2018). CAFs-secreted paracrine factors promote tumor virulence (Polnaszek, Kwabi-Addo et al. 2003, Chan, Hsu et al. 2016). This dissertation summarizes the putative role of oncogenic Kras-driven inflammation, in particular, CXCR2 signaling, in CAFs orientation and function, and explains how CXCR2 ligands and other paracrine factors such as FGF-2 shape up the tumor cells behavior and affects the overall tumor outcome. The first section of this chapter is a summary of the major findings and conclusions of this dissertation. The next section presents the future directions for this project by discussing the uninvestigated questions and suggestions of experiments to answer them.

MAJOR CONCLUSIONS

The role of FGF-2 and CXCL8 in tumor-CAFs interaction

I. Both CAFs and PDAC cells express FGF-2 and CXCL8.

II. Direct co-culture showed that CAFs increased the growth of the aggressive PDAC cells but inhibited the non-aggressive cells.

III. FGF-2 secreted by CAFs induced the growth stimulatory effect and the secretion of CXCL8 in the aggressive PDAC cells.

IV. CXCL8 produced by PDAC cells modulated CAFs growth and induced secretion of FGF-2 to create a feedforward loop.

V. Gene expression of PDAC-CAFs co-culture indicated that CAFs produce the bulk of the pro-tumor cytokines and chemokines.

VI. The co-culture of CAFs with PDAC cells increased the expression of FGF-2 and CXCL8.

VII. In addition to the pro-tumor cytokines and chemokines, CAFs co-culture with the aggressive PDAC cells indicated that CAFs promote tumor progression by upregulating genes such as *ELMO1* that promotes tumor cells motility and invasion.

The role of oncogenic Kras in regulating CAFs

I. CAFs paracrine factors promote the growth of PDAC cells with oncogenic Kras.

II. Paracrine factors secreted from PDAC cells with oncogenic Kras inhibits the growth of CAFs.

III. Oncogenic Kras-driven PDAC secreted factors alter myofibroblast markers.

IV. PDAC factors, in the context of oncogenic Kras, promote secretory function in CAFs portrayed as the secretion of CXCR2 ligands and pro-tumor cytokines.

The role of CXCR2 signaling in regulating CAFs function

I. PDAC cells with oncogenic Kras produce more CXCR2 ligands.

II. CXCR2 signaling induces an inhibitory effect on CAFs proliferation.

III. CXCR2 signaling in CAFs promotes phenotypic changes to CAFs by downregulating myofibroblast markers.

IV. In response to CXCR2 signaling, CAFs assume a secretory function by secreting CXCR2 ligands and pro-tumor immunosuppressive cytokines.

V. The CXCR2-induced secretory CAF phenotype is mediated through NF-κB activation.

VI. The secretory CAFs express CD10.

CAFs phenotypes distribution in PDAC

I. CD10 is expressed on myofibroblast in addition to αSMA; whereas; secretory CAFs only express CD10.

II. PDAC tissues displayed expression of both myofibroblasts and secretory CAFs.

III. Wildtype Kras tumors mostly presented myofibroblast phenotype (CD10 and αSMA present at the same location most of the time).

IV. In oncogenic Kras tumors, there is more secretory CAFs (more CD10 staining than αSMA staining).

V. CXCR2 inhibition in oncogenic Kras produced phenotype change to mostly myofibroblast and increased fibrosis.

VI. CXCR2 inhibition in wildtype Kras PDAC mouse models yielded less aggressive tumors.

The big picture

Based on our observations as well as findings from the literature, we compiled the following conclusions:

I. CAFs are recruited from different origins and express different markers.

II. CAFs display functional heterogeneity as myofibroblast or secretory phenotypes.

III. Myofibroblasts develop in response to fibrogenic factors such as TGF-β, PDGF and SHH.

IV. Myofibroblast CAFs produced ECM and promote tumor stiffness, hypoxia, chemotherapy hindrance and EMT, factors that enable invasion.

V. Secretory CAFs develop in the context of inflammation, in response to mediators such as CXCR2 ligands and DAMPs.

VI. Producing pro-tumor mediators such as CXCR2 ligands and IL-6, secretory CAFs enable tumor growth, immunosuppression, vascularity, and stemness-induced therapy resistance **(Figure 6.1)**.

VII. Oncogenic Kras that occur early in PDAC development is highly linked to inflammation; thus, expected to display an abundance of secretory CAFs in early stages.

