UMEÅ UNIVERSITY

THE IDENTIFICATION AND FUNCTIONAL EVALUATION OF NOVEL CANCER-ASSOCIATED FIBROBLAST SUBTYPES AND MATRISOME PROTEINS IN PANCREATIC CANCER

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“To go wrong in one’s own way is better than to go right in someone else’s”

Fyodor Dostoyevsky

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy characterized by an extensive desmoplastic stroma. The stroma is the site of intricate communication between malignant cells and their surrounding environment. This tissue microenvironment (TME) is populated by a heterogenous mixture of cell types and extracellular matrix proteins. Distinct stromal elements confer tumour-restraining or tumour-promoting influences on tumorigenesis. Characterizing stromal composition therefore represents an opportunity to identify candidates for therapeutic intervention to facilitate improved clinical outcomes. In this thesis we identify galectin-4 as an extracellular matrix protein which is upregulated in PDAC. We find that galectin-4 exerts a pro-tumorigenic influence in PDAC through promoting immune suppression, highlighting its potential as a novel therapeutic target. We subsequently provide a comprehensive characterization of mesenchymal cell diversity in PDAC including cancer-associated fibroblasts (CAFs) which represent one of the dominant stromal cellular components. We identify inflammatory CAF (iCAF) and myofibroblastic CAF (myCAF) subtypes in addition to defining a novel interferon-response CAF (ifCAF) subtype. In addition, we demonstrate that pancreatic stellate cells (PSCs) are capable of forming iCAFs, myCAFs and ifCAFs in response to tumour-derived signals using an organoid-based co-culture model and define biological pathways regulating CAF subtype formation. We then perform a high-throughput drug-screen using this co-culture model to identify compounds which can suppress tumour growth indirectly through modifying CAFs. One such compound is GNF-5 which we show can suppress cancer cell proliferation indirectly through manipulating CAF phenotype. Taken together, this thesis augments our understanding of the composition of the PDAC stroma and identifies potential therapeutic targets as well as developing an approach to discover drugs which yield a therapeutic benefit through targeting the PDAC stroma.

ORIGINAL PAPERS

Paper I

Tommy Lidström, **Joshua Cumming**, Rahul Gaur, Lars Frängsmyr, Ioannis S. Pateras, Matthias J. Mickert, Oskar Franklin, Mattias N.E. Forsell, Niklas Arnberg, Mitesh Dongre, Cedric Patthey, and Daniel Öhlund.
Extracellular galectin 4 drives immune evasion and promotes T-cell apoptosis in pancreatic cancer.
Cancer Immunol Res. 2023 Jan 3;11(1):72-92.

Paper II

Joshua Cumming, Kristian Pietras, Cedric Patthey and Daniel Öhlund.
Dissecting FAP^{+} mesenchymal cell diversity and regulation uncovers an interferon-response cancer-associated fibroblast subtype.
Manuscript under review.

Paper III

James Mason, **Joshua Cumming**, Anna U. Eriksson, Carina Binder, Mitesh Dongre, Cedric Patthey, Margarita Espona-Fiedler, Erik Chorell and Daniel Öhlund.
Potentiating the tumor-restraining properties of the stroma in pancreatic cancer with small molecules.
Manuscript under review.

ABBREVIATIONS

ADM	Acinar-to-ductal metaplasia
APC	Antigen-presenting cell
αSMA	Alpha smooth muscle actin
apCAFs	Antigen-presenting cancer-associated fibroblasts
CAF	Cancer-associated fibroblast
CAP	Cancer-associated perivascular cell
CTL	Cytotoxic T lymphocyte
CXCL	Chemokine (C-X-C) motif
DC	Dendritic cell
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
FACS	Fluorescence activated cell sorting
FAP	Fibroblast activation protein alpha
FSP1	Fibroblast-specific protein-1
Gal	Galectin
GEMM	Genetically engineered mouse model
HA	Hyaluronic acid
ICB	Immune checkpoint blockade
iCAFs	Inflammatory cancer-associated fibroblasts
ifCAF	Interferon-response cancer-associated fibroblasts
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IPMN	Intraductal papillary mucinous neoplasia
ISH	*in situ* hybridization
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
myCAFs	Myofibroblastic cancer-associated fibroblast
NGS	Next-generation sequencing
NK cells	Natural killer cells
PanIN	Pancreatic intraepithelial neoplasia
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PSC	Pancreatic stellate cell
PVL	Perivascular-like cell
scRNAseq	Single-cell RNA sequencing
STING	Stimulator of interferon genes
TAM	Tumour-associated macrophage
TGFβ	Transforming growth factor beta
Th cells	T helper cells
TIL	Tumour infiltrating lymphocyte
TME	Tumour microenvironment
Tregs	Regulatory T cell
UMAP	Uniform manifold approximation and projection
UMI	Unique molecular identifier

INTRODUCTION

Over the course of reading this introduction I hope to convey to you the reasons behind the work that was conducted during the course of my PhD. This work has been centred around understanding the composition of pancreatic tumours so that we might find features which can be targeted to help develop better treatments for pancreatic cancer. But what is pancreatic cancer? What on earth is a pancreas for that matter? I will first attempt to clarify key aspects of pancreatic cancer so that you might better understand the more specific goals of this thesis. These goals focus on better characterizing components of a tumour which are not the cancer cells and clarifying the roles such components play in the disease. Now you may be thinking that not focusing on the cancer cells sounds like a somewhat misdirected idea for finding new treatments for a cancer. A tumour is however, comprised of far more than just the cancer cells. Surrounding tumour cells is a complex and heterogenous environment consisting of diverse cell populations embedded in an extensive network of proteins. Distinct elements within this tumour microenvironment play contrasting roles on disease progression, with some elements aiding cancer cells and others attempting to resist their growth and spread. So, what are these cells and proteins which pervade a tumour and encapsulate cancer cells? Which components are helping the cancer and which ones are resisting it? Can we give the resistors a helping hand to fight the tumour and can we target elements which are assisting it? These questions are the reasons this thesis was written, but before I show my attempts to answer them, I must first try to clarify the scientific progress which has allowed us to ask such questions and to what extent we can address them currently.

STRUCTURE AND FUNCTION OF THE PANCREAS

For most of my life, indeed until I started this PhD, I had no clear concept of what a pancreas actually does. I believed it was a digestive organ but it lacked the purposeful clarity of its competitors, say the brain or lungs. I'm sure to many of the population, as with me, the word pancreas can exist in our consciousness only as a nebulous object we might use for a guess in a quiz where we aren't sure of an answer. Corroborating this, a survey conducted in the UK found that 40% of people did not know what the pancreas does and when shown it ~25% of people misidentified it as a penis. Fortunately, the pancreas itself is not lurking in our upper abdomen, hiding behind the stomach unsure exactly what it is there to do but is instead busy releasing digestive enzymes and hormones into our intestines and blood, respectively. Such functions facilitate our digestion of food and maintenance of blood sugar levels.

Structurally the pancreas can be divided into the head, body and tail with the head sitting immediately adjacent to the duodenum whilst the tail extends toward the spleen. Running through the pancreas are a series of intricately connected ducts which together converge on the main pancreatic duct. The main pancreatic duct runs through the full length of the organ, from the tail through the head, where it joins with the duodenum. These ducts facilitate the

transport of digestive enzymes away from the pancreas to orchestrate their functions within the gut (Figure 1).

Functionally and histologically the pancreas can be divided into the endocrine and exocrine components. The exocrine tissue of the pancreas is by far the most prevalent accounting for ~85% of the organ and is responsible for the synthesis and secretion of digestive enzymes[1]. Acinar cells, so named after the Greek word “acinus” translated as grape, due to their clustering together in groups of cells, are the cellular source of these digestive enzymes and secrete them into ducts. Once secreted into ducts, digestive enzymes are mixed with bile from the gall bladder at the entry to the duodenum and form digestive juices which help us break down proteins, fats and carbohydrates.

Interspersed amongst the exocrine tissue are the components of the endocrine system, pancreatic islets or islets of Langerhans. These islets are a heterogeneous mix of cells each synthesizing specific hormones for secretion into the blood stream. Insulin-producing beta cells are the most abundant whilst glucagon, somatostatin, pancreatic polypeptide and ghrelin are produced by alpha, delta, pancreatic polypeptide (PP) and epsilon cells, respectively[2]. These hormones regulate physiological processes throughout the body including the homeostasis of blood sugar levels.

Collectively the endocrine and exocrine tissue regulate digestive, metabolic and hormonal processes. The centrality of the pancreas to these processes augur the potential for its’ pathologic disruption to manifest in dire consequences.

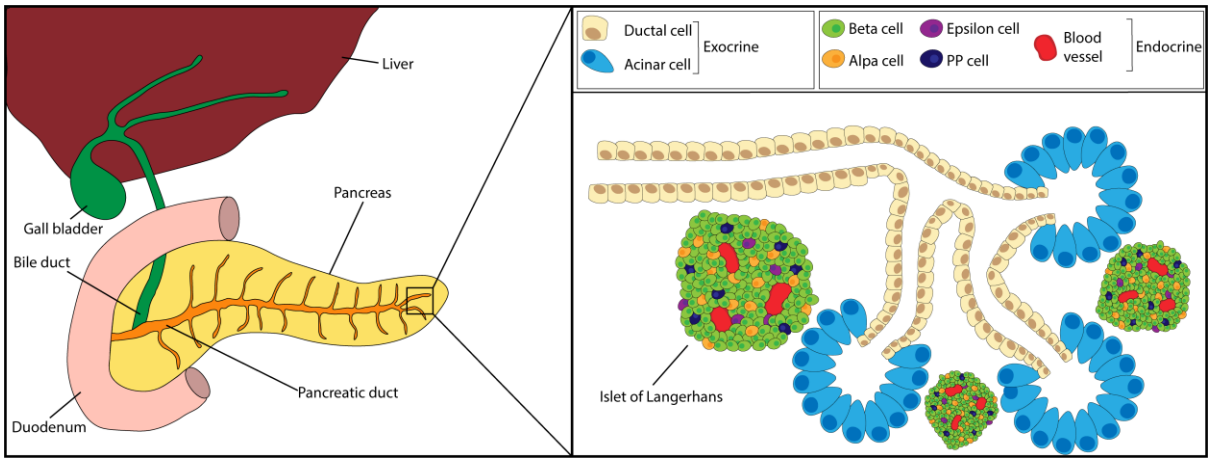

Figure 1. Schematic illustrating the location and composition of the pancreas. Created with Adobe Illustrator.

PANCREATIC DUCTAL ADENOCARCINOMA

Cancer is a leading cause of death worldwide accounting for 10 million deaths globally in 2020[3]. Carcinogenesis is a process through which cells undergo a transformation from normal to malignant. The causes and manifestations of this transformation are diverse and our comprehension of them are, like cancer, complex and constantly evolving. In 2000 Hannahan and Weinberg proposed six hallmarks of cancer as a means to codify the complex characteristics of a diverse and heterogeneous disease[4]. These include self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading cell death. Whilst these hallmarks have been expanded recently to encompass more shared characteristics[5], these six illustrate the collective devastating potential of cancer cells to deviate from their normal functions and gain a malignant potential to proliferate uncontrollably and spread to and compromise additional organs through metastasis.

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer representing ~90% of cases[6]. Acinar carcinomas and neuroendocrine tumours account for the majority of the remaining incidences of pancreatic cancer and occur through neoplastic transformations of acinar and endocrine cells of the pancreas, respectively. As the name would suggest, PDAC has been proposed to originate from ductal cells, however, more recent studies have called this notion into disrepute. The use of genetically engineered mouse models (GEMMs) has demonstrated that targeted mutations of oncogenes in acinar cells can also give rise to PDAC[7–9]. Anatomically PDAC occurs primarily in the head of the pancreas with ~76% of cases localized there in comparison to ~11% and ~13% in the body and tail, respectively[10].

Epidemiology and etiology

Whilst pancreatic cancer is not one of the most prevalent cancers, accounting for 2.6% of cases worldwide in 2020, it is one of the most lethal with a 5-year survival rate of just 12% reported in the US in 2023[3,11]. Pancreatic cancer's severe lethality is not projected to diminish and is instead predicted to become the second leading cause of cancer related deaths by 2030[12,13]. The causality of this grim projection is multifactorial with uncontrollable elements, such as the aging population, conspiring with a current lack of progress in disease detection and management which is expected to manifest in dire patient outcomes. It is therefore essential that we develop new treatments in an attempt to invalidate these ominous predictions.

The incidence of pancreatic cancer worldwide is highly variable. The human development index (HDI) is designed to reflect relative socioeconomic status of countries based on the United Nations Human Development Report[14]. Regions with a higher HDI, including Europe and North America, have higher incidences of pancreatic cancer than those with a lower

HDI, demonstrating age and lifestyle factors have a pronounced effect on developing pancreatic cancer. Regardless of incidence, the poor prognosis of pancreatic cancer is reflected by the similar rates of mortality which is irrespective of HDI.

The high mortality in pancreatic cancer underscores the significance of identifying risk factors associated with developing the disease. Such risks can be denoted as modifiable, those associated with lifestyle choices, and non-modifiable risks over which we have no control. Age represents one of the most significant non-modifiable risks with 70 and 72 years old being the median age of diagnosis and death from pancreatic cancer in the US, respectively[15]. Gender and ethnicity also influence incidence with men more likely to develop the disease and reports from the US of higher incidences in Native Hawaiians, African and Japanese Americans than Latino and European Americans[3,16].

An additional non-modifiable risk is hereditary pancreatic cancer. Hereditary pancreatic cancer is described as either familial, cases in which two or more first degree relatives have the disease, or genetic which describes cases in which inherited germline mutations which confer susceptibility to disorders associated with pancreatic cancer are identified. Around 80% of hereditary pancreatic cancer is familial and individuals with a family history of pancreatic cancer have been shown to be significantly more likely to develop the disease[17,18]. The remaining 20% of cases can be attributed to a genetic syndrome. Genetic syndromes and their corresponding genetic mutations linked to pancreatic cancer include Peutz-Jeghers syndrome (*STK11/LKB1*), familial atypical multiple mole melanoma (*CDKN2A*) and hereditary breast-ovarian cancer (*BRCA1/BRCA2*)[19–22]. Additional non-modifiable risk factors such as blood type and diabetes have also been reported[15].

Amongst the modifiable risk factors, smoking has the strongest association with promoting pancreatic cancer[23]. Heavy alcohol consumption has also frequently been associated with developing pancreatic cancer and it has been demonstrated that males are more susceptible than females to this increased risk[24–26]. Heavy drinking also severely increases the risk of developing chronic pancreatitis, a condition characterized by persistent inflammation and subsequent deterioration of the endocrine and exocrine pancreas with irreversible morphological changes including fibrosis and acinar and beta-cell loss[27–29]. Chronic pancreatitis has been shown to markedly increase the risk for pancreatic cancer, especially following a recent diagnosis of chronic pancreatitis as well as in smokers and elderly patients[30,31]. Obesity has also been associated with an elevated incidence of pancreatic cancer but one study suggested this may be reversible and switching to a low-fat diet could reduce the incidence of the disease in women who were overweight or obese[32,33].

Whilst some risk factors may be out of our control and others within our grasp, in reality, and in reference to Seinfeld, few of us are truly “masters of our domain” and irrespective of how one may arise at a pancreatic cancer diagnosis the opportunity to recover from one is of universal importance. As our understandings of the risks associated with developing PDAC deepens our capacity to screen for the disease and diagnose it at earlier stages will improve.

Diagnosis, staging and treatment

The high lethality of PDAC is largely driven by the late stage of diagnosis with ~50% of patients presenting at a stage where the disease has already metastasized[11]. The vague and nonspecific symptoms of PDAC make it particularly challenging to diagnose early but it is of great importance in the race to combat the malignancy.

Histopathology is the current best practise when it comes to diagnosing PDAC. Endoscopic ultrasonography (EUS) is the recommended modality to obtain guided fine-needle core biopsies. Biopsies can then be assessed by a pathologist for histological hallmarks of PDAC including atypical tubular glands and cribriform structures[34]. Computational tomography (CT) scans allow for the detection of suspected tumour masses within the pancreas. Furthermore, they can distinguish whether tumours are localized around major blood vessels and as such determine the suitability for surgical intervention. EUS also allows for pancreatic masses to be visualized directly and has been proposed to be a more accurate methodology than CT for diagnosing PDAC[35]. Additionally, EUS elucidates tumour vascularization and local lymph node involvement[6]. Magnetic resonance imaging (MRI) is an additional technique through which pancreatic masses can be identified. Whilst it is inferior to CT and EUS in assessing tumour vascularization, it is capable of more accurately discerning whether indiscriminate liver lesions may represent metastases.

Following diagnosis PDAC can be categorized into four stages based on the tumour nodes metastasis (TNM) system. This system delineates tumour stage through assessing (T) tumour volume and vascularization, (N) spread to lymph nodes and (M) presence of metastasis in other organs. Whilst the TNM staging system provides valuable prognostic information for predicting patients' outcome, clinically PDAC tumours are divided into four stages of resectable, borderline-resectable, locally advanced and metastatic. Such classifications are more relevant to treatment strategy for the patient and determine the suitability of surgical intervention based on localization of the tumour around vasculature of the pancreas and presence of metastatic spread to other organs.

Surgical resection is the most beneficial treatment strategy for patients with the capacity to raise 5-year survival rates from the dismal ~10% to 38% when performed on TNM stage I patients[36]. Patient survival is further enhanced when combined with adjuvant chemotherapy[37,38]. FOLFIRINOX, a multi-drug regimen, and gemcitabine, often used in combination with paclitaxcel, are two commonly used chemotherapeutic treatments in pancreatic cancer. In an adjuvant setting FOLFIRINOX was shown to improve survival relative to gemcitabine but at the cost of increased adverse side effects[39]. Unfortunately, only 10-15% of patients presenting with PDAC are suitable candidates for surgery and not all patients are capable of completing courses of adjuvant chemotherapy following surgery, further narrowing the window of opportunity to improve outcome for patients.

In borderline-resectable pancreatic cancer, surgery can sometimes be appropriate, but where it isn't neoadjuvant chemotherapy can be used in an attempt to shrink the tumour to facilitate resection. A meta-analysis observed that no benefit to survival was attained following neoadjuvant treatment in patients with resectable PDAC but demonstrated improved survival in patients with border-line resectable or locally advanced disease[40]. A randomized control study recently established that a combination of chemotherapy with radiation can lead to an increase in complete resection and overall survival of patients with borderline-resectable PDAC suggesting cautious optimism for this approach[41].

Unfortunately, the majority of patients present with metastatic disease. Whilst surgery is no longer a viable option in such patients, chemotherapeutic treatments including FOLFIRINOX and gemcitabine in combination with paclitaxcel have been shown to improve overall survival[42,43]. It is also critical to integrate supportive care to improve quality of life in all patients but especially those with advanced disease. Management of nutrition, psychological distress, pain and thromboses are vital to benefiting patient's quality of life.

The ominous outcomes of PDAC are a consequence of the lack of therapeutics and efficient screening approaches. There is a desperate need to augment our understanding of the disease and develop novel treatments and diagnostic approaches which can help to shift the balance of power from the disease to clinicians when combating PDAC.

Mutational landscape

Malignant transformation of normal cells into cancer cells is the result of accumulated mutational alterations in genes which results in the dysregulation of cellular function, instead driving cells towards a phenotype encompassing the aforementioned hallmarks of cancer.

The oncogene *KRAS* is mutated in ~95% of PDAC cases and is the most prominent mutation found in the cancer[44,45]. Typically, KRAS can exist either in an inactive guanosine diphosphate (GDP) bound or an active guanosine triphosphate (GTP) bound state. Activation is often regulated through receptor tyrosine kinases (RTKs) which upon ligand binding may activate KRAS through guanine nucleotide exchange factors. Such factors displace KRAS-bound GDP with GTP. Activated KRAS may then initiate signalling cascades, including the MAPK pathway, which can stimulate cell proliferation and metabolism. In the context of PDAC KRAS is transformed to a perpetually activated state through mutation, >90% of which are single amino acid substitutions at the glycine-12 (G12) position[44].

In addition to gain of function mutations in oncogenes, loss of function mutations in tumour suppressors can dictate tumorigenesis. *TP53*, encoding the tumour suppressor p53, is the

next most frequent mutation in PDAC occurring in 60-70% of cases[46]. p53 possess a DNA binding domain which allows it to directly associate with DNA and regulate expression of target genes. Such binding can regulate a variety of cellular processes including the cell-cycle, metabolism and stemness. p53 can become activated following cellular stress and DNA damage, triggering cell cycle arrest to suppress the accumulation of damaged cells[47]. Single amino acid mutations in *TP53* can lead to permanent inactivation of the tumour suppressor, catalysing PDAC tumorigenesis. *CDKN2A* is another critical tumour suppressor gene playing an essential role in regulating the cell cycle. *CDKN2A* inactivation has been reported in up to 98% of PDAC cases but estimates of frequency range from as low as 17%[44,48]. Hypermethylation of promoters, homozygous deletions and inactivating point mutations can all render *CDKN2A* inactive and contribute to the tumorigenic development of PDAC. Whilst a myriad of mutations has been reported in PDAC, another frequent genetic alteration observed is in the *SMAD4* gene. *SMAD4* represents an additional tumour suppressor gene which mediates signal transduction in the TGF-β pathway. Loss of function mutations in *SMAD4* occur in around half of PDAC cases and are frequently the result of homozygous deletions or chromosome allelic loss. Such loss of function mutations abrogates TGF-β/SMAD4 mediated cell cycle arrest and DNA damage repair which can serve to restrain carcinogenesis[49].

Collectively, oncogenic mutational burden dictates the tumorigenic potential of a cell. The sequential accumulation of such mutations can drive the tumorigenic development of PDAC from early stage lesions to advanced metastatic disease.

Tumorigenesis

A cell's transition from a normal helpful worker dutifully conducting a required function to a disruptive malignant entity does not occur instantaneously, instead defined tumorigenic stages underscore this metamorphosis.

The cellular origin of PDAC has, as previously described, more recently been expanded from exclusively a ductal origin to also include acinar cells. Acinar cells of the pancreas are not a terminally differentiated cell type and retain the plasticity to de-differentiate into stem-like ductal cells in the process of acinar-to-ductal metaplasia (ADM). ADM can occur in response to TGF-α and TGF-β signalling[50,51] and in response to pancreatitis[52]. During pancreatitis-driven inflammation it has been shown that macrophage-derived inflammatory signals can promote ADM and a recent study found a small molecule inhibitor of the inflammatory transcription factor STAT3 could reverse ADM[53,54]. It has been proposed that the de-differentiated ductal cells formed in ADM are susceptible to oncogenic mutations and that *KRAS* mutations can occur during this process, leading to the initiation of PDAC development. Significantly, it has also been demonstrated that induction of *KRAS* mutations in mature acinar cells can promote ADM and the formation of a putative precursor lesion of PDAC called pancreatic intraepithelial neoplasia (PanIN)[8,9,55,56]. It is therefore reported that ADM may either promote or occur as a result of *KRAS* mutations. Irrespective of the precise

order of events, what is clear is that *KRAS* mutations are a key tumorigenic event which initiate the development of PDAC.

Regardless of whether the exocrine cell is acinar or ductal, transformation of an individual cell represents the genesis of PDAC. The subsequent pathogenic events involve the formation of intraductal lesions which progress from low-grade to high-grade as they accumulate genetic alterations resulting in morphological and cytologic atypia. PanINs represent the most frequent precursor lesions in PDAC. They are observed in ~85% of cases and are proposed to result in more aggressive forms of PDAC than the ~15% which arise from a distinct precursor lesion called intraductal papillary mucinous neoplasia (IPMN)[57]. The scale of architectural and cytological atypia present in PanINs is reflected in a categorization of the lesions as PanIN-I (mild atypia), PanIN-II (mild-moderate atypia) and PanIN-III (pronounced atypia). Makohon-Moore *et al* used whole genome sequencing (WGS) to infer evolutionary relationships between PanIN lesions and PDAC tumours from human patients. This demonstrated that clonal expansion of a neoplastic cell could generate multiple PanIN lesions throughout the pancreas which could accrue mutations and lead to PDAC[58]. Augmenting the findings that progressive accumulation of mutations drives PDAC development from PanINs, GEMMs which promote pancreas specific induction of *KRAS* gain of function mutations lead to PanIN formation. These lesions rarely develop into PDAC but can do so where subsequent loss of function mutations occur in *TP53*, *CDKN2A* or *SMAD4*[59–61]. A study from Maitra *et al*, used tissue microarrays (TMA) to characterize the sequential aberrations of PDAC drivers in PanIN development. This demonstrated that loss of CDKN2A was frequently observed in low-grade PanINs whilst SMAD4 and p53 were frequently functionally incompetent in high-grade lesions prior to development of PDAC[62].

Once established PDAC is commonly referred to as a highly aggressive malignancy with a propensity for metastasis. In alignment with this, it has been demonstrated that metastases can occur in the liver at early stages of PDAC before the primary tumour has grown to a significant mass[63]. Furthermore, it has been shown that PanIN lesions can begin modulating the liver, promoting it to form a pre-metastatic niche even prior to the development of PDAC[64]. It has been proposed that accumulation of mutational and epigenetic modifications of tumour cells drive the metastatic potential of tumour cells but characterizing these changes has been a challenge[65,66]. However, a recent study found that metastatic tumours could be distinguished from their primary counterparts based on alternative-splicing profiles. Furthermore, the splicing regulator RBFOX2 was identified as the mediator of these changes and modulating expression of this protein was capable of diminishing or elevating the metastatic potential of tumour cells[67].

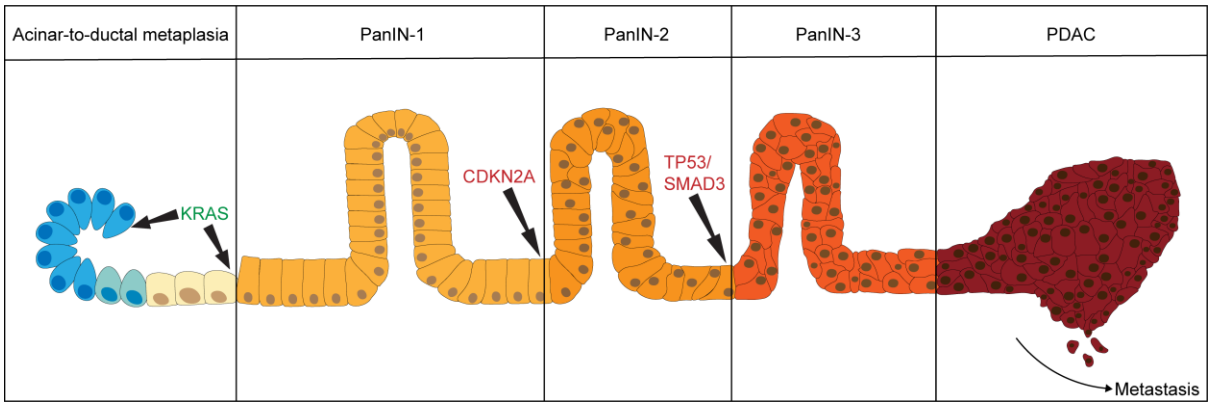

Figure 2. Schematic illustrating PDAC tumorigenesis. Arrows indicate stage at which mutations are commonly observed, red indicates loss of function mutations and green indicates gain of function mutations. Inspired from a review article by Liot et al[68]*. Created with Adobe Illustrator.*

Thus, PDAC tumorigenesis is a complex cascade of events initiating with the malignant transformation of an exocrine cell which, through clonal expansion and accumulated oncogenic mutational burden, can form lesions of increasing atypia which ultimately result in the formation of a highly invasive neoplasia (Figure 2).

Tumour heterogeneity

In the first line of Anna Karenina Leo Tolstoy writes “All happy families are alike; each unhappy family is unhappy in its own way”. This line illuminates the scrutiny with which we observe dysfunction comparatively to harmony. As much is true with cancer and, whilst the healthy pancreas is largely considered homogenous, PDAC is now understood to be a heterogeneous disease comprised of distinct forms each with their own characteristics.

A landmark study from Collisson *et al* profiled the transcriptomes of PDAC tumours and PDAC derived cell lines to categorize the disease into three subtypes termed “classical”, “quasimesenchymal” and “exocrine-like”[69]. Significantly, the PDAC subtypes identified responded variably to chemotherapeutic agents and patient survival was observed to change depending on the subtype present. The notion of PDAC heterogeneity was later reaffirmed by Moffit *et al*, also through an RNA sequencing approach to characterize PDAC tumours[70]. Such bulk transcriptional approaches look for global differences in gene expression between tumour samples through defining the expression profile of the total cellular milieu. Given that pancreatic cancer has an abundant stromal component, comprised of non-neoplastic cells, this can overwhelm the contribution of cancer cells to the bulk gene expression profile. Moffit *et al,* sought to circumvent this issue through virtual microdissection which was used

to deplete contributions of stromal cells to the overall gene expression profile of PDAC tumours[70]. Such an approach elucidated the presence of two PDAC cancer cell subtypes called “basal” and “classical” with the basal subtype associated with a worse prognosis. Bailey *et al*, subsequently employed an integrative genomic approach where transcriptional and genomic profiles were used to stratify PDAC tumours[71]. This identified four subtypes; “squamous”, “pancreatic progenitor”, “immunogenic” and “aberrantly differentiated endocrine exocrine (ADEX)”. Such subtypes were defined based on a combination of mutational and transcriptional profiles and again associated with variable patient survival.

Whilst these studies all represent prominent advancements in our understanding of PDAC heterogeneity, it raises the question of which categorization should be used to define distinct tumour subtypes? A review from Collisson and Bailey proposed a hierarchical ordering of subtypes rooted in the distinction of basal and classical PDAC subtypes characterized by Moffit *et al*[72]. Basal and classical subtypes could then be further subdivided into the additional aforementioned subtypes. In accordance with this, a spate of additional publications utilized laser capture microdissection (LCM) to extract tumour glands specifically for transcriptional profiling[73–75]. Such studies were able to validate the presence of basal and classical subtypes and largely corroborate the additional proposed subtypes, with the exception of the ADEX and exocrine-like groups identified by Bailey *et al* and Collisson *et al*, respectively. These subtypes have been suggested to be a result of elevated acinar contamination in certain tumour samples which skew the bulk transcriptional profile. Significantly, LCM approaches also demonstrated that distinct tumour glands within the same patient could represent either classical or basal subtypes.

Whilst PDAC classification is in its’ nascency the capacity for characterizing PDAC tumours based on a taxonomic framework which can inform treatment strategies is an exciting opportunity to better tailor therapeutics to individual patients.

