UMEÅ UNIVERSITY

IMMUNOSUPPRESSIVE AND METASTASIS-PROMOTING MATRISOME PROTEINS IN PANCREATIC CANCER

The role of galectin 4 and SERPINB5

Tommy Lidström

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Umeå 2022

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“What do you think science is? There's nothing magical about science. It is simply a systematic way for carefully and thoroughly observing nature and using consistent logic to evaluate results.

-Steven Novella

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Abstract

In Sweden 1200-1300 people are diagnosed with pancreatic ductal adenocarcinoma (PDAC) every year. Late diagnosis, together with poor treatment response and resistance to checkpoint inhibitor immunotherapy, contributes to the poor prognosis of the disease.

PDAC is characterized by abundant deposits of extracellular matrix, which mainly includes structural proteins including collagens, proteoglycans, cell-binding glycoproteins, carbohydrates, and secreted proteins, all constituting the matrisome of the tumor. The matrisome protects cancer cells and affects the outcome. Several highly expressed matrisome proteins are involved in oncogenesis, including the processes of immunosuppression and metastasis formation, therefore contributing to the poor prognosis. In this thesis the pathophysiological role of several matrisome proteins in PDAC tumor progression was studied. Unbiased analysis of matrisome proteins in PDAC tumors revealed increased levels of cancer cell-derived secreted proteins compared to normal healthy control tissue. Subsequently, differentially expressed candidate proteins, with known cellular functions in other disease but hitherto uncharacterized role in PDAC progression, were selected.

Serine protease inhibitor clade B member 5 (SERPINB5), agrin, and cystatin B (CSTB), were selected for the study described in paper I based on their known roles in the metastasis formation process in other types of cancers. SERPINB5, agrin and CSTB were found to increase metastasis in models of PDAC by affecting epithelial to mesenchymal transition, ECM degradation and extravasation. In PDAC tumors, high levels of extracellular SERPINB5 correlated to reduced overall survival.

Galectin 4 (Gal 4) was selected for the study described in paper II based on its known immunosuppressive effects. Gal 4 is highly expressed in PDAC and was found to inhibit T cell infiltration and induce apoptosis in CD8+ T cells by binding to CD3 on the surface of T cells. Gal 4 was associated with better survival in PDAC patients and correlated to higher activation and cytolytic effect of CD8+ T cells.

The relation between gal 4 and other immunosuppressive proteins was studied in paper III. Analysis of available datasets revealed that gal 4 expression correlates with other cancer cell-derived immunosuppressive proteins of the galectin family, galectin 3 and galectin 9, while negatively correlating with the stroma-derived factors galectin-1 and TGFBI.

Findings in this thesis show that targeting of matrisome proteins in PDAC can be a promising therapy strategy. Blocking extracellular SERPINB5 could result in reduced metastasis and increased survival. Blocking intracellular gal 4 could increase anti-tumor immunity and synergize with checkpoint inhibition therapy.

The identified co-expression and coregulation of different immunosuppressive proteins indicate that different tumors can be classified based on their predominant immunosuppressive mechanisms. Following this classification in individual patients, combinations of therapies against different immunosuppressive mechanisms could represent a promising strategy to introduce effective immunotherapies for PDAC patients.

Abbreviations

apCAF antigen presenting cancer-associated fibroblast

Breg regulatory B cell

CAF Cancer-associated fibroblast

CEA carcinoembryonic antigen

CRC colorectal cancer

CSTB Cystatin B

CT computed tomography **IFNγ** interferon gamma

ECM extracellular matrix

EMT Epithelial-Mesenchymal Transition

Gal 1 galectin 1

Gal 3 galectin 3

Gal 4 galectin 4

Gal 9 galectin 9

HA Hyaluronidase

HRP horseradish peroxidase

IC immune cells

iCAF inflammatory CAF

IFNγ Interferon gamma

IHC immunohistochemistry

ISH In situ hybridization

IL interleukin

IPMN intraductal papillary mucinous neoplasm

KRAS Kirsten rat sarcoma virus

MRI magnetic resonance imaging

myCAF myfibroblastic cancer-associated fibroblast

NK natural killer

PanIN pancreatic intraepithelial neoplasia

PD-1 programmed cell death protein 1

PDAC pancreatic ductal adenocarcinoma

PEGPH20 PEGylated hyaluronidase

PSCA prostate stem cell antigen

RTK Receptor tyrosine kinase

SERPINB5 Serine protease inhibitor clade B member 5

SHH sonic hedgehog

TAMs tumor associated macrophages

Tim-3 T cell immunoglobulin and mucin-domain containing-3

TMA Tissue microarray

Treg Regulatory T cells

VEGF vascular endothelial growth factor

αSMA alpha smooth muscle actin

Populärvetenskaplig sammanfattning

Bukspottkörtelcancer (eller pankreascancer) är en allvarlig sjukdom som drabbar mellan 1200 och 1300 personer per år i Sverige. Patienter som drabbas av pankreascancer har en 5-årsöverlevnad på endast 8%. Att cancern upptäcks vanligtvis sent samt att det finns få effektiva behandlingsmetoder bidrar till den dåliga prognosen. Operation av tumören ökar 5-årsöverlevnaden till mellan 20–25 % men är enbart möjligt om tumören upptäcks tidig innan den hunnit sprida sig till andra organ.

Pankreastumörer består mestadels av ett bindvävsrikt tumörstroma sammansatt av immunceller, nerver och blodkärl. Stromat bidrar till motståndskraft mot behandling och kan hjälpa till att försvara cancercellerna mot immunförsvaret, vilket tillsammans främjar spridning och tillväxt av cancern.

Immunoterapi, som använder patientens egna immunförsvar för att attackera cancern, har i andra cancerformer visat sig effektiv. För pankreascancer har dessa behandlingar dock ej visat någon större effekt. Det finns därför ett behov av att utveckla nya effektiva behandlingsmetoder för pankreascancer.

Cancerceller producerar faktorer, vanligtvis proteiner, som utsöndras till stromat och där kan förhindra immunceller från att ta sig in i tumören, och på så vis hindra att immuncellerna dödar cancerceller. Cancerproducerade protein bidrar även till att stimulera cancercellernas tillväxtförmåga och förmåga att sprida sig.

I denna avhandling har vi identifierat proteiner som producerats i höga nivåer av cancerceller och karaktäriserat dessa proteiners funktion och bidrag till tumörens tillväxt och spridning. Vi har identifierat proteiner (SERPINB5, CSTB och agrin) som ger ökad spridning av cancerceller samt ett protein, galectin 4, som hämmar immunförsvaret.

I framtiden kan läkemedel som blockerar effekten av dessa cancerproducerade proteiner leda till effektivare behandlingar mot pankreascancer.

List of scientific papers

Paper I
Chenxi Tian, Daniel Öhlund, Steffen Rickelt, **Tommy Lidström**, Ying Huang, Liangliang Hao, Renee T. Zhao, Oskar Franklin, Sangeeta N. Bhatia, David A. Tuveson, Richard O. Hynes.
Cancer Cell–Derived Matrisome Proteins Promote Metastasis in Pancreatic Ductal Adenocarcinoma.
Published in Cancer Research, 1 April 2020; 80 (7): 1461–1474.

Paper II
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.
Accepted for publication in Cancer Immunology Research.

Paper III
Tommy Lidström, Cedric Patthey, and Daniel Öhlund.
Coordination and cooperation of immunosuppressive mechanisms in pancreatic ductal adenocarcinoma.
Manuscript.

Not included in thesis

Kolan, Shrikant S., **Lidström, Tommy**, Mediavilla, Tomás, Dernstedt, Andy, Degerman, Sofie, Hultdin, Magnus, Björk, Karl, Marcellino Daniel, Forsell, Mattias N. E. *Growth-inhibition of cell lines derived from B cell lymphomas through antagonism of serotonin receptor signaling*. Sci Rep 9, 4276 (2019).

Introduction

The pancreas is a pinecone-shaped abdominal organ. It is a heterocrine gland whose main functions are regulation of blood glucose levels by endocrine section of insulin and glucagon, and digestion of food by exocrine secretion of enzymes. The pancreas consists mainly of exocrine tissue, which makes up nearly 95% of the organ, interspaced with endocrine islet cells (islets of Langerhans) that produce hormones released directly into the bloodstream.

Anatomically, the pancreas is divided into the head, body, and tail, with the head directly adhered to the duodenum of the small intestine (*see fig. 1*). Exocrine pancreas secretes pancreatic juice into pancreatic ducts. These highly intercalated ducts progressively drain into larger ducts and ultimately join the main pancreatic duct. Distally, the main pancreatic duct merges with the common bile duct, carrying hepatic secretions, such as bile and other digestive enzymes.

Because the pancreas is central to metabolism and hormone production, pathological disruptions can lead to severe clinical manifestations. For example, acute pancreatitis is inflammation of the pancreas caused mainly by premature activation of digestive enzymes. This can cause acute abdominal pain and may increase the risk of malignant transformation of pancreatic cells, especially the pancreatic ductal cells.

Early mutational events in proto-oncogenes and/or tumor suppressors lead to the formation of precursor lesions related to cancer. These lesions include pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasm (IPMN), but also the rare mucinous cystic neoplasm (MCN). These precursor lesions share some, but not all, gene abnormalities associated with the invasive pancreatic tumors.

Pancreatic ductal adenocarcinoma (PDAC), which is the most common type of pancreatic cancers, has one of the worst prognoses among all cancer types. The tumor consists of dense structures of extracellular matrix (ECM) proteins together with recruited fibroblasts known as cancer-associated fibroblasts (CAFs). These dense structures serve to reduce perfusion, protect the tumor from the immune system, and contribute to treatment resistance, a hallmark of the disease.

Characterizing the function of individual proteins of the ECM, followed by designing targeted therapies against these components, could unravel

ways to dismantle the cancer, resulting in more effective treatment strategies against pancreatic cancer.

Pancreatic ductal adenocarcinoma

About 90% of pancreatic tumors occur in the ductal part of exocrine pancreas and are referred to as pancreatic ductal adenocarcinoma (PDAC) [1]. Acinar cells, which produce and secrete digestive enzymes, can transform to acinar cell carcinomas, while the pancreatic endocrine cells can undergo malignant transformation into neuroendocrine tumors. These non-ductal pancreatic tumors are rare and clinically distinct from PDAC. Throughout this thesis, the term "pancreatic cancer" will refer to PDAC.

Anatomically, tumors of the pancreas arise most commonly in the head of the pancreas (76%). The remaining 24% occur in the body or tail region of the organ [2] (*see Fig. 1*).

Pancreatic cancer has historically been believed to originate from the ductal epithelial cells of the exocrine pancreas. Following accumulation of mutations, normal ductal epithelial cells slowly lose their cuboidal shape, increase mucin production, and become more elongated [3] (*see Fig.2*). New studies on the plasticity of pancreatic exocrine acinar cell have shifted this view and it is now speculated that pancreatic cancer originates from acinar cells. This hypothesis is based on the process acinar-to-ductal metaplasia, where acinar cells accrue ductal cell characteristics, followed by transformation. Transformation of acinar cells into ductal PanIN lesions have been demonstrated by activating *KRAS* and notch signaling [4-6].

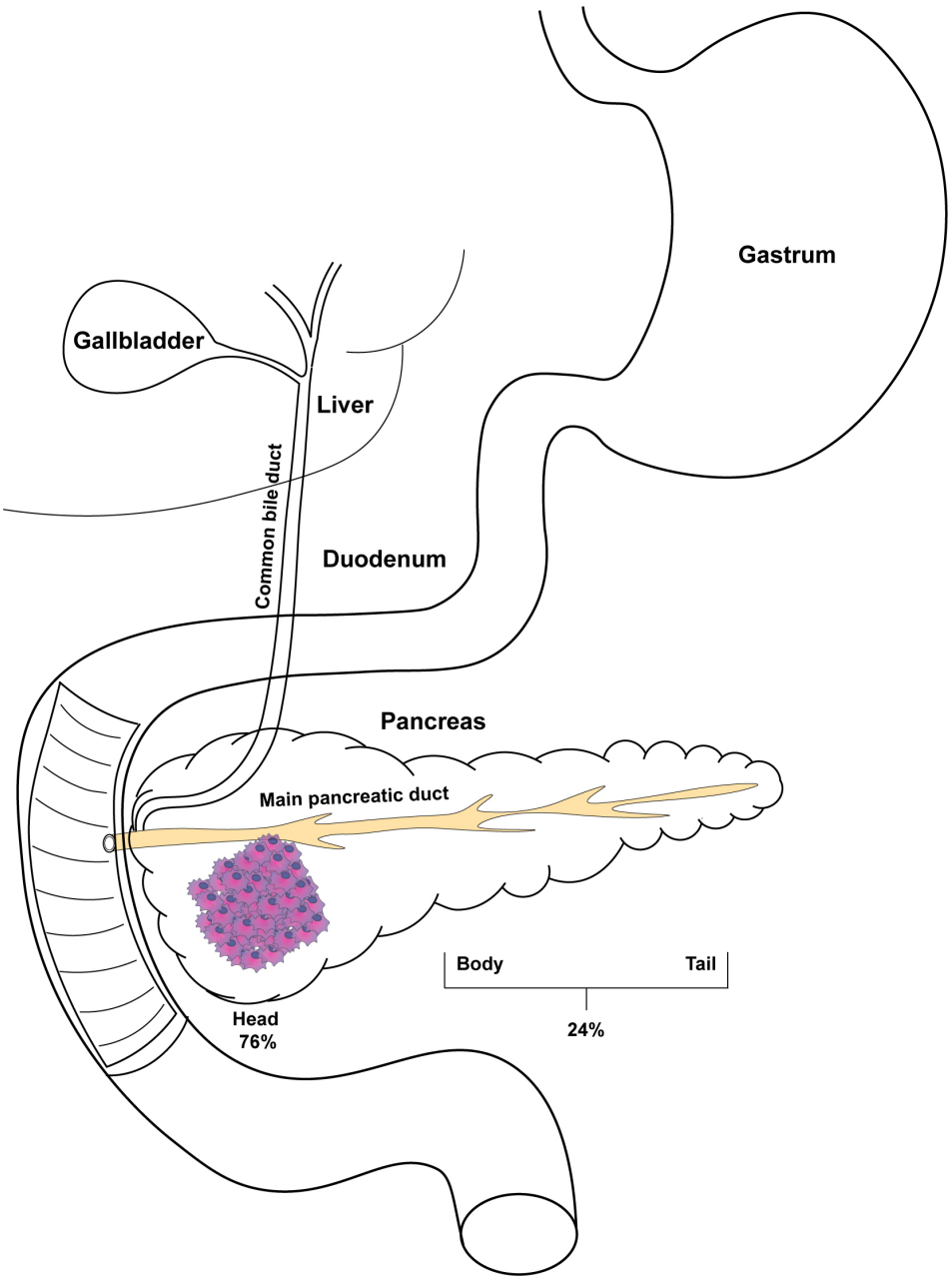

Figure 1. Illustration of the approximate location of the pancreas in relation to the liver and the small intestine. Percentages show the relative occurrence of pancreatic cancer (purple cells) in different anatomical parts of the pancreas. Figure by T. Lidström.