VIII. Mutations in tumor suppressors such as p53 and Smad4 are often late events and are linked to the increased desmoplastic reaction.

IX. The increased desmoplasia and the inactivation of the tumor suppressors could be the tipping point for invasive PDAC.

FUTURE DIRECTIONS

With what we currently know, based both on the current study and the literature, about the CXCR2-dependent role of CAFs in PDAC, there still many unanswered questions. In this section, we will discuss the major unanswered question and their potential future direction.

CAFs origin and CAFs functional role

CAFs compartment develops from fibrotic cells of different origins, including PSCs, tissue-resident fibroblasts, bone marrow-derived mesenchymal stem cells and fibrocytes (Öhlund, Elyada et al. 2014). Multiple markers, including αSMA, FAP, and PDGFR, have been heavily utilized to distinguish CAFs from other cells; however, these markers are not uniformly expressed on CAFs (Öhlund, Elyada et al. 2014). It is not clear if the lack of uniform expression is due to origin disparities, functional heterogeneity, differentiation stage, or a combination of multiple factors. We know that secretory CAFs in PDAC have low expression of αSMA (Ohlund, Handly-Santana et al. 2017); however, that is not the case in other cancer. The αSMA expression in the secretory CAFs of breast and lung cancers was not different; whereas, markers such as CD10 and GPR77 were used (Su, Chen et al. 2018). We showed that secretory CAFs express CD10, but so are myofibroblasts. CAFs compartment in PDAC is maybe different in term of the origins of cells compared to other cancer types. Stellate cells were reported in multiple organs, but, are mostly described in the context of the liver and the pancreas (Omary, Lugea et al. 2007, Apte, Wilson et al. 2013, Öhlund, Elyada et al. 2014, Moir, Mann

et al. 2015). Recently, a subset of CAFs in breast cancer was described as bone marrow-derived mesenchymal cells reported to have low expression of PDGFR and to be functionally important for tumor growth and angiogenesis (Raz, Cohen et al. 2018). This suggests that CAFs origin may be involved in functional heterogeneity. In addition to the involvement of CAFs origin or lack thereof, it is not clear if the distinct functional subsets of CAFs are the yield of disparities in differentiation stage, or it is just a dynamic process in response to milieu changes, and whether they are able to switch into one another remains unknown. It is not clear also if other functional subsets present. There is a need for a better characterization of CAFs marker based on their origin and differentiation stages and cross-referenced to their functional roles. Single-cell RNA-seq and other gene expression and the functional assays can be utilized to achieve this goal.

Temporal context of CAFs functional heterogeneity

There is a great similarity between cancer and wound healing process. Cancers are often referred to as “wounds that do not heal” (Dvorak 1986). As discussed before, wound healing occur as a coordinated process of inflammatory response followed by tissue repair and remodeling. Fibrogenic activity in the wound healing process is a late event that happens after the secession of the inflammatory response (Dvorak 1986, Midwood, Williams et al. 2004). Similarly, PDAC is associated with early pro-tumor inflammation that often happen in the context of oncogenic Kras and a late pronounced desmoplasia prior to the emergence of invasive PDAC that occur after inactivation of tumor suppressors including p53 and Smad4 (Hruban, Offerhaus et al. 1998, Hruban, Wilentz et al.

2000, Purohit, Varney et al. 2016). This temporal context of genetic alterations alongside the transformation from inflammation to desmoplasia suggests a timely CAFs phenotype conversion. We know that targeting CAFs have been attempted before with opposing outcomes (Olive, Jacobetz et al. 2009, Özdemir, Pentcheva-Hoang et al. 2014). It is possible that targeting the right subset of CAFs in the right time could yield a beneficial result. Materials required to achieve this purpose include PDAC progression models with inducible mutations.

CXCR2 in the context of PDAC mutations and progression

Similar to targeting desmoplasia, inhibiting CXCR2 produced opposing outcomes (Ijichi, Chytil et al. 2011, Purohit 2015, Steele, Karim et al. 2016), which may involve mutation and/or progression context. With only oncogenic Kras mutation, CXCR2 deletion was adverse; whereas, the beneficial outcome was observed when CXCR2 was inhibited in the context of tumor suppressors' mutations (Ijichi, Chytil et al. 2011, Purohit 2015, Steele, Karim et al. 2016). Thus, CXCR2 could have opposing roles and targeting CXCR2 in the right context can produce advantageous effects. We can utilize PDAC progression models.