THE TUMOUR MICROENVIRONMENT IN PDAC

We have thus far focused primarily on the cancer cells of PDAC which may, on the surface, seem a fairly reasonable approach for discussing a cancer. A tumour is however, comprised of far more than just cancer cells. The tumour microenvironment (TME) refers to the ecosystem of a tumour not just the neoplastic cells. The TME of PDAC encompasses a vast and diverse cellular milieu populated with immune cells, nerve cells, vasculature, cancer-associated fibroblasts (CAFs) and other mesenchymal cells, in addition to an abundance of dense extracellular matrix (ECM) (Figure 3). In the context of cancer, we collectively refer to the non-neoplastic cellular components and ECM of a tumour as the stroma. The stroma of PDAC is a hypoxic and hypovascular microenvironment serving as the site of intricate bidirectional communication between cancer cells and this surrounding environment. PDAC is unusually stroma rich, accounting for up to 90% of the total tumour volume. As such, this abundant stroma has become the topic of intense scientific scrutiny. Whilst our understanding of the heterogeneous composition of the stroma and the influence distinct elements confer on tumorigenesis is incomplete, a host of studies have equipped us with a developing comprehension I will endeavour to summarise here.

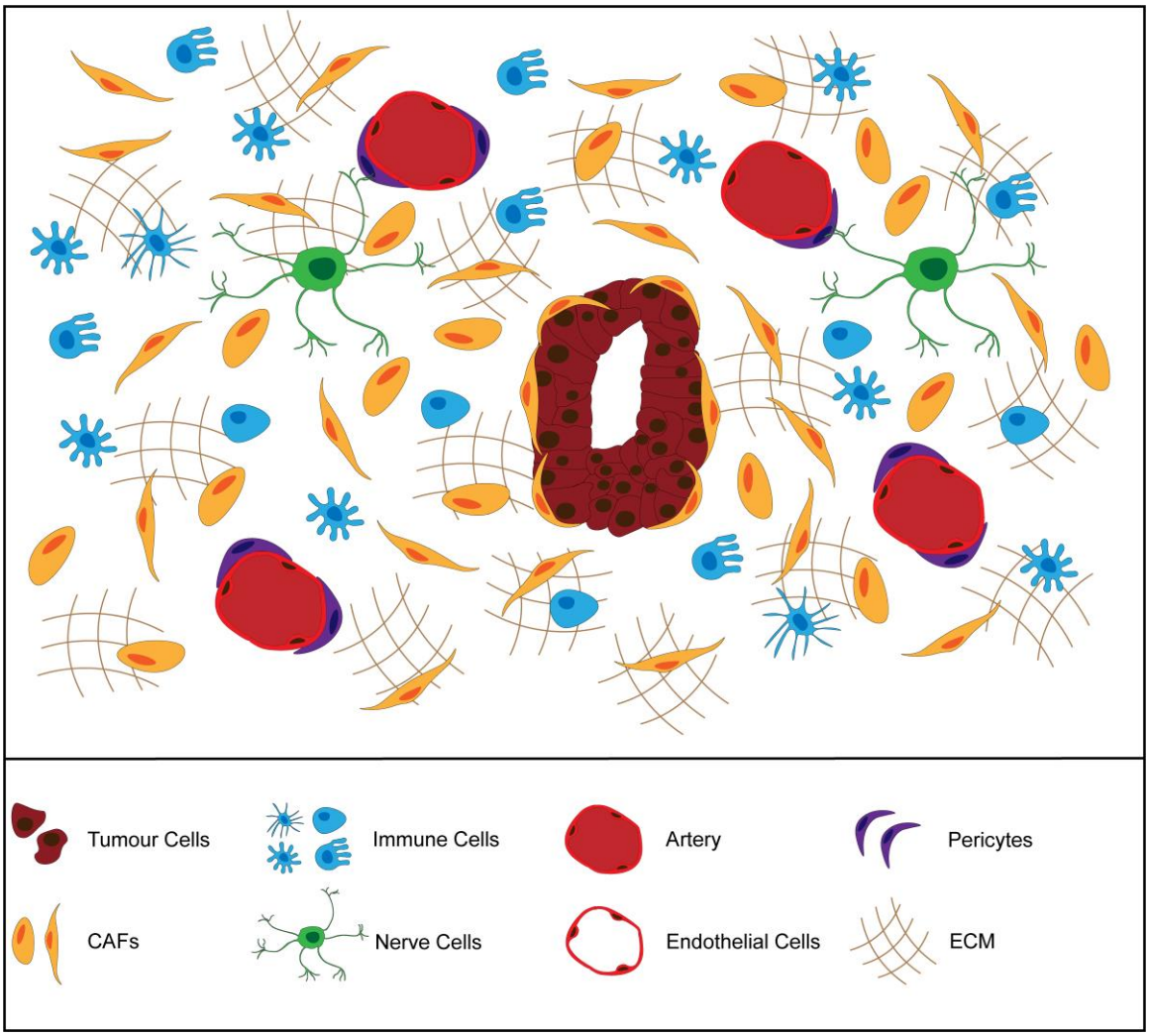

Figure 3. Schematic illustrating the cellular composition of the PDAC TME. Created with Adobe Illustrator.

Immune cells

A vital and extensively studied component of the TME are immune cells. PDAC has historically been considered as a "cold" or non-immunogenic tumour. Such a characterization may not be reflective of the paucity of immune cells within the TME but rather the promotion of an immunosuppressive environment to facilitate immune evasion by cancer cells, which is a feature of PDAC[76]. The balance of distinct immune cell populations present within the tumour can dictate the anti-tumour potential of the TME. An ever-expanding cornucopia of immune cell populations have been identified within the stroma of cancer but at the most basic level immune cell populations can be divided into innate and adaptive immune cells. Cells of the innate immune system are fast-acting and capable of recognizing pathogenic threats based on conserved features. In contrast, the adaptive immune response requires time to develop but involves the expansion of immune cells capable of recognizing specific features of a pathogenic threat. Both innate and adaptive cells are present in the stroma of PDAC and orchestrate diverse functions which regulate the growth and spread of disease.

Innate immune cells

Macrophages, monocytes, dendritic cells and granulocytes signify a subset of leukocytes together termed myeloid cells. Myeloid cells of the innate immune system are the most abundant immune cell populations present in the PDAC TME[77]. Myeloid cells can differentiate from haematopoietic stem cells in the bone-marrow before their distribution throughout the body through the circulatory and lymphatic systems. They may then be recruited to the site of infection or injury following the release of chemoattractants which draw them to a site of distress. In parallel, myeloid cells can be found residing in tissues in the absence of distress where they exert homeostatic, tissue-repair and developmental functions[78]. As with other stromal cell types, myeloid cells are susceptible to manipulation by neoplastic cells and their phenotypes can be perturbed by cancer cell-secreted factors present in the TME causing them to mediate tumorigenic functions.

Tumour-associated macrophages (TAMs) are a central myeloid population occupying the PDAC TME. TAMs can be activated and polarized in response to the biochemical atmosphere of the TME to distinct phenotypes which regulate their function. Such phenotypes have commonly been divided into a pro-inflammatory and tumour-restraining M1 phenotype and an anti-inflammatory tumour-promoting M2 phenotype[79,80]. Whilst such a binarization may not be reflective of the true phenotypic diversity of TAMs, it serves as a categorical framework to illuminate their capacity to supplement or confine tumorigenesis[81]. PDAC is proposed to direct macrophages towards a tumour-promoting M2 phenotype to abet their

evasion of tumour restraining immune populations[82]. TAMs abundance has been correlated with worse outcomes in PDAC and they are capable of lessening anti-tumour responses by T-cells, a component of the adaptive immune system[71,83]. M2 macrophages can suppress the activity of T-cells through competitively metabolizing L-arginine which is required for T-cells to mount an effective anti-tumour response[84]. Reprogramming macrophages towards a pro-inflammatory M1 phenotype has been shown to enhance a tumour-restraining immune response[85]. TAM phenotypes can also be influenced by their developmental origin. TAMs in PDAC can originate from tissue-resident macrophages or from bone-marrow derived monocytes and can concert immunogenic or pro-fibrotic roles, respectively, which modulate disease progression[86].

Oncogenic *KRAS* mutation has been shown to facilitate the recruitment of an immunosuppressive myeloid population, monocyte-derived suppressor cells (MDSCs), via the secretion of granulocyte macrophage colony-stimulating factor[87]. Once recruited, MDSCs promoted the progression of PDAC by negatively regulating tumour restraining T-cell subsets. Similarly to TAMs, MDSCs can suppress the activity of tumour restraining T-cell populations through sequestering biochemical components, including L-arginine and reactive oxygen species (ROS), required for their activation and survival[88,89]. Elevated infiltration of MDSCs at the tumour site and in circulation has been found to correlate with tumour progression and it has been observed that MDSCs can accumulate at PanIN lesions helping to establish an immunosuppressive microenvironment which promotes PDAC progression[90,91].

The efficacy of an anti-tumour immune response is reliant on the presence and functional integrity of dendritic cells (DCs) which prime subsets of T-cells to recognize and attack tumour cells via antigen-presentation. In PDAC a paucity and dysregulation of DCs has been reported which restrains the capacity for an effective immune response to be mounted against the tumour[92]. Restoring DC function was able to reverse these effects instead polarizing the immune landscape towards a tumour-restraining nature.

Natural killer (NK) cells are an innate lymphocyte that retain the ability to directly kill cells showing signs of distress, such as infection or neoplastic transformation. NK cells have been reported to be extremely rare in PDAC tumours accounting for <0.5% of the tumour infiltrating lymphocytes (TILs)[93]. Nevertheless, circulating levels of NK cells have been shown to correlate with a better outcome in PDAC[94]. Although circulating levels of NK cells are correlated with improved overall survival in PDAC patients, a number of studies have demonstrated that the cytolytic killing of tumour cells by NK cells can be supressed in the PDAC TME[93,95–97]. This suppression can be stimulated directly by cancer cells and indirectly through cancer cell driven modulation of other stromal cell types including MDSCs.

Adaptive immune cells

T-cells are a heterogeneous lymphocytic element of the adaptive immune system. They mature in the thymus and express uniquely rearranged T-cell receptors specific for independent antigens. T-cells are a common immune infiltrate of PDAC and encompass numerous distinct cell types including $CD4^+$ T helper (Th) cells, cytotoxic $CD8^+$ T-cells and regulatory T-cells (T_{regs}).

The primary subset of $CD8^+$ T-cells are interferon (IFN)-γ producing cytotoxic T lymphocytes (CTLs) which possess the capability to directly lyse target cells through the secretion of cytolytic proteins including granzymes and perforin. CTLs are critical facilitators of anti-tumour immunity given their capacity to directly recognize and attack cancer cells. Due to the fact PDAC is a highly aggressive and lethal malignancy it is apparent that CTL mediated killing of tumour cells is insufficient to prevent disease and may therefore be circumvented by the cancer cells.

A common feature of PDAC is CTL exclusion from tumours and those which possess increased numbers of $CD8^+$ T-cells are associated with better survival[98]. Cancer cell secreted factors such as C-X-C motif chemokine ligand (CXCL)-1 can encourage the exclusion of CTLs from the tumour[99]. CTLs are also frequently sequestered in the abundant desmoplastic stroma of PDAC, preventing their direct association with cancer cells[100,101]. In PDAC patients with $CD8^+$ T-cells detected in closer proximity to tumour cells a significant benefit in overall survival was observed[102].

Cancer cells can also mitigate killing by $CD8^+$ T-cells through reprogramming them towards an exhausted state in which their cytolytic activity is abrogated. Exhausted $CD8^+$ T-cells can be characterized through the co-expression of inhibitory receptors including programmed cell death-1 (PD-1), cytotoxic T-lymphocyte-associated antigen-4 (CTLA4), T-cell immunoglobin and mucin-domain containing-3 (TIM-3) and lymphocyte-activation gene-3 (LAG-3)[103]. Through directly upregulating expression of ligands for these receptors, or indirectly upregulating their expression in stromal cells, cancer cells can gradually diminish the activity of CTLs in the TME. Oncogenic *KRAS*, which is abundantly present in PDAC, has been shown to upregulate expression of the PD-1 ligand PDL-1 and subsequently reduce anti-tumour immunity[104].

T_{regs} are considered an immunosuppressive subset of T-cells with significant pathological relevance in PDAC. T_{regs} are associated with dampening immune responses to negatively regulate the immune system and help protect against auto-immune disease. In PDAC the prevalence of T_{regs} was significantly higher in tumours than in non-neoplastic inflammation and their increased abundance was associate with worse patient outcomes[105]. T_{regs} role in promoting immunosuppression in PDAC however, remains controversial. A study from Jang *et al*, observed that T_{regs} ablation in GEMMs could mitigate immunosuppression and evoke

an efficient anti-tumour immune response when PDAC cells were injected into mice[106]. T_{regs} were able to suppress $CD8^+$ T-cells anti-tumour activity through extended interactions with DCs which repressed the expression of co-stimulatory molecules on DCs which are required for the activation of $CD8^+$ T-cells. In refute of this, Zhang *et al* recently demonstrated that depletion of T_{regs} in a GEMM of PDAC was insufficient to ablate immunosuppression and instead resulted in accelerated carcinogenesis[107]. This was a consequence of T_{regs} depletion resulting in the enhanced recruitment of immunosuppressive myeloid cells and a reduction in other tumour-restraining non-immune stromal cell types. Whilst the literature contains contradictory evidence for the immunosuppressive tumour-promoting roles for T_{regs} in PDAC, their abundance has been described and associated with a worse overall outcome in patients[105,108]. Additional interrogation of their tumorigenic role within the TME of PDAC will better elucidate whether they are directly contributing to this or are an unfairly implicated bystander in the process.

Th cells are $CD4^+$ T-cells which can be further subdivided into subpopulations including Th1 and Th2 cells. Th1 cells secrete cytokines, including IFNγ, which mediate cellular type I immunity through stimulating priming, activation and recruitment of CTLs, M1 macrophages and NK cells, assisting immune targeting of intracellular pathogens and tumours. In contrast, Th2 cells coordinate humoral type II immunity through M2 macrophage polarization which regulate immune responses to extracellular pathogens and allergy[109]. Within the TME of PDAC Th2 cells are present at a higher frequency than Th1 cells[110]. Furthermore, the ratio of Th1:Th2 cells is predictive of patient outcomes with higher proportions of Th2 cells yielding a worse prognosis, implicating Th2 cells in carcinogenic progression[111]. Increased serum levels of the Th2 derived cytokine interleukin (IL)-4 was indicative of worse patient survival in PDAC, further connecting Th2 cells with a pro-tumorigenic role[112]. In addition, Th2 secreted cytokines IL-4 and IL-13 were capable of upregulating expression of the oncogene MYC resulting in enhanced cancer cell proliferation in a GEMM of PDAC, directly associating Th2 cells with PDAC tumorigenesis[113]. In contrast, treating a GEMM of PDAC with a combination of chemotherapy and immunotherapy was observed to polarize Th cells towards a Th1 phenotype, as well as macrophages towards an M1 state, and was associated with enhanced anti-tumour immunity[114].

B-cells are an additional lymphocytic component of the adaptive immune system capable of differentiating into antibody producing plasma cells. B-cells involvement in PDAC progression is controversial with independent studies defining beneficial, neutral and negative effects of B-cell infiltration on patient survival[115]. Through secretion of IL-35 B-cells were shown to promote PDAC development by stimulating tumour cell proliferation[116]. In contrast, it has also been shown that B-cell infiltration and plasma cell differentiation is associated with improved patient survival[117]. These findings were recently woven together in a study from Bhalchandra *et al* which found IL-35 could suppress B-cell differentiation into plasma cells, instead polarizing them towards an immunoregulatory phenotype[118]. Pharmacologically inhibiting this immunoregulatory phenotype and restoring plasma cell differentiation potentiated an anti-tumour immune response. Taken together, infiltrating B-cells can differentiate into plasma cells which mediate anti-tumorigenic functions within the TME but this process can be abrogated by cancer cells instead directing B-cell fate towards a tumour promoting phenotype.

Immune cells are vastly heterogeneous and functionally diverse (Figure 4). Their capacity to directly attack neoplastic cells make them invaluable allies in the campaign to combat cancer though their amenability to manipulation by tumour cells frequently render them incapable of doing so. As we better characterize the mechanisms through which cancer cells direct their fate towards a pro-tumorigenic phenotype our capacity to assuage this process will improve.

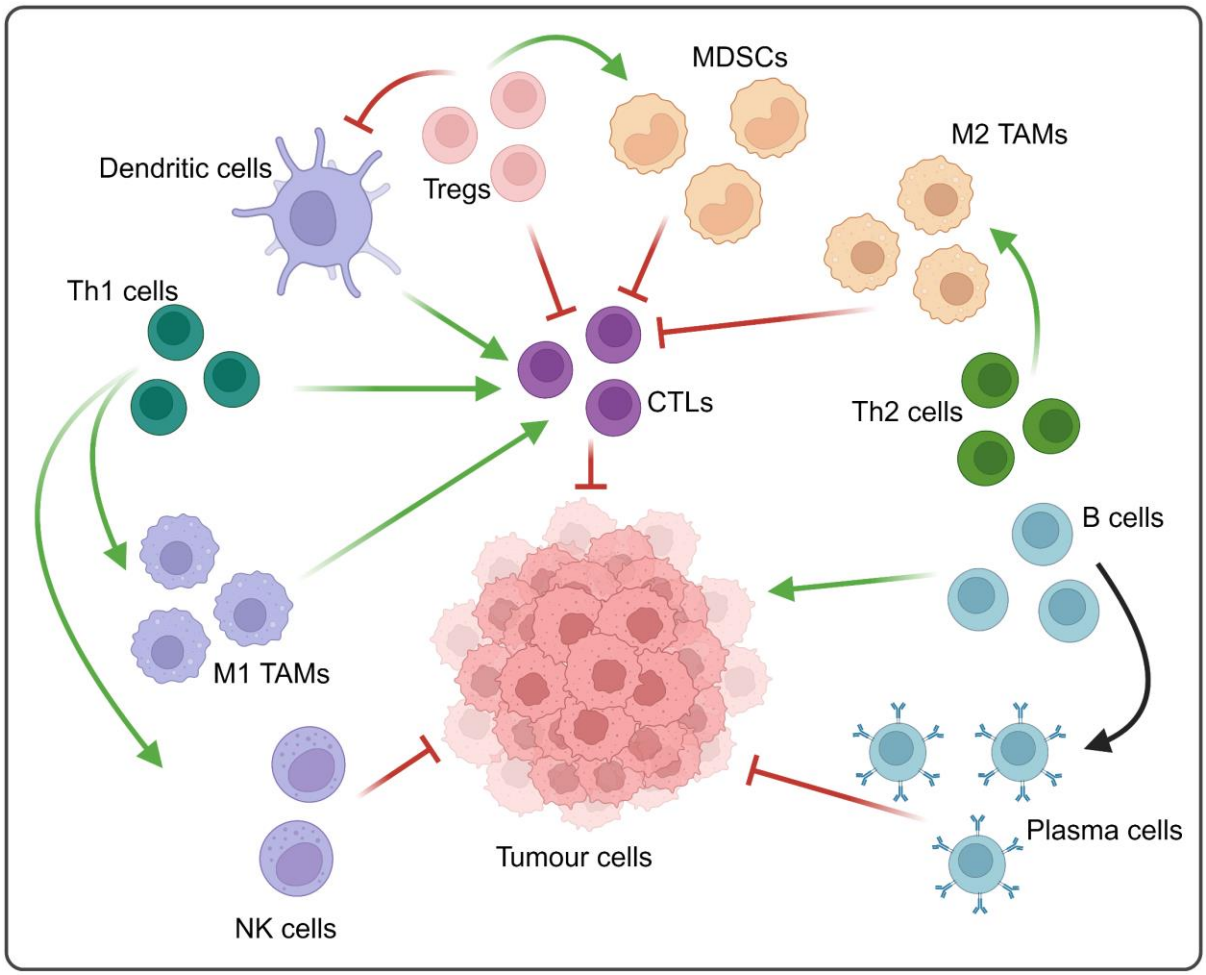

Figure 4. Schematic illustrating regulatory relationships between immune cell populations in the PDAC TME. Created with BioRender.

Extracellular matrix

One of the dominant characteristics of PDAC is a prominent desmoplasia. Such desmoplasia is the consequence of a fibrotic reaction in which dense deposits of ECM are secreted into the TME. This sustained fibrosis in PDAC, and more generally in cancer, has led to the ubiquitously used quote that cancers are "wounds that do not deal"[119]. ECM is a prominent non-cellular component of the PDAC TME comprised of structural proteins, enzymes, proteoglycans, glycoproteins and other secreted factors which may collectively be referred to as the matrisome. In healthy tissues and cancer, the ECM provides a structural framework for cells, also regulating the tissue stiffness and modulating biomechanical responses in cells. In healthy tissue the ECM provides an interstitial scaffolding support for cells of the stroma and also a basement membrane linking the epithelial and stromal compartments. In PDAC the aberrant deposition of ECM which occurs, modulates biophysical properties of the stroma including increased interstitial pressure which promotes collapse of vasculature and impedes drug delivery[120–122]. Despite studies demonstrating that enzymatic depletion of ECM components could yield improvements in drug-delivery it has also been demonstrated, in GEMMs of PDAC, that depletion of the ECM can lead to accelerated tumour growth and a reduction in overall survival[120,122,123].

The capability of structural proteins in the ECM to modulate tissue stiffness has key tumorigenic implications in PDAC. Collagens represent the most abundant structural proteins found in the PDAC ECM, specifically collagens I, III and IV[124]. Collagens are not strictly structural and also exert biological influences on tumour cells governing their proliferation and migration[125]. Elevated deposition of collagens and other structural ECM proteins does however, also directly increase the stiffness of tumour tissue. Increased stiffness has been shown to promote an invasive and metastatic phenotype in cancer cells and has been suggested to regulate all of the aforementioned hallmarks of cancer[126–128]. Stiffness can be augmented not only through the enhanced deposition of structural ECM proteins but also through their cross-linking. Cross-linking of ECM proteins, by the cancer secreted enzyme transglutaminase (TG2), and subsequent increases in tissue stiffness were shown to stimulate proliferation and growth of pancreatic tumours[129]. Despite the frequent observation that elevated stiffness can promote invasiveness and facilitate worse outcomes in PDAC it has also been demonstrated that reducing collagen deposition can lead to enhanced tumour growth and resultant worse outcomes in GEMMs of PDAC, evidencing tumour-restraining functions of ECM components[130].

ECM proteins are able to govern biophysical properties of the TME as well as biochemical responses. The diversity of matrisome proteins in PDAC make characterizing the overall contribution of the ECM to tumorigenesis complex. A study from Tian *et al*, identified around 200 ECM proteins which were aberrantly expressed in PDAC compared to the normal pancreas[131]. Interestingly, it was observed that cancer cell derived matrisome proteins were frequently associated with a negative prognosis whilst those derived from stromal cells may either improve or diminish survival. Specific cancer-cell derived ECM components including agrin (AGRN), serine protease inhibitor B5 (SERPINB5) and cytastatin B (CSTB) were shown to directly promote metastasis[132].

Galectins

Amongst the proteins upregulated in the ECM of PDAC, Tian *et al* identified a number of galectins. Galectins are a family of galactose binding lectins, a subset of which have been shown to regulate immune cell function and tumour cell phenotype in PDAC. Galectin (Gal)-1 expression is upregulated in the ECM of PDAC and was found to be primarily derived from stromal cells[133]. PDAC cell lines exposed to Gal-1 exhibited increased proliferation and invasion[133]. Gal-1 can also drive immunosuppression through suppressing $CD8^+$ T-cell recruitment and inducing T-cell anergy and apoptosis[134,135]. Elevated levels of Gal-3 have also been identified in the ECM of PDAC. Gal-3 is expressed by tumour cells and suppressing Gal-3 expression has been shown to repress the proliferative and invasive potential of cancer cells[136]. In addition, Gal-3 has been demonstrated to suppress $CD8^+$ T-cell effector function through binding the inhibitory receptor LAG-3[137]. Gal-9 expression is also elevated in PDAC[138]. Gal-9 is predominantly expressed by cancer cells and leukocytes and can promote T-cell exhaustion directly through interaction with TIM-3 and indirectly through the tolerogenic reprogramming of TAMs[138–140].

Gal-4 represents an additional aberrantly expressed component of the ECM in PDAC. Under normal physiological conditions Gal-4 is expressed by epithelial cells of the GI tract[141]. Gal-4 possesses distinct intracellular and extracellular functions. Previous findings have demonstrated that elevated expression of intracellular Gal-4 in PDAC tumour cells reduces their migratory and invasive capacity by inhibiting Wnt signalling[142]. Less is known about the function of extracellular Gal-4 in PDAC although some clues may be taken from studies defining the influence of extracellular Gal-4 in inflammatory conditions of the GI tract. Paclik *et al*, found that extracellular Gal-4 was capable of inhibiting T-cell activation and expansion *in vitro* and found that Gal-4 could ameliorate inflammation through promoting T-cell apoptosis in a mouse model of colitis[141]. However, these findings were contradicted by Hokama *et al*, who found that extracellular Gal-4 could exacerbate inflammation in a mouse model of colitis by stimulating IL-6 secretion in Th cells[143]. Whilst we do not currently know the role of extracellular Gal-4 in PDAC, its capacity to modulate inflammation in inflammatory conditions of the GI tract suggests it is a promising candidate for study in the disease.

The matrisome of PDAC is constructed from a sundry of proteins which together make-up the dense desmoplasia observed in the disease. The ECM of PDAC may orchestrate tumour-suppressive and tumour-promoting functions and it is likely that distinct ECM components regulate divergent pathological functions. Specific targeting of stromal ECM elements may therefore be required to promote a favourable outcome.

CANCER-ASSOCIATED FIBROBLASTS (CAFS)

Before we cover the cancer-associated part of CAFs let us first consider the fibroblast section. In 1858 the German pathologist Rudolf Virchow identified a population of cells he defined as "spindle shaped cells of the connective tissue" or "Spindelzelle des Bindegewebes"[144]. Fortunately, as that doesn't exactly roll off the tongue, Ernst Ziegler later proposed the term "fibroblast" to describe cells capable of replenishing connective tissue upon healing[145]. Within healthy tissues, fibroblasts occur normally as single cells located in the interstitial space where they reside embedded in the fibrillar ECM unattached to basement membranes[146]. The definition of fibroblasts is somewhat loosely characterized, and they may generally be referred to as a cellular component of connective tissue which are distinct from immune and epithelial cells which have likely originated from the mesenchyme. In accordance with this, a recent study from Han *et al*, found that the majority of tissue resident fibroblasts of the pancreas originate from the splanchnic mesenchyme[147].

The capacity of a tissue resident fibroblast to modify their phenotype in a pathological setting was first described in the wound-healing process[148]. In response to injury, fibroblasts could modulate the wound-healing process through increasing their synthesis of ECM which was described as activation[149,150]. Fibroblasts were subsequently observed to become activated in response to cancer to form CAFs[146]. Once activated, CAFs are the main cellular source for the fibrosis which is abundant in cancer including the extensive desmoplasia found in PDAC.

Fibroblasts populate the interstitial space with a community of cells which are not fibroblasts but also likely of mesenchymal origin. Such mesenchymal cells including stellate cells, mesenchymal stem cells and pericytes are proposed to be distinct from fibroblasts. These cells bare similarities to fibroblasts which has made it difficult to concretely distinguish them from fibroblasts and vice versa. There is also emergent evidence of separate lineages of fibroblasts which are phenotypically distinct as well as studies indicating cells of disparate developmental origins can adopt a fibroblastic phenotype. Our understanding of the origins and distinctions between mesenchymal cells of healthy tissue and the contribution of distinct populations of mesenchymal and non-mesenchymal cells to the mature CAF population in PDAC is a rapidly progressing area of study.

CAFs an origin story

CAFs presence in the TME is well-established, whilst the full extent of their origin remains undefined. Cancer type, developmental stage and histological grade are likely to influence the recruitment and differentiation of CAFs, posing additional complexity when characterizing CAF development. A growing body of work has however, elucidated a number of sources through which CAFs can arise in several malignancies including PDAC. Furthermore, it has been suggested that the diverse repertoire of cells from which CAFs can

arise influence the scale of their heterogeneity observed in the TME[151]. CAFs may be derived from the quiescent fibroblasts of healthy tissue prior to tumorigenesis. Stellate cells are a lipid storing fibroblast found in specific organs including the liver and pancreas. Such cells may also be transformed from a quiescent state into CAFs. Additionally, mesenchymal stem cells (MSCs) are thought be recruited from the bone marrow to the tumour site where they are integrated into the CAF population. Epithelial to mesenchymal (EMT) transition and endothelial to mesenchymal transition (EndMT) are also proposed sources of CAFs. Lastly, monocytes, pericytes and mesothelial cells are thought to differentiate into CAFs.

Pancreatic stellate cells

A source of CAFs in PDAC are pancreatic stellate cells (PSCs)[152]. PSCs are residents of the exocrine pancreas located in the periacinar, perivascular and periductal regions[153]. In the absence of diseases in the exocrine pancreas, such as chronic pancreatitis and PDAC, PSCs are maintained in a quiescent state. Quiescent PSCs can be distinguished from quiescent fibroblasts through detection of lipid droplets within the cytoplasm[154–157]. These vitamin A rich droplets were first used to identify PSCs in 1982 by Watari *et al* who described the presence of fibroblast-like PSCs and pericyte-like PSCs in the pancreas of mice[154]. The presence of lipid droplets within the cytoplasm allows for the isolation of quiescent PSCs through density centrifugation[156,157]. Isolation of PSCs facilitated their characterization and identification of markers for PSCs including desmin, vimentin, nestin and glial fibrillary acidic protein (GFAP)[153]. Within the pancreas GFAP promoter activity has been proposed to be unique to PSCs using a transgenic GFAP-LacZ mouse model[158].

Vitamin A rich lipid droplets are depleted when quiescent PSCs become activated. Traditionally PSC activation was inferred through the generation of a myofibroblastic phenotype characterized by αSMA expression. However, additional phenotypically distinct activated CAF subtypes derived from quiescent PSCs in PDAC have muddied the waters in terms of a defined generic activated phenotype[159]. Depletion of the cytoplasmic lipid droplets, however, does appear to be a universal symptom of PSC activation. In addition, increases in ECM production, cell migration and cell proliferation can occur during PSC activation.

Maintaining PSCs in a quiescent state *in* vitro has proved problematic due to the activation of PSCs in two-dimensional adherent cell culture. This activation of PSCs to the myofibroblastic state was noted through the upregulation of αSMA and depletion of cytoplasmic lipid droplets when quiescent PSCs were cultured following their first successful isolation from rats[156,157]. In order to assess the molecular mechanics of PSC activation Jesnowski *et al* were able to revert activated PSCs to a state of quiescence through cultivation of PSCs in Matrigel[160]. Reversion to quiescence was assessed through the formation of vitamin A rich cytoplasmic droplets and down regulation of αSMA and additional myofibroblastic associated genes.