Epidemiology and prognosis

Pancreatic cancer is one of the major causes of cancer related deaths worldwide [7]. Pancreatic cancer incidence varies between different geographical regions. In western Europe the age standardized incidence is 7.7 per 100 000 people. There is a slight difference in incidence between men and women, 5.5 vs. 4 per 100 000 people [8]. In Sweden between 1 200 and 1 300 people are diagnosed with pancreatic cancer each year, corresponding to about 2% of all cancers [9].

PDAC has a poor prognosis, with a 5-year year survival of 7-10% [1, 7]. In Sweden the corresponding figure is 7.9% [9]. Early diagnosis followed by surgical resection and adjuvant medical treatment, the only potentially curative therapy, increases 5-year survival, but only to 18 % [10]. Because of poor survival statistics combined with increasing age of the population and lack of effective therapy, PDAC is predicted to become the 2nd most lethal cancer in the US by the year 2026 and predicted to cause over 46 000 deaths worldwide in 2040 [11]. The poor survival is in part also due to late diagnosis. PDAC presents either with diffuse or no early symptoms. Moreover, presently there are no well-characterized PDAC-specific biomarkers, which can be used for the routine screening in general population [12]. In addition, pancreatic cancer cells can metastasize early during tumor progression, limiting the use of curative surgical intervention as a therapeutic option [13]. Only 20-25% of patients can be treated with surgical intervention.

Etiology

Factors influencing the development of PDAC are multifactorial. The most prominent behavioral risk factor is tobacco smoking and studies have found a dose-response between smoking and increased pancreatic cancer risk. The risk of developing PDAC is nearly two times higher in smokers compared to non-smokers and tends to correlate with earlier onset of the disease. In addition, smokers with PDAC show worse overall survival compared to non-smokers [14-16]. Dietary factors, like high meat consumption, have also been associated with a higher risk of PDAC [17]. However, meta-analysis of prospective studies on red meat consumption have been criticized for not considering confounding factors, including type II diabetes, exercise habits, and body mass index [18].

The largest increase in risk of developing PDAC is related to inheritable factors. It is estimated that about 5- 10% of pancreatic cancer cases are associated to hereditary factors [19, 20]. Familial pancreatic cancer risk varies with genetic changes. Specific mutations in certain genes, including

CDKN2A, a tumor suppressor, and PRSS1 and SPINK1, responsible for causing hereditary pancreatitis, have been identified to increase PDAC risk. However, in most cases the genetic alterations that lead to the increased risk are still unknown[13]. One study of ~5 000 individuals with familial pancreatic cancer found a 9-fold increased risk of pancreatic cancer[21]. Another study found that the increase in risk was 18 times higher in familial pancreatic cancer kindreds compared to the general population in [22].

Chronic pancreatitis patients show, on average, a 13-fold increased risk of developing pancreatic cancer during their lifetime. For hereditary pancreatitis, the risk is even greater [23].

Diagnosis and staging

Most pancreatic cancer patients show vague or no symptoms in the early stages of the disease. This delays initiation of treatment and reduces the likelihood of curative surgical intervention at diagnosis. The most common symptoms include fatigue, weight loss, anorexia and abdominal or back pain. Tumors developing in the head of the pancreas can result in jaundice from blockage of the biliary canal, which in turn can cause pruritis [24-26].

The diagnostic workup for PDAC involves imaging with computerized tomography (CT) and endoscopy. A “double duct sign”, i.e., a concurring dilation of the pancreatic and common bile duct, is considered a sign of malignancy and followed up by further testing [27].

After initial diagnosis, CT or magnetic resonance imaging (MRI) scans of the tumor and surrounding tissue is performed to determine involvement of blood vessels and presence of metastasis, and thereby set the stage of the tumor. This ultimately determines if the patient is a candidate for surgery [28].

According to the TNM classification system, the tumor (T) in the pancreas is categorized by size, involvement of main retropancreatic arteries, regional lymph node involvement (N) and the presence of distant metastases (M) [8, 28, 29]. Below is a table summarizing the TNM-classification of pancreatic tumors [29].

Stage	TNM	Description
Ia	T1 (N0 M0)	The tumor is limited to the pancreas and has a size of less than 2 cm (T1)
Ib	T2 (N0 M0)	The tumor is < 4 cm but > 2cm (T2)
IIa	T3 (N0 M0)	The tumor is confined to the pancreas and is bigger than 4 cm (T3)
IIb	(T1-T3) N1 (M0)	Regional lymph node metastases (no more than 3 nearby lymph nodes) (N1)
IIIa	(T1-3) N2 (M0)	Regional lymph node metastases (>3) (N2)
IIIb	T4 (N0-N2, M0)	Involvement of truncus celiacus or arteria mesenterica superior
IV	(T1-T4, N0-N1) M1	Distal metastases

Table 1. Staging of pancreatic tumor. T denotes tumor, N denotes spread to nearby lymph nodes, M denotes spread to distant sites.

At the time of diagnosis, about 20% of tumors are deemed resectable. 5-10% of the tumors are considered borderline resectable, meaning that if these patients undergo surgery they are at high risk for margin-positive resections [30]. Most tumors, about 50-60%, show distal metastasis (Stage IV) at the time of diagnosis and are ineligible for curative surgical intervention [31].

Subtype classification

PDAC is not a homogenous disease. In recent decades development of more advanced sequencing techniques has allowed attempts to classify pancreatic cancers into subtypes based on transcriptional programs.

Bailey et al. identified four molecular subtypes, squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated squamous pancreas cancer. The immunogenic subtypes showed upregulated immune gene programs, including B-cell signaling, antigen presentation, and CD8+ T cell signaling pathways [32]. This analysis was based on 32 commonly mutated genes, clustered in 10 pathways in PDAC.

Moffit et al. identified two subtypes, classical and basal-like, while also classifying the tumor stroma as either activated or normal [33].

Collinson et al. defined three subtypes of PDAC; classical (high expression of epithelial and adhesion-associated genes), exocrine like (tumor cell derived digestive enzyme genes) and quasimesenchymal tumors (mesenchyme associated genes). [34].

Wadell et al. classified PDAC into 4 subtypes; stable, locally rearranged, scattered, and unstable [35] by variation in chromosomal structure.

Collectively, these reports show that PDAC prognosis may differ between different subtypes, but the current treatment paradigm is not stratified based on molecular subtypes. However, well defined classification from different PDAC subtypes could benefit future targeted therapies against PDAC.

Genetic alterations

Normal ductal cells in the pancreas display a cuboidal well-ordered appearance. When transformed, normal ductal cells take on a more columnar appearance, show increased deposits of mucin, and accrue atypical histology and morphology [3, 36] (*Fig. 2*).

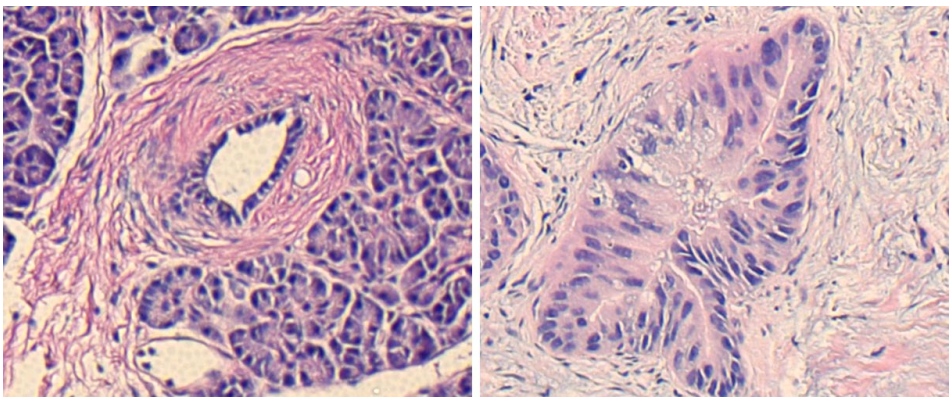

Figure 2. IHC H&E-stained images of normal pancreatic duct (left) and tumor duct (right). Image captured by D. Öhlund.

PanINs are the most common precancerous lesions preceding PDAC development. These lesions are characterized by increased accumulation of mutations transforming well-ordered ductal cells towards more dysplastic and invasive phenotypes. This process of genetic transition from normal tissue to cancer is predicted to span over up to 12 years [37]. Recent re-classifications have grouped PanINs into low-grade and high-grade PanINs, with low grade lesions described to have a lower risk of progression into PDAC [38].

Though PDAC is a heterogenous disease with multiple etiologies, several reports have implicated alterations in four major tumor suppressors and proto-oncogenes in the disease progression. These genes include *KRAS,* the main driver of proliferation, *TP53,* a tumor suppressor, *CDKN2A,* a tumor suppressor, and *SMAD4,* a tumor suppressor in TGFβ signaling pathway [39].

KRAS has been found mutated in more than 90% of all PDAC tumors. The mutation which causes G12D substitution, a change between glycine and aspartate on position 12, is found in 41 % of cases followed by G12V in 34 % and G12R in 16 % [35, 40-43]. Other less common substitutions make up the remaining percentages. Interestingly, the G12C variant, a target for FDA-approved anti-KRAS drug Sotorasib [44], is rare in PDAC [45] while accounting for the majority of KRAS mutants in lung adenocarcinoma[46].

Because of the high frequency of KRAs mutations, pancreatic cancer is considered the most *RAS*-addicted cancer. KRAS essentially acts as a

tightly regulated ON/OFF switch for cell proliferation by alternating between an active GTP bound, and an inactive GDP bound state[47]. In normal cells this switch is usually in the OFF position, GDP bound. When in this "OFF" position, KRAS requires a signal from cell surface receptor tyrosine kinases (RTKs) to activate into the GTP bound state [48]. In pancreatic cancer cells, mutation in the KRAS gene changes the conformation of the KRAS protein and locks the switch in the constitutively "ON" position, generating continuous proliferative signaling.

Experimental evidence has shown that inducing a G12D *KRAS* mutation is sufficient to induce the formation of low-grade PanIN precursor lesions [49, 50]. Further accumulation of mutations in cell cycle inhibitor gene *CDK2NA* [49], the tumor suppressor *TP53* [51], or *SMAD4* [52] accelerates the dysplasia of PanIN formation and transforms the lesion to PDAC.

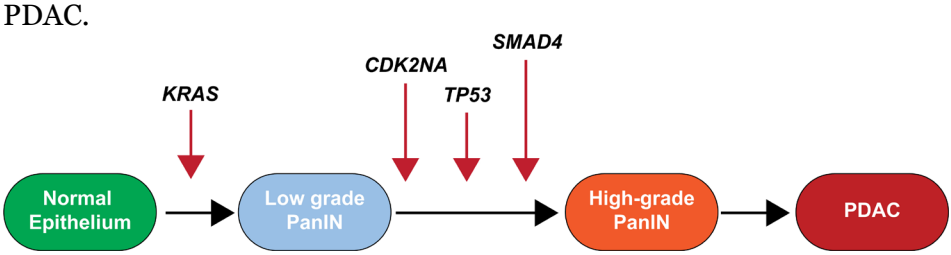

Figure 3. Timeline and progression of genetic alterations in PDAC. KRAS mutation by itself is enough to progress normal epithelium to low grade PanIN lesion. Accumulated mutations in CDK2NA, TP53 and SMAD4 in tumor suppressors increase dysplasia forming high-grade PanIN lesions which can progress to PDAC.

Since *KRAS* is the essential factor for PDAC cell proliferation, several approaches to inhibit the function of *KRAS* are currently being developed. This includes small molecule inhibitors targeting specific KRAS mutations and immunotherapy using designed CAR (Chimeric antigen receptor) T cell targeting. These emerging therapies for treatment of PDAC are described below.

The tumor microenvironment

PDAC is characterized by extensive desmoplasia, a dense fibrous stroma that can make up as much as 90% of the total tumor volume *(Fig. 4)*. The stroma is made up of a matrix of secreted extracellular proteins, cancer-associated fibroblasts (CAFs), blood vessels, nerve cells and immune cells.

The dense deposits of extracellular matrix (ECM) in the tumor have been linked to resistance to systemic therapy, because of increased interstitial pressure, poor perfusion, and the complex molecular composition of the extracellular matrix [53, 54].

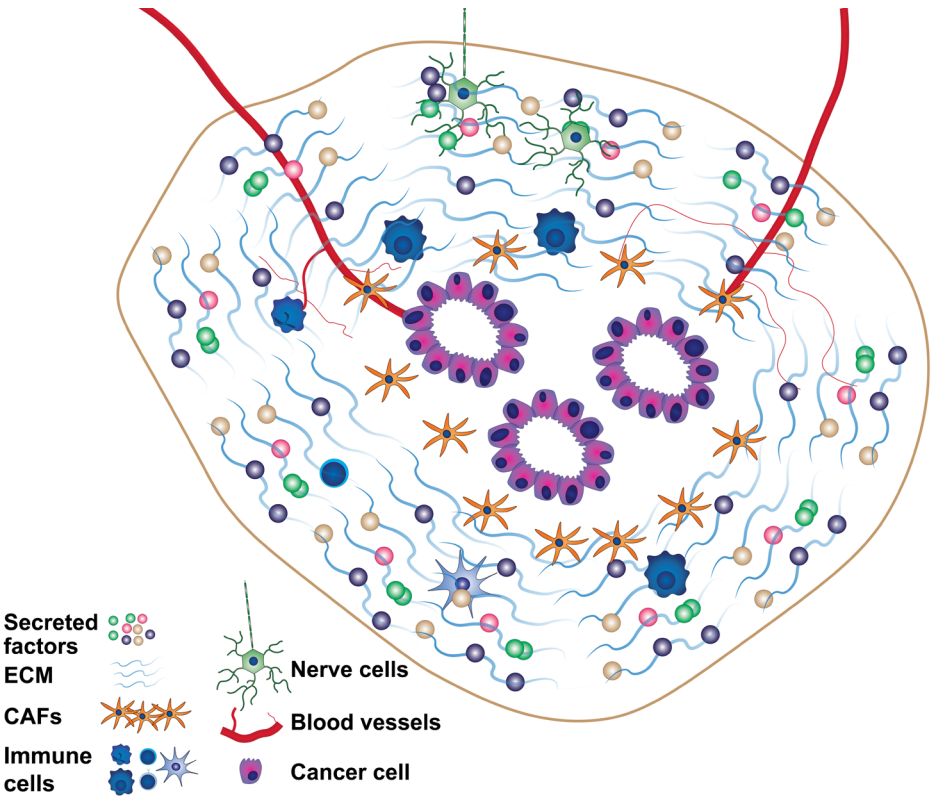

Figure 4. Illustration of a PDAC tumor with different cell types and components of the stroma. Illustration by T. Lidström.

Histologically, well differentiated PDAC form glandular clusters or tumor glands, surrounded by heavy deposits of ECM (*Fig. 5*). Aggressive pancreatic cancer forms tumor buds made up by migratory cancer cells at the invasive front of the tumor. Tumor buds have been reported as an adverse prognostic factor in pancreatic cancer [55-58]. Apparently, the process of forming tumor buds is similar to the epithelial-mesenchymal transition (EMT) process in pancreatic cancer cells. Tumor buds display reduced expression of the epithelial marker E-cadherin, and overexpression of other EMT biomarkers, like SNAIL, ZEB1 and ZEB2[59].