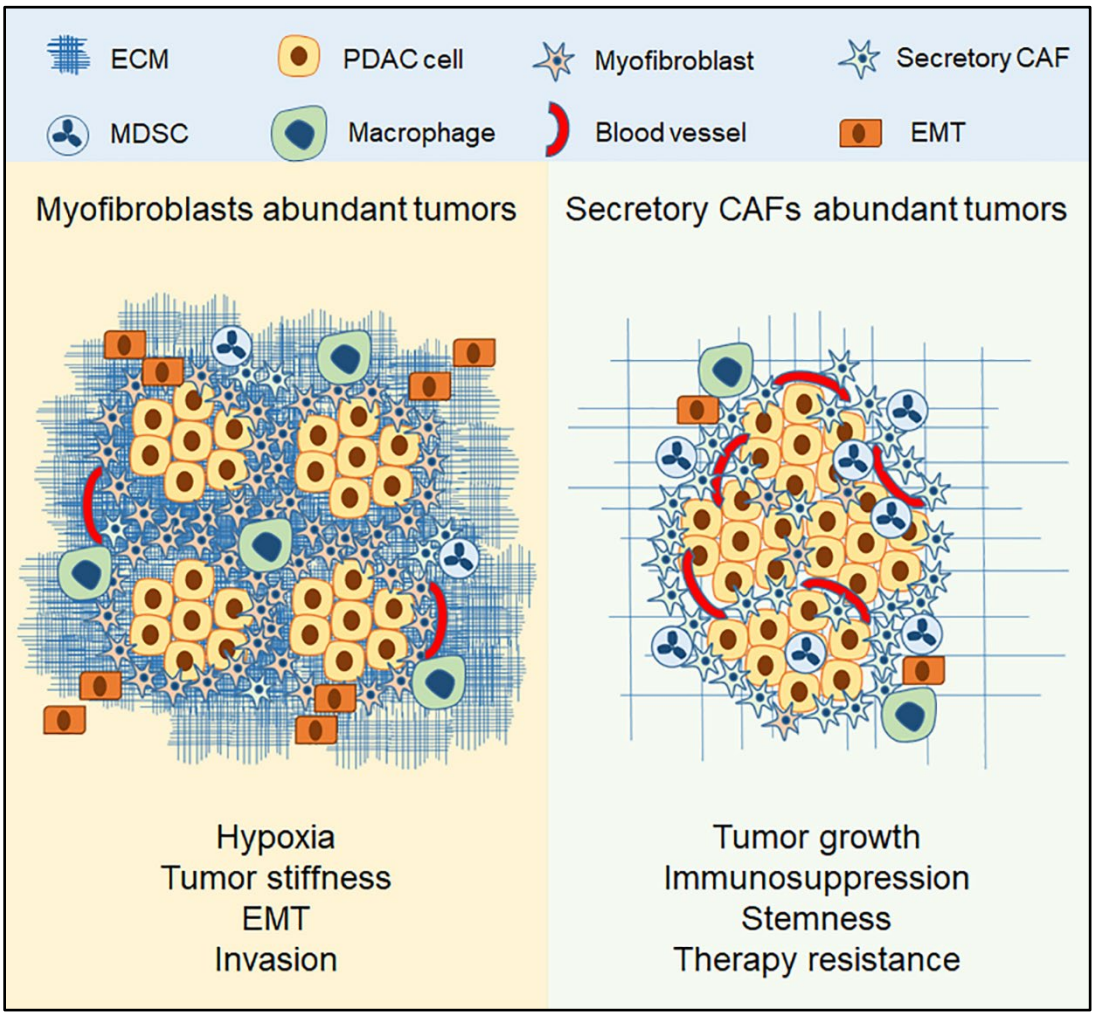

ECM
PDAC cell
Myofibroblast
Secretory CAF
MDSC
Macrophage
Blood vessel
EMT
Myofibroblasts abundant tumors
Secretory CAFs abundant tumors
Hypoxia
Tumor stiffness
EMT
Invasion
Tumor growth
Immunosuppression
Stemness
Therapy resistance

Figure 6.1: CAFs distribution affects the tumor outcome

Tumors abundant in myofibroblasts are characterized by increased ECM deposition that contributes to hypoxia and tumor stiffness, increased EMT, and a tendency for metastasis. The abundance of secretory CAFs contributes to tumor growth as a result of vascularization and proliferation cues, promotes immunosuppression by recruiting MDSCs, and enhances chemotherapy resistance by increasing stemness.

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