The extensive presence of GFAP positive cells in the stromal compartment of PDAC has suggested that PSCs are the dominant source of CAFs within the TME of PDAC[152]. However, this concept has more recently been challenged. Helms *et al* used an elegant lineage tracing system with genetically engineered mouse models (GEMMs) of pancreatic cancer to fluorescently label PSCs based on *Fabp4* expression[161]. The prevalence of PSC-derived CAFs throughout the stroma could therefore be quantified during cancer progression. In contrast to previous observations, they demonstrated that PSC-derived CAFs represented a minority of the total CAFs. Whilst they represented a functionally and transcriptionally distinct subset their abundance was dwarfed by non-PSC-derived CAFs. However, it is not established whether *Fabp4* is expressed by all PSCs or by a PSC subpopulation.

PSCs exist as a distinctive cellular composite of the pancreas which can be transformed from a quiescent to activated state by cancer cells to form CAFs. However, their precise characterization and contribution to the CAF population during PDAC progression remains an incompletely defined and somewhat opaque question.

Fibroblasts

In addition to PSCs, resident fibroblasts of the pancreas can be activated by neoplastic cells to form CAFs. Similarly to PSCs, fibroblasts located in healthy tissues are maintained in a quiescent state where they exhibit reduced transcriptional and metabolic activity but can be distinguished from PSCs through the absence of cytoplasmic lipid droplets. Quiescent fibroblasts can be transformed to an activated myofibroblastic phenotype by cancer cells although this phenomenon is also observed in response to tissue injury and inflammation[162–164].

Distinct subpopulations of resting fibroblasts have been defined in the healthy pancreas. A subset of Gli1 expressing fibroblasts are present in perivascular regions[162,165]. Such vasculature associated Gli1 positive cells have been described in several other tissues as mesenchymal progenitors which have the capacity to become activated to myofibroblasts in response to tissue injury[166,167]. An additional and distinct resident fibroblast population characterized by Hoxb6 expression is found not in the perivascular region but rather dispersed throughout the parenchyma[162]. Hoxb6 is a homeobox factor expressed widely in the developing mesenchyme but subsequently restricted to a subset of fibroblasts in the mature pancreas[162,168]. To delineate whether quiescent fibroblast subtypes contribute to the CAF populations of the desmoplastic stroma observed in PDAC, lineage tracing of $Gli1^+$ and $Hoxb6^+$ fibroblast was conducted in a mouse model of PDAC[162]. This demonstrated that fibroblast subtypes differentially contribute to the stroma of PDAC. $Gli1^+$ fibroblasts were found to proliferate far more in response to neoplasia compared to the $Hoxb6^+$ subset, with $Gli1^+$ cells accounting for around half of the myofibroblast population.

A study from Hutton *et al* further demarcated fibroblast heterogeneity in the healthy pancreas through the identification of two separate populations of fibroblasts which could be distinguished through CD105 expression[169]. These two populations of fibroblast were found to be non-interconvertible distinct lineages which both significantly contribute to the stromal CAF population present in PDAC. Furthermore, CD105 positive and negative CAFs were present in a myriad of other cancers and healthy tissues from which they arise. Interestingly, in the previous study from Garcia *et al* they observed a subpopulation of both $Gli1^{+}$ and $Hoxb6^{+}$ fibroblasts also expressed CD105. Thus, there may be an additional tier of heterogeneity within $CD105^{+}$ and $CD105^{-}$ fibroblasts.

Dominguez *et al* further strengthened the definition of tissue resident fibroblasts as a heterogeneous population of cells, through profiling healthy fibroblasts with single-cell RNA sequencing (scRNAseq)[170]. This elucidated the presence of two distinct fibroblast lineages within the normal pancreas. One gene used to define these separate lineages was *Eng,* which encodes CD105, suggesting that these fibroblast lineages may correspond to the $CD105^{+/-}$ populations also described by Hutton *et al*[169].

Whilst few markers exist to delineate fibroblasts from PSCs the absence of cytoplasmic lipid droplets is the current primary distinguishing factor. However, the capacity of distinct fibroblast subpopulations, to retain lipid droplets in their cytoplasm when cultured in Matrigel, as done with PSCs, is unknown. Whilst additional research will better define or diminish the boundaries between PSCs and fibroblasts, quiescent fibroblasts are a heterogeneous population which represent an additional cellular repertoire from which CAFs can arise.

Mesothelial cells

The mesothelium is a monolayer of tightly bound mesothelial cells which encapsulate our inner organs and serous cavities. The transcription factor Wilms' Tumour 1 (WT1) is highly expressed in mesothelial cells in addition to a subset of tissue-resident mesenchymal fibroblasts[171]. A subset of such $Wt1^{+}$ fibroblasts have recently been identified in the pancreas[172]. These $Wt1^{+}$ fibroblasts may be of mesothelial descent given that mesothelial to fibroblast transition has been described to occur in fibrosis[173]. Interestingly, Hutton *et al* observed expression of mesothelial associated genes, including *Wt1*, in $CD105^{-}$ fibroblasts suggesting this fibroblast subset may be of mesothelial origin[169].

It has been established through integrating multiple scRNAseq datasets and performing lineage tracing that mesothelial cells can form CAFs[174]. In response to PDAC mesothelial cells were found to proliferate and gain a fibroblastic phenotype contributing significantly to the CAF population. Interestingly, a subpopulation of mesothelial derived CAFs were found to possess a specific phenotype which could distinguish them from more abundant CAF subpopulations present in PDAC. However, it is unclear whether all mesothelial derived

CAFs form directly from the mesothelium during PDAC carcinogenesis or whether a subpopulation of mesothelial derived fibroblasts exist in the healthy pancreas and differentiate into CAFs during tumorigenesis. It is also possible that both these options occur simultaneously.

Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are stromal cells that retain the capacity for self-renewal and multilineage differentiation which are the hallmarks of a stem cell. They were first identified in bone-marrow but were later described in additional tissues such as adipose, endometrial polyps and umbilical cord[175,176]. However, much like fibroblasts and PSCs, specifically defining an MSC has been called into disrepute. It has been demonstrated that perivascular cells, principally pericytes, display the multilineage differential capacity, self-renewal capabilities and share the cell-surface markers of MSCs[177]. It has thus been theorised that in fact all MSCs are pericytes[178]. So, for the course of this section I will refer to the pericyte subpopulation which may represent all MSCs simply as MSCs. Such MSCs have been described in the healthy pancreas and may represent a cellular repertoire of CAFs[179].

In the context of cancer bone-marrow derived mesenchymal stem cells (BM-MSCs) were shown to differentiate into a CAF-like phenotype and display CAF functional properties when exposed to tumour cell conditioned media *in vitro*[180]. It was later demonstrated that adipose-derived MSCs exhibit the capability to differentiate into CAFs when co-cultured alongside pancreatic cancer cells *in vitro*[181]. MSC-derived CAFs have also been characterized in the stroma of PDAC *in vivo*[182,183]. Such MSC-derived CAFs were shown to have specific pathophysiological roles in PDAC.

MSCs like other mesenchymal stromal cell types are difficult to concretely define within the cellular milieu. However, whilst their difference from perivascular cells remains controversial, they represent an additional cellular population from which CAFs can arise.

Pericytes

Pericytes are broadly defined as mural cells surrounding the vasculature in pathogenic and healthy tissues. Pericytes develop from diverse origins including the mesenchyme, neural crest and mesothelium in tissue dependent processes[184]. The heterogeneous origins of pericytes have also complicated their precise definition. As previously mentioned, pericytes have been suggested to represent MSCs due to their capacity for multilineage differentiation and self-renewal[177]. It has also been demonstrated that *in vivo* pericytes retain their lineage and do not transdifferentiate into other populations[185]. This has led to the notion that some pericytes may represent MSCs but not all. Despite the debate regarding the distinction of

MSCs and pericytes, pericytes possess cellular markers which allow their characterization. These include chrondoitin sulfate proteoglycan 4/neural glial antigen 2 (NG2), regulator of G protein signalling 5 (RGS5) and melanoma cell adhesion molecule (MCAM) although these markers can be partially shared with glial precursor O2A and endothelial cells[184,186,187].

Pericytes are able to transdifferentiate into fibroblasts in a process called pericyte-to-fibroblast transition (PFT). Within the context of cancer Hosaka *et al* elucidated a role for tumour cell secreted platelet-derived growth factor BB (PDGF-BB) in promoting PFT *in vitro* and *in vivo*[188]. Pericytes exposed to PDGF-BB upregulated fibroblast-specific-protein-1 (FSP1) expression and were thus annotated as fibroblasts. Cells undergoing PFT were found to promote tumour invasiveness and enhance the metastatic potential of tumour cells. scRNAseq profiling of melanoma tumours identified a population of stromal cells with both fibroblast and pericyte markers[189]. This population was found to be localized not only around blood vessels but in peri-tumoral spindle-shaped cells providing more evidence of PFT *in vivo*.

In breast cancer pericytes that have been transformed by malignant cells but have been described as distinct from CAFs and instead categorized as cancer-associated pericytes (CAPs)[190]. Such CAPs were positive for fibroblast activation protein alpha (FAP) but did not express podoplanin (PDPN), a separate fibroblast marker. CAPs were functionally disparate from CAFs which expressed podoplanin. In PDAC, pericytes can be influenced by malignant cells to upregulate expression of α-smooth muscle actin (αSMA)[191]. This alteration of pericytes was associated with increased leakiness of the vasculature. Here pericytes were defined based on their co-localization to vasculature and it remains to be elucidated whether these transformed pericytes can undergo PFT and contribute to the CAF population.

Whilst pericyte transformation by neoplastic cells has been observed in PDAC, little evidence of PFT has emerged. Despite this, perivascular cell's presence in the pancreas and their capacity to form CAFs in other cancers makes them a reasonable candidate for an additional cellular source of CAFs in PDAC.

Epithelial cells, endothelial cells and monocytes

Epithelial cells have the capability of converting into a mesenchymal phenotype in the process epithelial-to-mesenchymal transition (EMT). During this process a polarized epithelial cell loses its interactions with the basement membrane, via the basal surface, and becomes more migratory and invasive whilst also increasing synthesis and secretion of ECM proteins. This process is well described in cancer where neoplastic cells undergo EMT and spread to different organs but has also been described in non-neoplastic epithelial cells during embryogenesis, organ development, tissue regeneration and organ fibrosis[192]. Cells undergoing EMT can gain expression of FSP1 and have therefore been considered as a repertoire for CAFs[193].

Similarly, to epithelial cells endothelial cells typically reside in a polarised form bound by tight junctions and anchored to a basement membrane. It has also been demonstrated that endothelial cells can transform into a mesenchymal phenotype through the process of endothelial-to-mesenchymal transition (EndMT). Endothelial cells were found to gain a mesenchymal phenotype and induce FSP1 expression following exposure to transforming growth factor-β (TGFβ)[194]. Furthermore, through lineage tracing experiments with GEMMs of pancreatic cancer it was shown that endothelial cells transdifferentiated into CAFs during tumorigenesis *in vivo*[194].

Another cell type implicated in the production of CAFs in the PDAC stroma are monocytes. Monocytes are leukocytes which originate in the bone-marrow before dissemination through the circulation to tissue where they can form macrophages and dendritic cells[195]. Monocytes capacity to give rise to CAFs in PDAC was proposed following the observation that transplantation of enhanced green fluorescent protein (EGFP) labelled haematopoietic stem cells into chimeric mice led to $EGFP^+$ cells being detected in the pancreas[196]. These haematopoietic stem cell-derived cells were characterized as monocytes. Significantly these monocytes had developed into a mesenchymal phenotype and lost expression of the pan-immune cell marker CD45 and gained expression of PSC markers GFAP, desmin, vimentin and αSMA. Ino *et al* therefore established that monocytes can be recruited to the pancreas where they form PSCs. Whilst not directly shown, it is reasonable to presume that these monocyte derived PSCs can become activated during tumorigenesis to form CAFs in PDAC.

An overview

The cellular origins of CAFs in PDAC is a constantly evolving area. Cells from diverse developmental origins have been proven to contribute to the CAF population observed in PDAC (Figure 5). However, a recent study from Han *et al*, used lineage tracing to simplify this notion and elucidate that the majority of CAFs in a GEMM of PDAC were formed from tissue resident fibroblasts[147]. Such resident fibroblasts had a conserved origin in the splanchnic mesenchyme. Furthermore, they demonstrated that bone-marrow derived cells and epithelium contributed very little to CAFs. Despite this study providing evidence for tissue resident fibroblasts giving rise to the majority of CAFs it lacked the capacity to characterize the heterogeneity of tissue resident fibroblasts which is still controversial.

The boundaries distinguishing mesenchymal cell types which act as CAF progenitors are often blurry. MSCs are located in perivascular regions and have suggested to in fact be a subset of pericytes[177,178]. PSCs can also be located in perivascular regions and share markers with pericytes and MSCs[153]. Quiescent fibroblasts may also be located in perivascular regions and share markers which are commonly used to define the cell type and yet are common to all the aforementioned cell types[162,165]. Furthermore, fibroblasts in the pancreas have been segregated into defined lineages. Hutton *et al* and Dominguez *et al* both identify CD105 positive and negative fibroblasts in the healthy pancreas, but it is unclear how these relate to the $Hoxb6^+$ and Gli^+ subsets described by Garcia *et al* which each may be either CD105

positive or negative[162,169,170]. To add more complexity some of these fibroblast subpopulations exhibit a perivascular localization and share markers of PSCs, pericytes and MSCs. CD105 itself is used as an MSC specific marker for their isolation[182].

When adhering to the nomenclature it can be confusing to concretely discern between pericytes, PSCs, fibroblast subtypes and MSCs. It reminds me of a quote from Jorge Luis Borges I have amended with a mesenchymal cell twist "No one is anyone, one single immortal mesenchymal cell is all mesenchymal cells". Whilst this is too extreme, there are certainly distinct populations of mesenchymal cells, I believe the nomenclature may not reflect the reality of cellular heterogeneity and further studies will either diminish or define the boundaries which distinguish mesenchymal cell populations. Despite this what is clear is that heterogeneity exists amongst mesenchymal lineages of the healthy pancreas and these lineages contribute to the CAF landscape of PDAC.

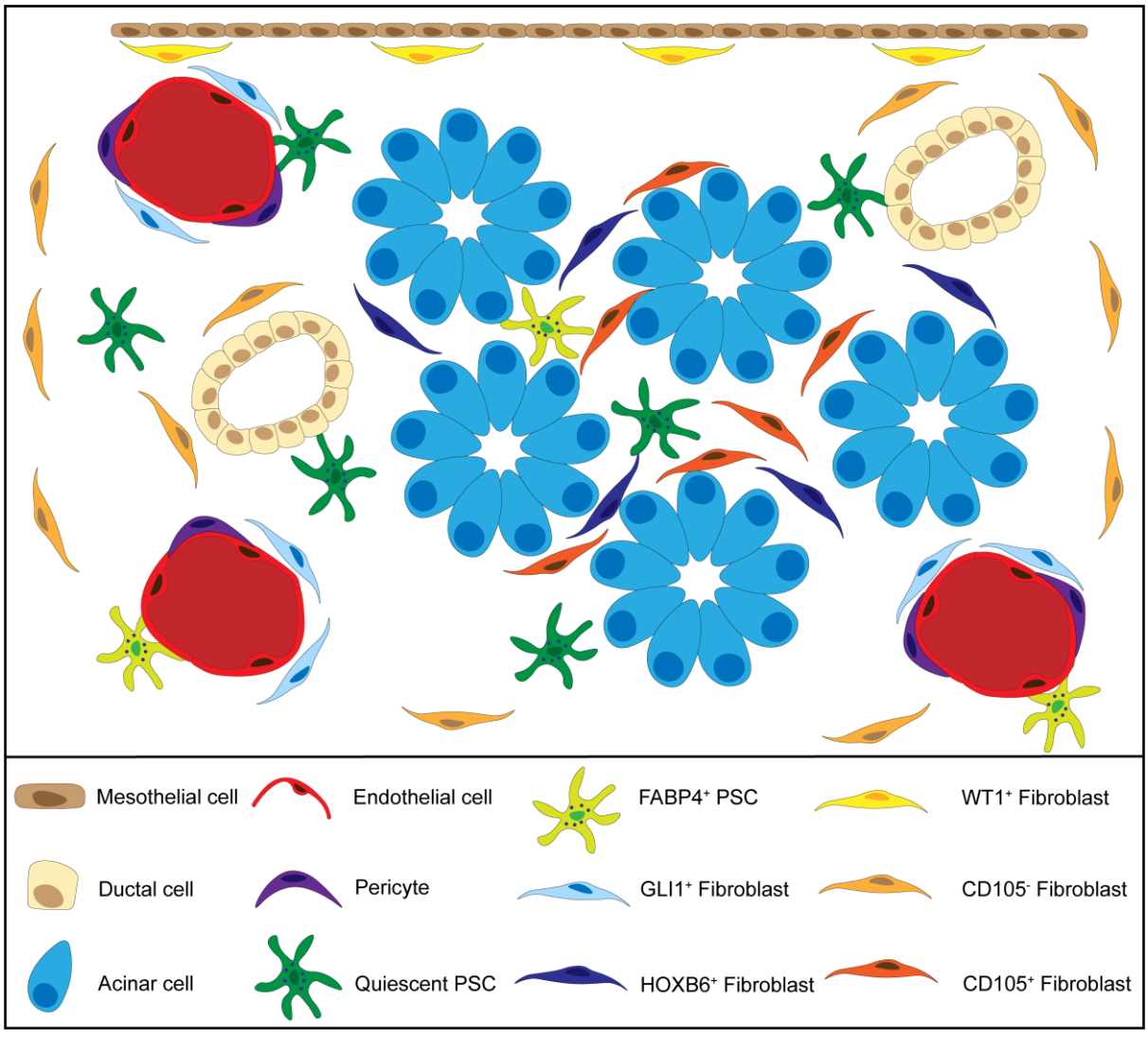

Figure 5. Schematic illustrating the cellular origins of CAFs in PDAC. Created with Adobe Illustrator.

CAF heterogeneity

Over the last decade, the conception of CAFs as a homogenous entity in PDAC has been rebuked largely through the advent and application of scRNAseq. CAF heterogeneity is a multifaceted constantly developing paradigm with multiple studies identifying distinct CAF subtypes. Consensus regarding the distinction of CAF subtypes remains elusive but a taxonomic framework and phenotypic stratification of defined subtypes is gradually emerging.

myofibroblastic CAFs (myCAFs)

One of the first identified and best characterized CAF subtypes are myofibroblastic CAFs (myCAFs). myCAFs were first described in PDAC by Öhlund *et al*, who noted that elevated αSMA expression was present in CAFs localized in close proximity to tumour cells[159]. Furthermore, they demonstrated that co-culturing PSCs with tumour organoids in an *in vitro* model system could recapitulate the formation of juxta-tumoral CAFs with enhanced αSMA expression. These tumour adjacent CAFs were labelled as myCAFs due to the high expression of αSMA. myCAFs were found to arise only when PSCs were co-cultured directly with tumour cells and could not be induced by tumour-derived signals suggesting a juxtacrine signalling component is required for their formation. Bulk transcriptional profiling of myCAFs identified upregulation of the myofibroblastic gene *Acta2* (encoding αSMA), TGF-β responsive genes (*Ctgf* and *Col1a1*) and genes related to ECM deposition (*Col1a1*, *Col2a1* and *Col5a1*).

Elyada et al, subsequently utilized scRNAseq to validate and characterize myCAFs in human and mouse PDAC[197]. The transcriptional profile of myCAFs from human PDAC closely resembled that which had been observed *in vitro* by Öhlund *et al*. This included high expression of *ACTA2* and additional genes related to ECM deposition, collagen formation and smooth-muscle contraction. A sequence of additional studies using scRNAseq to profile CAFs taken from human PDAC further validated the presence of myCAFs[198–200].

Dominguez *et al*, profiled tissue resident fibroblasts and CAFs from a GEMM of PDAC with scRNAseq[170]. As discussed earlier, tissue resident fibroblasts were separated into distinct lineages through *Eng* and *Dpp4* expression. *Eng*$^+$ fibroblasts differentiated into CAFs with a myCAF phenotype which could be distinguished through expression of *Lrrc15* and displayed increased TGF-β signalling activity. Although it should be noted the normal *Eng*$^+$ fibroblast progenitor also possessed a myCAF phenotype. Taken together, Dominguez *et al* proposed that a specific resident *Eng*$^+$ fibroblast subtype could differentiate into myCAFs through TGF-β signalling. The notion that TGF-β signalling promotes a myCAF phenotype had previously been established by Biffi *et al*, who demonstrated that inhibiting TGF-β signalling in a GEMM of PDAC could repress myCAF formation[201].

Hutton *et al*, used mass cytometry for single-cell profiling of tissue resident fibroblasts[169]. As previously described and similarly to Dominguez *et al*, they identified the presence of separate fibroblast lineages demarcated based on CD105 (encoded by *Eng*) positivity. In contrast to the above-mentioned concept that specific fibroblast lineages give rise to myCAFs, they demonstrate that $CD105^{+/-}$ fibroblasts are both capable of differentiating into mature myCAFs in the PDAC stroma of GEMMs. Interestingly, they find that $CD105^{+}$ fibroblasts are more responsive to TGF-β but demonstrate that prolonged exposure to TGF-β can promote myCAF formation in both $CD105^{+}$ and $CD105^{-}$ lineages.

The $Gli1^{+}$ fibroblast subtype identified by Garcia *et al* was also found to differentiate into myCAFs[162]. However, not all myCAFs were $Gli1^{+}$ and not all $Gli1^{+}$ CAFs were myCAFs. This suggests that $Gli1^{+}$ fibroblasts are a potential, but not exclusive, reservoir for myCAFs.

In addition to TGF-β signalling, and the unknown juxtacrine signalling component, it has been demonstrated that inhibition of hedgehog (Hh) signalling can lead to a decrease in myCAF formation *in vivo* using GEMMs of PDAC. Thus suggesting Hh signalling as an additional regulator of myCAF differentiation[202].

Whilst myCAFs are one of the best characterized and most thoroughly validated CAF subpopulation, their origin and differential regulation are not fully described. Furthermore, additional heterogeneity within myCAFs remains to be fully defined.

inflammatory CAFs (iCAFs)

In addition to myCAFs, Öhlund *et al* observed an additional population of CAFs which were distally located to tumour cells and interspersed throughout the stroma[159]. These CAFs possessed elevated expression of cytokines including *Il6, Il11* and *Lif*, and were therefore annotated as inflammatory CAFs (iCAFs). iCAFs spatial localization, more distal to tumour cells than myCAFs, was recapitulated using the co-culture *in vitro* model system comprised of PSCs and tumour organoids. Furthermore, it was demonstrated that diffusible signals derived from tumour cells were sufficient to induce PSCs to form iCAFs and direct contact with tumour cells was not required. Transcriptionally, iCAFs were distinguishable from myCAFs and quiescent PSCs through induction of genes associated with cytokine and chemokine signalling.

As with myCAFs, iCAFs were subsequently reported in human and mouse PDAC through scRNAseq[170,197–200]. Such studies helped characterize the transcriptional profile of iCAFs in PDAC, which resembled those profiled *in vitro*, identifying elevated expression of genes associated with mediating inflammation including cytokines (*IL6* and *LIF*), chemokines

(*CXCL12* and *CXCL1*), complement components (*C3* and *C7*) and prostaglandin D2 synthase (*PTGDS*).

The progressive characterization of iCAFs and myCAFs has occurred in relative synchronicity and as with myCAFs, Dominguez *et al* proposed that iCAFs differentiate from a distinct *Dpp4*$^{+}$ resident fibroblast subpopulation whilst Hutton *et al* demonstrated that CD105$^{+/-}$ resident fibroblasts could both give rise to iCAFs[169,170]. Furthermore, Dominguez *et al* identified IL-1 as a cytokine driving iCAF formation in *Dpp4*$^{+}$ fibroblasts where Hutton *et al* demonstrated prolonged exposure of CD105$^{+/-}$ CAFs to IL-1 could induce an iCAF phenotype in both. To complete the perfect mirroring of myCAF characterization in iCAFs, these findings were in accordance with the previous study from Biffi *et al*, which had discovered IL-1 was capable of promoting expression of inflammatory iCAF genes and inhibition of STAT3, a downstream effector of IL-1 signalling, depleted iCAF formation *in vivo*[201].

It has more recently been demonstrated that hypoxic conditions can also promote iCAF formation[203]. Fibroblasts with a higher level of hypoxia isolated from a GEMM of PDAC were found to be enriched for an iCAF gene signature. Furthermore, co-culturing PSCs with tumour organoids under hypoxic conditions *in vitro* promoted an iCAF phenotype. A recent study observed that iCAF formation could also be promoted via the cytokine IL-17A[204]. A subset of CD8+ T-cells which secrete IL17A were identified as the cellular reservoir for the cytokine and implicated in directing iCAF differentiation.

In the above summary the word myCAFs could easily be swapped for iCAFs with respect to our current understanding of their biology. Whilst they are commonly accepted, well validated and well characterized the precise mechanics of their formation, extent of heterogeneity within iCAFs and defined cellular origins remain to be fully elucidated.

antigen-presenting CAFs (apCAFs)

Antigen-presenting CAFs (apCAFs) were a later addition to the CAF subtype family than iCAFs and myCAFs. apCAFs were identified in PDAC by Elyada *et al*, through scRNAseq profiling of GEMMs of PDAC[197]. As the name would suggest, apCAFs could be discerned from iCAFs and myCAFs through elevated expression of genes associated with major histocompatibility complex (MHC) class II antigen presentation, including *H2-Aa*, *H2-Ab1* and *Cd74*. MHCII expression was subsequently validated at the protein level in a subset of CAFs in both human and murine PDAC. Additionally, unlike iCAFs or myCAFs, apCAFs could induce a modest activation of T-cells through antigen-presentation. This activation of T-cells by antigen-presentation in apCAFs was however, far less effective than the activation of T-cells by professional antigen-presenting cells (APCs) of the immune system. The lack of co-stimulatory molecules (CD40, CD80 and CD86) in apCAFs, which are present in professional APCs, is suggested to account for this difference.

apCAFs have subsequently been detected in human PDAC through scRNAseq[200], although they have not been detected with as much regularity as iCAFs and myCAFs with certain studies only reporting a modest barely detectable enrichment for genes involved in MHCII antigen-presentation in a subset of CAFs[198,199]. Our understanding of the origin of apCAFs has progressed rapidly since their identification. Huang *et al* observed, through lineage-tracing in GEMMs of PDAC, that apCAFs are formed predominantly from mesothelial cells[174]. Mesothelial cells gained a fibroblastic phenotype as PDAC developed and could be reprogrammed to adopt an apCAF phenotype in response to tumour-cell derived IL-1 and TGF-β[174]. Similarly, Dominguez *et al* identified a population of mesothelial-like cells which were enriched for the apCAF signature. A previous study from Buechler *et al* observed that mesothelial cells could be distinguished from fibroblasts based on platelet-derived growth factor receptor α (PDGFRα) expression[172]. Whilst mesothelial cells express PDPN, commonly referred to as a fibroblast marker, they did not express PDGFRα, which was specific to fibroblasts. Bulk RNA profiling of mesothelial cells and fibroblasts identified specific gene expression profiles to distinguish mesothelial cells from fibroblasts. A subset of these genes, including *Nkain4*, *Upk3b* and *Gpm6a*, were identified as apCAF markers by Elyada *et al*, providing further evidence that apCAFs may have a mesothelial origin[197]. Finally, Hutton *et al* noted that CD105$^-$ CAFs but not CD105$^+$ CAFs contained a subset of antigen-presenting cells[169]. As mentioned above, Hutton *et al* detected mesothelial-associated genes in the CD105$^-$ CAFs. Thus, perhaps CD105$^-$ fibroblasts are mesothelial derived and can form apCAFs explaining why the apCAFs were only present amongst CD105$^-$ CAFs.

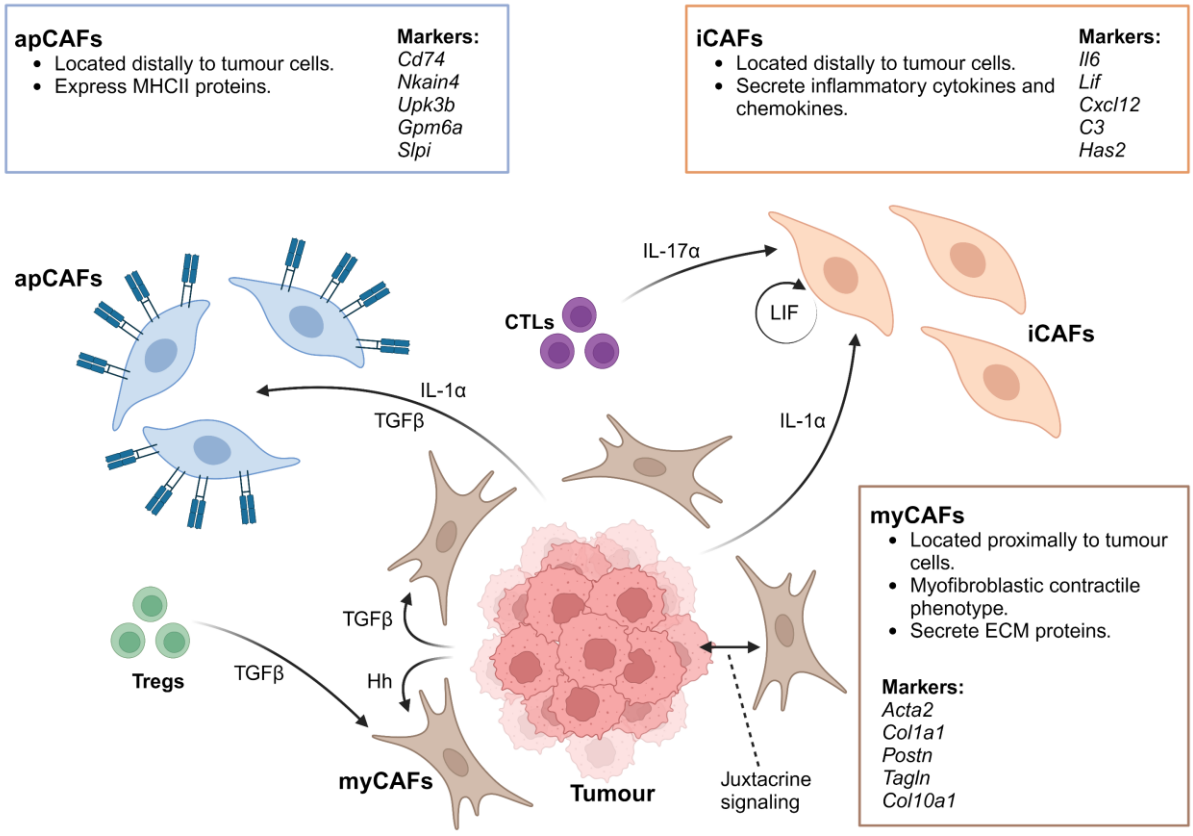

Figure 6. Schematic illustrating the phenotypic hallmarks and key regulators of CAF subtypes. Created with BioRender.