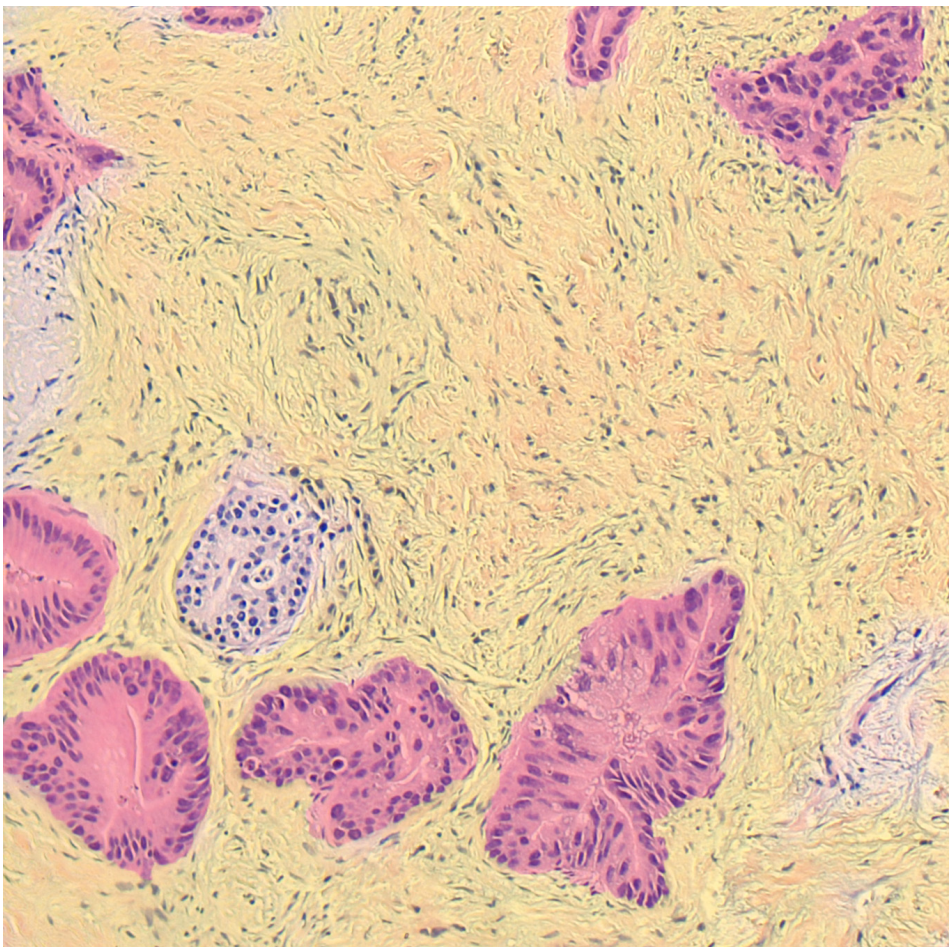

Figure 5. H&E-stained human PDAC. Tumor glands (red) surrounded by secreted ECM proteins and stromal cells (yellow). Image captured by D. Öhlund. Shading by T. Lidström.

Nerve cell infiltration into the tumors has been shown to be present in nearly all PDAC specimens [60]. Moreover, studies done in model systems of PDAC [61], and analysis of recurrence after surgical treatment, have linked neural invasion to metastasis, and thus to poor prognosis [62, 63]. Induction of Sonic hedgehog (SHH) signaling by hypoxia [64] has also been shown to promote neuronal invasion by stimulating pancreatic stellate cells (PSCs) to secrete factors that attract neurons to PDAC cancer cell colonies and facilitate metastasis formation [65].

The extracellular matrix and the matrisome

PDAC tumors show extensive deposits of ECM proteins, resulting in a dense fibrotic stroma. All ECM proteins are collectively referred to as the matrisome. The core matrisome consists of structural ECM proteins like collagens, proteoglycans, and glycoproteins. Besides core matrisome proteins, the matrisome also consists of matrisome-associated proteins. The matrisome-associated proteins can in turn be subcategorized into ECM regulators (proteases and cross-linking proteins), ECM-associated proteins (mucins, lectins, etc.), and secreted factors. In a tumor, all matrisome tumors are synthesized and secreted by cancer cells and stromal cells, with the major contributing cell being CAFs [66, 67].

Matrix metalloproteases (MMPs) belong to a group of proteases in the matrisome with the ability to degrade components of the extracellular matrix. There are 24 distinct MMPs identified in humans, they are divided into 6 categories based on their cleavage targets: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others. Overexpression of several MMPs has been linked to poor overall survival in PDAC [68].

Cancer cell-ECM interactions stimulate several signaling pathways, including proliferation, survival, differentiation, and migration [69]. The biophysical properties of the matrix also contribute to high interstitial pressure resulting in the collapse of intratumoral capillaries. This results in poor perfusion which contributes to decreased drug delivery and hypoxia, resulting in increased resistance to systemic treatment [53]. Therefore, high levels of collagen and hyaluronan in PDAC tumors have been linked to poor prognosis [70].

Cancer-associated fibroblasts

Cancer is classically described as a wound that never heals [71]. Pancreatic cancer is characterized by the involvement of wound healing cells, fibroblasts. Pancreatic stellate cells (PSCs), a fibroblast-like cell found in the pancreas, contain lipid droplets with vitamin A. These cells are normally present in a quiescent state, however, upon injury they become activated, lose the vitamin A droplets, and secrete ECM proteins [72]. In tumors, quiescent PSCs are stimulated by cancer cell-derived secreted proteins and juxtacrine interactions, to a state of constitutive activation, triggering excessive secretion of ECM proteins, which in turn, supports cancer cell proliferation [73]. Activation of PSCs is characterized by elevated expression of αSMA, a contractile stress fiber that confers contractile function important for wound closure in the wound healing

process [74, 75]. The activated PSCs in tumors are referred to as cancer-associated fibroblasts (CAFs), which is a collective term describing all fibroblasts in the tumor ECM [76]. CAFs were initially described as tumor promoting cells. However, complete depletion of αSMA expressing CAFs, as well as reduction of the desmoplasia by deleting sonic hedgehog, lead to worse overall survival and accelerated malignant progression [77, 78]. This highlighted CAFs as heterogenous populations of cells, and that different subtypes of CAFs may contribute to tumor progression, while others might act as tumor suppressors.

Initially two subtypes of CAFs were characterized in PDAC: αSMA expressing myofibroblast cancer-associated fibroblasts (myCAFs), and IL-6 and CXCL12 -producing inflammatory cancer-associated fibroblasts (iCAFs). Differentiation of CAFs into different subtypes is governed by multiple factors. iCAF differentiation is dependent on IL-1 signaling, while myCAF formation requires TGF-β [79-81]. These two subtypes are also spatially heterogeneously distributed in the tumor. myCAFs tend to localize directly adjacent to the cancer cells, while the iCAFs show a more distal distribution *vis-a-vis* the cancer cells [81].

Hypoxia is common in the TME of many solid tumors including PDAC [82]. Recently, hypoxia has been linked to iCAF formation through an increase in IL1α signaling. It was found that hypoxia can increase IL1α expression in the tumor and these hypoxic regions in the tumor were enriched for iCAFs [83].

CAFs have been generally described as tumor promoting cells. However, recent studies have shown that myCAFs can restrain tumor growth[78]. Moreover, specific types of tumor-restrictive CAFs have also been described. *Djurec et al.* found that Saa3 negative CAFs restricted tumor growth in an orthotopic mouse model [84]. Collagen 1 expression also contributes to the tumor restrictive phenotype of CAFs [85].

In addition to myCAFs and iCAFs, transcriptome analysis at a single cell level has led to characterization of new CAF subtypes. *Elyada et al.* found a CAF subtype expressing the antigen presenting receptor, MHC-II, but not activating co-receptors. The subtype was named antigen presenting CAF or “apCAFs” and is believed to be immunosuppressive [80]. Other transcriptomic studies have further classified CAFs into additional subtypes [86, 87] based on the gene expression programs, nevertheless, the iCAFs and myCAFs are still considered as the primary classification categories.

Current and emerging therapy

Surgery

PDAC tumors detected in earlier stages can be treated by surgical resection. By a pancreaticoduodenectomy, a Whipple procedure, the head of the pancreas, the first part of the duodenum, the gall bladder, and the bile duct are removed [88]. Surgery is currently the only curative procedure for PDAC. Unfortunately, only about 20% of patients present with a localized occurrence, qualifying for surgical intervention [89].

Chemotherapy

Postoperative adjuvant chemotherapy for 6 months following surgery is a routine treatment for PDAC in Sweden [90]. The chemotherapeutic agent gemcitabine has been considered the standard agent either as monotherapy or in combination with capecitabine. The combination therapy FOLFIRINOX, consisting of 5-FU, oxaliplatin, irinotecan and folinic acid, have shown to increase disease free survival in the adjuvant setting but is limited to patients with good performance status [91]. Patients unable to receive FOLFIRNOX are offered combination therapy of gemcitabine and capecitabine [92].

Neoadjuvant or preoperative treatment with FOLFIRINOX for borderline resectable tumors, conversion therapy, has shown to increase the number of patients that qualify for curative resection [93].

For non-resectable tumors palliative chemotherapy is standard therapy. Recommended first line treatment for patients with good performance status is FOLFIRNOX/ [94] or gemcitabine together with nab-paclitaxel [95]. Frail patients could be offered monotherapy of gemcitabine or gemcitabine/capecitabine combination therapy [96, 97].

Chemotherapy gives some survival benefits to PDAC patients with metastatic disease. About 30% show objective response to FOLFIRNOX therapy but only about 10% to gemcitabine. Palliative FOLFIRNOX therapy increased median survival to 11.1 months compared to 6.8 months in patients receiving gemcitabine in monotherapy [94]. Combination therapy of gemcitabine and nab-paclitaxel increased median overall survival to 8.5 months compared to 6.7 months in gemcitabine monotherapy treated patients with advanced pancreatic cancer [95].

Chemotherapy resistance is common in PDAC, and even very toxic combination therapies only provide small survival benefits for most of the patients. This emphasizes the need for new effective treatment strategies against pancreatic cancer.

Targeting the extracellular matrix

As mentioned above, one of the biggest hurdles in developing an efficient treatment regimen for pancreatic cancer is treatment resistance. This is in part due to the abundant deposits of secreted proteins in the ECM which shields the cancer cells from systemic treatment [54]. To dismantle this barrier, trials have been performed with broad spectrum inhibitors of MMPs, which are widely considered as facilitators of PDAC progression and metastasis [98, 99] and linked to poor overall survival [100-102]. While the early trials of these ECM-targeting drug candidates were promising, no clinical benefit was observed in randomized controlled trials using the MMP inhibitors [103-105].

Hyaluronic acid (HA) in the ECM has been associated with reduced survival in PDAC. PEGylated hyaluronidase (PEGPH2O) can digest hyaluronic acid in the ECM, reducing the barrier which protects the cancer cells. Initial phase II trials showed promising results. 6 patients classified as having high HA levels in the tumor stroma were treated with PEGPH2O in combination with gemcitabine, and the results showed increased overall survival (13 months) [106]. Unfortunately, phase III trials evaluating PEGPH20 + gemcitabine and nab-paclitaxel failed to confirm any improvement in overall survival in HA high classified patients [107].

Sonic HedgeHog (SHH) signaling has also been shown to be upregulated in PDAC [108]. SHH signaling stimulates myCAF proliferation, the CAF subtype which secretes most of the ECM components [109]. Studies in experimental PDAC models further verified that inhibition of SHH signaling pathway reduce tumor stroma and improve chemotherapy delivery [109]. Following these positive initial observations, phase 1b/II trial using the SHH inhibitor Vismodegib, in combination with gemcitabine, failed to demonstrate a benefit to either overall survival or progression free survival [110]. Another trial using a different SHH inhibitor, Saridegib, was stopped prematurely after primary analysis showed that the trial would not yield expected improvement in overall survival[111].

Immunotherapy

Treatment with checkpoint inhibitors, though successful in other cancer forms, has not been successful in PDAC [112]. Monotherapy using CTLA-4 or PDL1 blocking antibodies has not shown any benefits in PDAC treatment. However, a few limited clinical trials using combinations of two checkpoint inhibitors have shown some optimism [113]. The only partial success of checkpoint inhibition therapy was observed in a small subset of mismatch repair deficient PDAC tumors, where neoantigen frequency and presentation were found to have increased [114].

A new research approach suggests that the poor antitumor immunity is caused by poor T cell priming [115]. Antigen presenting cells (APCs), like dendritic cells (DCs), B cells, and myeloid cells, express the surface marker CD40. It has been demonstrated that agonistic stimulation of CD40, which promotes T cell priming, could overcome resistance to anti-PD1 therapy in T cell PDAC tumors [116].

The failure of checkpoint inhibition in PDAC contrasts with the remarkable response observed in other types of cancers [117]. This lack of response is likely due to the presence of multiple immunosuppressive proteins and mechanisms in the tumor microenvironment. These mechanisms will be discussed further in this thesis. Combination therapy targeting these immunosuppressive proteins, together with checkpoint inhibitors, could therefore prove effective against PDAC.

CAR T cells

CAR T cells are engineered T cells with specific antigen receptors targeting tumor antigens, coupled with co-stimulatory signaling proteins. The construction process consists of extraction of patient lymphocytes from peripheral blood, adding specific antigen receptors targeting tumor antigens, expanding, and reintroducing T cells into the patients. CAR T cells can self-renew, directly target, and destroy tumor cells expressing the targeted antigen. Early results using CAR T cells to target metastatic lesions in melanoma patients have shown promising results [118].

In PDAC several highly expressed proteins have been identified as potential targets for CAR T cell development. CAR T cells targeting mesothelin, a highly expressed protein in several cancers including pancreatic cancer, have shown some ability to halt the cancer progression but not eradicate the tumors [119]. CAR T anti-mesothelin are still undergoing clinical trials, with a recent study using lentivirus to transduce the anti-mesothelin antibody chain (NCT03323944).

PSCA is the prostate stem cell antigen expressed in about 60% of primary PDACs, but not in normal ductal tissue [120]. PSCA is also highly expressed in PanIN precursor lesions which makes it an attractive target for CAR T therapy [121]. Preclinical data has shown encouraging results and the therapy has entered clinical trials (NCT02744287).

CEA (carcinoembryonic antigen) is a cell surface glycoprotein currently in use to follow treatment response in pancreatic cancer. CEA is highly expressed in the majority of pancreatic cancer cells[122]. Unfortunately, trials using CAR T cells directed against CEA have led to acute respiratory toxicity in preclinical trials making further development unlikely [123].

As described above the most common driving mutation of pancreatic cancer are KRAS gain-of-function mutations. The most common amino acid substitution being G12D. Recently it was reported that a single patient showed regression of visceral metastatic lesion following infusion of T cells targeting the *KRAS* G12D mutation, with the neoantigen expressed on the MHC-1 of the cancer cells [124]. The effectiveness of this type of genetically engineered T cell in a larger cohort remains to be seen.

Other targets being pursued for CAR-T cell therapy, which are in earlier stages of development, include CD24, CD47 (prevents phagocytosis), tyrosine kinase growth factor receptors (including IGF1R, FGFR, PDGFR), fibroblast activation protein (highly expressed in stromal cells) and HER2 [125].