Taken together apCAFs represent a phenotypically distinct CAF subtype distinguished through MHCII antigen presentation. Substantial evidence exists that apCAFs are derived from mesothelial cells. However, it still remains to be defined whether subsets of tissue resident fibroblasts can be derived from the mesothelium, exist in the healthy pancreas and subsequently act as a cellular reservoir for apCAFs during PDAC or if mesothelial cells are directly converted to apCAFs during PDAC. It is also conceivable that both of these possibilities occur in parallel but this question requires further investigation.

Additional CAF subtypes

Whilst iCAFs, myCAFs and apCAFs have been widely reported there are many additional CAF subtypes which have been proposed. Wang *et al* profiled human PDAC tumours using scRNAseq, in addition to iCAFs and myCAFs they identified a population of CAFs with enhanced glycolytic and translational activity which were therefore labelled as metabolic CAFs (meCAFs)[199]. meCAFs were observed to be more abundant in samples taken from patients who had a "loose" stroma with reduced desmoplasia.

An additional study using scRNAseq to profile human PDAC identified three subpopulations of CAFs referred to as PSCs, classical CAFs (cCAFs) and complement secreting CAFs (csCAFs)[205]. The PSC cluster showed elevated expression of markers associated with pericytes and perivascular-like cells (PVLs) including *NOTCH3* and *RGS5* suggesting such cells may be PVLs or pericyte-like PSCs[154]. The cCAFs had elevated expression of collagens including *COL1A1*, *COL10A1* and *COL11A1* suggesting they may correspond to myCAFs. It has been noted above that complement secretion has previously been described as a feature of iCAFs, thus the csCAFs showing induction of the complement genes *C7* and *C3* may correspond to iCAFs. Thus, the three CAF subtypes described here show features of previously defined CAF subtypes.

Helms *et al* identified PSC-derived CAFs as a distinct subpopulation which were phenotypically distinct from CAFs with a proposed non-PSC origin[161]. PSC-derived CAFs in a GEMM of PDAC had elevated expression of markers including *Tie1*. A subpopulation of CAFs were observed to express *TIE1* in human PDAC and it was found that PSC-derived CAFs frequently contributed 10-20% to the total overall CAF population, although up to 60% was observed in some patients. Genes upregulated in PSC-derived CAFs were enriched in biological pathways including cell adhesion, angiogenesis and ECM organization. This study used bulk RNA sequencing to profile CAFs so it is unclear whether additional heterogeneity exists within PSC-derived and non-PSC-derived subpopulations.

Our understanding of CAF heterogeneity is still in its' infancy. The relationship between CAF progenitors and mature CAF subtypes remains incompletely understood. Consensus is building around the presence of iCAFs, myCAFs and apCAFs although there remains contention regarding the origin and additional heterogeneity within these subpopulations.

Regardless of whether we have arrived at the most accurate categorization of CAF subtypes, what is apparent is that phenotypic variance is widespread amongst CAFs and such variance signifies the potential for subpopulations to orchestrate divergent functions within the TME of PDAC.

Tumour-promoting roles for CAFs in PDAC

Historically, CAFs have been regarded as accomplices to tumour cells which support their malign intent. Certainly, the case against CAFs is a compelling one and a slew of studies have implicated them as promoters of disease progression. CAFs have been shown to promote tumorigenesis in PDAC through a variety of mechanisms which collectively potentiate tumour cells ability to grow, spread and evade attack from immune cells and therapeutics. However, the characterization of CAF subtypes illuminates the potential for defined subtypes to mediate discrete influences on tumorigenesis. The mechanisms through which CAFs orchestrate pro-tumorigenic roles can therefore provide clues as to whether such influences are common to all CAFs or may be attributed to phenotypically distinct subtypes.

Proliferation

FAP expression by CAFs is widely reported and the prevalence of FAP^+ CAFs in PDAC has been correlated with worse prognosis[206,207]. However, it has been shown that not all CAFs are FAP^+ and co-culture of PDAC cancer cells with FAP^+ CAFs promoted proliferation of the tumour cells whilst FAP^- CAFs did not[208]. PSC-derived CAFs have been suggested to represent a FAP^+ subpopulation[159]. Using an orthotopic mouse model of PDAC, where PSCs were injected alongside tumour cells, it was demonstrated that co-injection of increasing proportions of PSCs promoted the growth of tumours[209]. Inflammatory features of CAFs have also been proven to promote tumour growth. Co-culture of tumour cells and CAFs revealed that IL-1α secreted by cancer cells induced the secretion of pro-inflammatory cytokines by CAFs which could directly increase proliferation of the neoplastic cells[210]. Given that iCAFs are defined as a subpopulation of CAFs expressing pro-inflammatory cytokines this suggests a role for iCAFs in promoting tumour cell proliferation.

Metastasis

Roles for CAFs in driving metastatic spread of PDAC have also become well-established. Co-injection of PSCs with cancer cells not only enhanced the proliferation but also the metastatic spread of cancer cells[209]. Furthermore, cancer cells co-cultured with PSCs were found to down-regulate epithelial markers and gain a fibroblastic phenotype, a hallmark of EMT[211]. CAFs may also regulate the invasive potential of tumour cells through remodelling of the ECM. CAFs mediate the re-alignment of ECM proteins including collagens and

fibronectin to provide tracks which cancer cells use for directional migration[212,213]. They may also degrade the ECM allowing for tunnels to form which metastatic cancer cells can use as paths to migrate and invade through[214]. The mechanisms through which CAFs promote metastasis can signify the involvement of defined subpopulations. IL-6 has been demonstrated to induce EMT in PDAC cells, IL-6 is an established iCAF marker suggesting that iCAFs may promote metastasis[215]. However, a recent study from Chen *et al* found that close proximity of αSMA$^+$ CAFs was correlated with worse survival in PDAC[216]. Furthermore, they demonstrated that juxtacrine homophilic ATP1A1 interactions between tumour adjacent CAFs and neoplastic cells induced activin A secretion from CAFs which promoted metastasis. As myCAFs are αSMA$^+$ CAFs residing in a peritumoral area, this provides convincing evidence that they are enhancing metastatic spread in PDAC.

Metabolism

The chronic proliferation of cancer cells dictates their requirements for elevated metabolic activity. CAFs have been implicated in supplementing tumour cells with metabolites that can sustain their growth. A key feature in tumour metabolism, characterized as early as 1924 by Otto Warburg, noted that tumours metabolise glucose glycolytically to form lactate, rather than through oxidative phosphorylation to form pyruvate, even in the presence of oxygen[217]. This process was named "the Warburg effect". Significantly, Pavlides *et al* later demonstrated that tumour cells were capable of reprogramming CAFs to conduct the Warburg effect, allowing them to secrete energy rich metabolites including lactate under normoxic conditions which could sustain tumour growth[218]. CAFs were also shown to promote glycolysis in tumour cells through the secretion of chemokines. CAFs with low expression of focal adhesion kinases (FAKs) were found to secrete elevated levels of chemokines which enhanced the glycolytic capacity of tumour cells allowing for them to boost their metabolic activity[219]. CAFs were also found to augment the glycolytic potential of tumour cells in PDAC through the secretion of hepatocyte growth factor (HGF)[220]. In addition, CAFs have been associated with providing additional metabolites to tumour cells including amino acids such as aspartate, asparagine and alanine which can sustain cancer cell proliferation[221–223]. CAFs supplementation of tumour cells with metabolites for their growth represent a mechanism through which they can abet tumorigenesis, although the contribution of defined CAF subtypes in this process is less well-comprehended.

Chemoresistance

One of our most effective tools in combatting cancer are chemotherapeutic agents which target highly proliferative cell types including cancer. Development of chemoresistance in PDAC contributes to the poor prognosis of the disease and CAFs have been implicated in assisting the development of chemoresistant tumours. CAFs are the primary source of the dense desmoplasia observed in PDAC. This desmoplasia can serve as a barrier preventing drugs from reaching their target cells and mitigating their activity. Stromal depletion approached in GEMMs of PDAC have encouraged better drug delivery improving overall

survival and reducing tumour growth[224]. Provenzano *et al* improved drug delivery by specifically targeted hyaluronic acid (HA) in the ECM through enzymatic degradation. Interestingly, Elyada *et al* identified HA synthase genes as markers for iCAFs, suggesting a role for iCAFs in promoting chemoresistance[197]. iCAFs were further incriminated as facilitators of chemoresistance through the antibody blockade of IL-6 , an iCAF derived cytokine, in GEMMS of PDAC resulting in elevated efficacy of chemotherapeutics in suppressing tumour growth[225]. In addition, an increased prevalence of iCAFs was defined in human PDAC specimens isolated from chemotherapy treated in comparison to naïve untreated tumours which, the author's suggest, could be indicative of a chemoprotective function of this subtype[226]. Finally co-culture of primary patient-derived tumour organoids and patient-matched CAFs enhanced chemoresistance in tumours cells compared to mono-cultures of tumour cells[227]. Co-cultured CAFs were found to be more inflammatory than those in mono-culture, again suggesting iCAFs may aid chemoresistance, although myCAFs were also induced in co-culture so it is difficult to disentangle which subtype is culpable. iCAFs were given a reprieve in the case against them as chemoprotective by Grünwald *et al* who observed that stromal microenvironments which were more inflammatory were less chemoresistant than "deserted" environments with low-cellularity and increased deposition of collagens[228]. Given that myCAFs are attributed to be the primary depositors of collagen in the ECM, this suggests they could also be aiding tumours to alleviate the effectiveness of chemotherapeutics. CAFs are heavily associated with promoting chemoresistance in cancer cells, iCAFs are thought to be a major component of this phenomenon but are not universally castigated as the moderator.

Immunosuppression

CAFs can directly interact with tumour cells to modulate their phenotype, directing them towards a state which encourages tumorigenesis but they can also regulate disease progression indirectly through interactions with other stromal cells. Our understanding of the immunomodulatory properties of CAFs is expanding and defined CAF subtypes have been associated with differentially contributing to the immunosuppressive microenvironment in PDAC.

FAP^+ CAFs have been suggested to contribute to immunosuppression through the secretion of the chemokine CXCL12[229]. Feig *et al* highlighted that CXCL12 was secreted primarily by FAP^+ CAFs and was found to coat cancer cells promoting the exclusion of $CD8^+$ T-cells. Genetic depletion of FAP^+ CAFs or treatment with a CXCL12 inhibitor dramatically increased tumour infiltration by $CD8^+$ T-cells.

Hartmann *et al* elucidated that a low-density collagen matrix could enhance T-cell infiltration whilst a high-density collagen matrix resulted in T-cell exclusion[101]. Given that myCAFs are characterized as a dominant producer of collagens in PDAC, it suggests that a high abundance of myCAFs could lead to dense deposits of collagen which may prevent T-cells from accumulating in tumours. Tumour responsiveness to immunotherapy can signify

the extent of infiltration by CTLs. Dominguez *et al* identified a TGF-β responsive CAF subtypes, which resembled myCAFs and possessed elevated expression of *Lrrc15*[170]. Elevated levels of the LRRC15$^+$ CAF signature in human PDAC correlated with worse response to immunotherapy suggesting that myCAF-like LRRC15$^+$ CAFs negatively regulate anti-tumour immune responses.

iCAFs have been widely reported to influence immune response in PDAC, and as with their previously described tumorigenic effects, the weight of evidence largely points towards a pro-carcinogenic role for the subtype. iCAFs represent a prominent cellular source of CXCL12 suggesting the above described immunosuppressive functions of the chemokine are driven by iCAFs[197,229]. IL-6 is an additional iCAF derived cytokine with immunomodulatory properties relevant to PDAC progression. IL-6 has been shown to increase glycolytic flux in tumour cells stimulating elevated lactate secretion into the TME which drives macrophage polarization towards an M2 immunosuppressive phenotype[230]. Furthermore, in response to caloric deficit, a feature of cachexia which is commonly observed in PDAC, IL-6 induced elevated production of glucocorticoids which promoted exclusion of CTLs from the TME[231]. CAF-derived IL-6 has also been shown to be capable of inhibiting NK cell activity and enhancing PDAC progression[232]. STAT3 signalling in CAFs is associated with iCAF differentiation[201]. Disruption of STAT3 signalling in stromal fibroblasts from a GEMM of PDAC resulted in a decrease in M2 macrophages and increase in CD8$^+$ T-cells resulting in increased survival[233]. Lastly, Gorchs *et al* found that CAFs could stimulate upregulation of inhibitory receptors in CD8$^+$ T-cells and suppress their proliferation[234]. This was partially mediated through secretion of prostaglandin E_2 (PGE_2), due to the fact enzymes involved in the synthesis of PGE_2 have been characterized as iCAF markers, this again suggests an immunosuppressive capacity of the subtype[197].

As discussed previously apCAFs retain the capability to interact with CD8$^+$ T-cells but are incapable of strongly activating them[197]. This has led to the hypothesis that apCAFs may sequester CD8$^+$ T-cells in the stroma, acting as decoys for the tumour cells. Huang *et al* identified mesothelial cells as the pre-cursor population for apCAFs. Such mesothelial-derived apCAFs were capable of augmenting immunosuppression by inducing the differentiation of T_{regs}[174].

CAFs profusion within the TME of PDAC portends their capacity to play a profound influence on the development of the disease. Such influences frequently enhance the destructive power of cancer cells (Figure 7). CAF subtypes exhibit specific phenotypic properties which can be related to pro-tumorigenic roles in PDAC. This has implicated apCAFs, myCAFs and iCAFs as collaborators with tumour cells, although the most abundant evidence incriminates iCAFs as a tumour-supporting subtype. In addition, generic features of CAFs have been associated with facilitating disease progression. Despite the profuse evidence for CAFs orchestrating pro-tumorigenic roles in PDAC, our attempts to mitigate these roles have uncovered the intriguing prospect that CAFs may not universally support tumorigenesis.

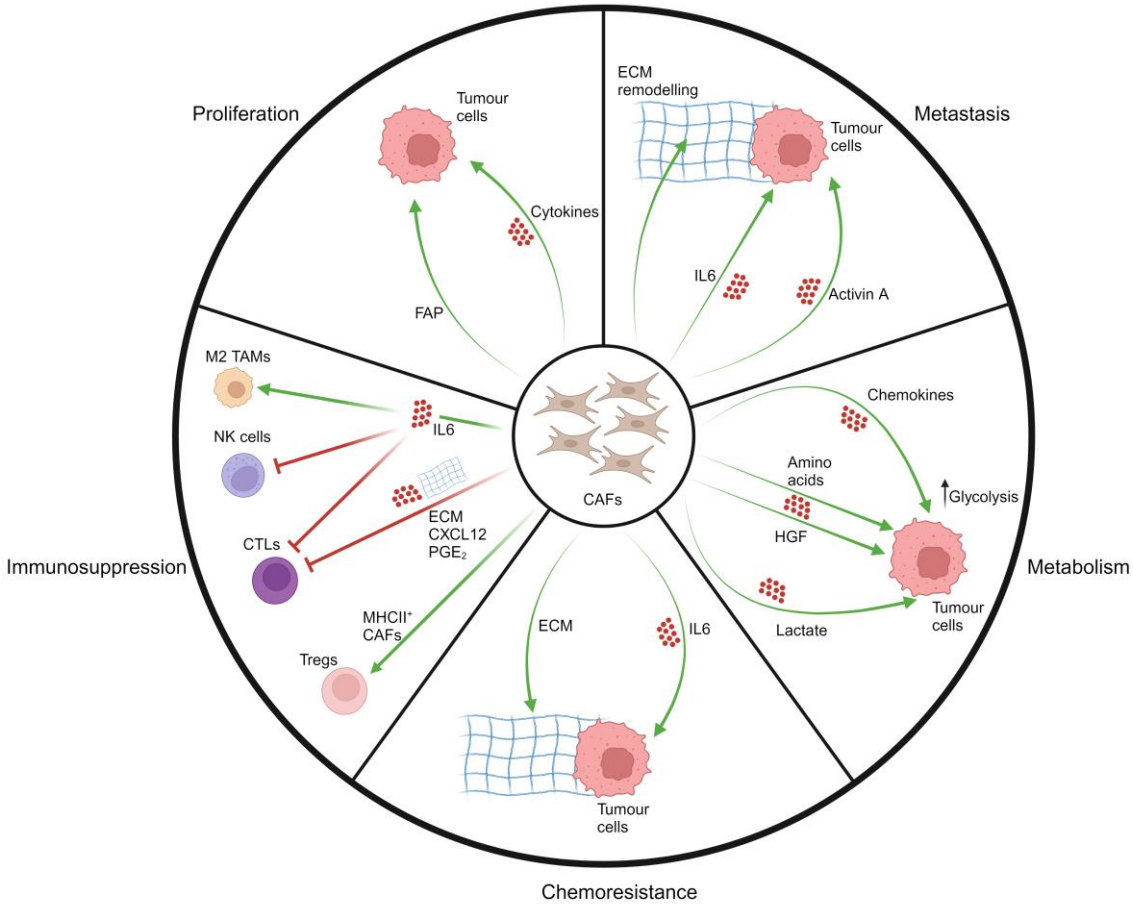

Figure 7. Schematic illustrating tumour-promoting roles for CAFs in PDAC. Created with BioRender.

Tumour-restraining roles for CAFs in PDAC

The idea that CAFs have been unfairly maligned as a universal tumour supporting entity has gained increasing traction. The characterization of phenotypically distinct CAF subpopulations augurs the possibility that they can exert independent influences on the TME and emerging evidence suggests that some of these influences can serve to restrain tumorigenesis.

A breakthrough study from Özdemir *et al* helped kick start the reformation of CAFs image from universally cancer promoting to potentially restraining cancer growth[235]. Through selective deletion of proliferating αSMA$^+$ myofibroblasts in a GEMM of PDAC, they observed an overall decrease in survival finding more aggressive less differentiated PDAC developed. αSMA$^+$ myofibroblasts depletion was associated with reduced collagen I deposition in tumours, reduced vascularization, increased hypoxia and decreased stiffness of the ECM. Interestingly, this reduction in ECM and tumour rigidity did not enhance the delivery of

chemotherapeutic agents. Finally, an increased proportion of myeloid cells and T_{regs} and a reduction in CTLs in the TME occurred following abrogation of αSMA$^+$ myofibroblasts suggesting a more immunosuppressive microenvironment. However, when combined with immunotherapy αSMA$^+$ myofibroblast depleted mice showed better overall survival suggesting that the negative effects on tumour growth could be mitigated. Given that myCAFs are associated with elevated αSMA expression, this highlights a potential tumour suppressing role for this subtype.

In the same year Rhim *et al* provided evidence for an anti-tumorigenic role of CAFs in PDAC[236]. In a GEMM of PDAC hedgehog (Hh) signalling was repressed through the selective knocking out of *Shh* in the pancreas. This led to a decrease in overall survival and promoted the formation of metastases. Tumours were also found to be hypervascularized and less differentiated. A drastic reduction in αSMA$^+$ cells and immune infiltration was observed following suppression of Hh signalling. Significantly, it was shown that Hh signalling was restricted to mesenchymal stromal cells. A similar study from Lee *et al* demonstrated that ablating Hh signalling could promote tumorigenesis and restrain αSMA$^+$ stromal cell formation[237]. Given that Hh signalling has been linked to driving myCAF formation[202] and a reduction in αSMA$^+$ cells was observed, this provides more evidence for myCAFs restricting tumour development.

A recent and elegant study from Chen *et al*, provided further evidence of a tumour restraining role for CAFs[130]. Pancreas specific deletion of type I collagen (Col1) in αSMA$^+$ myofibroblasts in a GEMM of PDAC resulted in accelerated disease progression and reduced survival. By profiling tumours lacking Col1 in αSMA$^+$ myofibroblasts with scRNAseq, they demonstrated increased infiltration of immunosuppressive MDSCs and suppressed T- and B-cell recruitment to the TME. *Col1a1* has been shown to be highly expressed by myCAFs[159], evidencing a role for this CAF subpopulation in promoting a favourable immunogenic niche to obstruct PDAC development.

Mizutani *et al* also proposed that a subpopulation of CAFs could inhibit tumour growth[238]. They suggested that a subpopulation of CAFs, which originated specifically from PSCs, expressed the protein meflin. Meflin expression in CAFs was found to correlate with increased patient survival and genetic deletion of meflin$^+$ CAFs caused more aggressive less differentiated tumours to develop in a GEMM of PDAC. Interestingly, and in contrast to previous findings, they observed that meflin$^+$ CAFs were αSMA low whilst tumour promoting CAFs expressed high levels of αSMA. Thus, Mituzani *et al* present a tumour restraining role for CAF subpopulations but also contradict an anti-tumorigenic function for αSMA$^+$ cells.

Finally, Hutton *et al* observed that independent CAF populations differentially mediated tumour growth in PDAC[169]. By isolating CD105$^+$ and CD105$^-$ fibroblasts from the pancreas and co-injecting them with tumour cells they observed that CD105$^-$, but not CD105$^+$, CAFs could restrain tumour growth. Given that apCAFs are specific to the CD105$^-$ population, they sought to determine whether MHCII expression in apCAFs was driving the inhibition of

tumour growth. However, they found that depleting MHCII expression in CD105$^-$ CAFs had no effect on the tumour restraining properties. They did observe in GEMMs that lacked T- and B-cells that CD105$^-$ CAFs lost their protective effect on cancer cell growth, suggesting CD105$^-$ CAFs mediate their protective effect through adaptive immune cells.

An emergent body of work has proposed that we reimagine the role of CAFs in PDAC tumorigenesis. Rather than viewing CAFs as a homogenous population of tumour promoting accomplices to cancer cells we should instead regard them as a heterogeneous population of resistors fighting the spread and followers assisting it. This assertion is grounded in findings from a litany of studies which demonstrate that CAFs, like other components of the stroma, may be both tumour promoting or tumour restraining (Figures 7 & 8). Despite the potential for CAF subpopulations to mediate anti-tumorigenic functions within the TME, what is clear is that left to their own devices these tumour suppressive effects are insufficient to quell the aggressive nature of PDAC, as demonstrated by the dismally low survival rate. But what if they were not left to their own devices and instead given a helping hand in the effort to combat PDAC development? Therapeutic intervention targeting the stroma contains the potential to minimize the pro-tumorigenic and maximize the tumour-suppressive influences of CAFs, and other stromal components, of the PDAC TME.

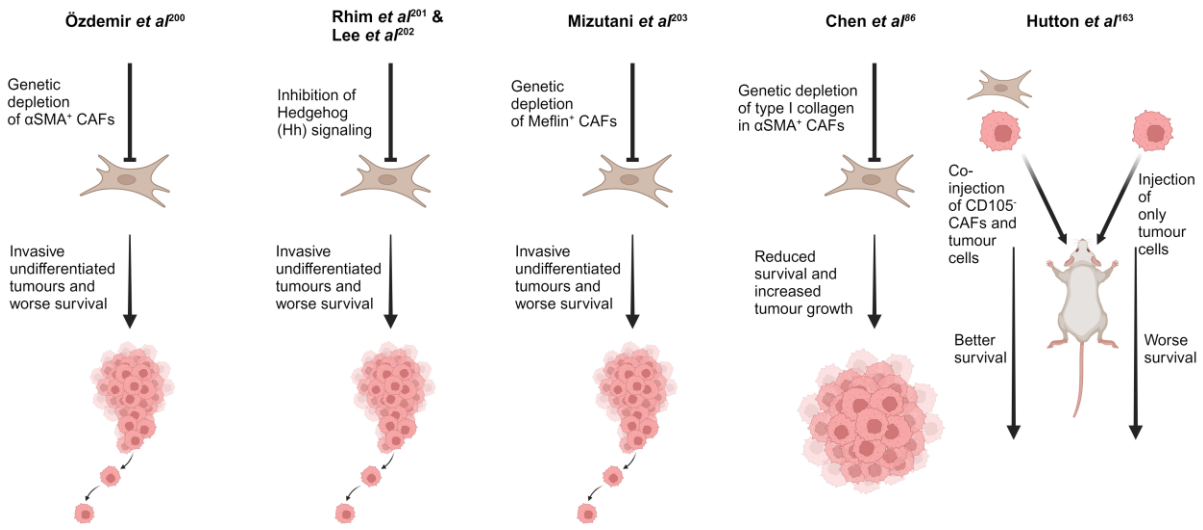

Figure 8. Schematic summarizing landmark studies defining tumour-restraining roles for CAFs in PDAC. Created with BioRender.

THERAPEUTIC TARGETING OF STROMAL COMPONENTS

The abundance of desmoplastic stroma in PDAC makes it an appealing target for therapeutic intervention. We have thus far discussed some of the dominant stromal components including CAFs, ECM and immune cells and how they may play divergent pathophysiological roles in facilitating the development of PDAC. Efforts have been made to identify components of the stroma which we can manipulate, using therapeutics, to shift the stroma towards a tumour restraining phenotype. Such efforts have had mixed success but, regardless of outcome, they enhance our understanding of how stromal elements can be manipulated and with what result.

ECM

The copious desmoplasia present in PDAC has made it the frequent target of therapeutics with specific targeting of ECM components yielding varied outcomes. Such approaches allow us to discern the influence of discrete ECM proteins on the regulation of PDAC carcinogenesis.

HA is a high molecular weight glycosaminoglycan pervasive in the PDAC stroma. In PDAC HA is heavily bound to water leading to the formation of a gel-fluid interstitial phase which increases interstitial pressure, driving collapse of vasculature and impeding drug delivery[121,224,239]. Hyaluronidase is able to degrade HA and has been encapsulated in polyethylene glycol (pegylated recombinant human hyaluronidase 20 (PEGPH20)) to generate a therapeutic agent capable of degrading HA with an improved *in vivo* half-life. GEMMS of PDAC treated with PEGPH20 showed a reduction in interstitial pressure increasing vascularization and synergizing with chemotherapy to reduce tumour growth[224]. On the back of such promising pre-clinical data, a clinical study was carried out using either PEGPH20 in combination with FOLFIRINOX or FOLFIRINOX alone to treat metastatic PDAC[240]. Unfortunately, the combination treatment containing PEGPH20 reduced overall survival from 14.4 months in the FOLFIRINOX arm to 7.7 months. Whilst these results were disappointing, it was noted that 4 patients in the combination treatment group had a complete response whereas 0 patients did in the control arm. An additional clinical trial attempted to identify patients who were more suitable for PEGPH20 treatment by identifying patients who had high levels of HA in the ECM[241]. Patients with elevated HA were treated with PEGPH20 in combination with gemcitabine and nab-paclitaxcel or the chemotherapeutic agents alone. Disappointingly, no benefit in overall survival was detected in patients receiving the combination treatment and the study was abandoned. Thus, whilst degradation of HA in the stroma of PDAC showed promise in pre-clinical testing of GEMMs it does not appear to be a useful candidate in treating metastatic human disease.

Lysyl oxidase-like 2 (LOXL2) is an enzyme involved in remodelling the ECM of PDAC through cross-linking collagen fibres. Lysyl oxidase (LOX) activity was found to promote

metastasis of cancer cells through cross-linking of collagens in the hypoxic and fibrotic stroma observed in breast cancer[242,243]. Abrogation of LOX activity could prevent fibrosis-enhanced metastasis in pre-clinical GEMM models. Additionally, in gastric cancer high expression of LOXL2 was found to negatively correlate with patient survival and promote metastasis in cancer cell lines[244]. A monoclonal antibody against LOXL2 was found to ablate metastasis in GEMM of gastric cancer. This prompted the development of a clinical trial to assess the utility of an antibody against LOXL2 (simtuzumab) in combination with chemotherapy in treating metastatic PDAC[245]. Again, the promising pre-clinical findings did not translate into the clinical setting and the trial was abandoned as patients showed no increase in overall survival following the combination therapy.

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes capable of degrading components of the ECM. MMPs have frequently been associated with enabling metastatic spread of cancer cells and as such have been identified as potential therapeutic targets of the ECM[246]. However, the use of two broad inhibitors of MMPs have had disappointing results in clinical trials. Marmistat and Tanomastat were compared against gemcitabine with both MMP inhibitors yielding worse outcomes than chemotherapy[247,248]. It has been suggested that targeting specific MMPs may be preferential to these broad inhibitors as certain MMPs', including MMP8, expression levels are positively correlated with survival[249]. In contrast, antibodies which target MMP9 specifically can synergize with chemotherapeutics to improve overall survival in mouse models of PDAC.

Targeting the ECM has shown promise in pre-clinical models but so far, such findings have not been translated effectively into a clinical setting. This does not exclude the possibility that such approaches are valuable and as our comprehension of the composition of the ECM and the precise tumorigenic functions of proteins within it augments, therapeutic intervention may yet improve survival in PDAC.

Immune cells

Immune cells represent one of the most obvious candidates for therapeutic intervention. The obviousness of their candidacy is derived from the native capacity for immune cell populations to directly attack and destroy cancer cells. PDAC fosters an immunosuppressive microenvironment through recruiting and polarizing classes of immune cells, and other stromal cells, which abet the cancer cells growth and spread by suppressing the activity of tumour restraining immune cell populations. Through immunotherapy we can attempt to remodel the immune composition of the stroma turning the "cold" immunosuppressive microenvironment into a "hot" tumour-suppressive one.

Some of the most familiar and effective immunotherapies established are checkpoint inhibitors. PD-1 is an inhibitory receptor on $CD8^+$ T-cells which when ligated can suppress T-cell activation and proliferation. The PD-1 ligand, PDL-1, is frequently expressed on cancer

cells and is a mechanism through which they can evade killing by CTLs, including in PDAC where PDL-1 expression levels are negatively correlated with survival[250,251]. An additional inhibitory receptor on T-cells is CTLA4 which can compete with the co-stimulatory receptor CD28 for the stimulatory ligands CD80 and CD86 on APCs. This inhibits T-cell priming by APCs and reduces their anti-tumour potential. CTLA4 expression on $CD8^+$ T-cells has also been established as a marker of poor prognosis[252]. Immune checkpoint blockade (ICB) uses antibodies which prevent the binding of inhibitory receptors to their ligands by competitively binding PD-1, PDL-1 and CTLA4. Antibodies targeting these components can be administered as monotherapies, where only one antibody is administered, or as combination therapies where multiple antibodies targeting individual components are administered simultaneously. Despite ICB proving an effective treatment in other solid malignancies, trials in PDAC have yielded disappointing results[253–255]. ICB monotherapy has not provided any benefit in survival and combination immunotherapy alongside radiation or chemotherapy have only pointed to modest improvements[255].

Given the poor response to ICB observed in PDAC alternative strategies have emerged which attempt to augment the efficacy of ICB through reprogramming immunosuppressive myeloid populations which are abundant in the TME of PDAC. Myeloid populations in the TME of PDAC including TAMs and MDSCs can negatively regulate the activity of CTLs[256]. Pharmacological intervention to polarize myeloid populations towards a phenotype which promotes $CD8^+$ T cell infiltration and activity have shown promise in pre-clinical models. Small molecule inhibitors of the CSFR1 receptor on myeloid cells can polarize TAMs towards a pro-inflammatory state which can enhance CTL infiltration and activity and synergize with ICB to suppress tumour growth in GEMMs of PDAC[257]. Response to ICB was also shown to be improved in mouse models of PDAC when combined with an agonist for the myeloid receptor CD11b. CD11b agonism could repress accumulation of immunosuppressive myeloid populations in the stroma and reprogram myeloid cells from an immunosuppressive state towards a phenotype which boosted CTL activity[258]. Despite the promising pre-clinical data, a clinical trial involving a CD11b agonist was abandoned as no clear benefit was observed (NCT04060342) whilst we await data from clinical trials of CSFR1 inhibitors.

An alternative strategy for improving ICB response in PDAC emerged through the observation that treating mouse models of cancer with a monoclonal antibody agonist for CD40 triggered downregulation of PD-1 on $CD8^+$ T-cells[259]. PD-1 expression on $CD8^+$ T-cells was previously shown to indicate responsiveness to checkpoint inhibitors[260]. Initial results in clinical trials showed promise, demonstrating that CD40 agonists could enhance $CD8^+$ T-cells infiltration, activity and proliferation in the PDAC TME[261]. However, the phase II trial was abandoned as the primary endpoint of 1-year overall survival was not met when CD40 agonists were administered in combination with chemotherapy or chemotherapy and ICB[262].

An alternative approach from ICB to immunotherapy involves the generation of chimeric antigen receptor (CAR) T-cells. This methodology involves the isolation of T-cells from patient's blood or tumour tissue and their subsequent genetic manipulation to express a CAR which recognizes a specific tumour-associated antigen[263]. Thus, CAR T-cells can preferentially target tumour cells. In addition, co-stimulatory receptors can also be

expressed on CAR T-cells, enhancing their activation and capacity for self-renewal[264,265]. Once these super-soldier T-cells have been engineered in the lab, they can be reintroduced into patients with such approaches proving efficient in treating haematologic malignancies[266]. Pre-clinical studies in GEMMs of PDAC transplanting CAR T-cells specific for tumour-associated antigens overexpressed in PDAC including mesothelin (MSLN), trophoblast cell-surface antigen 2 (TROP2), carcinoembryonic antigen (CEA) and PDL-1 have shown the capacity to reduce tumour burden[267–270]. The results of CAR T-cell therapy in a clinical setting of PDAC are eagerly awaited.

Immunotherapy has emerged as a pivotal weapon in combating several haematologic and solid malignancies. Despite this, the efficacy of these approaches in PDAC have predominantly been underwhelming. Here we have focused on some of the most promising immunotherapies but these represent only a fraction of the strategies which have shown promising pre-clinical applications. The failure of auspicious pre-clinical studies to translate into clinical solutions is likely reflective of the complexity of the PDAC TME where a heterogeneous milieu of cells and proteins exert compensatory influences on immune evasion. The development of combinatorial immunotherapeutic approaches will hopefully directly improve patient outcomes but should at least provide mechanistic insight on the contribution of stromal elements to immune evasion.

CAFs

The saturation of the PDAC TME with CAFs make them an actionable target for therapeutics. Furthermore, the conflicting capacity of CAFs to augment and restrain tumorigenesis highlights the potential for therapy to manipulate CAFs towards a tumour-supressing phenotype.

CAR T-cells can be employed not only to directly attack tumour cells but also to target stromal cell populations. FAP is highly expressed in the stroma of a number of solid malignancies, including PDAC. FAP^+ CAFs have been reported to enhance tumorigenesis in PDAC[207,229]. Treating GEMMs of various cancers with CAR T-cells which attack FAP^+ cells was capable of bolstering the endogenous anti-tumour $CD8^+$ T-cell response[271]. Furthermore, FAP-specific CAR T-cells could restrain tumour growth and reduce formation of desmoplasia in a GEMM of PDAC[272]. A small phase I clinical trial using FAP-CAR T-cells to treat pleural mesothelioma reported that no serious side effects were observed[273]. A more recent clinical trial (NCT03932565) using CAR T-cells to target FAP and Nectin4, a cell surface molecule overexpressed in cancers including PDAC, is currently underway[274]. FAP^+ stromal cells can also be targeted through the administration of antibody-drug conjugates (ADCs). ADCs are capable of binding specific surface proteins triggering the internalization of the cytotoxic agent which can then facilitate cell death. A FAP specific ADC (OMTX705) has been developed and treating GEMMs of PDAC with OMTX705 abolished tumour burden when combined with chemotherapy[275].

CAF phenotype can be directly modulated using compounds which modulate vitamin D receptor (VDR) activity. As PSCs are activated to form CAFs they lose the presence of cytoplasmic lipid-rich droplets. Sherman *et al*, found that treating CAFs with the VDR ligand calcipotriol could revert them to quiescent PSCs with characteristic cytoplasmic lipid droplets[276]. Treating GEMMs of PDAC with the VDR agoinst inhibited CAF activation, reducing desmoplasia, enhancing vascularity and synergized with chemotherapy to inhibit tumour growth. A number of clinical trials are ongoing to assess the utility of VDR agonists and vitamin D supplementation in resectable and advanced PDAC.

Chronopoulos *et al* also investigated the possibility of reverting CAFs to a quiescent state[277]. Cytoplasmic droplets in quiescent PSCs are rich in vitamin A. Treating PSCs with all-trans-retinoic-acid (ATRA), a vitamin A metabolite, was able to induce mechanical quiescence in PSCs. Mechanical quiescence was defined as the inhibition of PSC-derived CAFs to generate high traction forces and respond to mechanical signals. This suppressed the ECM remodelling capabilities of PSC-derived CAFs, resulting in an inhibition of local cancer invasion in a 3-dimensional organoid model system. The inhibition of ECM remodelling by PSC-derived CAFs through promoting PSCs to a quiescent-like state with ATRA treatment is also currently being assessed in clinical trials.

A recent and extremely promising pre-clinical study from Koikawa *et al* identified peptidylprolyl cis/trans isomerase NIMA-interacting 1 (Pin1) as a druggable target in PDAC[278]. Pin1 expression was negatively correlated with survival and positively associated with a desmoplastic and immunosuppressive TME. Inhibiting Pin1 suppressed CAF activation and reduced the formation of desmoplasia. It also directly acted on cancer cells upregulating PDL-1 and the gemcitabine transporter ENT1. This led to the complete eradication of tumours in GEMMs of PDAC when combined with immunochemotherapy. Building on these findings Liu *et al*, found that conjugating a Pin1 inhibitor to anti-FAP antibodies to selectively target CAFs was able to co-operate with a novel immunotherapy, using aptamers to engage CTLs, to also eradicate tumours in a GEMM of PDAC[279]. Clinically available drugs which inhibit Pin1 already exist and their capacity in a clinical setting to treat PDAC will be of much interest given the promising pre-clinical studies.

CAF subtypes show differential plasticity and can be manipulated, through pharmacological intervention, to switch between phenotypes. Biffi *et al* demonstrated that iCAF formation could be suppressed through the use of JAK/STAT inhibitors[201]. Treating a mouse model of PDAC with JAK/STAT inhibitors polarized CAFs towards a myCAF phenotype and resulted in repressed tumour growth. Although inhibiting myCAF formation with a TGF-β receptor inhibitor did not affect tumour growth, suggesting repression of iCAFs was responsible for diminishing tumour burden. The IL-1 receptor was demonstrated to be responsible for the signalling cascade which promoted iCAF formation and clinical trials are currently being conducted using IL-1 receptor antagonists to suppress iCAF formation, and promote myCAF formation, in a clinical trial.

Since iCAFs have been associated with promoting tumorigenesis, treatment approaches targeting iCAF-derived cytokines are also being developed. A study found that targeting IL-6, an iCAF secreted cytokine, with an antibody blockade could trigger suppression of tumour growth[280]. IL-6 blockade alone did not have a pronounced effect but could effectively synergize with ICB to reduce tumour growth in a $CD8^+$ T-cell dependent manner. An additional study found that treating PSCs with an inhibitor targeting heat shock protein 90 (Hsp90) could reduce IL-6 secretion by PSCs[281]. Administering the Hsp90 inhibitor to a GEMM of PDAC in combination with ICB depleted both iCAFs and myCAFs *in vivo* and was associated with increased $CD8^+$ T-cell infiltration and a reduction in tumour burden. The utility of combining IL-6 inhibitors with ICB is also under current assessment in clinical trials.

An additional CAF secreted factor which is the subject of therapeutic intervention is the chemokine CXCL12, which has been reported as an iCAF marker[197]. As described above Feig *et al* characterized a tumour supporting role for CAF-derived CXCL12 in excluding $CD8^+$ T-cells from the PDAC TME[229]. Through inhibiting the CXCL12 receptor, C-X-C motif receptor 4 (CXCR4), they could mitigate the immunosuppressive effect of the chemokine and elevate the efficiency of ICB. A phase II trial involving combination treatment of a CXCR4 inhibitor with immunochemotherapy demonstrated elevated $CD8^+$ T-cell intrusion and a reduction in MDSC accumulation in tumours of patients receiving the treatment, suggesting the regime should advance to later phases of testing[282].

Whilst pre-clinical results targeting CAFs and a number of ongoing clinical trials are cause for optimism in the battle to combat PDAC, past trials have highlighted the need for such optimism to be tempered. Inhibiting Hh signalling in a GEMM of PDAC could reduce desmoplasia and increase drug delivery in pre-clinical models of PDAC. However, in clinical trials Hh inhibtors have failed to benefit patients with one trial being abandoned early due to a reduction in overall survival (NCT01130142, NCT01195415)[283,284]. As described above, additional pre-clinical studies subsequently demonstrated that Hh signalling could also suppress PDAC tumorigenesis and enhance myCAF formation[202,237]. This highlights the need to comprehensively define the tumorigenic functions of drugs in pre-clinical models and proceed with caution where contrasting findings are observed.

One of the most promising clinical results from therapeutically targeting CAFs has come from the use of an angiotensin inhibitor, losartan. In a GEMM of PDAC losartan was found to deplete profibrotic stromal properties in CAFs[285]. Interestingly, losartan downregulated expression of both myCAF markers (*Col1a1*) and iCAF markers (*Has1* and *Has2*). The depletion of fibrosis enhanced vascularization of the tumours allowing more efficient delivery of chemotherapeutic agents. In a phase II study patients with untreated locally advanced and unresectable PDAC were treated with losartan and FOLFIRINOX[286]. Significantly, 86% of patients were able to undergo surgical resection following the neoadjuvant therapy with R0 resection (surgical margin microscopically negative for residual tumour) achieved in 69% of patients. The hugely encouraging results of this trial provide optimism in the capacity to therapeutically target CAFs in the stroma of PDAC as a measure to treat PDAC.

CAFs domination of the stroma and capacity to regulate tumorigenesis make them appealing candidates for therapy. Through accurately characterizing the regulation, phenotypic heterogeneity and tumorigenic contributions of distinct subtypes we can advance the formation of desperately needed novel therapeutics to facilitate beneficial patient's outcomes.

INTRODUCTION IN SUMMARY

If I were to summarize what we have covered so far in a sentence, I would state "PDAC is a highly lethal malignancy characterized by an extensive desmoplastic stroma comprised of a heterogeneous milieu of cells and proteins which exert contrasting influences on disease progression and therefore represent candidates for therapeutic intervention". A little bit of a mouthful but in my defence, we are on page 45. Really, what I hope to have accentuated is that the stroma of PDAC represents both an impediment and an opportunity in the development of therapeutics to treat the disease.

The aberrant deposition of ECM proteins in PDAC, which collectively form the matrisome, generates a pathogenic niche which can regulate biophysical properties of tumours directly influencing the phenotype of cancel cells. The matrisome also directs cell fate of stromal cell populations with distinct elements recruiting and regulating cellular components which can both restrain and augment tumorigenesis.

Immune cells are fundamental to resisting the growth and spread of cancers. Despite this, they are vulnerable to manipulation by cancer cells which seek to evade their anti-tumour properties and coerce them towards a pro-tumorigenic phenotype. This can be accomplished directly through the secretion of immunosuppressive proteins and indirectly through the modulation of other stromal elements including cancer-associated fibroblasts (CAFs).

CAFs are prevalent in the PDAC TME and originate from diverse cellular origins. Cancer cell-derived signals orchestrate the differentiation of spatially and phenotypically distinct subpopulations of CAFs. These subpopulations mediate diverse pathophysiological roles in the TME through directly interacting with neoplastic cells, secreting matrisomal elements and modulating the phenotypes of other stromal cells.

Whilst stromal elements may disrupt the aggressive development of PDAC they are fighting a losing battle. Without intervention cancer cells will proliferate and metastasise to devastating effect. The accessibility of the stroma to therapeutics make it more easily druggable than the malignant cells sheltering within. The plasticity of cell types in the TME and their capacity to interfere with tumorigenesis represent a therapeutic opportunity and stromal-targeting approaches have yielded therapeutic benefit. In order to capitalize on this

opportunity, we need to better comprehend the composition, regulation and pathophysiological contributions of stromal components in PDAC.

In this thesis we identify galectin-4 as a matrisomal protein upregulated in PDAC which promotes immunosuppression and could therefore represent a druggable target. We subsequently provide a comprehensive characterization of CAF subtypes in the PDAC stroma. In addition, we validate the utility of an *in vitro* three-dimensional co-culture model system in recapitulating this CAF heterogeneity to study the regulation of CAF subtype formation. Furthermore, we demonstrate that the differential fate of CAFs can be pharmacologically manipulated within this model system and perform a high-throughput drug screen to identify compounds which suppress tumour cell growth indirectly through the action of CAFs. Together these findings equip us with the potential to develop desperately needed novel therapeutics in PDAC.

AIMS OF THIS THESIS

The overall aims of this thesis were to better characterize the composition of the stroma in PDAC in order to define potential therapeutic targets as well as identify small molecules which can restrain tumorigenesis indirectly through modulating the stroma.

The specific aims of the thesis were as follows:

Paper I

- Determine how the overexpressed matrisome protein galectin-4 modulates tumorigenesis in PDAC.

Paper II

- Characterize the landscape of CAF heterogeneity in PDAC.
- Recapitulate CAF heterogeneity *in vitro* using a three-dimensional co-culture model system to investigate regulation of CAF subtype formation.

Paper III

- Determine whether CAF differentiation can be manipulated *in vitro*.
- Identify pharmacological agents which can suppress tumour growth through manipulating CAF phenotype with a high-throughput drug-screen.

MATERIALS AND METHODS

Our capacity to understand reality is restrained by the modalities through which we can interrogate it. Here we will briefly summarize the principles of the methodologies we have employed in this thesis. Detailed accounts of the materials and methods are present in Papers I, II and III.

Immunohistochemistry (IHC) – Paper I

IHC allows for the detection of specific antigens in tissues or cells through the binding of an antigen-specific antibody. To allow antibodies to recognize antigens in tissues or cells fixation allows for the maintenance of the structural integrity of tissues and antigens. This can be achieved with organic solvents which dehydrate cells and precipitate proteins, including antigens, or cross-linking agents which chemically bond them to other macromolecules within cells. However, fixation also impedes the access of antibodies to the antigen which is circumvented through heat or enzymatic digestion. Furthermore, if the antigen is intracellular the cell membrane needs to be permeabilized using detergents which perforate the plasma membrane. Primary antibodies which directly bind antigens can then be incubated with the tissue before a secondary antibody which binds to the primary antibody is added. During addition of the primary antibody normal serum, from the species the secondary antibody is taken from, is added to block non-specific binding of the antibody to active sites. The secondary antibody is labelled with an enzyme which can catalyse a substrate to facilitate its' detection. Here, we use secondary antibodies labelled with horseradish peroxidase (HRP). To prevent endogenous peroxidases from also reacting with the substrate their activity was blocked with H_2O_2. The substrate DAB was then added which becomes oxidized by HRP to form a brown colour at the sites where antigens are detected by the antibodies. To visualize the tissue structure, we used a haematoxylin stain which non-specifically stains cellular components. Whilst, IHC can be used to validate the presence of an antigen it cannot be used to quantify the expression levels of antigens.

We use IHC to detect the presence of Gal4 and markers of distinct immune cell populations in murine tumour tissue in **Paper I**. Stained tissue samples were scanned to generate images which could then be quantified using imaging software.

In situ hybridization (ISH) – Papers I and II

The principles of ISH are similar to those covered in IHC, although rather than using antibodies specific to antigens, gene specific probes which hybridize with RNA molecules are used. Fixed and permeabilized tissue sections are incubated with gene specific probes and to aid detection hybridized probes are targeted with hybridization amplifiers which provide more sites for detection agents to bind. Hybridized probes are then labelled either with an

enzyme **(Paper I)** or a fluorescent molecule **(Paper II)**. For the enzymatic approach taken in **Paper I** two enzymes were bound to specific probes each targeting a different gene which gave either a blue or red colour when exposed to a substrate allowing for the visualization of two genes. The fluorescent approach taken in **Paper III** ligates independent gene specific probes with differently coloured fluorescent molecules facilitating the independent detection of up to four genes. To identify cells the fluorescent nuclear stain DAPI was added to tissue sections. The expression of genes could then be detected using brightfield **(Paper I)** or fluorescent microscopy **(Paper II)**. ISH allows for the simultaneous detection of multiple genes in a single cell.

We utilized ISH in **Paper I** to visualize *Lgals4* expression alongside the epithelial cell marker *Krt19* or CAF marker *Fap* in murine PDAC tumours. In **Paper II** ISH was used to detect *IFIT3* and *RSAD2* expression alongside the CAF marker *DCN* and epithelial cell marker *KRT19* in human PDAC tumour samples.

Enzyme-linked immunosorbent assay (ELISA) – Papers I and II

ELISA can be used to quantify the concentration of a soluble antigen in solution. In **Papers I and II** we use a sandwich ELISA. The basic principle of this is to coat wells of a plate with an antibody for a specific protein. Non-specific binding of the antibody to proteins is subsequently blocked using bovine serum albumin (BSA). A solution containing the antigen of interest can then be incubated in the coated wells where the antigen will bind to antibodies coated on the surface of the well. A detection enzyme, specific to a separate epitope on the antigen, bound with a chromogenic enzyme, is then added to bind to antigens attached to the coated antibody. Addition of the substrate chromogen to wells results in a colour change in the presence of the enzyme. The intensity of this colour change can be quantified through measuring the optical density in each well. A standard curve is generated from the optical densities of a serial dilution of the antigen at known concentrations. Optical densities for samples with unknown concentrations can be interpolated into the standard curve to quantify the concentration of antigen in solution.

In **Paper I** we use ELISA to assess the circulating levels of Gal4 in patient serum and in **Paper II** we quantify the levels of Cxcl10 secreted by PSCs following drug treatment.

The cancer genome atlas (TCGA) data analysis – Paper I

The cancer genome atlas (TCGA) is a publicly available genomics program containing molecular characterizations of tumour samples and matched controls with complementary clinicopathological data. We used the Xenobrowser platform to download normalized gene counts from bulk RNA sequencing data profiling pancreatic tumours[287]. We selected only

cases of pancreatic cancer which had been annotated as PDAC by the authors. To interrogate the influence of *LGALS4* on survival we divided patients into high and low expression groups and used a Cox proportional hazards regression model to assess statistical significance between groups. Patients were split into two groups at percentiles and log-rank scoring was used to assess P-values at each percentile. The percentile with the lowest P-value was used to select the cut-off between high and low groups. Kaplan-Meier survival analysis was then used in **Paper I** to test for significant differences in survival between *LGALS4* high and low groups.

We obtained gene signatures for cytolytic effect and $CD8^+$ T-cell activation scores from Azizi *et al*[288]. To quantify how gene expression for multiple genes differs between samples we calculated Z-scores, number of standard deviations from the mean, for each gene in a signature as follows:

$Z = (x - \mu) / \sigma$, where x = normalized gene count, μ = mean gene count, σ = standard deviation.

The mean for all genes of a signature was used as the signature Z-score. In **Paper I** we compared cytolytic effect and $CD8^+$ T-cell activation signature Z-scores between the previously defined *LGALS4* high and low groups.

Read counts derived from bulk RNA sequencing of tumours give a global gene expression profile across all cells present in the sample. It can therefore be used to identify differential gene expression between tumours but cannot elucidate the contribution of different cell populations to overall expression. Variable cellularity between tumour samples can therefore also drive differences in gene expression meaning results inferred from it should be treated with caution.

Mouse models – Paper I & II

All animal experiments were given ethical approval from the Swedish board of agriculture. Animals were housed in accordance with Swedish rules and regulation on medical research. Animal experiments that were conducted in Cold Spring Harbour laboratories adhered to guidelines from the institutional animal care and use committee. We obtained C57BL6/J and $RAG1^{-/-}$ mice from Jackson Laboratory, USA and they were housed at Umeå University UCCB. In **Paper I and II**, C57BL6/J and $RAG1^{-/-}$ mice were used for orthotopic transplantation of tumour and control cells and immune cell isolation.

Isolation and culture of murine cell lines – Papers I, II and III

Murine PDAC and normal organoid and 2D cell lines used in **Papers I, II and III** were a kind gift from Professor David Tuveson at Cold Spring Harbour Laboratory, USA. PDAC cell lines were derived from the KPC mouse model (Pdx1-Cre, LSL-Kras$^{G12D/+}$, LSL-Trp53 $^{R172H/+}$) of PDAC.

Organoid cell lines were isolated using a method Boj *et al* established for growing organoids from normal and cancerous pancreatic tissue[289]. In this method tissues are first enzymatically digested to obtain a cell suspension. Rather than attaching cells to plastic, as in adherent 2D cell culture, organoids are grown by seeding dissociated tissue in 3D using Matrigel, a heterogeneous mix of ECM proteins, which facilitates the formation of multicellular spheroid structures mimicking the formation of tumour glands embedded in ECM observed *in vivo*. Tumour organoid cell lines are grown in a specific murine feed culture medium comprised of a cocktail of growth factors and compounds which stimulate their aggregation and proliferation[289]. To passage organoids, they are fragmented using a fire-polished pipette, in a reduced culture media lacking the growth factors in feed culture media, and a fraction of these fragments are re-seeded in a Matrigel dome. Adherent 2D cell lines were grown in 2D culture media, DMEM with FBS and antibiotics (penicillin/streptomycin).

PSCs used in **Papers II and III** were isolated using a method established by Apte, *et al*[156]. This involves enzymatically digesting the pancreas before using density centrifugation to separate biological components. During density centrifugation PSCs segregate into a distinct fuzzy layer from which they can be isolated. Once isolated PSCs can be grown in adherent 2D culture in 2D culture medium.

For our co-culture model system used in **Papers II and III** we co-cultured tumour organoids alongside PSCs. To generate co-cultures, we first mechanically fragmented tumour organoids or enzymatically dissociated them to single cells. PSCs were then mixed with tumour organoids in Matrigel and seeded together in co-culture or separately in monoculture. Culturing PSCs in Matrigel prevents them from gaining a myofibroblastic phenotype through plastic activation and instead maintains them in a quiescent state. Co-cultures and monocultures were grown in either reduced culture medium or 2D culture medium.

Splenocytes used in **Paper I** were isolated by surgically removing spleens from C57BL6/J mice. Spleens were finely minced in PBS with FBS to generate a cell suspension and tissue fragments removed by filtering the cell suspension. The cell suspension was then centrifuged and resuspended in ammonium-chloride-potassium (ACK) lysis buffer to lyse red blood cells. To isolate T-cells used in **Papers I and II** we use magnetic beads coated in antibodies specific for all immune cell populations except the desired T-cells. Cells are mixed with the

magnetic beads in a tube and then placed in a magnet causing for immune cells which are bound to the beads to adhere to the side of the tube. T-cells which do not bind the beads can then be isolated from the solution. Once isolated T-cells were activated using beads coated in antibodies which ligate the CD3/CD28 receptors on T-cells, stimulating their activation. To enhance proliferation and maintain activation of T-cells they are cultured in media containing recombinant IL-2. T-cell media also contained FBS, HEPES buffer (maintains a physiological pH), GlutuMAX (L-glutamine supplement), antibiotics and β-mercaptoethanol which is added daily to prevent accumulation of oxygen radicals from reaching toxic levels.

Transfection of fluorophores and immortalization of cell lines – Papers I, II and II

In this thesis we employ lentiviral transfections to immortalize and generate fluorescent cell lines. Lentiviral transfections utilize the capacity of retroviral lentiviruses to integrate exogenous genetic material into a host cell genome. A lentiviral vector, a modified form of the viral genome lacking the essential sequence for replication, containing the target gene, promoter elements and a viral packaging sequence is transfected into a packaging cell using a transfection method such as electroporation or lipid-based transfection. In this thesis, we use third generation technology where packaging cells are co-transfected with separate viral packaging plasmids, each of which contain genes encoding essential viral structural proteins, which generate infectious viral particles. Third generation lentiviral technology improves the safety of using lentiviruses by introducing the structural plasmids separately which ensures the virus can only replicate in the packaging cell.

Once packaging cells, here HEK 293T cells, have been transfected they are left for a period of viral production before the infectious viral particles are collected in the media. The infectious lentiviral particles can then be added to the target cell for transduction, to integrate the gene of interest to the host genome. In addition to the gene of interest we introduce a gene which confers resistance to an antibiotic which allows us to select for cells which have successfully been transfected by treating the cells with high concentration of an antibiotic, which is normally toxic to cells.

We use lentiviral transfections to generate immortalized PSCs by integrating the Simian virus 40 (SV40) into the genome. This immortalizes cells through inactivating tumour suppressor genes and activating cell cycle regulators and telomerases. We also generate fluorescently-labelled PSCs (expressing mKate2 and mCherry) and tumour organoids (expressing GFP and turboGFP) using lentiviral transfections.

Lentiviral transfections allow us to introduce target genes into a cell of interest which are resistant to other transfection methods such as electroporation. However, target genes insertions into the host cell DNA can lead to unintended insertional mutations which can modify cell phenotype.

Generation of Gal4-KD organoid and Gal4-KO 2D cell lines – Paper I

We also use the above described lentiviral transfection method to modulate Gal4 expression in tumour cell lines in **Paper I**. To generate Gal4-knockout (Gal4-KO) 2D cell lines we use the CRISPR-Cas9 method. Briefly we transfect target cells to stably express Cas9. We then introduce small guide RNAs (sgRNAs), specific to regions in Exon1 and Exon2 of Gal4, through lentiviral transfection to the target cell line genome. sgRNAs associate with Cas9 and guide it to the site, here the Gal4 gene, where it can cleave the DNA and inactivate the gene, in our case introducing frameshift mutations which truncate the protein. In addition to sgRNAs, plasmids contained GFP which could be used to verify where successful transfection had occurred. We use sgRNAs targeting an inactive region of the genome, the Rosa26 locus, as a control cell line for the GAL4-KO cell lines to control for the effects of the lentiviral transfection and Cas9 expression in the target cell line and maintain normal expression of the target protein.

Lentiviral transfections were also used to knock-down Gal4 expression (Gal4-KD) in tumour organoid cell lines in **Paper I**. This involves introducing a short-hairpin RNA (shRNA) expression cassette into the host cell genome. Transcribed shRNA sequences form a hairpin structure which is targeted and degraded by the host cell machinery to generate small-interfering RNAs (siRNAs) which are complementary to the target genes mRNA. siRNAs associated with the target gene's mRNA preventing translation and resulting in degradation of the transcripts resulting in reduced expression of the protein. In addition to sequences specific to the target, scrambled unspecific sequences are introduced to control for the effects of the lentiviral transfection in the target cell line and maintain normal expression of the target protein.

Orthotopic transplantations – Paper I

In order to assess how inhibiting Gal4 expression impacted tumorigenesis *in vivo* **(Paper I)** we orthotopically injected Gal4-KD and Gal4-KO cell lines and relevant controls into C57BL6/J mice. Mice aged between 42 and 52 days were sedated for surgery and monitored post-surgery according to ethical guidelines. Gal4-KO and relevant control cell lines suspended in culture media were injected into the splenic lobe of the pancreas. For the organoid Gal4-KD and relevant controls the cells were re-suspended in 30% Matrigel and culture media prior to injection. To prevent surgical error from influencing the results, mice were excluded from studies if excessive bleeding, leakage or no clear injection bubble were present. Orthotopic transplants allow for us to introduce modified cell lines into the relevant biological niche which is essential for augmenting our understanding in a relevant *in vivo* disease context. It is however, an invasive and technically difficult procedure, when using the pancreas, which can result in animals being sacrificed without enrolment and the trauma of the procedure may also impact the findings.

Flow cytometry and fluorescence activated cell sorting (FACS) – Papers I, II and III

Flow cytometry allows for the rapid classification of high numbers of individual cells based on the expression of cell-surface or intracellular molecules. Flow cytometry requires a single-cell suspension, as such *in vitro* cultures or tissue taken from *in vivo* must first be dissociated. To prevent cells from re-aggregating in solution the chelating agent EDTA can be added which binds and sequesters calcium preventing cadherins from joining cells. Flow cytometry facilitates the detection of antigens through antibodies. If the antigen of interest is at the cell-surface then cells can be kept alive for antibody staining. Where intracellular antigens are being profiled then cells must be fixed and permeabilized, in a similar manner to IHC. To detect the antigen of interest, live or fixed cells are incubated with a primary antibody specific to the antigen. Prior to or during incubation with the primary antibody non-specific binding can be blocked using BSA or foetal bovine serum (FBS). Certain immune cells also possess Fc receptors, which can non-specifically bind antibodies and must therefore also be blocked with proteins that ligate these receptors prior to incubation with the primary antibody. The primary antibody may be directly conjugated with a fluorophore or can be bound with a secondary antibody, specific to antibodies from the species the primary was created in, which is conjugated with a fluorophore. A cocktail of antibodies labelled with discrete fluorophores can be added to a cell suspension to identify the expression of multiple antigens simultaneously. In addition to antibody staining, fluorescent compounds which interact with intracellular components can be used to assess cell viability. Such compounds can only access intracellular components in dead cells where the integrity of the plasma membrane is lost.