Targeting receptor tyrosine kinases

To grow and spread tumors need to promote the formation of new blood vessels and receive continuous proliferative receptor signals. These are two of the hallmarks of cancer [126]. Bevacizumab which neutralizes VEGF has been effective in the treatment of other cancers (reviewed in [127]). In PDAC, patients did initially show some promise in phase II clinical trials [128]. However, bevacizumab inhibition failed to improve survival when tested on 602 patients with advanced pancreatic cancer [129]. In contrast, the EGFR tyrosine kinase inhibitor, erlotinib, commonly used for treatment of non-small cell lung cancer, did show a small significant effect (median survival 6.24 months versus 5.91 months) on overall survival in advanced pancreatic cancer patients [130].

Mutational Targeting

The most common *KRAS* mutation in PDAC is the G12D mutation, though other variants exist. Because of the prevalence and importance of the

KRAS mutation to drive proliferation, numerous compounds have been developed to target specific *KRAS* mutations.

Small molecule inhibitors targeting G12D mutations are currently undergoing preclinical development. In early trials involving xenograft model of PDAC the in vivo response rate was 73% [131].

Clinical trials using G12C targeting inhibitors, though rare in PDAC, have also demonstrated increased progression free survival. In 10 advanced PDAC patients treated with the inhibitor progression free survival increased to 6.6 months compared to less than three months in controls [132].

Novel matrisome derived targets for therapy development

CAFs and tumor cells produce and secrete several stromal proteins, including core ECM proteins, ECM regulators, and ECM-affiliated proteins, which, together with other secreted factors, make up the matrisome [66].

By purifying the ECM from PDAC tumors, taking advantage of the unique solubility features of the ECM [133], followed by proteomic analysis, have revealed several matrisome as potential targets for future therapy development. Categorically, ECM components including glycoproteins and structural proteins like collagens, were found to be upregulated in the tumors when compared to the ECM of normal pancreatic tissue. Interestingly, numerous ECM-affiliated proteins, proteins secreted by CAFs or epithelial cells and trapped in the ECM, were also found to be upregulated. Subsequent survival analysis using publicly available databases found that many of the cancer cell derived ECM-affiliated proteins were correlated with poor survival [133].

After further analysis of this dataset, we have identified several protein candidates that are upregulated in the ECM of PDAC tumors when compared to normal pancreatic tissue. These candidates include the protein galectin 4, which is described in the literature as immunosuppressive [134]; SERPINB5, a proteinase inhibitor which is described in the literature as a tumor suppressor [135]; Cystatin B, (CSTB) a cysteine protease inhibitor described as having both tumor promoting and suppressing functions [136]; and agrin, which is known to be involved in invasion and metastasis of different cancer types [137]. These proteins

were selected as matrisome candidate proteins which could be potential therapeutical targets in pancreatic cancer.

Characterizing these highly expressed cancer cell-derived matrisome proteins is the basis for the results presented in this thesis. Below is the described function of SERPINB5, CSTB, agrin and galectin 4.

SERPINB5

Serine (or cysteine) proteinase inhibitor clade B (SERPINB5) is physiologically a negative regulator of endopeptidase activity. In cancer, it is described as a tumor suppressor, blocking growth invasion and metastatic activity in mammary cancer [135]. Tissue micro array analysis in pancreatic cancer identified SERPINB5 upregulation early in tumor progression, and that it is negatively related to postoperative survival [138]. Together with four other genes, a meta-analysis used SERPINB5 expression as a marker to distinguish normal tissue from pancreatic cancer [139]. Further studies based on transcriptome analysis, using both xenograft and microarrays, verified SERPINB5 as an upregulated protein in pancreatic cancer [140, 141]. Analysis of signaling pathway in pancreatic cancer cell lines has showed that SERPINB5 is involved in AKT signaling pathway and found to be one of 22 downregulated proteins in the secretome of these cancer cell lines [142]. Mass spectrometry analysis of dissociated ECM proteins found that SERPINB5 is overexpressed in pancreatic tumors and that high expression of SERPINB5 is linked to poor survival in PDAC [143].

Agrin

Agrin is a heparan sulfate proteoglycan and is a component of the basement membrane. In several cancer types, agrin has been linked to invasion and metastasis. In liver cancer agrin is upregulated by increased ECM stiffness and transduce signals via YAP protein translocation. Agrin also increases invadopodia (cell protrusions linked to ECM degradation) and EMT transition in hepatocellular carcinoma [137, 144] .

CSTB

Cystatin-B (CSTB) is an intracellular cysteine proteinase inhibitor. In cancer, both tumor promoting and tumor suppressive roles of CSTB have been described. In gastric cancer cell lines, downregulation of CSTB resulted in increased proliferation and migration [145]. CSTB is an inhibitor of cathepsin, which is upregulated in many cancers and correlated to poor prognosis [136, 146].

The galectin family of proteins

Galectins, galactose binding lectins, are a family of proteins with a carbohydrate recognition domain (CRD) [147]. Galectins are grouped into three subfamilies based on spatial structure of the CRD [148], with the numerical names are based on the order of discovery. Galectin 1, 2, 5, 7, 10, 11, 13, 14 and 15 are monomer galectins with one CRD. Tandem repeat galectins include galectin 4, 6, 8, 9 and 12. The third subfamily is the chimera type, forming multimers, and includes galectin 3.[149]. (See *fig. 6*)

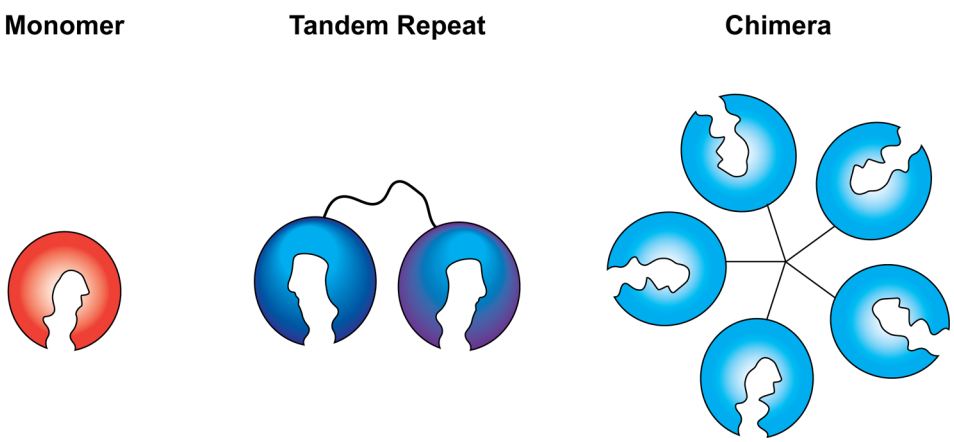

Figure 6. Classification of the galectin family of proteins. Galectin divides structurally into monomeric, tandem repeat or chimeric configurations. Figure by T. Lidström.

The function varies between family members, but they all have a common theme of immunomodulation[150-152]. Galectin-1 (gal 1) and 3 (gal 3) have been widely studied in PDAC and have been shown to affect tumor progression and metastasis[153-157] while contributing to immunosuppression [158-162].

Upregulation of gal 1 in pancreatic cancer has been linked to PSC activation and promoting fibrosis. Gal 1 expression also relates to poor survival. Moreover, TGFβ has been found to upregulate expression of gal 1 in PSCs [163]. Depleting gal 1 results in impaired tumor formation and metastasis in a murine model of pancreatic cancer. Tumors with reduced gal 1 expression also show increased numbers of CD4+ and CD8+ T cells and decreased number of CD11b+ and GR1+ myeloid-derived suppressor cells (MDSCs) [164].

Gal 3 modulates T cell function by binding to tumor infiltrating lymphocytes (TILs). This binding result in a reduction of IFNγ secretion which can be restored by detaching bound gal 3 from the T cell [165].

Galectin 9 (gal 9) is a ligand of the TIM-3 receptor, a known exhaustion marker of tumor infiltrating lymphocyte. TIM-3 expression, as well as other inhibitory immune cell receptors, immune checkpoint receptors, are highly expressed on immune cells in the PDAC tumor microenvironment[166]. TIM-3 signaling induces apoptosis in exhausted T cells and generally polarizes immune cells towards a Th2 fate [167-169]. Blocking the interaction between gal 9 and its binding partner dectin-1 on macrophages improved intratumoral T cell activation in PDAC. The inhibited interaction also inhibited macrophages from forming a tolerogenic phenotype[170].

Galectin 4

Galectin 4 (gal 4) is a tandem repeat galectin consisting of two carbohydrate recognition domains connected by a flexible linker. Gal 4 is normally expressed in epithelial cells lining the antrum, ileum, colon, and rectum[134]. *In vitro* experiments show that intracellular gal 4 regulates proliferation, apoptosis and differentiation, while extracellular gal 4 is linked to cell adhesion [171] and immunomodulation [134].

Since gal 4 expression is high in enterocytes of the small intestine, the role of gal 4 in intracellular processes in this cell type has been studied extensively. It has been shown that gal 4 is involved in trafficking of glycoproteins to the apical side of enterocytes, by binding complex-type N-glycans [172]. The ability of gal 4 to bind N-glycans is also central to its effect on immunity. Interestingly, it has been shown that both gal 4 and gal 8 can recognize and bind to blood group antigens (similar to N-glycans) expressed on pathogens [173].

Gal 4 seems to have contrasting roles in inflammation. Studies have shown that gal 4 can both ameliorate[134] and exacerbate intestinal inflammation by increasing CD4+ T cell IL-6 production[174]. Similarly, *Nishida et al.* identified an O-linked glycan expressed on CD4+ T cells which serves as a binding target for gal 4. When bound, it stimulates expansion of CD4+ memory T cells and exacerbates intestinal inflammation[175].

In addition to these contradictory findings, gal 4 is also associated with increased intestinal epithelial wound healing [176]. *Paclik et al.* showed that extracellular gal 4 binds the CD3 receptor of T cells and prevents

cycling and expansion which eventually lead to calpain-mediated T cell apoptosis [134]. The immunosuppressive role of gal 4, together with its expression pattern in the GI-canal, indicates that gal 4 is involved in immune tolerance towards food antigens.

Previous work in PDAC has confirmed the distinctly different cell-intrinsic and extracellular roles of gal 4 in PDAC.

The mechanism of secretion of gal 4 has not been defined. The protein lacks signal sequence for ER transport, but its externalization has been linked to tyrosine phosphorylation by *src* family of kinases [177].

In PDAC, gal 4 is linked to reduced migration and metastasis [178, 179] This effect has been linked to modulation of the *wnt/beta-catenin* pathway[178]. In studies on resected PDAC tumors, gal 4 expression was found to be linked to recurrence and differentiation of the tumor [180]

The intracellular effect of gal 4 on migration/differentiation has also been noted in other forms of cancer.

In studies on colorectal cancer (CRC) derived cell lines, abrogated gal 4 expression was found to promote tumorigenesis by increasing proliferation. High gal 4 expression reduced cell migration and motility and was linked to increased differentiation in colorectal cancer [181, 182]. In contrast, high gal 4 expression in lung, gastric, and prostate cancer, is instead linked to metastasis and poor clinical outcome [183-185].

Metastasis promoting properties of the stroma

SERPINB5, CSTB and agrin have been linked to signaling pathways related to metastatic processes including EMT, invasion/migration and invadopodia. Preventing metastasis would result in major survival benefit in PDAC since metastasis is a major cause of mortality and morbidity in pancreatic cancer.

To be able to metastasize, cancer cells need to become motile (EMT, invasion/migration), form new blood vessels (angiogenesis), invade adjacent tissue, and prepare the new site for colonization (metastatic niche). All these processes have been found to be influenced by different factors in the stroma.

EMT is a process in which cancer cells lose expression of genes related to an epithelial phenotype and increase expression of markers related to a mesenchymal phenotype. This process is coupled with increased motility of cancer cells leading to metastasis. Analysis of resected PDAC tumors have shown that classifying tumors on the ratio of the EMT marker vimentin to the epithelial marker E-cadherin could predict prognostic features of PDAC. Mesenchymal tumors are also associated with increased metastasis and portal vein invasion. Taken together, patients with more epithelial tumors show better survival compared to patients with more mesenchymal tumors [186].

Analysis of animal models of PDAC metastasis using a tag and track method found that metastasis occurs early in tumor progression and begins with EMT [187]. EMT can be promoted by stromal cells. For instance, signaling between epithelial cells and immunosuppressive cells, such as TAMs, can promote EMT. M2 tumor-associated macrophages (TAMs), when co-cultured with pancreatic cancer cells, have been shown to stimulate upregulation of EMT markers and decrease expression of the epithelial marker E-cadherin. These changes are driven by TLR4 and IL-10 signaling in TAMs [188].

Extravasation and angiogenesis are key processes of successful metastasis formation. Cancer cells can stimulate vascular endothelial growth factor (VEGF) expression in many ways to form new blood vessels. This includes expression of STAT3 [189], induction of hypoxia [190] , and activation of NFκB [191]. Indirectly, cancer cell-derived IL35 can recruit TAMs to tumors. These macrophages express CXCL1 and CXCL8 which promote angiogenesis [192]. In addition, TAMs can stimulate angiogenesis directly by secreting VEGF [193].

Metastasis also occurs through lymphatic vessels. Lymphatic vessel density in PDAC has been shown to inversely affect patient survival and has also been linked to M2 macrophages. Recruited M2 TAMs increase lymphatic vessel density and accelerate lymphatic metastasis [194].

Besides, PSCs in the stroma can also produce factors that influence metastasis. CD10+ PSCs were shown to increase expression of a matrix metalloproteinase, MMP3, which remodels the ECM by catabolizing stromal components and destroying the basement membrane [195]. CD10+ PSCs were linked to shorter survival and lymph node metastasis in PDAC [196]. Gal 1 production by activated PSCs can also affect pancreatic cancer cell metastasis and is another example of cancer cell paracrine signaling. Cancer cell-produced TGFβ stimulate activated PSCs

to produce gal 1 [163]. Gal 1 expression, in turn, increases production of SDF-1 (CXCL12), which promotes metastasis [157] and prevents immune cell infiltration [197].

Furthermore, mechanosignaling between the stroma and cancer cells has been linked to cancer cell invasion. ECM stiffening creates an environment conducive to cancer cell invasion via CAFs or integrin signaling [198, 199]. A study of primary tumor and matching liver metastases found similar amounts of desmoplasia [70] and increased levels of solid stress in the metastasis compared to the primary tumor [200]. Certain signaling pathways have been shown to affect mechanosignaling of the PSCs present in the stroma. ATRA, a vitamin A metabolite, has been shown to reduce the ability of PSCs to generate high traction forces, which lead to a reduction in cancer cell invasion in model systems of pancreatic cancer [201].

Invasion and migration of cancer cells are important for metastases. Cancer cells can migrate into adjacent blood vessels, using a process of extravasation to access distal sites. Extravasation can be influenced by stromal cells. TAMs secrete pro-inflammatory proteins that can influence MMP expression by cancer cells, degrading ECM and promoting invasion. Secreted MIP-3a signals cancer cells to increase MMP9 expression. This increased expression of MMP9 has been shown to increase invasion of cancer cells into collagen IV in an invasion model of PDAC [202, 203].

To metastasize to distal sites, cancer cells adhere to the endothelial membrane and continue with extravasation and transendothelial migration [204]. Circulating pancreatic cancer cells express very high levels of ECM proteins which may assist in the establishment of metastasis. High SPARC expression in the circulating epithelial cells was also linked to increased cell migration and invasiveness [205].