The basic principle of flow cytometry relies on the flow of a cell suspension through a small nozzle which funnels individual cells into a narrow stream that passes across an interrogation point. At the interrogation point distinct fluorophores bound to the cells are excited by lasers which emit light at variable wavelengths. Excited fluorophores in turn emit light which passes through a series of mirrors and filters which facilitate the detection of light at a wavelength specific to the fluorophore. In the absence of fluorophores cells also scatter light which allows for quantification of biophysical properties of cells such as size and granularity. The scatter and emission of light from cells is detected by sensors which convert emitted photons into an electrical current. This allows the quantification of antigen expression as the amount of light emitted is proportional to the abundance of the antigen on a cell.

Flow cytometry allows the simultaneous detection of multiple fluorophores depending on the number of lasers and filters available on the flow cytometer. In order to do so a variety of controls must be applied. Compensation controls correct for spectral overlap between independent fluorophores, unstained controls elucidate background autofluorescence of cells, isotype controls demonstrate non-specific background signals and fluorescence minus one (FMO) controls can be used for positive and negative gating of cell populations.

FACS is a specialized form of flow cytometry in which distinct cell populations are identified and separated based on the expression of a panel of antigens. In FACS, once cells pass the interrogation point the stream is broken into droplets which encapsulate individual cells. An electrical charging ring is used to charge droplets based on the detection of fluorescence at the interrogation point. Deflection plates then attract or repel charged droplets splitting differentially charged droplets into separate streams. Cell populations characterized by different fluorescence profiles, corresponding to variable antigen prevalence, can thus be segregated into streams which flow into collection vessels for each population.

In this thesis we use flow cytometry to characterize immune cell infiltrates in mouse tumours and observe exhaustion and apoptosis of T-cells and tumour cells *in vitro* **(Paper I)**. We measure MHCII expression in PSCs *in vitro* **(Paper II)**. In addition, we use flow cytometry to assess how CAF subtype formation can be manipulated *in vitro* **(Paper III)**. We use FACS to isolate tumour, immune and CAF populations from murine tumours in **Paper I** and mesenchymal cells from human tumours in **Paper II**. We also use FACS to separate CAFs and tumour cells from co-cultures *in vitro* in **Papers II and Paper III**.

Single-cell dissociations – Papers I, II and III

For methodologies used in **Papers I, II and III** including flow cytometry, cell-cell interactions must be disrupted to generate a single-cell suspension. Single-cell dissociations are variable depending on the composition of the starting material. Adherent 2D cultures can easily be dissociated to single cells using trypsin, a proteolytic enzyme which cleaves arginine and lysine residues at the C-terminus, to break down proteins which facilitate cell-cell interactions and adherence to the culture plate. To dissociate cells grown in Matrigel, including tumour organoids, ECM proteins present in Matrigel must first be broken down. This is achieved with dispase, a protease which cleaves components of Matrigel including fibronectin and collagens. Once the Matrigel has been dissolved from cells they can be trypsinized to form a single-cell suspension. This is done in the presence of DNase which prevents cells from clumping together due to the presence of extracellular DNA, released by lysed cells.

To dissociate tumour samples the tissue is first finely minced in media supplemented with DNase. It is then re-suspended in a digestion media that contains DNase, collagenases and dispase and incubated with gentle agitation for a sustained period to break down the dense ECM and free cells to suspension. To prevent over digestion and quench the activity of digestion enzymes, as well as proteases secreted by pancreatic tissue, FBS and defined-trpsin inhibitor are added. The cell suspension is then thoroughly triturated through a pipette tip to further disintegrate clumps of residual tissue into single cells. The cell suspension is passed through a 100μM filter to remove any remaining clumps of tissue. Finally, red blood cells are removed from the cell suspension, to prevent them from interfering with flow cytometry, by lysing them with ACK lysis buffer.

SMART-Seq library generation – Papers I, II and III

SMART-seq can be employed to generate complementary DNA (cDNA) from RNA taken from individual cells or groups of cells. In this thesis we utilize SMART-Seq2 as a bulk RNA sequencing tool to generate cDNA libraries[290]. We employ FACS to directly sort 500 cells from a desired cell population into a lysis buffer which contains triton-x100 to lyse the cells and an RNase inhibitor to prevent degradation of the RNA. Cell lysates are also immediately frozen on dry ice after collection to minimize degradation.

To generate cDNA libraries from independent cell lysates using SMART-seq a number of steps are conducted (Figure 9). First oligo-dT adapters are ligated to the polyadenylated tail of mRNA molecules, from lysed cells, followed by reverse transcription using a specific reverse transcriptase (MMLV) which adds extra cysteine (C) residues to the synthesised first strand to form an overhang. The overhanging untemplated C nucleotides are recognized by a template-switching oligo (TSO) which promotes template switching by the MMLV reverse transcriptase. The TSO oligo also adds a common sequence to the 5' ends of the double stranded DNA which can be recognized by an ISPCR primer. This ISPCR primer is used for cDNA amplification with PCR. The amplified cDNA is then tagmented, a process in which the DNA is simultaneously fragmented and adapters inserted at cleavage sites. This is accomplished using a Tn5 transposase that randomly cleaves the DNA and inserts adapters. The tagmentation is conducted over 5 minutes in an attempt to limit the number of cleavages to generate fragments of between 200 and 800 base pairs. The Tn5 adds two separate adapters at the 5' end which are recognized by read 1 and read 2 custom sequencing primers, used later in sequencing. Only cleaved DNA with fragments containing separate adapters complementary for read 1 and read 2 sequencing primers inserted at the 5' ends of double-stranded cDNA are amplified through semi-suppressive PCR. The tagmented cDNA libraries can then be amplified using indexed adaptors which recognize those inserted during tagmentation. Indexing adapters add conserved barcode sequences to the cDNA which allow them to hybridize with the flow cell during sequencing. In addition, different indexing adapters add distinct conserved sequences to the cDNA which can be used to label independent samples with a known sequence. This allows for multiple cDNA libraries with unique indexes to be sequenced simultaneously and reads to be mapped to independent samples. Once cDNA libraries have been constructed the concentration and distribution of fragment sizes can be quantified using a Bioanalyzer instrument.

SMART-seq is a cost-effective method we use to profile FACS sorted cell populations. One limitation is that as all cells sorted are lysed and a bulk transcriptional profile is generated from the lysate. If cells from a different population, than the one desired, contaminate the sample they contribute to bulk profile. Differentially expressed genes identified in down-stream analysis could therefore potentially be derived from cellular contaminants and artificially influence interpretation of the data.

We use SMART-seq to profile tumour cells from Gal-4KD and control cell line orthotopic transplants in **Paper I**. PSCs treated with IFNγ and DMXAA were profiled using SMART-seq in **Paper II**. In **Paper III** we use SMART-seq to profile tumour organoids and PSCs from mono-cultures and co-cultures following treatment with recombinant proteins and small molecules.

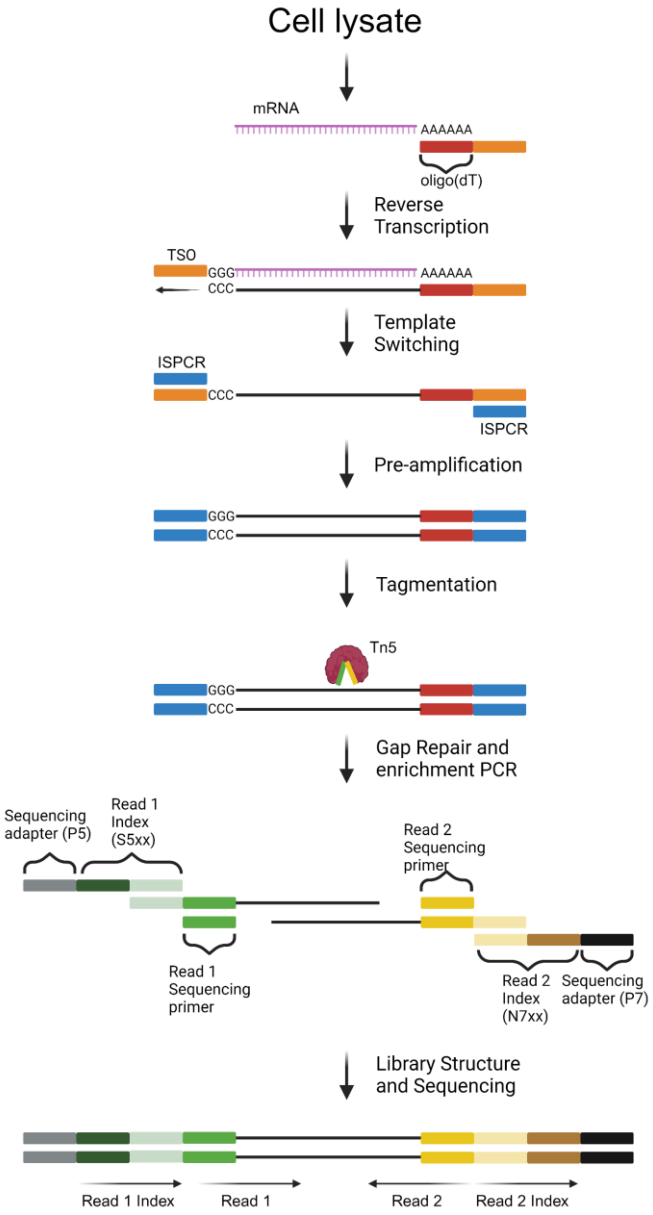

Figure 9. Schematic illustration of the steps to generate and final structure of a SMART-seq library. Created with BioRender.

Single-cell RNA sequencing – library generation Papers I and II

Single-cell RNA sequencing (scRNAseq) has revolutionised are ability to define cellular heterogeneity in complex biological systems. It allows for the generation of transcriptional profiles of individual cells. In this thesis we utilize droplet-based scRNAseq methodologies including the 10x Genomics **(Papers I and II)** and ddSeq Bio-Rad **(Paper II)** platforms.

The basic principle of droplet-based scRNAseq approaches are to use a microfluidics device to encapsulate individual cells, microbeads coated in primers and lysis buffer in sub-nanoliter droplets using a partitioning oil. The primers coated on the microbeads possess a poly(dT) sequence which binds mRNA from the lysed cells within the droplet. Within the lysis buffer are enzymes to conduct reverse transcription which are primed by the poly(dT) to form cDNA complementary to the mRNA. The microbead adapters also contain a conserved sequence which is complementary for the read 1 sequencing primer used later in sequencing. In addition, each microbead adapter has a unique barcode which adds a specific sequence to all cDNA molecules generated within the droplet. Each microbead is attached with multiple adapters each of which has an individual unique molecular identifier (UMI). This sequence allows for each individual mRNA molecule to be differentially labelled. In summary, a cell is lysed within a droplet causing the mRNA molecules to attach to an adapter labelled microbead within the droplet. Reverse transcription generates cDNA which has a cell-barcode common to all cDNAs within the droplet and a UMI which labels cDNAs from individual mRNA molecules separately.

First strand synthesis occurs within the droplet allowing for cDNAs from different cells to be uniquely labelled. After this, droplets are lysed and cDNAs from the same sample pooled. The subsequent steps are similar to those described in the SMART-seq section. Double stranded cDNA libraries are synthesized, amplified and tagmented (ddSeq) or fragmented (10x). During or after these processes indexing adapters, sequencing adapters and the read 2 sequencing primer are integrated into cDNA libraries (Figure 10).

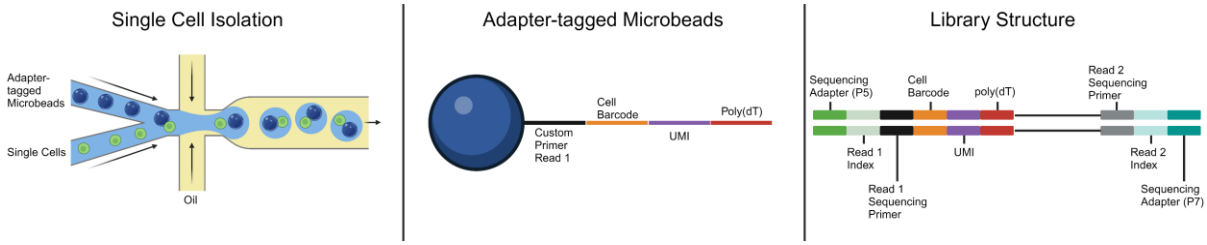

Figure 10. Schematic illustration of cell droplet encapsulation, microbeads adapter structure and final library structure during single-cell RNA sequencing. Created with BioRender.

scRNAseq allows for thousands of individual cells to be transcriptionally profiled within a sample. This circumvents the issues of contamination previously mentioned with bulk RNA-seq as each cell can be independently characterized. However, fewer unique transcripts are identified per cell making it more difficult to detect low abundance transcripts than in bulk RNA-seq. In addition, technical limitations such as multiple cells being isolated in a single droplet and ambient RNA from lysed cells contaminating droplets can confound analysis.

scRNAseq was used to characterize orthotopic transplants of Gal4-KD and control cells lines in **Paper I**. In **Paper II** we profile FAP^+ mesenchymal cells from human PDAC tumours and PSCs co-cultured with tumour organoids *in vitro* through scRNAseq.

Next generation sequencing (NGS) – Papers I, II and III

The nucleotide sequences present in cDNA libraries are defined using NGS. Within this thesis we use the Illumina sequencing platform. Prior to sequencing a size selection is performed using magnetic beads to retain cDNA fragments of similar length and remove contaminations of adapter dimers. Multiple cDNA libraries can be sequenced simultaneously, providing they have unique indexing adapters and common sequencing primers. Equal concentrations of cDNA libraries are pooled together before denaturation to separate dsDNA to single strands.

Denatured libraries are added to flow cells, glass slides with lanes composed of channels coated with two types of oligos. The oligos hybridize to conserved adapters at the 5' and 3' ends of single strands of the cDNA. A complementary strand to flow cell bound single strands is then PCR amplified yielding dsDNA in which a single strand is bound to the flow cell. The dsDNA is then denatured and the unbound strand of DNA washed away. The adapter at the unbound end of each DNA molecule is complementary to the other hybridization oligo on the flow cell. A bridge building step subsequently anneals the unbound end of the DNA to the complementary oligo on the flow cell forming a bridge structure. The DNA is subsequently amplified and denatured, though both complementary strands of the DNA are now tethered to the flow cell at the 5' and 3' ends. This process of bridge amplification is repeated giving rise to regions on the flow cell comprised of clonal replicates of a single DNA fragment referred to as clusters. Following this clonal amplification, the reverse strands are washed off leaving only the forward strands. Sequencing is initiated with the extension of the first sequencing primer to produce the first read. Complementary fluorescently-tagged nucleotides are then sequentially added to the single stranded DNA template in cycles. During each cycle the newly added fluorescently-tagged nucleotides are excited with a light source causing them to emit light with different colours defining each of the four nucleotides. The identity of bases in each cluster can be determined from the colour emitted at each cycle. The number of cycles therefore determines the length of the read. After the completion of the first read the read product is washed away and the read 1 indexing adapter is read in the same manner. After the first read the unbound end is folded to bind the complementary oligo on the flow cell. The DNA is then amplified and denatured and the forward strands are removed leaving only the reverse strands. The read 2 index is then sequenced before the read 2 sequencing primer is added to generate the second read in the previously described manner. Through this process both ends of the DNA fragments are sequenced in two separate reads (Figure 11).

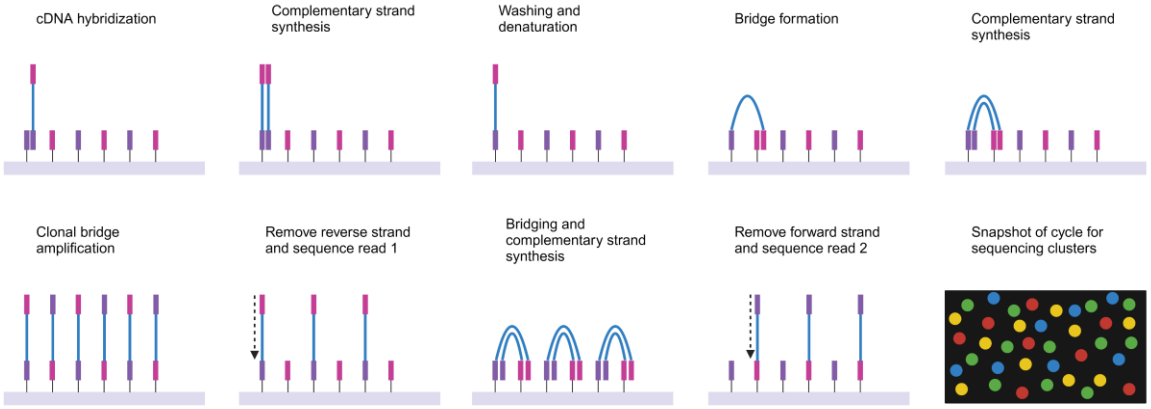

Figure 11. Schematic illustration of steps during next generation sequencing. Created with BioRender.

RNA-seq analysis – generating read counts – Papers I, II and III

To transform the sequencing data into an expression matrix, in which read counts are quantified for genes across individual samples or cells, a number of steps are conducted. During sequencing the Illumina software stores the base call identities for each cluster in binary files called BCL files. The next step is to generate FASTQ files which contain the nucleotide sequence for each read from BCL files. During this process the reads are demultiplexed through identifying sample specific index sequences in individual reads and aggregating them into a single file containing only reads from a specific sample. The reads can then be aligned to a reference genome to map reads to genes. The total number of reads per gene can then be quantified for each sample.

In scRNAseq each sample is comprised of reads mapping to individual cells. Given that each read contains a unique sample index and cell barcode, reads with a matching barcode and index can be classified as belonging to an individual cell from a sample. In addition, specific UMIs are present for cDNA fragments generated from a single mRNA molecule. This allows for the quantification of the absolute number of detected transcripts for a gene, cell or sample. The low amount of starting material in scRNAseq makes it susceptible to biases in PCR amplification. UMIs allow for the removal of PCR duplicates instead counting only the number of reads which correspond to distinct mRNA molecules.

Bulk RNA-seq analysis – differential expression, gene-set enrichment analysis and inferring transcription factor activity – Papers I, II and III

To interrogate biological differences between samples based on an expression matrix the fundamental step is to identify differentially expressed genes. However, technical inconsistencies between samples can generate difference in quantification of gene expression. The expression matrix is therefore pre-processed to mitigate technical sources of variation and instead determine how gene expression varies between samples due to their biological state.

The most crucial step in pre-processing is to normalize the data. Normalization allows for sources of technical variation between samples such as sequencing depth, gene length and RNA composition to be accounted for. Sequencing depth represents the number of reads each sample receives during sequencing. Independent samples may differentially cluster on the flow cell, due to differences in library size, concentration and complexity, and therefore can receive variable numbers of reads. If one sample receives twice the number of reads than another sample, then without normalization, genes will appear to be expressed two-fold higher. Gene length needs to be accounted for when comparing gene expression between genes within a sample. Longer genes are susceptible to PCR bias in amplification during library preparation as they can be tagmented more times. They also have a larger region, therefore more opportunities, for reads to be aligned to them during mapping. Finally, RNA composition can impact differential expression analyses between samples through skewing certain types of normalization. For example, let's consider a normalization approach that aims to mitigate differences in sequencing depth by dividing the expression of each gene within a sample by the total number of reads for that sample. If a sample (sample 1) has a few differentially expressed genes which are detected at very high levels, all genes would be divided by the total number of counts which are inflated by the very high levels of a few genes. This would cause the majority of genes to gain very low normalized expression values. If this was to be compared against another sample (sample 2) without the extremely highly expressed genes then the majority of genes in sample 1 would appear to be expressed at lower levels than sample 2, even if this is not the case.

In this thesis, we use a normalization approach (relative log expression) from the software package DESeq2 for pre-processing the bulk RNA-seq data[291]. Given that we are interested in identifying differentially expressed genes between samples, not different gene expression within samples, gene length is not accounted for by this method. Instead, this normalization approach first calculates a pseudoreference sample defined as the geometric mean of each genes' expression across samples. The ratio of each sample's expression to the pseudoreference is then calculated for each gene. We expect the majority of genes not to be differentially expressed so this ratio should be close to 1 in the absence of technical variation. We then take the median ratio across all genes to calculate the normalization factor. Through taking the median we circumvent the influence of differentially expressed genes as the median should not be affected by them. By dividing the raw counts for each gene in a sample by the sample specific normalization factor we obtain a normalized read count that can

reduce technical differences in gene expression derived from differences in sequencing depth and RNA composition.

Once read counts have been normalized we can identify differentially expressed genes between samples using the DESeq2 package. During this process the package can also regress out unwanted sources of biological variation from the differential expression analysis such as sample replicates. Once we have identified differentially expressed genes between two conditions we can further interrogate biological differences between them.

Gene-set enrichment analysis allows us to define biological pathways which show variable activity between conditions following differential expression analysis. Gene-set enrichment analysis is reliant on previously defined and publicly available gene-sets. These gene-sets are comprised of lists of genes which are differentially expressed during a biological process. During gene-set enrichment analysis genes are ranked by fold-changes or a combination of fold-changes and p-values obtained during the differential expression analysis. We can then determine if genes relating to a biological pathway are overrepresented at the top or bottom of the ranked list of genes. If genes relating to a pathway are overrepresented at the top or bottom of the list, the pathway will be identified as being differentially active. In contrast, if genes from a pathway are absent or are randomly distributed throughout the ranked list then the pathway will not be considered as differentially active. This process allows us to identify biologically relevant pathways from huge lists of genes which are difficult to interpret.

We can further interrogate biological differences from transcriptomics data through inferring transcription factor activity from gene expression. To estimate differences in transcription factor activities through gene expression with bulk RNA-seq data we use Integrated Motif Activity Response Analysis (ISMARA)[292]. ISMARA aims to identify gene regulatory interactions which are underlying the differences in gene expression changes. To do so ISMARA uses a database in which the number of binding sites for a transcription factor in the promoter regions of all genes are computationally modelled. Binding sites are identified based on the presence of conserved sequence motifs in gene promoters which transcription factors are known to recognize. ISMARA can then model gene expression in terms of transcription factor activity. If groups of genes with common transcription factor binding sites in the promoters are upregulated the inferred activity of the transcription factor is higher. The inferred transcription factor activity is given as the expected log fold-change in expression which would result from removing a single binding site from the promoter. This allows for key transcriptional regulators of differential gene expression to be identified helping to better characterize transcriptional programs that differ between conditions.

Single-cell RNA-seq analysis – differential expression, gene-set enrichment analysis and inferring transcription factor activity – Papers I and II

The count matrix generated from a scRNAseq experiment can consist of tens of thousands of cells with expression values for tens of thousands of genes. In order to interpret such complex data a series of data processing steps are conducted which we will summarize here. In this thesis we primarily use the R package Seurat v4 to analyse scRNAseq data[293].

The data is first subject to quality controls steps to remove low quality cells from the analysis and modulate the gene expression matrix to account for technical sources of variation in the data. Prior to droplet encapsulation, cells may die and lyse in the single-cell suspension causing for mRNA to be released into solution. When a cell is captured in a droplet, the droplet may also therefore contain ambient mRNA present in the solution. To account for this we use the package SoupX to estimate the contamination of ambient RNA in each cell and modify read counts accordingly[294]. We then remove low quality cells which have a low number of mRNA transcripts detected, low numbers of genes detected and a high proportion of reads mapping to mitochondrial genes, which can be a sign of cell death. Finally, we use the package DoubletFinder to estimate which cells are doublets, multiple cells encapsulated in a single droplet, and remove them from further analysis[295].

Once we have pruned the read count a global-scaling normalization is performed to account for differences in sequencing depth between cells. The normalization divides the counts for a gene by the total number of counts for all genes, multiples it by a scaling factor and log transforms it for each cell. Once gene expression has been normalized, genes which exhibit high cell-to-cell variation are identified for down-stream data processing steps. Through focusing on these highly variable genes biological signal can be more easily detected in scRNAseq data. Normalized read counts are then scaled for each cell such that the mean of each gene is 0 and the total variance of each gene is 1 across all cells. This gives equal weighting to all genes in down-stream analysis. During this step unwanted sources of variation can be removed by regressing out differences corresponding to certain genes. Here, we remove heterogeneity driven by genes involved in the cell-cycle to reduce the separation of cells that have similar transcriptional profiles but are distinguished by whether or not they are replicating. Once highly variable genes have been identified and scaled we can then perform dimensionality reduction.

Principal component analysis (PCA) is a dimensionality reduction technique that facilitates the transformation of a large number of variables (genes) to a smaller number of variables (principal components) which account for the majority of the variance within the data and can be more easily visualized and analysed. Principal components (PCs) are linear combinations of the original variables which can be ranked based on the proportion of variability they capture within the data. Another way to think of a PC is as a “metagene” which combines the information of multiple correlated genes which are highly variable

within a dataset. Each cell can therefore have values, similar to expression values, for each PC. We determine the dimensionality of the data, the number of principle components, by defining the last point at which the percentage change of variation is more than 0.1 between PCs. To account for technical differences between samples and datasets we perform PCA independently on each sample and integrate the PCA analyses together using a reciprocal PCA (RPCA) which can extract shared variation between datasets.

Our main goal when analysing scRNAseq is to group similar cells together in clusters. To generate clusters, we use the Euclidean distances between cells in the PCA space to embed cells in a graph structure. With Seurat we use a K nearest neighbour graph in which a defined number of edges (K) are drawn between each cell and the closest cells in PCA space. The graph is then partitioned into highly connected communities of cells. We then iteratively group the cells into clusters to optimize the detection of groups of cells with more internal edges than would be expected in a random network. In Seurat the number of clusters detected is determined by a modifiable parameter which can impact how we classify cells. To determine whether clusters are different enough to be considered separate populations of cells we can identify differentially expressed genes between clusters. Here we define the integrity of clusters by ensuring that there are >20 significantly differentially expressed genes with a fold change of >1.5 in a cluster. Once established clusters were visualized in two-dimensions using the non-linear dimensional reduction uniform manifold approximation and projection (UMAP).

Cells which form distinct clusters can be considered as transcriptionally distinct subpopulations of cells. We can determine how clusters differ transcriptionally by identifying differentially expressed genes between clusters. As described in the bulk RNA-seq section we can also identify biological pathways which are enriched in clusters using gene-set enrichment analysis, although we use the package SCPA for scRNAseq data[296]. In addition, we can infer transcription factor activity from gene expression although we use a slightly different approach to the one described for bulk RNA-seq. Instead we use the decoupleR package[297]. decoupleR uses a database of transcription factors and known gene targets to infer transcription factor activity. Each transcription factor target is given an interaction weight such that positive interaction weights indicate that a transcription factor increases a genes expression and negative interaction weights where a transcription factor decreases gene expression. The transcription factor activity for each cell can then be estimated by correlating the expression value for transcription factor target genes and their interaction weights in a linear model.

Finally, we can use scRNAseq data to project a trajectory of cell differentiation. To construct a trajectory the previously mentioned dimensionality reduction and clustering steps are conducted. The root of the trajectory is then specified in a cluster of cells which corresponds to the progenitor population. Cells are then linked based on their transcriptional similarity to estimate pseudotime. Pseudotime values increase as cells become more transcriptionally distinct from the progenitor population. It therefore serves as an estimate of the differential state of a cell. Trajectories can also identify branch points where cells diverge into separate

lineages or cellular states where they begin to develop along distinct differential paths. In this thesis we use the Slingshot package to construct trajectories and estimate pseudotime[298].

Quantitative PCR – Papers I and II

Quantitative polymerase chain reaction (qPCR) facilitates the accurate measurement of gene expression levels through amplification of specific DNA sequences. Prior to qPCR cDNA samples are generated from mRNA. To do so, we first lyse cells to released RNA and then isolate the RNA using a column with a silica membrane that binds the RNA. DNA contamination is removed using a DNase to digest any DNA bound to the membrane. Once RNA has been isolated it is reverse transcribed and amplified to generate cDNA. The PCR reaction is then conducted using cDNA, gene-specific primers, nucleotides and a fluorescent dye which can bind to double stranded DNA. During the PCR reaction the cDNA is first denatured by heat, the primers then anneal to their complementary sequence before a DNA polymerase synthesizes the complementary strand to generate an amplification of the target region. This process is repeated in cycles to exponentially amplify target regions within genes of interest. Binding of the fluorescent dye to the amplified DNA is proportional to the starting concentration and can be used to quantify differences in gene expression between samples. To account for differences in the concentration of total cDNA input to the PCR reaction gene expression is normalized relative to house-keeping genes. Such genes are considered to be uniformly expressed by cells and should therefore be consistent between samples if equal concentrations of cDNA are loaded into the reaction.

We use qPCR to quantify expression of EMT associated genes in tumour cell lines with reduced Gal4 expression in **Paper I**. In **Paper II** we use qPCR to measure differences in inflammatory and interferon-responsive genes in PSCs treated with a stimulator of interferon genes (STING) agonist.

Proliferation assays – Paper III

Proliferation assays allow us to measure cell division and growth over a defined time period. In this thesis, we measure proliferation using fluorescently labelled cell lines. By culturing fluorescently labelled cells in wells of a plate, we can quantify the fluorescence intensities of wells over a time period using a plate-reader (Figure 12). As cells proliferate the fluorescence intensity of a well will increase allowing us to quantify cell growth over time. Each well is normalized to the start point to account for differences in cell numbers of seeded cells. We quantify proliferation of fluorescently labelled PSCs and tumour organoids grown separately in monocultures or together in co-cultures. Prior to seeding, cells were enzymatically dissociated to single-cells before being seeded in Matrigel diluted with reduced culture medium. Once plated, cells were cultured in reduced culture medium at 37°C and fluorescent intensities of wells imaged every 24 hours for 7 days.

Reazurin is a cell-permeable dye which can be reduced by mitochondrial flavoproteins and oxidoreductases to induce a colour change. The intensity of the colour change is proportional to the metabolic activity of cells. We add reazurin to cultures at the end-point of the proliferation assay to quantify metabolic activity. We employ proliferation assays to quantify differences in cell proliferation in response to TGFβ signalling manipulation in **Paper III**. Proliferation assays were also used in the high-throughput drug screen of the co-culture model system in **Paper III.**

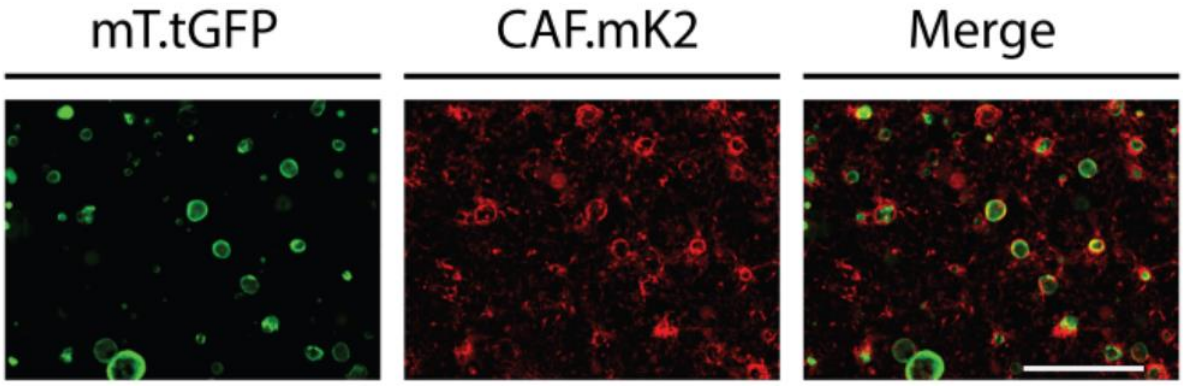

Figure 12. Fluorecent imaging of murine co-cultures. (Left image) Fluorescent illumination of tumour organoids, (centre) fluorescent illumination of CAFs, (right) merged detection of tumour organoids and CAFs. Taken from Paper III.

High-throughput drug-screen of the co-culture model system – Paper III

Drug-screens can be used to identify potential therapeutic agents by testing a large number of chemical compounds against a specific biological assay. In **Paper III** we use the co-culture model system comprised of fluorescently labelled PSCs and tumour organoids to screen a library of compounds. Co-cultures were prepared using the same approach described for proliferation assays. We obtained a library of ~36,000 compounds from the Chemical Biology Consortium Sweden (CBCS SciLifeLaf, Karolinska Intitute). Co-cultures were seeded in reduced culture media containing compounds at 10μM in 384 well plates. Each plate had single wells of co-cultures treated with 352 compounds alongside internal controls. These controls included 8 wells of co-cultures treated the with vehicle control DMSO, 4 wells of co-cultures treated with the compound cycloheximide as a cytotoxic control, four wells of organoid mono-cultures in growth media, four wells of PSCs seeded in reduced culture media and four wells of Matrigel without cells in reduced media. Plates were incubated at 37°C for five days before being sequentially profiled using scanned well spectrophotometry of total fluorescence and imaged using the ArrayScan and Spectrmax platforms.

High-throughput drug screen – analysis and hit selection – Paper III

To identify hits from the screen, we utilized the spectrophotometry total fluorescence data alongside an additional 71 imaging metrics acquired from the high content image analysis. Given that we have ~36,000 wells containing discrete compounds, alongside controls, with multiple variables quantified in each well, we were able to process the drug-screen data using multivariate analysis in a manner similar to the previously mentioned scRNAseq data.

Instead of a matrix with columns of cells and rows of genes, as in scRNAseq, we have a matrix where columns are compounds and rows are imaging metrics but the steps to process the data are comparable to the ones previously described. First low-quality wells are filtered with a non-stringent outlier removal of those with values outside 3 times the interquartile range from the median measure of each metric for each plate. We then performed a scaling for each metric for PCA and PC component filtering. Through plotting wells based on their PC1 and PC2 scores we observed that compounds which induced desired phenotypic characteristics such as reduced organoid growth, unaffected PSC growth and increased proportion of PSCs in proximal regions to tumour cells had elevated PC1 and PC2 values. We therefore selected compounds using the PCA by identifying those with the highest product for PC1 and PC2.

We then used the PCA data to perform a K-means clustering. K-means clustering partitions the data into a predefined number of clusters (K). We use a gap statistic algorithm to select the number of clusters by identifying the point at which increasing the number of clusters diminishes the performance of clustering. K-means clustering segregates data points into clusters with the nearest mean (centroid) using Euclidean distances in the PCA space. Once clusters were generated, Z-scores, calculated for each imaging metric, were used to define the properties of each cluster. We could subsequently identify clusters which were characterized by specific imaging metrics to identify compound clusters which induced a desirable phenotype. Once we had identified the cluster of interest we could select the top compounds within this cluster based on their total Euclidean distance from the origin in all PCs used in the K-means clustering.

In addition to selection of compounds using the PCA and K-means clustering approach we applied an approach which is more traditionally used in drug-screens. For this approach data was filtered in the same manner described above and each metric was standardized plate-wise as a fold change of the mean value of the DMSO vehicle control cultures of each plate. Compounds were then selected according to the criteria outlined in Table 1.

Overall, we used three approaches (PCA, K-means and traditional) to identify hit compounds. We could therefore identify compounds which were shared between identification methods. This allowed us to select 368 compounds that were identified by two methods. We had a pre-specified number of compounds to identify as hits (704), we selected

the remaining compounds equally from each method based on their identification specific ranking.

Metric	Condition	Action
Organoid Count	<50% DMSO co-culture control	Reject
Organoid Count	>155% DMSO co-culture control	Reject
PSC object count	<100% PSC mono-culture control	Reject
"Extra-large" Organoid (>15,000μm²) proportion of total organoid area	>30% of DMSO co-culture control	Reject
Total Organoid Area	<60% DMSO co-culture control	Select; Rank order from smallest to largest

Table 1. Traditional selection criteria from the initial screen.

High-throughput drug screen – hit verification and final compound selection – Paper III

During the initial screen we use only one technical replicate per compound which can lead to a large number of false positives being identified as hits. It is therefore necessary to repeat the screen under identical conditions, now using the 704 selected compounds at three concentrations (5, 10 and 20μM), to verify effects on co-cultures. To compare between the initial screen and the verification screen we filtered and standardized data for each metric in a standardized plate-wise manner, although now as a fold change of the mean value of the DMSO vehicle control cultures of each plate. This was due to the fact that we had selected hit compounds which could skew plate-wise scaling between the initial screen and hit verification screen. We then employ OPLS modelling to validate the multivariate analysis which models the relationship between predictor variables and response variables. To train the OPLS model we used the DMSO vehicle controls, CHX cytotoxicity controls and 704 selected compounds from the initial screen. We used the OPLS model with the verification screen data to predict whether test compounds were classified as vehicle controls, cytotoxic controls or test compounds. Top compounds from the OPLS approach were chosen by rank ordering compounds by their average predictive score to be a test-compounds and selecting the most likely. For the more traditional approach we selected compounds through first selecting those which reduced organoid growth without reducing overall CAF growth. Compounds which met these criteria were then ranked by the area of organoids and those which reduced organoid area the most, at the lowest tested concentration, were taken. (Table 2).

Metric	Condition	Action
Total number PSC	<50% DMSO co-culture control at any two concentrations	Reject
PSC Object count	<85% PSC mono-culture control at any concentration	Reject
Resazurin	<20% DMSO co-culture control at any concentration	Reject
"Extra-large" Organoid (>15,000μm²) proportion of total organoid area	<40% DMSO co-culture control at 10 and 20 μM	Select
Total Organoid Area	<37% DMSO co-culture control at any concentration	Select; Rank order by 5μM condition

Table 2. Traditional selection criteria from the hit verification screen.

Therapeutic window calculations – Paper III

To calculate therapeutic windows, we treated tumour organoids in co-culture with PSCs and normal organoids in monoculture with titrated concentrations of compounds. We measured the metabolic activity, using reazurin, and total organoid covered areas with a plate reader. To compare data for each culture type we scaled the imaging metrics according to the average values of DMSO and cycloheximide controls. We could then observe the effects of compounds on culture types in a dose response to determine concentrations at which a beneficial effect was observed on tumour co-cultures and a modest effect was observed on normal organoids. Therapeutic windows were identified for the top selected compounds from the drug-screen in **Paper III**.

RESULTS AND DISCUSSION

EXTRACELLULAR GALECTIN 4 DRIVES IMMUNE EVASION AND PROMOTES T-CELL APOPTOSIS IN PANCREATIC CANCER

Galectin-4 is aberrantly expressed in PDAC

The desmoplastic stroma of PDAC is partially comprised of ECM proteins collectively referred to as the matrisome. We sought to better characterize protein components of the PDAC matrisome to identify elements which mediate tumorigenesis and could therefore represent therapeutic targets.

A previously published mass spectrometry dataset quantifying differential expression of ECM proteins in normal and PDAC tissue was analysed to identify proteins which were upregulated in cancer[131]. A number of galectins were present amongst the upregulated proteins in PDAC including galectin-4 (Gal4) (Paper I, Figure 1). Gal4 is known to exhibit immunoregulatory properties in the gastrointestinal (GI) tract but, unlike other galectin family members, its' function in the context of PDAC had not been well described[141,143]. We therefore aimed to discern the pathophysiological role of extracellular Gal4 in PDAC.

We identified epithelial cells as the cellular source of Gal4 through IHC, ISH and analysis of a previously published scRNAseq dataset profiling human PDAC (Paper I, Figure 1 and 2)[299]. Significantly, we were also able to detect higher levels of circulating Gal4 in serum taken from PDAC patients compared to healthy controls (Paper I, Figure 1). Gal4 may therefore represent a potential novel biomarker for diagnosis of PDAC or as a marker to measure patient response to treatment. However, we did also observe elevated Gal4 levels in patients with inflammation of the GI tract which would need to be considered if utilizing Gal4 as a biomarker of PDAC (Paper 1, Figure S1).

Galectin-4 is a prognostic marker in PDAC

We subsequently analysed *LGALS4* (encoding Gal4) expression in the cancer genome atlas (TCGA) pancreatic cancer dataset which profiled PDAC tumours through bulk RNA sequencing[287]. Elevated *LGALS4* was associated with significantly worse survival in moderately differentiated cases of PDAC (Paper I, Figure 2). Since Gal4 possesses immunomodulatory functions and $CD8^+$ T-cells are key regulators of anti-tumour immunity, we quantified the expression of gene signatures reflecting $CD8^+$ T-cell activation and cytolytic activity in PDAC patients with varying *LGALS4* expression levels. Patients with

higher *LGALS4* expression exhibited reduced Z-scores for $CD8^+$ T-cell activation and cytolytic activity gene signatures indicating a reduction in anti-tumour immunity (Paper 1, Figure 2). This evidenced an immunomodulatory role for Gal4 in PDAC although this data was obtained from bulk RNA sequencing data so should be interpreted with caution due to potential confounding factors such as varying cellularity between samples.

Inhibiting Galectin-4 expression influences stromal composition in mouse models of PDAC

Due to the association between variable *LGALS4* and immune cell related gene expression, we aimed to better characterize immunomodulatory functions of Gal4. To do so, we generated murine PDAC cell lines with eliminated (Gal4-KO) and tumour organoids with reduced (Gal4-KD) expression. Such cells were injected alongside control cells, which retained Gal4 expression, orthotopically into mice. Orthotopic transplants of Gal4-KO cell lines generated tumours with increased $CD4^+$ and $CD8^+$ T-cell infiltration demonstrated through IHC staining and flow cytometry (Paper I, Figure 3). We also utilized scRNAseq to profile tumours established from orthotopic transplants of Gal4-KD tumour cells and controls. This corroborated our previous findings, with Gal4-KD tumours demonstrating a higher proportion of T-cells amongst immune cells (Paper 1, Figure 5). Gal4-KD tumours were also associated with macrophage polarization exhibiting an increase in M1 TAMs and decrease in M2 TAMs. Populations of antigen-presenting immune cells were found at a higher proportion in GAL4-KD orthotopic transplants. In addition, Gal4-KD tumours showed altered CAF composition with a decrease in iCAF prevalence and increase in myCAFs. Changes in immune cell composition in response to inhibiting Gal4 expression such as increased $CD4^+$ and $CD8^+$ T-cell infiltration, polarization of macrophages towards an M1 phenotype and higher prevalence of antigen-presenting immune cells represent a shift towards anti-tumour immunity[85,92,98].

Given that we observed a shift towards anti-tumour immunity, following a reduction in Gal4 expression, we measured survival in mice orthotopically transplanted with Gal4-KD and control cell lines. Survival was significantly longer in Gal4-KD transplanted mice than controls and a significant reduction in tumour volume was observed (Paper 1, Figure 4). In addition, we injected Gal4-KD and control cancer cells into immunodeficient $RAG1^{-/-}$ mice, which lack mature B- and T-cells. In $RAG1^{-/-}$ mice no significant difference in survival was observed demonstrating that the adaptive immune system was fundamental to prolonging survival in response to Gal4-KD. Gal4 is thus capable of repressing anti-tumour immunity and can be inhibited to improve survival which is mediated through the adaptive immune system.

Galectin-4 mediates immunosuppression through promoting T-cell apoptosis in PDAC

We proceeded to mechanistically interrogate how Gal4 can modulate immune cell function in PDAC. To do so we utilized an *in vitro* model where tumour organoids and cell lines were co-cultured with immune cells. We found that apoptosis was significantly higher in cancer cells with reduced Gal4 expression when they were in direct co-culture with immune cells (Paper 1, Figure 6).

Previous findings have demonstrated that Gal4 is capable of inducing T-cell apoptosis in inflammatory conditions of the GI tract[141]. In corroboration of this we defined a significant reduction in apoptosis of $CD8^+$ T-cells co-cultured alongside Gal4-KO tumour cells compared to controls (Paper 1, Figure 6). Paclik *et al*, demonstrated that T-cell apoptosis can be mediated by binding of Gal4 to the CD3 receptor of $CD8^+$ T-cells[141]. Gal4 is a lectin which binds glycosylated residues which led us to hypothesise binding to the CD3 receptor may occur through the heavily glycosylated CD3δ/ε chains. We were able to validate preferential binding of Gal4 to CD3δ/ε chains through surface plasmon resonance (SPR)-binding assays. Furthermore, we directly implicated the glycosylated residues in mediating Gal4 binding through ablating binding with N-linked glycosidase treatment. We were able to validate these findings in the *in vitro* co-culture model system by reversing the significant reduction in apoptosis seen in $CD8^+$ T-cells exposed to Gal4-KO tumour cells through addition of soluble CD3 chains. These findings are consistent with a growing body of evidence that $CD8^+$ T-cell apoptosis can be induced through CD3-ligating antibodies[300,301].

My work within this study included the ELISA profiling circulating levels of Gal4 (Paper I, Figure 1). In addition, I conducted the analysis of the TCGA dataset including survival analysis and Z-scoring of immune cell signatures (Paper I, Figure 2). Finally, I performed qPCR experiments determining the effect of inhibiting Gal4 expression on tumour cells by quantifying expression of EMT associated genes (Paper I, Figure S3).

DISSECTING FAP$^+$ MESENCHYMAL CELL DIVERSITY AND REGULATION IN PANCREATIC CANCER UNCOVERS AN INTERFERON-RESPONSE CANCER-ASSOCIATED FIBROBLAST SUBTYPE

Discrete FAP$^+$ mesenchymal cell populations exist within the PDAC stroma

Mesenchymal cells represent a heterogenous population of stromal cells which perform divergent tumorigenic functions within the PDAC TME. We sought to characterize the landscape of mesenchymal cell heterogeneity in human PDAC using scRNAseq profiling by positively selecting cells based on FAP expression, a mesenchymal cell marker[159].

When clustering the cells at a low resolution we identified three distinct CAF populations in addition to cells which displayed a perivascular cell phenotype which we describe as cancer-associated perivascular cells (CAPs) (Paper II, Figure 1). CAF populations could be distinguished by markers which have previously been described to define distinct subpopulations including *WT1*, *ENG* and *LRRC15*[169,170,172]. We therefore labelled the CAF subpopulations as WT1$^+$ CAFs, ENG$^+$ CAFs and LRRC15$^+$ CAFs.

FAP$^+$ mesenchymal cell populations in PDAC relate to mesenchymal cells in the healthy pancreas

WT1 has been described as a marker of a subpopulation of fibroblasts in the normal pancreas[172]. WT1 is also described as a mesothelial cell marker which have been shown to differentiate into CAFs during PDAC[174]. *ENG* encodes CD105 which has been demonstrated as a marker of fibroblast lineages in the normal pancreas[169,170]. Given that we identified CAF populations which express *ENG* and *WT1* we hypothesized that CAF subpopulations might correspond to mesenchymal cell states in the normal pancreas.

To test this, we dissected mesenchymal cell heterogeneity in a recently published scRNAseq dataset profiling the developing human pancreas (Paper II, Figure 1)[302]. We identify a mesenchymal population which has elevated expression of pan-CAF markers and engagement of myCAF pathways relating to collagen deposition and contractility. In addition, we find a cluster of mesenchymal cells which show elevated expression of inflammatory genes and iCAF associated pathways including interleukin signalling. Interestingly, this suggests that mesenchymal cell subpopulations in the normal pancreas may be predisposed to a more inflammatory or myofibroblastic phenotype. This supports findings that fibroblast subpopulations in the normal mouse pancreas can display an iCAF or myCAF phenotype and differentiate into distinct CAF subtypes[170]. We also characterize a

mesenchymal cell subpopulation in the normal pancreas which displays a perivascular cell phenotype.

We therefore proceeded to determine whether the mature CAF subpopulations we identified in PDAC corresponded to the mesenchymal cell populations characterized in the normal pancreas. To do so, we integrated our PDAC dataset with the normal developing pancreas dataset. Interestingly, we found that mesenchymal populations identified in the normal developing pancreas clustered together with ENG$^+$ and WT1$^+$ CAF subpopulations and CAPs from PDAC (Paper II, Figure 1). This suggests that distinct mesenchymal cell populations in the normal pancreas may serve as cellular reservoirs for mature CAF subpopulations found in PDAC. Hutton *et* al, found that CD105$^{+/-}$ fibroblasts lineages exist in the normal pancreas and give rise to distinct CAF subtypes. Furthermore, they demonstrated that CD105$^-$ CAFs express higher levels of *WT1*. As we identified ENG$^+$ and WT1$^+$ CAF subtypes in PDAC which correspond to mesenchymal subpopulations in the normal pancreas these may correspond to CD105$^{+/-}$ fibroblasts. WT1 has been reported as marker of a subpopulation of fibroblasts and of mesothelial cells which can differentiate to form CAFs[172,174]. We found that WT1$^+$ CAFs are abundant in PDAC and correspond to a population of inflammatory mesenchymal cells in the normal pancreas but further work is required to better discern whether this population is mesothelial cells or fibroblasts. We demonstrated that LRRC15$^+$ CAFs were only identified in the context of PDAC and may therefore be considered as a PDAC specific phenotype. These findings provide novel insights into the heterogeneity of mesenchymal cells in health and PDAC through scRNAseq in a human setting for the first time. Such findings may help stabilize a taxonomic framework for mesenchymal cell subpopulations in the pancreas.

FAP$^+$ iCAF and myCAF subtypes populate the PDAC stroma

We proceeded to uncover the full extent of CAF heterogeneity through digitally extracting CAF populations and clustering them at a higher resolution (Paper II, Figure 2). We identified clusters as subtypes of iCAFs and myCAFs based on differential expression of iCAF and myCAF markers and through calculating previously published CAF subtype signature scores within clusters[197,199]. We classified iCAF and myCAF subtypes through expression of *WT1*, *ENG* and *LRRC15*. WT1$^+$ CAFs possessed an iCAF phenotype, LRRC15$^+$ CAFs were myCAFs and ENG$^+$ CAFs could be divided into iCAFs and myCAFs.

We then employed gene-set enrichment analysis and inferred transcription factor activities within iCAF and myCAF subtypes to better characterize them. LRRC15$^+$ myCAFs and ENG$^+$ myCAFs were both enriched for canonical myofibroblastic pathways relating to collagen deposition, contractility and ECM organization. Likewise, WT1$^+$ and ENG$^+$ iCAFs shared engagement of iCAF associated inflammatory pathways including JAK/STAT signalling and the complement cascade. LRRC15$^+$ myCAFs were strongly enriched for TGFβ signalling responsive transcription factors, SMAD3 and SMAD4, compared to ENG$^+$ myCAFs. This is consistent with previous findings that TGFβ signalling promotes formation of LRRC15$^+$ CAFs[170,303].

Interestingly, WT1$^+$ iCAFs also had elevated activity of SMAD3 and SMAD4 and demonstrated engagement of myofibroblastic pathways relating to collagen deposition, ECM organization and contractility. WT1$^+$ iCAFs also exhibited high activity of inflammatory transcription factors including STAT3 and NFκB1 signifying this subtype has both iCAF and myCAF features. Interestingly, mesothelial derived WT1$^+$ CAFs have been shown to form in response to TGFβ and IL1α treatment which coincides with enhanced NFκB1, STAT3, SMAD3 and SMAD4 transcription factor activities observed in WT1$^+$ CAFs in our data[174]. WT1$^+$ iCAFs were also strongly enriched for hypoxia associated pathways and transcription factors which has been shown to drive iCAF formation[203]. In contrast ENG$^+$ iCAFs had weaker activity of inflammatory transcription factors than WT1$^+$ iCAFs, although they were still higher than in myCAFs. ENG$^+$ iCAFs also had high activity of SMAD7 which can inhibit TGFβ signalling and did not demonstrate engagement of myofibroblastic pathways.

Overall, WT1$^+$ iCAFs demonstrated strong engagement of inflammatory pathways and transcription factors but also exhibited myofibroblastic phenotypic features whilst ENG$^+$ iCAFs showed weaker inflammatory pathway engagement and transcription factor activity but did not display any myofibroblastic features. LRRC15$^+$ myCAFs can be distinguished from ENG$^+$ myCAFs through enhanced TGFβ signalling activity.

scRNAseq identifies an interferon-response CAF subtype

In addition to iCAF and myCAF subtypes, we also identified CAF subtypes which we annotated through expression of previously described markers including *PI16* (PI16$^+$ CAFs)[304], *VEGFA* (VEGFA$^+$ CAFs)[305] and a subset of WT1$^+$ CAFs with reduced expression of iCAF markers (Paper II, Figure 2). An additional CAF cluster was defined by elevated expression of CAP markers, in addition to expressing CAF markers, and was therefore labelled as CAP-like CAFs. Finally, we identified a CAF subtype defined by expression of interferon-response genes including *RSAD2*, *IFIT3*, *CXCL10* and *DDX58* which we labelled as ifCAFs. We further corroborated this interferon-responsive phenotype through the detection of interferon signalling associated pathways and transcription factors within this cluster. We then used *in situ* hybridization to validate the presence of a subset of CAFs with expression of interferon-response genes *IFIT3* and *RSAD2*.

CAFs exhibiting an interferon-response have previously been described in a mouse model of breast cancer and labelled as interferon-licensed CAFs (ilCAFs)[305]. Such ilCAFs shared expression of interferon-inducible genes and activation of interferon signalling pathways observed here in ifCAFs. ilCAFs could be induced through blocking TGFβ signalling with an antibody and were associated with enhancing T-cell infiltration and activation. However, ilCAFs were associated with MHCII antigen presentation which was not detected in ifCAFs and is a feature of the previously established CAF subtype apCAFs. ilCAFs may therefore represent apCAFs rather than ifCAFs. An additional study profiling human breast cancer with scRNAseq identified a population of CAFs with a type I interferon-response phenotype which did not exhibit MHCII antigen presentation[306]. These CAFs may correspond to ifCAFs

identified in this paper, suggesting this subtype is observed in different cancer types. In a mouse model of PDAC it has been shown that tumour cells with high levels of interferon signalling can transmit signals to CAFs, in turn inducing an interferon-response in CAFs which stimulates tumour growth[307]. Thus, evidencing a potential pro-tumorigenic role for ifCAFs, although this was attributed to global changes in CAF phenotype and not attributed to a specific subpopulation of CAFs. Here we described a subpopulation of CAFs with an interferon-response phenotype in human PDAC for the first time.

Tumour cell derived signals induce iCAF, myCAF and ifCAF phenotypes in a three-dimensional in vitro model system of PDAC

PSCs are a proposed cellular reservoir for CAFs, although the extent to which they contribute to the total CAF population in PDAC remains controversial[159,161]. We therefore sought to investigate whether PSCs can differentiate into CAF subtypes following exposure to tumour-derived signals. To do so, we used three-dimensional co-cultures of fluorescently labelled PSCs and tumour organoids, followed by FACS and scRNAseq profiling (Paper II, Figure 3). PSCs were monocultured in Matrigel, to prevent plastic activation giving rise to a myofibroblastic phenotype and instead maintain them in a quiescent state[160], or co-cultured with tumour cells prior to transcriptional profiling.

Clustering analysis of the scRNAseq data was able to distinguish PSCs in monoculture from those which had been activated to form CAFs in co-culture. Quiescent PSCs were characterized by gene markers and engagement of pathways involved in lipid metabolism. CAFs formed in co-culture segregated into distinct clusters which could be annotated as iCAFs, myCAFs and ifCAFs based on enrichement of markers, pathways and transcription factors relating to myofibroblastic, inflammatory and interferon-response phenotypes, respectively (Paper II, Figure 3). Therefore, tumour cell-derived signals were sufficient to induce iCAF, myCAF and ifCAF phenotypes in PSCs in a co-culture model system and such phenotypes are not dependent on signals from other stromal cell types.

PSC-derived CAFs formed in vitro correspond to FAP$^+$ CAF subtypes identified in vivo

We next sought to determine whether PSCs in monoculture and PSC-derived CAFs in co-culture related to mesenchymal subpopulations we identified in the human pancreas or PDAC, respectively. We calculated PSC-derived CAF subtype signature scores across *in vivo* CAF clusters and found that Z-scores for iCAF, myCAF and ifCAF signatures from co-culture were enriched in their corresponding subtypes *in vivo* (Paper II, Figure S3). We then integrated scRNAseq data of PSCs from mono-culture with mesenchymal cells from the normal pancreas. Interestingly, we find that PSCs form a cluster with a subpopulation of mesenchymal cells in the normal pancreas which have a myofibroblastic phenotype (Paper II, Figure 3). We then integrated co-cultured PSCs with FAP$^+$ mesenchymal cells in PDAC and found that co-cultured

PSCs clustered together with ENG$^+$ CAFs and LRRC15$^+$ CAFs but not with CAPs or WT1$^+$ CAFs. We have shown above that mesenchymal cells with a myofibroblastic phenotype in the normal pancreas correspond to ENG$^+$ CAFs in PDAC. Here, we found that quiescent PSCs relate to the myofibroblastic mesenchymal cluster and PSC-derived CAFs relate to ENG$^+$ CAFs, further strengthening this association and demonstrating that PSCs correspond to specific subpopulations of mesenchymal cells and CAFs. Interestingly, we also found that PSC-derived myCAFs group with LRRC15$^+$ CAFs suggesting that PSCs are the cellular source of this subtype. This is consistent with findings that a subtype of fibroblasts in the normal mouse pancreas give rise to LRRC15$^+$ CAFs[170]. A recent study from Helms *et al* demonstrated that PSCs only contribute a small amount to the total CAF population in PDAC[161]. Here, we reaffirm that PSCs represent a CAF subpopulation in PDAC however, we find that they contribute more significantly to the total CAF population. Taken together, we show that quiescent PSCs represent a myofibroblastic mesenchymal subtype that give rise to ENG$^+$ and LRRC15$^+$ CAFs in PDAC but not WT1$^+$ CAFs or CAPs, such findings will help better define a taxonomic framework for CAF subtypes in PDAC.

A time-course profiling PSC differentiation *in vitro* elucidates the dynamics of CAF subtype formation

We next aimed to profile PSC differentiation using scRNAseq over a time-course to discern the dynamics and regulation of CAF subtype formation. After clustering we identified iCAF, ifCAF and myCAF subtypes using CAF subtype signature scores (Paper II, Figure 4). iCAFs formed earliest during the time-course with an early iCAF cluster comprised of cells predominantly from days 2, 3 and 4 of co-culture. iCAF phenotype changed over co-culture and an additional cluster with a mixed iCAF and ifCAF phenotype was found later in the time-course in addition to a late iCAF cluster comprised of cells from day 6. myCAF and ifCAFs clusters formed later in the time-course and were found to contain cells mainly from days 5 and 6 of co-culture.

We then used Slingshot to order cells through the differentiation process by assigning cells pseudotime values which correspond to their differentiation status[298]. Slingshot was also employed to separate cells into lineages with different lineages corresponding to cells undergoing differentiation to distinct subtypes. We define three lineages corresponding to cells differentiating into iCAFs, ifCAFs and myCAFs (Paper II, Figure 4).

CAF subtype signature scores increased over pseudotime in their corresponding lineage. We therefore correlated inferred transcription factor activity and gene expression with pseudotime to define correlated transcription factors and perform gene-set enrichment analysis between lineages. Transcription factors and pathways associated with inflammation and interferon-response were present in both iCAF and ifCAF differentiation likely because both lineages pass through the early iCAF cluster 1 and the iCAF/ifCAF intermediate cluster 2. However, the iCAF lineage demonstrated engagement of additional canonical inflammatory pathways, including hypoxia, complement and TNF signalling as well as exhibiting stronger correlations with STAT3 and HIF1A activity. We also reaffirmed the role

of TGFβ in driving myCAF differentiation by showing that TGFβ responsive transcriptions factors SMAD2 and SMAD3 were strongly correlated with pseudotime in the myCAF lineage specifically. myCAFs have been shown to form only in direct contact with tumour cells and not when exposed to tumour cell derived signals, suggesting a juxtacrine signalling component is required for their formation[159]. We uncovered a potential role for NOTCH1 signaling as a juxtacrine signaling component associated with myCAF differentiation by identifying NOTCH1 and HES1 activity in the myCAF lineage specifically. Overall, we validated the utility of the co-culture model system in recapitulating CAF differentiation and used it to define key pathways and transcription factors governing subtype formation.

Type I and type II interferon pathways govern interferon-response CAF (ifCAF) and antigen-presenting CAF (apCAF) formation

We next aimed to better characterize the interferon-response in ifCAFs. ifCAFs displayed an interferon-response phenotype but were not characterized by MHC class II antigen presentation which can be induced by the type II interferon, IFNγ. We therefore hypothesized that agonists targeting a regulator of type I IFN signalling, stimulator of interferon genes (STING), could induce an ifCAF phenotype. Through treating PSCs in monoculture and co-culture with a STING agonist (DMXAA) we induced an ifCAF phenotype specifically, as demonstrated through qPCR, ELISA and bulk RNA sequencing (Paper II, Figure 5). Interestingly, STING agonists have been shown to induce tumour-restraining immune responses in mouse models of PDAC[308,309]. Given that STING agonists induce an ifCAF phenotype, it is possible that ifCAFs may be involved in promoting this anti-tumour immunity. However, it could also be a direct consequence of the compounds on immune cells. Further work will help discern the role of ifCAFs in the TME of PDAC.