Circulating epithelial cancer cells forms when cells extravasate into the bloodstream. These circulating cancer cells have been described as the “seed” of metastasis. The “soil” is the metastatic site where the cancer cells adhere and establish a metastasis. The metastatic site can be prepared by the primary tumors by secreted factors, which influences the microenvironment to be more beneficial for metastases growth, creating the pre-metastatic niche [206].

The main site of metastasis for pancreatic cancer cells is the liver. To form a pre-metastatic niche in the liver, cancer cells can increase proliferation and expression of collagen and c-fibronectin in hepatic stellate cells [207].

Primary tumor produced exosomes have also been shown to affect hepatic stellate cell gene expression to create the metastatic niche [208].

Identified matrisome candidate proteins agrin, CSTB and SERPINB5 have been linked to metastasis processes. Agrin has been shown to be involved in mechanosignaling in liver cancer [137] . The role of CSTB and SERPINB5 are more contrasting, both proteins have been described as inhibiting tumor progression, but are also linked to signal pathways related to metastasis.

Immunosuppressive properties of the stroma

Cancer cell-derived secreted matrisome proteins secreted to the stroma in the stroma contributes to immunosuppression in PDAC [153, 161, 167]. Several immunosuppressive galectin family proteins have been found upregulated in the stroma of PDAC tumors, contributing to immunosuppression [133]. Secreted matrisome proteins are however, only one part of immunosuppression conferred by the stroma.

Desmoplasia in PDAC leads to a dense macroscopic barrier of fibrotic stroma and forms the first level of immunosuppression. Increased interstitial pressure collapses the few intratumoral capillaries and reduces perfusion to form another physical barrier and further prevents immune cell infiltration.

The mechanical properties are not the only suppressive feature of PDAC. Numerous secreted factors, including cancer cell-derived matrisome proteins, stromal- and cancer cell-derived chemokines, etc., contribute to immune resistance.

In addition, metabolic regulation by amino acids catabolism and generation of inhibitory nucleosides further inhibits the immune response.

Lastly, suppressive immune cells, a part of the stroma, infiltrate tumors in response to stromal factors, and mediate immunosuppression. Stromal factors may also polarize tumor resident immune cells into suppressive immune cells providing another mechanism of immunosuppression.

All these different systems co-operate to inhibit immunosurveillance, resulting in immune evasion of cancer cells.

Immunosurveillance of cancer cells

Resurgence of old ideas is a common phenomenon in science. In 1866 the German scientists Fehleisen and Busch found that there was significant tumor regression following erysipelas infection (cutaneous streptococcal infection). In 1901 Paul Ehrlich presented the idea that the immune system could repress a potential "overwhelming frequency" ("in einer ungeheurer frekvenz") of carcinomas [209]. Following the maturation of the immunology field, researchers formulated a hypothesis that cancer cells were distinguishable from healthy cells by postulating the existence of "tumor antigens" [210, 211]. This led to the concept of immunosurveillance, i.e., the immune system monitors and removes cancerous cells.

Development of inbred mouse models enabled researchers to experimentally evaluate immunosurveillance. Immune system mediated rejection of tumors gained interest in the 1990s when it was discovered that tumors grew faster in mice treated with anti-IFNγ antibodies [212]. Mice lacking functional T-, B- and NKT -cells (RAG-2), also develop more sporadic neoplasm, highlighting the importance of these cell types for immunosurveillance[213]. Interestingly, transferring sporadic neoplasms from immunocompromised mice to immunocompetent mice clears the tumor [214] while the similar transfer between two immunocompetent mice does not. This contrasts the ability of the immune system to clear transformed cells, while immune mediated pressure on cancer cells selects for immune evasion proficient cancer cells.

The role of the immune system in pancreatic cancer

The immune system consists of two distinct entities, the innate and the adaptive immune system. The role of innate immunity is to provide a fast and generalized attack against invading pathogens while adaptive immunity is slower and attacks more specific targets. Innate immune cells are made up of macrophages, neutrophils, NK cells, mast cells, eosinophils and basophils. The adaptive immune system comprises T cells and B cells. There are also cells bridging the innate and adaptive immune system, including NKT cells and dendritic cells.

The different types of immune cells have been described to have both tumor-promoting and tumor suppressive features in pancreatic cancer. *Table 1* below, lists some of the well-studied effects of innate and adaptive immune cells on progression of pancreatic cancer.

Immune cell types	Tumor-promoting	Tumor-suppressive
Macrophage	M2 TAMs accelerate metastasis [194]. M2 macrophages in close proximity to cancer cells result in poor prognosis[215]. Gal 9 binding to dectin-1 on macrophages promotes tolerogenic phenotype [216]	
Dendritic cells (DC)	Crosstalk between dendritic and Tregs, restrain CD8+ T cell activation [217]	Better overall survival (OS) regardless of stage at Diagnosis. Circulating DCs related to better prognosis [218-220]
Myeloid derived Suppressor cells	Numerous studies show MDSC promoting tumor progression. Depleting L-arginine by expression IDO, inhibiting T cell function [221-223].	
Neutrophils	Linked to poor prognosis [224, 225] and checkpoint inhibition resistance [226]. Promoting metastases[227]. Depletion of granulocytic MDSCs increases CTLs and apoptosis in tumor cells [221].	
NK cells		Circulating NK cells in treatment-naive patients show improved OS

		survival[228]. Post-resection quantities are linked to better OS [229]
T regs	Promoting tumor progression [230]. apCAFs induce Treg formation suppressing immunity, associated with shorter survival and immunosuppression [231-233]	
Thelper, CTLs		Numerous studies related to improved overall survival [234-237]

Table 2. Tumor-promoting and Tumor-suppressive roles of innate and adaptive immune cells in pancreatic cancer.

Immunoediting is a dynamic process resulting in selection of immune system resistant cancer cell clones. The immune system targets and destroys tumors without sufficient immunosuppression. Tumor cells expressing factors enabling evasion from immune destruction progress. Below is a summary figure of immunoediting in pancreatic cancer showing the effect of suppressive immune cells, metabolic inhibition and cancer cell derived immunosuppressive factors that aid escape from the immune mediated destruction *(Fig. 7)*.

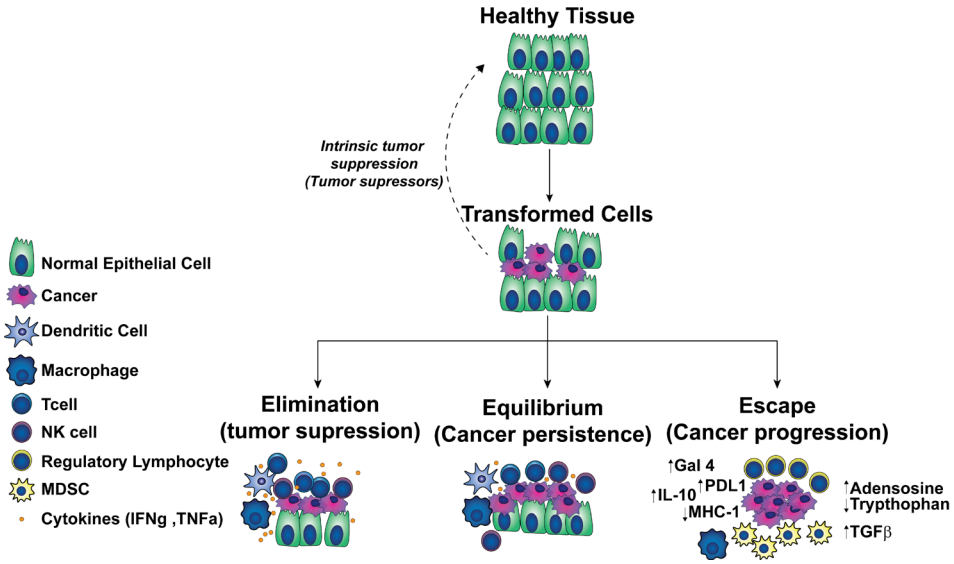

Figure 7. Pancreatic cancer immunoediting. Normal cells acquire mutations and overcome intrinsic tumor suppression. The immune system responds, however numerous metabolic, suppressive cells, cytokine or protein derived immunosuppression enables immune system evasion. Figure by T. Lidström.

T cells in Pancreatic cancer

To attack cancer cells T cells need to be primed, commonly in an adjacent lymph node, by presentation of tumor neoantigens by antigen presenting cells (APCs). When activated, T cells differentiate into antigen-specific cytotoxic T cells (CTLs). CTLs are proliferative, express IFNγ, and can differentiate into memory CTLs that are very long lived. Following the attack of tumor cells, neoantigen specific CTLs commonly undergo apoptosis. Memory CTLs can renew the population of neoantigen specific CTL clones to extend anti-tumor immunity. Tumor cell elimination takes place via cytotoxic granzyme release, production of cytokines TNFα and IFNγ, or binding of cell surface ligands, like FasL to the targets' death receptor FasR [238, 239]

To effectively target and clear cancer cells, CD8+ T cells need to be in direct contact, and the presence of direct contact between T cells and cancer cells has been shown to be a positive predictor of survival in PDAC. In pancreatic cancer, the spatial arrangement of CD8+ cells establish in the periphery of the tumor in the fibrotic stroma because of the influence of tumor defenses [240, 241]. In general, T cells are rare in PDAC.

Infiltrating cells are intercepted by secreted chemokines, like CXCL12 secreted by PSCs [241], which can repel infiltration. [237].

Studies pertaining to long-term survivors of PDAC found that the quality of T cells, rather than the total neoantigen load, was determinant of survival [242]. A similar observation was made in a cancer vaccine study where CD8+ T cell abundance in general did not correlate with survival but a subpopulation of granzyme B+ CD8+ T cells did [235].

Macroscopic structures observed in PDAC have also been linked to favorable prognosis. Tertiary lymphoid structures (TLS) contain many different types of immune cells, including T cells, B cells, and APCs. TLS in pancreatic cancer patients have been linked to increased survival [243, 244].

To successfully grow and spread cancer cells need protection from immune mediated destruction. This can be accomplished by producing factors that recruit cancer protective cells, directly disrupting infiltrating cells with secreted factors or upregulating membrane bound ligands.

Mechanism of Immune evasion

Pancreatic cancer is historically considered non-immunogenic due to numerous immunosuppressive systems. Though, transcriptome classifications have recently shown that a subset of PDAC tumors can be immunogenic.

PDAC tumors use different mechanisms to protect against attack from the immune system; recruitment of regulatory immune cells, secretion of immunosuppressive chemokines, cytokines, expression of cell-surface proteins, and productions and secretion of immunosuppressive proteins, such as galectins.

The tumor microenvironment also confers resistance by high interstitial pressure, poor perfusion, hypoxia, and metabolic inhibition.

Expression of cell-surface proteins and secreted proteins

PDAC tumors express immune cell checkpoint inhibitors that when stimulated cause apoptosis in activated immune cells. Early studies found that the checkpoint inhibitor PDL1 was highly expressed in pancreatic cancer and that high expression was linked to poor prognosis [245] later studies also verified this link in a bigger patient cohort using modern

techniques [236]. CTLA-4, which is expressed on the surface of activated immune cells, were also shown to be correlated with poor outcome[236]. Cancer cells can also downregulate MHC-1 on the cell surface to avoid binding of MHC-1 dependent cytotoxic cells, like CTLs. Tumor cells can still be targeted for destruction by NK cells, detecting the absence of MHC-1, a hypothesis that has been named "the missing self" hypothesis[246]. MHC-1 downregulation in PDAC have been described as a mechanism in several papers. *Yamamoto et al.,* described that autophagy degradation of the cell surface protein-presenting receptor MHC-1 contributed to pancreatic cancer immune evasion [247]. IHC and cell line analysis of human pancreatic cancer was also consistent with this finding. Cell lines showed low expression of MHC-1, and associated antigen transporter proteins [248].

To prevent another way CD8+ T cells target and destroy cancer cells, "the kiss of death". Cancer cells downregulate, or express non-functional Fas receptors, on the cancer cell surface. In addition, cancer cells can express Fas ligand on the cell surface enabling counterattacks against attacking immune cells [249].

Suppressive and regulatory immune cells

Pancreatic cancer recruits and polarizes infiltrating immune cell populations into immunosuppressive cells that help reduce anti-tumor immunity. Regulatory T cells (Tregs), tumor-associated macrophages (TAMs), myeloid suppressor cells (MDSCs), and regulatory B cells (Bregs), make up the primary immunosuppressive cells in PDAC [231, 250-253]

Regulatory T cells (Tregs) suppress the activity of cytotoxic T cells and have been associated with poor prognosis in pancreatic cancer[230]. Typically, Tregs assert their effect by producing IL-10 and TGFβ, or by interacting with inhibitory receptors on effector T cells [254, 255]. Tregs can also assert metabolic inhibition on effector immune cells by elevating kynurenine concentration and tryptophan catabolism. Increased kynurenine concentration lowers the available tryptophan in the TME, which is required for effector T cell metabolism [256].

Bregs, a subset of B cells have been described as having a regulatory function by secreting tolerogenic cytokines, IL-10 and TGFβ [257], in a similar way to Tregs.

Th17 lymphocytes (IL17 expressing T cells) infiltration have shown contrasting results. Analysis of tissue sections showed greater numbers of Th17 cells in pancreatic cancer tissue compared to adjacent normal tissue. Patients with lower number of Th17 cells had increased survival while patients with higher numbers of Th17 cells were associated with increased invasion and lymph node metastasis [258]. Paradoxically Th17 cells have also been shown to promote cytotoxic T cell activation in lung melanoma, a mouse model of poorly immunogenic B16/F10 melanoma that colonizes the lung [259].

Tumor-associated macrophages, TAMs, are reported to be a large part of the immune cell population in PDAC tumors [32, 236]. They are categorized into two subtypes: the classically activated M1 macrophages, expressing tumor necrosis factor, inducible nitric oxide synthase, and MHC-II; M2 macrophages, which are immunosuppressive cells expressing CD163, CD204, and CD206 [260]. Survival analysis also shows that an increased number of M2 macrophages in PDAC tumors result in accelerated metastasis [194]. Polarization of M2 macrophages also drives tumor growth in animal models of PDAC [261].

MDSCs are a heterogenous group of myeloid immunosuppressive cells. MDSCs can develop from two different progenitor cell lineages, either poly morphonuclear cells or monocytes. The markers, Ly-6C and Ly-6G are used to differentiate the different lineages, with common markers CD11b+ and Gr-1+ for both[262]. MDSCs use arginase 1 to deplete the essential amino acid L-arginine from the TME as the mechanism of immunosuppression. Reduction in the levels of L-arginine results in T cell inhibition [223, 263]. It's been demonstrated that reducing tumor derived GM-CSF (granulocyte macrophage stimulating factor), which reduces recruitment of MDSCs, and depleting MDSC using zoledronic acid in mouse models resulted in reduced tumor growth and enhanced survival [264].