MHCII antigen presentation is a hallmark of the previously defined antigen-presenting CAF (apCAF) subtype which has been shown to account for around 10% of CAFs in a mouse model of PDAC[197]. We did not detect apCAFs in our *in vitro* profiling of CAFs in PDAC. We reasoned that their absence *in vitro* may be a consequence of the lack of IFNγ in our co-culture model system. We tested whether apCAFs could be formed from PSCs in response to IFNγ and found that they were capable of acquiring MHCII presentation in response to IFNγ (Paper II, Figure 5). Furthermore, we showed that activated T-cells were able to induce an apCAF phenotype in PSCs. We also reaffirmed previous findings that tumour-cell derived signals and TGFβ can suppress IFNγ-induced apCAFs[169]. Together we showed that PSCs do not form apCAFs *in vitro* when exposed to tumour cells but can do so in response to recombinant IFNγ or activated T-cells but tumour cell-derived factors inhibit this process.

Interestingly, we also did not identify apCAFs amongst FAP^+ mesenchymal cells *in vivo*. apCAFs have been proposed to originate from mesothelial cells which gain a CAF phenotype in PDAC[174]. The author's find that mesothelial derived CAFs contribute substantially to the total CAF population and that mesothelial cells gain a fibroblastic phenotype in response to tumour cells. They find that apCAFs are most similar to the mesothelial population but that

mesothelial-derived CAFs can develop hallmarks of other CAF subtypes including expression of *IL6* and *ACTA2*. One reason for their absence in our data could be that apCAFs, which are similar to mesothelial cells, have not yet acquired FAP expression which was the marker we used to sort cells. However, we found CAFs that express *WT1* and thus may be of mesothelial origin in our data. Perhaps such cells are derived from mesothelial cells but have deviated more substantially from their origin and acquired FAP expression which could not be the case for apCAFs. Further research will help better clarify these ideas.

This study was one of the major components of my thesis. I generated all the data, alongside Dr Cedric Patthey, and performed all the data analysis present in the study. In addition, I compiled the data and constructed the manuscript.

POTENTIATING THE TUMOR-RESTRAINING PROPERTIES OF THE STROMA IN PANCREATIC CANCER WITH SMALL MOLECULES

CAF subtype formation can be manipulated *in vitro*

Delivery of drugs directly to tumour cells in PDAC is a challenge, partially due to the abundant stroma present in tumours which can serve as a physical barrier. To circumvent this issue, we sought to identify compounds which could suppress tumour cell growth despite the presence of CAFs and indirectly through modulating CAF phenotype, as CAFs are more accessible than tumour cells to pharmacological intervention. To do so, we used a high-throughput drug-screen targeting the co-culture model system to test for compounds which suppress tumour growth and modify CAF phenotype.

To establish the utility of the co-culture model system for a drug-screen we first aimed to determine whether we could manipulate CAF phenotypes. To do so we modulated the TGFβ signalling pathway which, in this thesis and in other studies, has been associated with driving myCAF formation[201]. We treated co-cultures, comprised of fluorescently labelled PSCs and tumour organoids, with recombinant TGFβ and an inhibitor of the TGFβ type I receptor (A83-01) and profiled CAF phenotype with flow cytometry, transcriptomics and microscopy.

We used canonical iCAF and myCAF markers IL-6 and αSMA, respectively, to assess CAF subtype prevalence with flow cytometry (Paper III, Figure 1). We observed that treating co-cultures with recombinant TGFβ significantly reduced iCAF prevalence and increased the proportion of myCAFs, leading to a significant increase in the myCAF to iCAF ratio relative to vehicle controls. In contrast, inhibiting TGFβ signalling with A83 led to a significant reduction in myCAF formation leading to a significant reduction in the myCAF to iCAF ratio.

We profiled CAFs with bulk RNAseq following TGFβ modulation of co-cultures (Paper III, Figure 1). We calculated Z-scores for previously published gene signatures for myCAFs, iCAFs and quiescent PSCs (qPSCs) at days 4 and 6 of co-culture. Recombinant TGFβ promoted a significant increase in the myCAF signature at both time-points and a significant reduction in the iCAF signature at day 4. In contrast, A83 treatment led to a significant decrease in the myCAF signature score after 6 days of co-culture. Both treatments also induced an increase in the qPSC signature at day 6 which may be a result of culture exhaustion.

In addition, we assessed how TGFβ manipulation affected the spatial distribution of CAFs in co-culture (Paper III, Figure 1). It has previously been established that CAF subtypes have discrete spatial localizations relative to tumour cells with myCAFs forming proximally to

tumour cells and iCAFs forming distally[159]. We used fluorescent microscopy to define the spatial organization of CAFs relative to tumour cells. We labelled CAFs in close proximity to tumour cells as proximal CAFs and those which are not adjacent to tumour cells as distal CAFs. Proximal CAFs served as a proxy to estimate the prevalence of myCAFs, and distal CAFs as a proxy for non-myCAFs. Given that we have previously established an additional CAF subtype, ifCAFs, with an unknown localization in co-culture this classification can only be regarded as an estimate of subtype identity. We determined that treatment with recombinant TGFβ was able to significantly augment the number of proximal CAFs per organoid whilst A83 was able to significantly reduce their prevalence. Thus, the spatial localization is reflective of the observations made through flow cytometry and transcriptomics.

Taken together we demonstrated that through modulating TGFβ signalling we can manipulate CAF subtype identity in the co-culture model system.

Manipulating CAF subtype formation alters cancer cell phenotype

We next aimed to determine how modulating CAF phenotype influences cancer cells. To do so, we profiled tumour cells with transcriptomics and quantified proliferation in response to TGFβ signalling moderation. To distinguish the direct influence of recombinant TGFβ and A83 on tumour cells from those orchestrated by CAFs we treated tumour cells in monoculture and co-culture with CAFs.

Inhibiting TGFβ signalling with A83 significantly reduced tumour cell growth in monoculture (Paper III, Figure 2). In contrast, tumour cell proliferation was significantly higher in A83-treated co-cultures. As we have previously established that A83 is capable of reducing myCAF formation, this suggests that myCAFs may be restraining tumour growth. In support of this, we observe that recombinant TGFβ did not significantly alter cancer cell proliferation in monoculture but when applied to co-culture significantly repressed proliferation.

We next aimed to assess the influence of directing TGFβ signalling on therapeutically relevant cancer cell features through transcriptomics. We performed bulk RNAseq profiling of tumour cells and calculated Z-scores for proliferation and EMT gene signatures in response to treatment with recombinant TGFβ and A83 (Paper III, Figure 2). In contrast to the proliferation assay, we found that A83 had no significant influence on the proliferation score of tumour cells in monoculture or co-culture. However, we profiled tumour cells at day 4 of culture, where a significant difference had not yet been observed in the proliferation assay, which may account for this. Recombinant TGFβ treatment demonstrated a significant reduction in proliferation scores for tumour cells in co-culture, reflecting the proliferation assay. However, we also observed a significant reduction in monoculture, suggesting the effect may be a direct result of TGFβ on tumour cell's transcriptomes. Whilst reducing

tumour cell proliferation is therapeutically beneficial we also observed an increase in the EMT score in cancer cells in both monoculture and co-culture. Thus, while TGFβ treatment impairs tumour cell proliferation it can have the therapeutically undesirable effect of enhancing the EMT.

Our goal is to identify compounds which are capable of altering cancer cells both despite the presence of CAFs and through CAFs, by manipulating their phenotype. We therefore aimed to determine whether the effects of TGFβ pathway manipulation on tumour cell transcriptomes were occurring directly through the action of the treatment or indirectly through the modulation of CAF phenotype. To do so, we analysed the transcriptomic data to identify genes that were significantly differentially expressed between tumour cells treated with TGFβ or A83 and vehicle controls. We selected genes that were significantly differentially expressed in tumour cells in monoculture, co-culture or both (Paper III, Figure 2). Genes which have a similar fold-change, relative to the vehicle control, in both monoculture and co-culture were classified as being CAF independent and thus directly induced by the treatment. We also classified genes as being CAF enhanced if the fold-change in co-culture was ≥ 2-fold higher than it was in monoculture or CAF suppressed if the fold-change in monoculture was ≥ 2-fold higher than it was in co-culture. We observed that the majority of differentially expressed genes in response to treatment with recombinant TGFβ or A83 were CAF independent and therefore directly modulated by the treatment. Significantly, we were also able to detect genes which were enhanced by CAFs in response to TGFβ pathway manipulation. In addition, we found that TGFβ treatment induced a large number of changes in gene expression in tumour cells that were suppressed by CAFs.

Overall, we observed that modifying the TGFβ pathway has co-culture specific effects on tumour cell proliferation. However, transcriptional profiling of tumour cells revealed that compounds which modulate the TGFβ signalling pathway have strong direct effects on the tumour cells. Thus, most transcriptional changes are a direct consequence of the treatment rather than being modulated by differences in CAF phenotype induced by the compound. However, we were able to identify transcriptional changes in tumour cells which were mediated indirectly through TGFβ pathway manipulation induced changes in CAF phenotype. Furthermore, whilst recombinant TGFβ suppressed tumour cell proliferation it also promoted metastasis suggesting it is not a promising therapeutic strategy.

A high-throughput drug screen in a co-culture model identifies compounds which modulate CAF phenotype and suppress tumour growth

We have established above that CAF phenotype can be manipulated in co-culture and compounds which do so can induce CAF dependent and CAF independent changes on tumour cell phenotype. We therefore performed a drug-screen using a library of ~36,000 compounds in the co-culture model system to identify compounds which can suppress tumour cell growth in the presence of CAFs. Fluorescently labelled tumour organoids and

PSCs were co-cultured for five days following addition of compounds or under control conditions. Controls included DMSO as a vehicle control and cycloheximide, a cytotoxic compound, as a control for cytotoxicity. We also included A83, as we have established the compound is capable of modulating CAF phenotype through disrupting the formation of proximal CAFs and enhancing tumour cell proliferation. After five days of co-culture we utilized a high content image-based analysis to characterize the influence of compounds on co-cultures. After image acquisition we retained >70 imaging metrics quantifying morphological parameters relevant to tumour cell and CAF phenotype. To identify hit compounds which promote a desirable effect on co-cultures we performed a traditional analysis as well as a multivariate analysis incorporating all imaging metrics.

We first used principle component analysis (PCA) to analyse the data from the initial screen (Paper III, Figure 3). Vehicle controls clustered separately from cytotoxic controls and were primarily distinguished along principle component (PC) 1. In contrast vehicle controls could be distinguished from A83 treated co-cultures by PC2. In accordance with this, the variable loadings for PC1 were primarily associated with general cell viability whereas PC2 loadings associated more with the cell composition of co-cultures (Paper III, Figure S6). A83 treated co-cultures clustered in the lower left quadrant of the PCA and were characterized by increased organoid area, modestly diminished CAF growth and a reduction in proximal CAFs per organoid. Conversely, we found that co-cultures in the upper right quadrant of the PCA were characterized by an increase in proximal CAFs per organoid and a reduction in tumour cell growth. In addition, there was only a modest effect on CAF proliferation in this region indicating that compounds in this area were primarily disrupting tumour cell growth. This region of the PCA plot was therefore used to select compounds of interest, given that it modulated CAF phenotype by increasing proximal CAF formation and reduced cancer cell proliferation without being general toxic to non-malignant cells. As this region of the plot contained high PC1 and PC2 values, we selected the top 704 compounds (selected to allow us to validate on 6 full plates) based on the product of their PC1 and PC2 scores to identify those which were most characteristic of the desired phenotype.

The PCA analysis incorporated two PCs which cumulatively explained 54% of the variance within the dataset. However, an additional 11 PCs were identified which together accounted for 88% of the variance. We therefore employed a K-means clustering analysis to incorporate additional explainable variance into the analysis (Paper III, Figure 3 & S7). We repeated the PCA using only test compound treated cultures, to identify clusters of compounds with desirable characteristics. This identified 13 PCs explaining 90% of the variance, we performed a K-means clustering using the 13 PC scores which had been scaled to give each PC equal weighting. We obtained 14 clusters which we could then characterize by examining the Z-scores for imaging metrics of cultures present in a cluster. We identified a cluster which possessed cultures defined by a desirable phenotype including reduced tumour organoid growth with altered CAF localization but little change in CAF growth. We selected the top compounds from this cluster by determining those which were furthest from the origin of all 13 PCs used in the analysis. This will yield compounds which are most dissimilar to the average and therefore those which induce the most unusual phenotypes which could be of most therapeutic benefit.

We also employed a more established screening analysis which incorporates fewer of the imaging metrics (Paper III, Figure 3). This analysis aimed to identify compounds which reduced tumour growth without exhibiting general cytotoxicity. Cultures with reduced organoid area without increased total number of organoids or having a significant contribution of extra-large organoids to the total area, which could indicate an escape mechanism from the activity of the compound, were selected. We again selected the top 704 compounds using this approach by selecting cultures which met the aforementioned criteria and ranking them by total organoid area.

We compared the top 704 compounds from the three analytical approaches to identify hits and selected compounds which were present in ≥ 2 analyses giving 482 compounds. The remaining compounds were selected evenly from the top ranked compounds of the separate selection methods. All three selection approaches identified hit compounds in a similar area of the PCA with reduced organoid growth, increased proximal CAFs per organoid and no significant modification in CAF growth.

Through incorporating CAFs into the screening process we were able to better distinguish compounds which were generally cytotoxic from those which are preferentially targeting tumour cells. Furthermore, it has been described that CAFs and CAF-derived ECM components can serve as a physical barrier to impede drug delivery[120]. By identifying compounds which are capable of disrupting tumour cell growth in the presence of CAFs we aimed to enrich for those which are capable of circumventing these effects. Furthermore, it has been demonstrated that CAF subpopulation can differentially restrain tumour growth[130,159,169,201,235,236]. Our multivariate analysis allowed us to identify compounds which modulate CAF phenotype, which we have previously established is indicative of CAF subtype prevalence. We therefore aimed to enrich for compounds which can manipulate CAF phenotype and indirectly suppress tumour cell growth. This would be advantageous in an *in vivo* setting as CAFs are more accessible to therapeutic intervention. However, as the screen only incorporates co-cultures we were only able to determine how the compound modulates CAF and tumour cell phenotype. We could not determine whether changes in CAF phenotypes indirectly modulate tumour cell phenotype or whether the compound is acting directly on the tumour cells. Furthermore, we could only make limited assertions of how CAF phenotypes change due to the fact we only assess morphological features.

Hit verification screen of selected compounds identifies GNF-5 as a potential anti-cancer therapeutic

We next aimed to validate the capacity of the 704 selected compounds to induce the selected phenotype by repeating the drug-screen, this time using compounds at three concentrations (5, 10 and 20μM) (Paper III, Figure 4 & S8). OPLS modelling demonstrated that the majority of compounds were clustered together with the selected hits from the initial screen, most favourably at the 10μM concentration, whilst at lower concentrations they shifted closer to the vehicle controls and at higher concentration towards CHX treated controls. Out of the

704 compounds those which were predicted to be DMSO (false positives) or CHX (cytotoxic) at any concentrations were omitted, leaving 412 compounds. We then selected the top 44 compounds based on the OPLS modelling approach as well as a more traditional approach as with the initial screen. We identified 19 compounds that were present in both the traditional and OPLS modelling approaches and selected the remaining 25 evenly from the top-ranked compounds in each approach.

Once we had identified the top 44 compounds we cross-referenced them with the screening library annotations to determine if any were established drugs. Cross-referencing identified GNF-5, an allosteric inhibitor of the Bcr-Abl fusion protein, as one of the top 44 compounds. Due to GNF-5 being readily available as an established therapeutic agent, we proceeded to better characterize its' influence on co-cultures. We first characterized the morphological phenotype induced by GNF-5 in co-cultures and found that it promoted CAF density around organoids whilst inhibiting cancer cell growth (Paper III, Figure 4). We subsequently performed a dose-response assay on three biological replicates to calculate the IC_{25}, IC_{50} and IC_{75} of GNF-5 through measuring organoid covered area by fluorescence and metabolic capacity of co-cultures with the resazurin reduction assay.

Through the validation screen we were able to validate compounds which induced a desirable phenotype in co-cultures including GNF-5. GNF-5 is an inhibitor of the Bcr-Abl fusion protein and the ABL1 proto-oncogene is aberrantly expressed in various cancer types including PDAC[310,311]. ABL1 is expressed both in CAFs and tumour cells and is associated with ECM organization[312]. ABL1 has been shown to be aberrantly activated in additional cancer types including chronic myeloid leukaemia and lung adenocarcinomas and targeting ABL1 has demonstrated therapeutic benefits in mouse models of lung cancer[313]. This led us to hypothesize that it may also be of therapeutic benefit in PDAC. However, ABL1 inhibitors can trigger vascular toxicity *in vivo*[314]. Increased Rho-associated kinase activity is associated with increased vascular toxicity in patients with chronic myeloid leukaemia treated with ABL tyrosine kinase inhibitors[314]. It may therefore be possible to mitigate the negative impact of ABL1 inhibitors on vascular toxicity through treatment with Rho-associated kinase inhibitors. Overall, whilst GNF-5 may prove a promising candidate as a therapeutic in PDAC it is known to also induce unwanted side effects *in vivo*. We reasoned that as a well characterized compound which is readily available it would serve as a suitable compound to further interrogate how it regulates tumour cell and CAF phenotypes in co-culture.

GNF-5 modulates tumour cells through manipulating CAF phenotype

We next aimed to determine whether GNF-5 was orchestrating its influence on tumour cells directly or indirectly through the action of CAFs. We used proliferation assays to measure tumour organoid growth in co-cultures and monocultures treated with GNF-5 (Paper III, Figure 5). We observed that tumour organoid proliferation was preferentially repressed in co-culture compared to monoculture, evidencing that CAFs are involved in suppressing tumour cell growth in response to the compound. We also demonstrated that proliferation of

organoids from normal tissue were not influenced by GNF-5 in monoculture, signifying the compound is not targeting non-malignant cells.

We next examined the influence of GNF-5 on CAF phenotype. We found that GNF-5 did not influence CAF proliferation in co-culture using proliferation assays (Paper III, Figure 5). We found that GNF-5 increased the proportion of proximal CAFs per organoid in a dose-dependent manner. The increased proportion of proximal CAFs led us to hypothesize that GNF-5 may be inducing myCAFs, which are found proximally to tumour cells. In contrast, we found that GNF-5 decreased the proportion of αSMA^{+} cells using flow cytometry, which is indicative of a reduction in the proportion of myCAFs. ABL1 can mediate TGF-β signaling in fibroblasts and αSMA expression in CAFs is modulated by TGF-β signaling[201,315–317]. This led us to believe that whilst αSMA expression may be inhibited, perhaps GNF-5 induced other myCAF associated features. We performed bulk transcriptomics of co-cultures and surprisingly found that GNF-5 induced a significant reduction in the total myCAF gene signature, despite the increase in the proportion of proximal CAFs with the compound. However, whilst we demonstrate that the proportion of proximal CAFs per organoid increase with GNF-5, we have previously shown that the proportion of proximal CAFs out of all CAFs decrease with GNF-5 (Paper III, Figure 4). This may be a result of the fact that GNF-5 reduces the total organoid area, meaning there is less area available for CAFs to be proximal to tumour cells. Given that we perform bulk profiling of CAFs, if there is a reduction in proximal CAFs relative to the total number of CAFs we are more likely to get fewer proximal CAFs in the bulk sample which could reduce the myCAF signature score. However, whilst the imaging data shows better encapsulation of organoids by CAFs in response to GNF-5 we cannot assert that this is an overall myCAF induction if their contribution to the total CAF population is diminished.

Using transcriptomics and flow cytometry we also demonstrated that GNF-5 had no effect on iCAF formation and suppressed the quiescent PSC gene signature. Furthermore, gene-set enrichment analysis showed that interferon-response pathways were down-regulated, suggesting that it also repressed an ifCAF phenotype. Instead, we observed that metabolism and lysosome pathways were upregulated in CAFs exposed to GNF-5. Taken together, we found that GNF-5 either did not affect or suppressed formation of CAF subtypes defined in co-culture and instead induced an artificial phenotype characterized by increased metabolic activity.

To better characterize how changes in CAF phenotype impacted tumour cells we compared differentially expressed genes in tumour cells following exposure to GNF-5 in co-cultures and monocultures (Paper III, Figure 5). We again categorized differentially expressed genes in tumour cells as being CAF independent, CAF enhanced or CAF suppressed following GNF-5 treatment, as we have described above for TGFβ pathway manipulation. Interestingly, we found that GNF-5 induced a higher proportion of CAF enhanced changes, amongst the differentially expressed genes, in tumour cells than were observed for TGFβ pathway manipulation. We performed gene-set enrichment analysis using the CAF enhanced genes and found that proliferation associated pathways were downregulated. In addition, we observed that the EMT pathway was upregulated. To reaffirm these findings, we quantified

Z-scores for proliferation and EMT gene signatures in tumour cells in monoculture and co-culture following GNF-5 treatment. We found that GNF-5 had no significant effect on EMT or proliferation signatures in monoculture but significantly decreased the proliferation score and significantly increased the EMT score in co-culture. Therefore, GNF-5 exerts effects on tumour cell phenotype indirectly through the action of CAFs by suppressing tumour cell proliferation and enhancing EMT. The capacity for GNF-5 to modulate CAF phenotype and indirectly suppress cancer cell growth is of potential therapeutic benefit. However, we also demonstrated that GNF-5 induced changes in CAF phenotype have the unwanted effect of inducing a metastatic phenotype in tumour cells. It has been suggested that CAFs in direct contact with tumour cells can promote metastasis[216]. It is therefore a concerning possibility that CAFs encapsulating tumour cells may suppress their growth but increase their metastatic potential. Characterizing more compounds which promote proximal CAF formation around tumour cells will help address this possibility.

The work in this study constituted a major part of my thesis. I developed assays, conducted and analysed all the flow cytometry, transcriptional profiling and proliferation assays present in Figures 1 and 2. In addition I performed the transcriptomics data acquisition and analysis present in Figure 5. I also helped with the conceptualization of the multivariate analysis used in the screen and the construction of the manuscript.

LIMITATIONS AND FUTURE PERSPECTIVES

In **Paper I** a limitation was that the functional evaluations of Gal4 were largely based on knocking-down or knocking-out Gal4. It is common practice in cell biology to disrupt the expression of a protein and then validate that it has been reduced. However, inhibiting expression of a protein can alter cell phenotype and expression of a multitude of other proteins within a cell. It can therefore be questioned whether the effect of reducing expression of a protein is as a direct consequence of inhibition or an indirect consequence, caused by downstream effects on the expression of other proteins. We are currently developing antibodies capable of blocking the activity of extracellular Gal4. By targeting extracellular Gal4 specifically we can reduce off target effects that arise from knocking-down or knocking-out Gal4 within cells and more accurately assess the effect of targeting extracellular Gal4 specifically. Through treating mouse models of PDAC with Gal4 blocking antibodies we aim to validate the role of extracellular gal4 in the stroma and asses the use of antibodies targeting Gal4 as a potential novel therapeutic.

In **Paper II** we relied on scRNAseq exclusively to define cell phenotypes. Supplementing our annotations with ATAC-seq or proteomics would better substantiate our labelling of subtypes. We were able to integrate mesenchymal subpopulations from the normal pancreas with those found in PDAC. Whilst this provides evidence that mesenchymal subpopulations in the normal pancreas act as the cellular reservoirs for their corresponding subpopulations in PDAC, lineage tracing experiments are required to concretely assess these findings. In addition, we showed that PSCs and PSC-derived CAFs characterized *in vitro* corresponded to specific mesenchymal subpopulations in the normal pancreas and PDAC, respectively. Given that we have cultured our PSCs for a sustained period of time it is possible that the cellular composition has changed over time and that certain PSC subtypes have been lost during this period. Freshly isolating PSCs and comparing them to our cell lines would clarify this. In addition, we identified a type I interferon response in ifCAFs and showed that it can be replicated through drug treatment but were not able to establish how tumour cells induced this phenotype. Finally, whilst we identified and characterized mesenchymal subpopulations and CAF subtypes further work is required to assess their pathophysiological role in disease. We are currently developing a project which will use bulk RNA sequencing to profile epithelial, immune and CAF components of a tissue-microarray containing PDAC tumour samples from over 100 patients. We can then employ bulk deconvolution to define the composition of CAF subtypes within each tumour sample and relate their abundance to patient survival and immune cell and tumour cell phenotypes. This will facilitate a better understanding of the pathophysiological role of CAF subtypes in PDAC.

In **Paper III** we were able to identify compounds which suppressed tumour growth through influencing CAF phenotype. However, given that the screen only included co-cultures, when we were selecting compounds, we could not be certain that the compound was not acting directly on the tumour cells. We were able to validate that compounds were indirectly modifying tumour cells through the manipulation of CAF phenotype later but could not do so when first selecting a hit. In addition, we used PSCs in the drug-screen which we have established may not represent the full extent of mesenchymal cell heterogeneity observed *in*

vivo. We therefore cannot know the impact compounds will have on other mesenchymal cell types, and other stromal cell types, which are absent from the co-culture model. Developing model systems with a more diverse population of stromal cells will help improve the use of organoid-based *in vitro* models in drug-screening. In addition, we are in the process of validating whether the biological activity of compounds identified in the screen are replicated in an *in vivo* setting. This will help substantiate the utility of our co-culture model screening approach. Finally, we set out to identify compounds which disrupt the balance of CAF subtypes to shift the stroma towards a more tumour-suppressive state. Specifically, we selected for compounds which increased the proportion of CAFs proximal to tumour cells, which might represent myCAFs. myCAFs have been suggested to repress tumour growth but have also been implicated in promoting metastasis. We must therefore better clarify the influence of proximal CAFs on tumour cells to ensure we are not selecting compounds which induce an undesirable tumour cell phenotype such as metastasis. Another consideration with our approach is that whilst substantial evidence exists that CAF subtypes can suppress tumorigenesis what is apparent is that all CAF subtypes are present in tumours and that PDAC is still able to progress rapidly. This raises the question of how competent CAF subtypes can be at inhibiting disease progression. Perhaps a more radical approach to modifying CAF phenotype is required to help improve patient outcomes and rather than trying to manipulate the abundance of existing CAF subtypes we should be aiming to transform CAFs into a tumour-restraining phenotype which is not present normally. Indeed, we found that GNF-5 induced a phenotype not associated with defined CAF subtypes in co-cultures and this phenotype could suppress tumour organoid growth. Therefore, artificially inducing phenotypes may be of therapeutic benefit.

CONCLUSIONS

The primary aim of this thesis was to augment our understanding of the composition of the stroma in PDAC to facilitate the identification of novel therapeutic targets.

In **Paper I** we identified galectin-4 (gal4) as a tumour cell-derived matrisome protein which is upregulated in PDAC. We found circulating levels of gal4 are elevated in PDAC patients and that high expression of gal4 is correlated with worse survival and diminished activity of $CD8^+$ T-cells. Using mouse models of PDAC we demonstrated that gal4 inhibits $CD4^+$ and $CD8^+$ T-cell infiltration of tumours resulting in worse survival. Finally, we showed that gal4 is able to bind the CD3εδ receptor and induce apoptosis in $CD8^+$ T-cells.

In **Paper II** we provided a comprehensive characterization of FAP^+ mesenchymal subpopulations in human PDAC using single-cell RNA sequencing (scRNAseq). We found that cancer-associated perivascular-like cells (CAPs) correspond to perivascular cells in the normal developing pancreas whilst ENG^+ CAFs and $WT1^+$ CAFs relate to myofibroblastic and inflammatory mesenchymal cells in the normal pancreas, respectively. We also identified a $LRRC15^+$ CAF subpopulation which is only present in the context of PDAC. We then defined iCAF and myCAF subtypes. iCAF subtypes shared expression of inflammatory genes and pathways but $WT1^+$ iCAFs could be distinguished from ENG^+ iCAFs based on enhanced TGFβ, HIF1A and STAT3 signaling. In contrast, myCAF subtypes commonly expressed myofibroblastic genes and pathways but $LRRC15^+$ myCAFs could be distinguished from ENG^+ myCAFs based on enhanced TGFβ signalling. We also identified a novel interferon-response CAF (ifCAF) subtype. Using an *in vitro* organoid-based co-culture model we demonstrated that pancreatic stellated cells (PSCs) could be transformed from a quiescent state to form iCAFs, myCAFs and ifCAFs in response to tumour-derived factors. We showed that quiescent PSCs correspond to a myofibroblastic mesenchymal subpopulation in the normal pancreas whilst PSC-derived CAFs relate to ENG^+ and $LRRC15^+$ CAF subpopulations in PDAC but not $WT1^+$ CAFs or CAPs. By profiling PSCs over a time-course we identified TGFβ and NOTCH signalling as regulators of myCAF differentiation, STAT3 and HIF1A as regulators of iCAF differentiation and transcription factors associated with interferon-response as regulators of ifCAF formation. In addition, we showed that the T-cell-derived type II interferon, IFNγ could induce antigen-presentation in PSCs but that tumour cells suppressed this. Finally, we showed that a STING agonist which promotes a type I interferon response could induce an ifCAF phenotype in PSCs. These findings are summarized in Paper II, Figure 6.

In **Paper III** we demonstrated that CAF subtype formation in the co-culture model could be manipulated by modulating the TGFβ signalling pathway. We found that inducing or repressing myCAF formation repressed or promoted tumour cell growth, respectively. In addition, we were able to characterize whether transcriptional changes in tumour cells were occurring due to the direct action of a compound on tumour cells or indirectly through changing CAF phenotype. We developed a high-throughput drug screen using the co-culture

model and employed multivariate analyses to identify compounds which repress tumour growth and manipulate CAF phenotype. Finally, we identified GNF-5 as a compound which is capable of suppressing tumour cell growth in co-culture specifically through modulating CAF phenotype.

Overall, the findings of this thesis provide us with a better understanding of mesenchymal cell heterogeneity in PDAC, including the discovery of an interferon-response CAF subtype, and substantiate the utility of a co-culture model system in replicating it. We developed a high-throughput drug-screen with the co-culture model and demonstrated its' capacity to identify compounds which can modulate tumour cell phenotype indirectly through the action of CAFs. Finally, we defined galectin-4 as a pro-tumorigenic matrisome protein which could serve as a novel therapeutic candidate.

When googling science quotes to sound profound I stumbled across one from John Green "What I love about science is that as you learn, you don't really get answers. You just get better questions." When thinking about this thesis we have, I think, answered some of the questions we asked but the more exciting aspect is the questions we can ask from the findings. Can you develop therapeutics which target extracellular galectin-4 and can this help alleviate the immunosuppression imposed by cancer cells in PDAC? What roles do CAF subtypes defined in this thesis play in mediating disease progression and can we modulate their abundance *in vivo*? Is the drug-screen on the co-culture model system a more accurate methodology to identify compounds which are more effective *in vivo* and can this pave the way for developing organoid-based drug-screening approaches which entirely encompass the complexity of the PDAC stroma? I, for one, will be eagerly anticipating the answers to such questions.

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