Tumor cells also recruit innate immune cells through the secretion of chemokines. Pancreatic cancer cells secrete CXCL8 and CXCL16 which attracts neutrophils [265]. Notably, TGFβ, which is highly expressed in PDAC, also recruits neutrophils[266]. Neutrophils or tumor-associated neutrophils (TANs) have been associated with poor survival in PDAC [233, 267]. IL-17 production by CD4+ cells and gamma delta T cells in the TME recruit neutrophils to the tumor [226]. Tumor promoting neutrophils express MMP-8, MMP-9, ROS, vascular endothelial growth factor (VEGF), and inflammatory cytokines TNFα and GM-CSF which favor chronic inflammation [265, 268]. One notable feature of neutrophils

is the release of neutrophil extracellular traps (NETs). NETs consist of decondensed chromatin, DNA, and intracellular proteins. NETs negatively correlate with survival in PDAC patients receiving adjuvant chemotherapy [269]. Neutrophils derived from the circulation of PDAC patients also show an increased NET formation[270]. Since neutrophil infiltration has detrimental effect on PDAC tumor progression and patient survival, it is likely that neutrophils suppress anti-tumor immunity, possibly due to NET formation.

Immunosuppressive chemokines/cytokines

To attack tumor cells, the tumor stroma must be traversed by immune cells. To defend against immune mediated attacks, CAFs and tumor cells can secrete chemokines and cytokines to induce apoptosis, inactivation or prevent infiltration of anti-tumor immune cells. Description of the function of some of the most prevalent cytokines/chemokines produced by cancer cells or stromal cells follows below.

CAF produced chemokines have evolved to shield the tumor from immune cell attack. CXCL12 is a CAF produced chemokine that binds CXCR4. *Feig et al.* found that treating a PDAC mouse model with AMD3100, a CXCL12 receptor inhibitor, rapidly accumulates T cells in the tumor. Clinical trials of AMD3100 have been performed but haven't shown any conclusive benefits on overall survival in PDAC patients. Clinical trials with combinations of AMD3100 together with PDL1 inhibitors are now ongoing (NCT04177810).

Tumor cells and inflammatory cells in the microenvironment produce IL-6, IL-10, IL-13 and TGFβ [271]. TGFβ and IL10 cooperate to produce an immunosuppressive environment in the tumor. TGFβ is produced by tumor cells, M2 macrophages and Th2 polarized T cells.

IL-6 has a multifunctional role in pancreatic cancer. It correlates to worse prognosis of pancreatic cancer[272]. Progression of PanIN precursor lesions to PDAC have also been demonstrated to involve IL-6 signaling via the stat3/socs3 pathway [273]. IL-6 can regulate the secretion of VEGF and stimulate angiogenesis in the tumor[274], and thereby contributes to metastasis. Physiologically, high IL-6 levels have also been linked to cachexia, which contributes to poor prognosis in PDAC [275].

IL-10 is a potent immunosuppressive cytokine involved in regulating inflammation. It is expressed by regulatory immune cells which produce IL-10 to reduce tissue damage caused by an active inflammatory reaction.

The mechanism of IL-10 involves downregulation of proinflammatory cytokines through activation of the JAK-STAT signaling pathways [276]. IL-10 is highly expressed in PDAC [272]. Cellular source of IL-10 in PDAC are MDSCs; TAMs, which stimulate Treg activation and proliferation [277]; and regulatory T cells, which promote non-responsiveness of macrophages [278].

TGFβ acts on cancer cell proliferation and can also affect immune response in pancreatic cancer. Normal pancreatic epithelial cells proliferation is inhibited by TGFβ [279]. In PDAC, mutations in TGFβ signaling pathway, most commonly SMAD4 [280], abolish this suppressive effect. TGFβ together with IL-10 has been shown to stimulate immune cell polarization to a Th2 fate [281]. TGFβ promotes immunosuppression by reducing DC cell proliferation and promoting formation of tolerogenic DCs [282]. As previously described, TGFβ also increases gal 1 expression in stromal cells [163]. Late in tumor progression, TGFβ is associated with EMT induction and metastasis [283].

Inducing T cell exhaustion

T cell exhaustion is a state of T cells which occurs when subjected to chronic antigen exposure, leading to functional changes which range from lack of effector function to altered functionality [284]. In melanoma great benefit has been demonstrated with therapeutic intervention targeting exhausted cells [285].

Exhausted T cells express surface checkpoint inhibitor proteins, decreased production of antitumor cytokines, increased production of tumor-promoting cytokines and a high apoptosis rate [286, 287].

To effectively target tumor cells and survive, T cells require the amino acids tryptophan and L-arginine [222, 288]. Cancer cells can inhibit the available levels of trypthophan by expressing high levels of IDO2 [289]. IDO2 is an enzyme that break down and deplete trypthophan and L-arginine in the TME [290]. Inhibiting IDO enzyme can reduce the immunosuppressive effect of the restriction in trypthophan and L-arginine and reduce infiltration of Tregs and MDSCs [291].

The nucleoside adenosine asserts metabolic inhibition on proliferation and activation of T cells as well as many other anti-tumor immune cells [292]. Regulatory T cells express the surface protein CD73 on its surface and can cleave ATP to free adenosine and inhibit effector T cell cytokine

expression. In a pancreatic disease model, CD73 knockdown was correlated to better survival and increased gemcitabine sensitivity [293].

PD-L1 and PD-1 play a major role upholding immune tolerance by suppressing the activity of activated T cells [294]. In PDAC PD-L1 is expressed by cancer cells [295] or on macrophages or dendritic cells. Binding of PD-L1 and PD-1 on activated T cells can induce exhaustion [296].

Introduction in summary

PDAC is characterized by numerous cancer cell derived immunosuppressive matrisome proteins cooperating to efficiently defend cancer cells against the immune system. Matrisome proteins can also promote the metastasis process in PDAC. Both metastasis and immunosuppression contribute to the poor prognosis of PDAC.

The processes of metastasis formation and immunosuppression are linked. Cancer cell-derived proteins recruit suppressive cells which promote metastasis by stimulating ECM degradation and neoangiogenesis. The linked signaling events between cancer cells, immune cells and CAFs in PDAC tumors emphasize the difficulty of broad treatment approaches, broadly depleting one compartment can result in unintended effects resulting in poor outcomes.

To address the tumor promoting functions of the stroma initial attempts to broadly depleted the ECM has been tested. The rational being that reducing the protective barrier would also reduce treatment resistance. With this approach, complex signaling interaction between the stroma and cancer cells was interrupted, which resulted in poor outcomes.

Recent studies of the stroma, focusing on individual components and signaling networks, has progressed the view on the stroma as having both tumor inhibitory and tumor promoting components. Currently, therapy development is focused on specific parts of the stroma, targeting the specific components contributing to poor prognosis.

Similarly, broad checkpoint inhibition in PDAC has failed. By focusing on specific stomal components, suppressive cell types or specific well characterized cell derived matrisome proteins, greater success might be achieved.

In this thesis we have studied several cancers cell-derived matrisome associated proteins that are highly expressed in PDAC tumors and contribute to poor prognosis by promoting metastasis or immunosuppression in PDAC. Therapeutic targeting of these proteins could result in effective treatments extending survival of pancreatic cancer patients.

Aims of this Thesis

The overall aim of this thesis is to identify cancer cell-derived matrisome proteins and determine their potential as treatment targets.

Specific aim I

To evaluate the function of the cancer cell-derived matrisome proteins SERPINB5, Agrin and CSTB and their effect on metastasis formation and association to patient survival.

Specific aim II

To determine the immune suppressive function of the cancer cell-derived matrisome protein galectin 4 and its mechanism of action.

Specific aim III

To investigate coregulation and co-expression of epithelial and stromal immunosuppressive factors and identify general mechanisms and networks driving immunosuppression in PDAC.

Materials and Methods

A brief description of all the materials and methods used in this thesis is listed below. A detailed description of the methods used can be found in the material and methods sections of the respective papers, attached to the thesis.

In situ hybridization (Paper I-II)

Formalin fixed paraffin embedded tissue was sectioned and analyzed for expression of *Lgals4*, *Krt18*, *KRT19 Fap* and *SERPINB5* using ViewRNA ISH tissue 2-plex assay. Tissue sections were deparaffinized and washed followed by boiling and enzymatic digestion to make the tissue permeable for probes directed at the target mRNAs. Probes were hybridized to the targets followed by amplifier hybridization and signal amplification. Finally, the substrates were applied which resulted in either a blue or red color. Sections were mounted with prolong gold antifade with DAPI. Sections were captured on a Zeiss LSM 710 microscope.

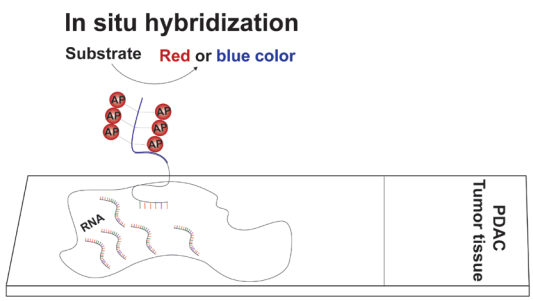

Figure 8. Illustration of in situ hybridization. Figure by T.Lidström.

Immunohistochemistry (Paper I and II)

Formalin fixed paraffin embedded tissue was sectioned, deparaffinized and rehydrated in xylene and gradually decreasing concentrations of ethanol. Antigens were unmasked by pressure cooking slides in antigen retrieval solution in a low pH sodium citrate buffer. For intracellular staining tissue was permeabilized using a 0.2% Triton-X detergent solution. The tissue was blocked with normal serum followed by incubation with primary antibody, in optimized dilutions, at 4°C overnight. Sections were blocked with H_2O_2 to deactivate endogenous peroxidase. Sections were incubated with species specific biotinylated-

secondary antibodies in buffer supplemented with 1% BSA, followed by ABC kit incubation which adds the avidin-linked enzyme horseradish peroxidase (HRP) to the secondary antibody. DAB substrate was then added to the tissue to generate the brown color of specific stains. Sections were counterstained in hematoxylin, mounted and scanned in a 3DHISTECH pannoramic 250 Flash III.

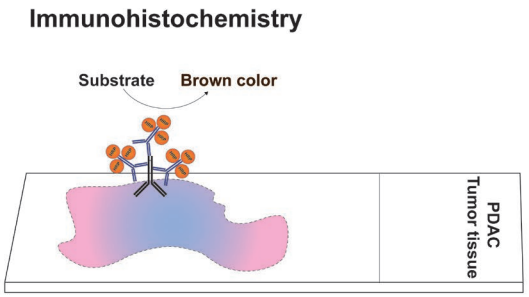

Figure 9. Illustration of immunohistochemistry. Figure by T. Lidström.

Immune cell infiltration analysis of immunohistochemistry sections (Paper II)

Tissue sections were analyzed in Qupath 0.1.2. The section was cleared of empty space, lymphoid infiltrates, and normal tissue. Tumor tissue was annotated for surface area. Qupath function to "estimate stain vectors" was used followed by the "fast cell counts" function. Threshold was manually adjusted depending on the background, and number of immune cells per surface area was determined. For the epithelial cleaved caspase 3, high powered field (400x) manual counting was performed to estimate the number of positive cells.

Photon-Upconversion Nanoparticles (Paper II)

Photon-upconversion nanoparticles are 100 nm particles with several unique photophysical properties. In this assay we used hexagonal $NaYF_4$ host lattice doped with Yb^{3+} and Tm^{3+}. Excitation by 980 nm light is absorbed by Yb^{3+} and transferred to the activation Tm^{3+} that emits the light at 810 nm. The brightness of each nanoparticle was bright enough for detection in a upconversion whole slide scanner developed by Lumito.

The staining procedures for tissue sections were performed in a similar way as described above for IHC tissue sections. Following the incubation

with secondary antibody, incubation with nanoparticles linked to streptavidin was performed. Slides were sealed with nail polish and signals were detected in a slide scanner.

Analysis of upconversion nanoparticles staining (Paper II)

For the analysis, fluorescent images were analyzed in qupath. Single isolated tumor glands, which were separated by at least 200 μm separated from adjacent glands, were selected to prevent signal spillover. Glands were annotated with an approximate 100 μm border. Annotations were divided into 25 μm tiles and 5-10 tiles each were selected for epithelial, proximal, and distal regions surrounding the gland. Signal was quantified in each tile and the average signal for each category was calculated for 5 tumor glands.

The cancer genome atlas dataset (Paper II, III)

The cancer genome atlas (TCGA) dataset was downloaded from the Xenabrowser website [297]. The dataset was controlled for samples so that all patients were verified with PDAC tumors. 74 patients were annotated with moderate differentiation degree and PDAC. These patients were included in the analysis of survival, CD8+ activation and cytotoxicity described in paper II.

The colon cancer dataset TCGA COAD was downloaded from the Xenabrowser website [297]. Since differentiation was not annotated in this dataset, all patients were included in analysis in paper II.

Breast cancer (BRCA), stomach cancer (STAD), bladder cancer (BLCA), Cervical cancer (CESC), Lung cancer (LUAD) and prostate cancer (PRAD) datasets were downloaded from the Xenabrowser website [297] and included in the comparative analysis included in paper II.

In paper III the PDAC TCGA dataset was used for correlation analysis. Patients with a verified PDAC diagnosis (n=174) were included in the heat map and correlation analysis between immunosuppressive factors in figure 1. Correlation values and p values were calculated using non-parametric spearman correlation.

For hierarchical clustering used in the heat map in figure 1, 8 genes of interest were clustered with the hclust function using the “complete” method on Euclidean distances between pairwise correlation estimates in R 4.1.1.

LGALS4 survival analysis (Paper II)

Kaplan-Meier survival analysis was performed in R using the survival package (v3.2-7; Therneau, 2020 [298]). Z scores, deviations from the mean, were calculated for all patients in the TCGA dataset. Z score was calculated as follows:

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

The cut off for high and low LGALS4 expression groups was fitted using a Cox proportional hazards regression model. Log-rank scoring was used to assess the P values at different cut-off percentiles. The percentile with the greatest difference in survival between the high and low group was selected.

Cytolytic effect and CD8 activation scoring (Paper II)

TCGA data analysis of the cytolytic effect and CD8 activation gene signatures described in paper II were obtained from *Azizi et al.* [299]. Z-scores for individual genes were calculated as described above. The mean of all composite gene z-scores for cytolytic effect and *CD8* activation score was compared between the high and low gal 4 expression groups of the same patients with moderate differentiation PDAC used in the survival analysis above.

ELISA analysis of blood sera (Paper II)

Serum samples of controls and PDAC patients were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit from R&D systems, precoated with antibodies against gal 4, according to the manufacturer's instructions.

Mouse Models (Paper II)

C57BL6/J and RAG1-/- mice used for transplantation and immune cell isolation in paper II were obtained from Jackson Laboratory, USA and housed in the Umeå University UCCB facility. Ethical approval was acquired from the Swedish board of agriculture, animals were housed according to Swedish rules and regulation on medical research. Experiments performed in Cold Spring Harbor laboratories were conducted in accordance with the institutional animal care and use committee.

Establishing monolayer (2D) and organoid (3D) cell lines from KPC mouse model (Paper II)

Organoid derived 2D cell lines and organoid cell lines were kindly provided by Professor David Tuveson at Cold Spring Harbor laboratory, USA. Cell lines were derived from the KPC (Pdx1-Cre, LSL-KrasG12D/+, LSL-Trp53R172H/+) mouse model of pancreatic cancer. Tumors were excised and tissue digested using a combination of collagenase XI and dispase II. This was followed by further digestion in TrypLE supplemented with DNAse I. Digest was resuspended in Matrigel and cultured with complete mouse feed media, containing defined growth factors and supplemented with apoptosis inhibitors. Following the establishment of organoid cell lines, the cells were dissociated to form a single cell suspension and plated on plastic with 2D cell media to generate the 2D cell lines. All cell lines were regularly tested for mycoplasma infection using 16s rRNA specific primers. For transplantation experiments, further sterility testing was performed (IDEXX impact sterility testing).

Culturing conditions of murine cell lines (Paper II)

KPC derived 2D tumor cells were cultured in high glucose DMEM supplemented with 5% FBS and 1% penicillin/streptomycin. KPC derived organoids were cultured in murine feed media [300]. For passaging, Matrigel domes were dissolved using ice cold triple plus media (DMEM F12 with 1x glutamax, 1x Hepes and 1% penicillin/streptomycin). Organoids were mechanically dissociated using fire polished pipettes, glass pipettes with a narrowed opening, and seeded in fresh Matrigel, usually in ratios of 1:4 or dependent on organoid density. When thawed and plated from frozen stock, media was supplemented with apoptosis inhibitors to improve viability. All cells were grown at 37°C with 5% CO_2.

Generation of gal 4-ko cell lines using CRISPR/Cas9 (Paper II)

To generate R26 controls and gal 4-ko 2D cell lines, MSCV-Cas9-puro plasmid, a gift from Christopher Vakoc were transfected into 293T cells together with plasmids coding for lentivirus structural components to generate Cas9 lentivirus particles for cell transduction. The assembled lentivirus were used to infect the KPC derived 2D cell lines, that after two days of puromycin selection, generated cell lines with stable CAS9 expression.

mRNA sequences for gal 4 were downloaded from NCBI and guides targeting these sequences were constructed using the tool at:

http://crispr.mit.edu. Targeting sequences were constructed and cloned into an LRG vector with GFP expression, forming (Lenti-sgRNA-EFS-GFP). The target vector was transformed into Stbl2 competent cells, grown on agar plates. To verify successful transformation 6 clones were picked from the agar plates and sequenced with pU6 forward sequencing primer. Lentivirus (with sgRNA-EFS-GFP) were constructed in the same way described above. The Lentivirus producing 293T cells were grown in the media for 24h, media was changed and after an additional 24h incubation, virus containing media was collected. Media was filtered and used to infect the previously constructed CAS9 expressing cell lines described above. In addition, sgRNA directed against RPA3 was cloned and amplified, as described above, to generate lentiviruses with RPA3-GFP, this was then used to transduce CAS9 expressing cells. The RPA3-ko cell line served as a positive control for proliferation inhibition.

Following lentivirus infection, cells were bulk sorted for GFP positivity and genomic cleavage detection kit was used to verify the deletion.

Following generation of cell lines, proliferation competition assays were performed. Cell lines with sgRNAs directed against gal 4 exon 1, exon 2 and RPA3 were used in bulk. Cells were grown for 23 days in 6 well plates, GFP fluorescence was measured over 23 days and normalized against itself and R26 control cells. In parallel, cells were single cell sorted on GFP positivity into separate wells and expanded into separate cell lines. In addition, bulk sorted GFP positive cells were compared for proliferative capacity using fluorescence intensity over 5 days.

Sequencing validation of CRISPR/Cas9-modified Gal 4-ko cell lines (Paper II)

Genomic DNA was extracted from the R26 control, and Gal 4-ko cell line. Exon 1 of the gene coding for gal 4 was amplified with specific primers. PCR products were sequenced using sanger sequencing in both forward and reverse direction. Resulting electopherograms were manually analyzed to determine the sequence of both alleles, of 3 different clones used in experiments in paper II. The inferred sequences were aligned to the corresponding mouse reference genome using MAFFT.

Generation of gal 4-kd 3D organoid cell lines (Paper II)

To generate Gal 4-KD and scramble control organoid cell lines we used glycerol stocks of stbl2 cells with plasmids coding for hairpins against gal

4 (primers can be found in the materials and methods section of paper II) or scramble controls together with puromycin resistance. We amplified the plasmid and generated Lenti-hairpin-puroR lentivirus in the same manner as described above for the 2D cell lines. Confluent organoids were dissociated into single cell suspensions and tumor cells were infected with the generated lentivirus. After infections, cells were collected and seeded in Matrigel domes, as mentioned above. Organoids were selected for puromycin resistance (1µg/ml). After 2 days of selection, organoid cells with stable expression of shRNA against gal 4 or scramble was generated.

To verify similar proliferative rate in the derived organoids following transduction CellTiter-Glo 2D cell viability assay was performed according to manufacturer's instructions. Luminescence measurements on the cells were taken at 0, 72 and 120 hours.

Orthotopic transplantation (Paper II)

To study the effect of gal 4 on tumor progression cell lines were transplanted into immunocompetent C57BL6/J mice aged between 42 and 52 days. Mice were sedated with isoflurane and $2.5x10^5$ cells, R26 controls or Gal 4-ko, were suspended in triple plus media and 50 µl was injected into the splenic lobe of the pancreas. Transplants were included in the study if no leakage occurred, a clear injection bubble formed, and no excessive bleeding took place during the injection or surgery. Mice were monitored post-surgery according to ethical guidelines. At 26-28 days mice were sacrificed, necropsy was performed, and the tumor and spleen were collected.

For organoid cell lines, cells were dissociated and resuspended in 30% Matrigel and triple plus media. Transplantation procedure was then performed as described above.

All surgeries were performed in an alternating fashion, transplanting one KO/KD cell line, followed by control cell lines.

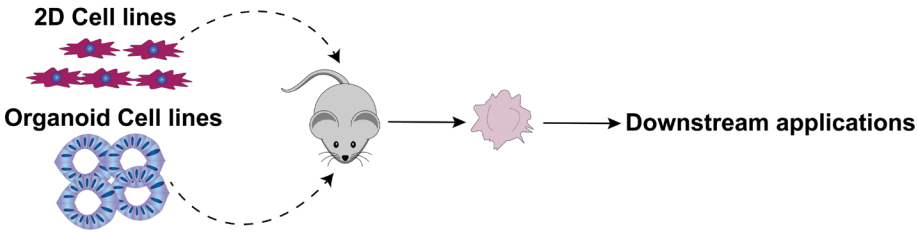

Figure 10. Illustration of orthotopic transplantation. Figure by T.Lidström.

Flow cytometry analysis of orthotopic transplants (Paper II)

Excised tumors were finely minced and digested in DMEM media with collagenase D, liberase DL and DNase I. Following digestion, digest was filtered through a 100 µm filter, and the solution was harvested and resuspended in ACK lysis buffer to lyse red blood cells. Cells were then counted, and viability determined before proceeding to FACS staining.

Up to $1x10^6$ cells were blocked with anti-CD16/32 antibodies (Fc receptor blocking). Followed by staining with antibody panels, at predetermined concentrations, for 30 min on ice. To remove dead cells from the analysis, cells were stained with Live/dead fixable red stain. For intracellular staining cells were fixed in 4 % PFA followed by permeabilization with 0.1 % saponin. For apoptosis experiments, Annexin V-7AAD apoptosis detection kit was used. Stained samples were analyzed on BD LSRII or Bio-Rad ZE5 flow cytometer with flow jo (V10) software.

For compensation, a combination of cells and Ultracomp ebeads was used. For markers with low expression, gating placement was determined with the aid of fluorescence minus one (FMO) stainings.

Western blot (Paper II)

Organoid cells in Matrigel were collected using cell recovery solution and incubated with rotation for 1h at 4°C to remove the Matrigel. Cells were spun down and lysed in 0.1% triton-x supplemented with protease and phosphatase inhibitor. Cells were incubated on ice for 30 minutes to complete the lysis; lysate was spun and supernatant containing protein collected. Protein was separated on an SDS-page gel and transferred to a

PVDF membrane. The membrane was blocked with 5% BSA in TBST and incubated with primary antibodies overnight at 4°C. The membrane was incubated with secondary HRP conjugated antibody followed by detection.

Splenocyte Isolation (Paper II)

Spleens from C57BL6/J mice were minced, suspended in PBS with 2 % FBS and filtered through a 70 µm filter. Cells were spun down and suspended in ACK lysis buffer to lyse red blood cells. Immune cells were washed and counted before proceeding with downstream applications.

Flow cytometry sorting for RNA sequencing of Gal 4-kd orthotopic transplantation (Paper II)

Tumors were dissociated in the same way described above. Followed by blocking with CD16/32 antibodies. Cells were then stained with anti-EPCAM-PE, anti-PDPN-biotin coupled to streptavidin-Alexa 647 and anti-CD45-FITC. Cells were stained in 1 µg/ml DAPI for live/dead and doublet discrimination. DAPI negative live cells positive for the different markers were sorted with a BD Aria-III sorter.

Single cell RNA sequencing (Paper II)

PDPN+ CAFs and CD45+ immune cells were collected in chilled PBS with 2 % FBS. Sorted cells were collected at a density of 1000 cells/µl in 0.2 % BSA in PBS. Equal numbers of CAFs and immune cells were pooled together. 7500 cells were loaded per well in a 10x chromium cartridge. This was followed by cDNA library construction according to manufacturer's instructions, described in Chromium Single Cell 3' dual indexing v3.1 protocol. Libraries were sequenced in-house on an Illumina Nextseq 500/550 v2 reagents to a depth of 50-60 thousand reads per cell with 28 bp for read 1 and 44 bp for read 2. Details on normalization, filtering, integration, and clustering can be found in the material and method section in paper II.

Clusters of CAFs or immune cells were determined using the "FindClusters" function and markers were identified using the "FindMarkers" function with default parameters. The proportion of a subtype was calculated using the 100*n/N, where n equates the number of cells in the shRNA group (scramble controls or Gal 4-KD) and N is the total number of CAF or immune cells. For the signatures, the mean value of the z-scores for the genes included in the signature was used. Further details are described in the material and methods section of paper II.

Bulk RNA sequencing (Paper II)

Organoids of the T5 line (scramble and gal 4-KD) were dissociated in the same manner described above. The cells were stained and sorted in the same manner described above in single cell sequencing. 500 EPCAM+ cells were sorted directly into 5.5 µl of lysis buffer with 0.2 % triton-x and 2U/µl RNase inhibitor. Samples were then frozen on dry ice. The samples were thawed, and RNA sequencing libraries were constructed using Smart-seq2 protocol [301]. Libraries were sequenced with Illumina Nextseq 500/550 V2 reagents with a sequencing depth of 6-27 million 75 bp single-end reads per sample. rRNA reads were removed, and remaining reads were trimmed andaligned to a reference genome. Reads mapped to protein coding genes were counted. Read counts were normalized using transcripts per million and relative log expression. Lgals4 downregulation was tested with default settings in DESeq2.

Re-analysis of published PDAC single cell data (Paper II, III)

Previously published data from Peng et al, 2019 [86] was reanalyzed to confirm the major contributing cell type of *LGALS4* expression. Using the authors' annotation, 1000 cells were randomly selected per patient with a maximum of 100 cells for each cell type cluster. Data was then merged into a single object using Seurat and normalized with the logNormalize function.

Similarly, to correlate LGALS4 expression with expression of other immunosuppressive proteins in paper III, *Peng et al.* dataset was re-analyzed. Tumor cells in the cluster annotated by the authors as "Ductal cell type 2", from 23 PDAC tumor samples, were extracted resulting in 11,315 cells. Correlation between different immunosuppressive protein-coding genes was assessed with spearman test using the cor.test function in R version 4.1.1. Expression levels of gene pairs were plotted by random selection of 1,000 cells with non-zero expression.

For pseudobulk analysis in paper III, figure 2, cell-types expressing target proteins were pooled into pseudo-bulk samples. UMI count normalization was performed using scaling normalization described by *Robinson et al.[302]*. Correlations were determined as described above.

Ex Vivo Experiments using 2D or Organoid (3D) cancer cell lines (Paper II)

2D tumor cells were seeded on a 24 well plate in Matrigel or on plastic. 2D cells were seeded and allowed to adhere for 24h. Organoid cells were

seeded for 24h in Matrigel in complete murine feed media. Splenocytes from C57BL6/J were isolated as described above. Immune cells were added to the liquid phase in T cell media. Every 48h for organoid experiments, immune cells in liquid phase were collected and spun down, resuspended in fresh T cell media.

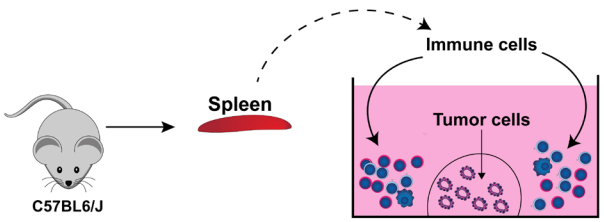

Figure 11. Illustration of Ex vivo experiments. Figure by T.Lidström.

For analysis immune cells were collected from the 2D *ex vivo* cell experiments, tumor cells adhered to the surface were detached with TrypLE and merged with immune cells before FACS analysis.

For imaging experiments, organoids were labeled with CFSE solution to make cells fluorescent. Immune cells were labeled with deep red cell tracker solution. Images were captured on a Zeiss LSM 710 confocal microscope. At the start of the experiment the $2x10^5$ immune cells were added to each well of both R26 and gal 4-ko cell lines. Experiments were repeated with 2 clones of the gal 4-ko cell lines.

For the CD3 heterodimer blocking *ex vivo* experiment cells were added to 6- or 96-well plates at $1.25x10^5$ cells per ml at timepoint 0 h. Splenocytes were harvested as described above, and CD8+ T cells were isolated using EasySep™ Mouse CD8+ T Cell Isolation Kit. CD8+ T cells were activated for 24 h with 2ul of anti-CD3, anti-CD28 activation beads. At timepoint 21h, CD3 heterodimers, CD3 epsilon and delta, CD3 epsilon and gamma, were added at 60 µg/ml. After 24 h the activated CD8+ T cells were labeled with CFSE solution and added to the tumor cell containing wells. After 24 h the immune cell containing media was collected and cells were stained

with 7AAD and AnnexinV and analyzed for apoptosis using flow cytometry.

For analysis of the 3D organoid ex vivo experiment immune cells were labeled with CFSE solution as described above. When cells were harvested, domes surfaces were washed with PBS to prevent transfer of non-infiltrating immune cells. Organoids were harvested in cell recovery solution. Cells were dissociated into single cell suspension with TrypLe and DNaseI, as described above. Cells were mechanically disrupted and strained through a 70 µM strainer. Cells were stained with 7AAD solution, separated based on CFSE positivity and apoptosis determined in epithelial cells with 7AAD positivity.

RNA Isolation (Paper II)

Tissue and tumor cells containing Matrigel domes were dissolved with TRIzol and stored at -80°C until RNA extraction. Extraction of RNA was performed according to manufacturer's instructions. Chloroform was added to samples in Phasemaker tubes. Samples were centrifuged and the aqueous phase was transferred. Isopropanol was added to the solution to precipitate nucleic acids. Solution was centrifuged and the pellet was washed with 70 % ethanol. RNA was dissolved in RNase free water and concentration determined with qubit fluorescence measurement. For tissue, an additional column purification, using purelink RNA mini kit, was performed to ensure good quality.

Real time quantitative polymerase chain reaction (Paper II)

400 ng of RNA was used for cDNA synthesis with Revertaid H minus reverse transcriptase according to manufacturer's instructions. cDNA was diluted 1:4 and 1µl was used per RT-qPCR reaction.

Primers were diluted to an effective concentration of 5 µM. SYBR green master mix was used in RT-qPCR reactions according to manufacturer's instructions. A pipetting robot was used to transfer mastermix and cDNA solution from a 96- to a 384-well plate. qPCR reactions were run in a Quantstudio 6 flex system, a melting curve was recorded to verify the specificity of the primers. HPRT was used to normalize gene expression.

Ethical considerations

Tissue and serum samples were obtained from biobank at Umeå university hospital in Sweden. The work presented herein was conducted according to the Declaration of Helsinki and was approved by the ethical

review board at Umeå university (09-175M, 2019-00399). All study subjects provided written informed consent before taking part in the study.

Data and statistical Analysis (Paper II)

Flowjo was used for flow cytometry data analysis. Fluorescence minus one was used to determine gate placement on markers with low fluorescent signal in multicolor FACS panels. Statistics between KO and controls were calculated using relative cell populations. Animal group-size (n) were determined by power analysis to achieve sufficient statistical power. Statistical analysis was performed in GraphPad prism v10. Unpaired nonparametric analysis (Mann-Whitney) statistical tests were used to compare groups unless otherwise indicated. Statistical significances were annotated as * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

Results and Discussion

Matrisome proteins are mainly produced by pancreatic cancer cells and CAFs. Several factors in the matrisome promote tumor progression. These factors range from growth factors, which stimulate tumor growth and/or formation of new blood vessels, to immunosuppressive factors, which prevent infiltration and/or induce apoptosis in activated immune cells and hence promote immunosuppression and metastasis.

By analyzing an unbiased mass spectrometry dataset [133], we identified matrisome proteins that are differentially expressed in the ECM of human PDAC tumors compared to the ECM of the normal pancreatic tissue. Three of these proteins, namely CSTB, SERPINB5 and agrin, were further analyzed for their metastasis promoting effects (Paper I). Concurrently, we also examined the function and mechanism of action of an immunosuppressive protein galectin 4 (Paper II). Finally, since immunosuppression is defined by a dynamic interplay between multiple cellular factors and mechanisms, which collectively form the immunosuppressive microenvironment seen in PDAC tumors, we characterized how different immunosuppressive factors correlate in PDAC (Paper III).

Cancer cell-derived matrisome proteins CSTB, SERPINB5 and agrin are upregulated in PDAC

By analyzing the expression of CSTB, SERPINB5 and agrin in a mass spectrometry dataset of purified PDAC ECM [133] we observed significant upregulation of three matrisome proteins, CSTB, SERPINB5 and agrin in PDAC patients. mRNAs of all the three proteins were also upregulated in KPC and KC PDAC mice mouse models. *In-silico* analysis of the TCGA database showed increased overall survival linked to low expression of *CSTB*, *SERPINB5,* and *AGRN* (Paper 1, Fig 1).

Knockdown of agrin, CSTB and SERPINB5 reduce tumor size

To study the role of Agrin, CSTB and SERPINB5 in PDAC metastasis, the proteins were knocked down in the BxPC3 cell line. Two knockdown (KD) cell lines each were generated for Agrin and SERPINB5, and one for CSTB. These derivative BxPC3 cell lines, which are notable for lung metastasis, were then used in orthotopic injection experiments in mice. While the weight of transplanted tumors was significantly reduced in all KD cell line

transplants, there was also a significant reduction in lung and liver metastases in animals transplanted with KD cell lines (Paper 1, Fig. 2).

SERPINB5 and agrin overexpression increases metastases

To further validate these results, we proceeded to produce cell lines overexpressing CSTB, SERPINB5 and agrin, followed by orthotopic injections of constructed cell lines. Overexpression of recombinant CSTB and SERPINB5 did not affect primary tumor weight when compared to the controls. However, metastases were significantly increased in mice injected with SERPINB5 and agrin overexpressing cell lines (Paper 1, Fig. 3). This observation was consistent with previously reported results. In hepatocellular carcinoma cell lines agrin complexes have been shown to activate the FAK pathway and drive EMT [144], which could result in increased metastases.

On the other hand, our data on SERPINB5 was in contrast to a previous report where SERPINB5 was shown to inhibit cancer cell migration and invasion and induce apoptosis in breast cancer [303]. Expression levels of SERPINB5 vary between breast cancer and pancreatic cancer. In breast cancer SERPINB5 is downregulated while the mass spectrometry dataset on purified PDAC ECM, showed that SERPINB5 is upregulated in PDAC, which indicates that SERPINB5it might confer different effects in PDAC compared to breast cancer. *Mardin et al.* showed a correlation between *SerpinB5* mRNA levels and increase in metastasis potential in PDAC [304] which corresponds with our results.

Agrin overexpression increases epithelial to mesenchymal transformation

EMT is a process in which epithelial cells undergo transformation to a more mesenchymal-like phenotype. This phenotype is characterized by reduced epithelial differentiation and increased motility. EMT also increases the ability of the tumor cells to degrade ECM by increasing expression of MMPs, which in turn promotes cell migration.

We determined the expression levels of EMT markers in mouse tumors developed from overexpression and knockdown cell line transplantation models of SERPINB5, CSTB and agrin. IHC analysis of agrin KD transplants showed significantly decreased quantities of EMT marker ZEB1+ and VIM+ cells, but a significant increase in the epithelial marker CDH1+ cells. The effect was reversed in the tumors developed in the agrin overexpression model which showed higher levels of ZEB1+ and VIM+

cells, and lower CDH1+ cells. However, our results suggested that SERPINB5 and CSTB do not affect EMT marker expression (Paper 1, Fig. 4). These experiments indicated that agrin is associated with an increase in EMT. An increase in EMT markers is generally associated with poor prognosis in PDAC [186]. In hepatocellular carcinoma, agrin is upregulated in response to an increased ECM stiffness which accelerates metastatic processes including EMT [137]. Our results are consistent with the observation in hepatocellular carcinoma.

Although, in contrast, high levels of agrin were not associated with reduced survival in analysis of PDAC tissue micro array (TMA) (unpublished data). Interestingly, high expression of galectin 4, also found to be upregulated in PDAC tumors, have been linked to increased differentiation in CRC [182], and gal 4-kd cell lines show higher levels of EMT markers (paper II, Fig. S3). Since both agrin and gal 4 were found to be upregulated in PDAC, it is possible that the expression levels of these proteins cooperate to determine EMT status of pancreatic cancer cells.

SerpinB5 and CSTB increase extravasation and extracellular matrix degradation

Next, we determined the effect of the upregulated proteins on extravasation, a process intimately associated with metastases. Tail vein injections of cells overexpressing agrin, CSTB and SERPINB5 increased the number of lung metastasis, while respective KD cell lines reduced lung metastases formation (Paper I, Fig. 5).

Extravasation assays further demonstrated that SERPINB5 and CSTB significantly induced extravasation while overexpression of agrin had no effect. To evaluate the ability of SERPINB5, CSTB and agrin to remodel ECM a gelatin degradation assay was used. Overexpression of SERPINB5 and CSTB increased gelatin degradation while agrin had no effect on gelatin degradation. This was further verified by determining protease cleavage rate of quenched fluorescent peptides by MMPs and other proteases produced in the overexpressing cell lines. We found that both overexpression of CSTB and SERPINB5 induces higher MMPs-mediated cleavage rate, which is consistent with the results from the gelatin degradation assay (Paper I, Fig. 5).

SERPINB5 has previously been shown to both promote and suppress metastasis [303, 304]. In tissues expressing either physiological or relatively lower levels of SERPINB5, the function of the protein could be different when compared to tissues expressing higher levels of protein.

This could explain the contrasting phenotype reported in PDAC, where the SERPINB5 expression levels are high, compared to breast and prostate cancer, where the expression levels are relatively low [305]. Similarly, CSTB exhibits variable cellular responses that could be related to differences in its transcriptional status in different cancer types. Besides MMPs, another cancer cell derived proteases, cathepsins, have also been associated with invasion and metastasis and their expression in the tumors is commonly connected to poor prognosis [306]. CSTB is described as a cathepsin inhibitor [307]. Thus, in tumor progression affected by cathepsins, CSTB could serve as a beneficial prognostic indicator.

Low stromal SERPINB5 is associated with better survival

To investigate a possible association between SERPINB5 levels and better survival in PDAC patients, we proceeded to verify expression of *SERPINB5* in epithelial cells using ISH assay on tissue samples from PDAC patients which showed *SERPINB5* was exclusively transcribed in epithelial cells. Since SERPINB5 was detected and found upregulated in the ECM of PDAC tumors compared to healthy tissue, we surmised that SERPINB5 is secreted into the ECM by epithelial cells. To determine if the levels of SERPINB5 in the ECM is associated to patient outcome, we semi-quantitively scored stromal levels of SERPINB5 using IHC analysis of a tissue microarray (TMA) of human PDAC samples and correlated the results with patient survival. We found that low extracellular SERPINB5 was related to better overall survival in PDAC patients (Paper I, Fig. 6).

Our results from the ISH and IHC analysis indicate that SERPINB5 is secreted from the cancer cells into the ECM of PDAC tumors. This is consistent with the fact that extracellular SERPINB5 was detected in the mass spectrometry analysis of the purified ECM [133]. SERPINB5 is also detectable in cell culture media of BxPC3 cells overexpressing SERPINB5 (Paper I, Fig.S6D), which further suggests active secretion of the protein by the cells over passive release from dying epithelial cells. Our results indicate that high levels of SERPINB5 expression can contribute to increased metastasis and poor prognosis.

A hypothesis for why extracellular SERPINB5 is a poor prognostic indicator relates to the metastatic niche. Secreted factors, like SERPINB5, could provide a microenvironment which is beneficial for metastasis spread to other organs. This is similar to a previous study in PDAC, which has shown that cancer cells influence hepatic stellate cells to make a more

beneficial microenvironment for metastasis [207]. In a similar manner secreted SERPINB5 could influence cell types in nearby organs and promote metastasis. Increased metastasis from secreted SERPINB5 would explain the survival benefit observed in patients with low stromal levels of SERPINB5. This hypothesis needs to be tested to determine how SERPINB5 affects PDAC tumor progression.

Gal 4 is highly expressed in epithelial cells in PDAC tumors

Further analysis of the mass spectrometry dataset identified several galectin family members as being upregulated in the ECM of PDAC tumors. Galectin 1, 3 and 9 have previously been described in the literature as having immunosuppressive roles. Interestingly, we also identified upregulation of galectin 4 (gal 4) in PDAC tissue compared to normal tissue. (Paper II, Fig. 1). The role of gal 4 in PDAC progression has been less studied compared to other galectin family members. We therefore proceeded to understand the significance of higher levels of gal 4 in PDAC progression.

IHC analysis of human PDAC tumors confirmed high gal 4 expression in epithelial cells. Interestingly, gal 4 is also highly expressed in metastatic lesions, which potentially indicates that gal 4 could be an immunosuppressive factor involved in metastasis (Fig. 1).

With ISH, we also observed that *LGALS4,* the gene coding for gal 4, was transcribed in epithelial cells, and not in fibroblasts (Paper II, Fig.2). Moreover, the fact that gal 4 is detected in the ECM, suggested that the protein is secreted. We used a sensitive image staining methodology, UCNP, to confirm the secretion status of gal 4, and observed higher ECM levels of gal 4 proximal to epithelial cells (Paper II, Fig.1).

Gal 4 is detectable in PDAC patient blood

Since gal 4 is secreted and detectable in the ECM, we proceeded to determine if gal 4 can be detected in blood samples. Analyzing blood sera, we could detect higher levels of gal 4 in pancreatic cancer patients compared to healthy controls. Interestingly, patients with IBD in the control group also showed high levels of Gal 4, indicating that inflammation alone is enough for the protein to leak into the bloodstream (Paper II, Fig.1, S1B).

Since high levels of gal 4 were detected in PDAC patient sera, gal 4 is a potential biomarker in PDAC. The idea of using gal 4 as a biomarker has been proposed in other publications [180]. Previously published data

from *Yu et al.* showed a variability in inflammation induced leakage of gal 4 into the blood circulation. However, in contrast to our results, *Yu et al.* [308] could not detect any significantly different levels of gal 4 in the blood of patients with inflammatory bowel disease. Gal 9 has also been reported as a potential biomarker of PDAC [166]. Though gal 9 concentrations were much lower compared to gal 4 concentrations in our data.

High levels of gal 4 decrease survival and immune activity

Gal 4 has distinct intracellular and extracellular functions. In CRC, high intracellular levels of gal 4 are linked to higher differentiation and increased survival[182]. Other studies have found immunosuppressive functions of secreted or extracellular gal 4 [134]. The contrasting functions of extracellular and intracellular gal 4 complicates analysis since beneficial intracellular effects on cellular differentiation would be in direct opposition to the extracellular effects on immunosuppression. Since numerous studies have shown a prognostic benefit of CD8+ T cell infiltration in PDAC tumors, we analyzed the expression of genes denoting cytotoxicity and activation of CD8+ T-cells in moderately differentiated TCGA patients with high or low expression of gal 4.

We observed significantly higher CD8+ T cell activation, and cytolytic effect in PDAC patients with low gal 4 expression and could also note a longer survival in this group (Paper II, Fig. 2). The survival benefit of low gal 4 expression contrasts the findings made in CRC patients where high gal 4 expression is associated with longer overall survival [309]. Possibly since T-cells are more dominant in anti-tumor immunity in PDAC compared to CRC, since the latter has been described as B-cell driven cancer [310]. It is also possible that, unlike pancreatic cancer, the survival in CRC patients is more dependent on cellular differentiation, which is an intracellular effect of gal 4.

Reducing gal 4 increase immune cell infiltration

To characterize the effect of gal 4 on tumor progression and immunity, we constructed gal 4 knockout (gal 4-ko) and knockdown (gal 4-kd) in KPC derived 2D cell lines and organoid cell lines. Gal 4-ko were constructed using CRISPR-CAS9 knockout techniques while gal 4-kd were constructed with short hairpins directed against gal 4. After verifying gal 4-ko status with sequencing and gal 4-ko/kd with western blot protein expression, we transplanted gal 4-ko and isogenic R26 control cell lines into the pancreas of immunocompetent C57BL6/J mice (Paper II, Fig.3).

Our data from the gal 4-ko model system showed a rapid infiltration of both CD4+ and CD8+ cells into the tumor when gal 4 is removed from the tumor microenvironment. A similar increase in CD8+ T cell infiltration was observed by *Feig et al. [197]* when CXCL12, another immune modulating protein, was removed from the tumor environment. Incidentally, increased infiltration, as well as the spatial distribution of CD8+ T cells, is linked to survival benefits of PDAC patients [234, 236, 237].

Single cell dissociation of the transplanted tumors, followed by flow cytometry analysis of immune cell subpopulations, verified that the infiltrating CD4+ and CD8+ cells were T-cells. The proportion of CD8+ T-cells were significantly higher in gal 4 ko tumors (Paper II, Fig.3). However, functional characterization of these cells revealed that a significantly higher proportion of CD8+ T-cells were positive for both PD-1 and Tim-3, which are known exhaustion markers (paper II, Fig.3). Though T cell exhaustion is generally high in the TME of PDAC tumors, it is unclear why the number of exhausted T-cells were higher in gal 4-ko tumors. It is possible that this could be due to the rapid cancer cell growth in this specific transplantation model system or caused by a subsequent increased infiltration of myeloid suppressive cells [264]. It is also possible that the lack of gal 4 in the knockout tumors has led to a compensatory upregulation of other immunosuppressive proteins, like other galectin family member proteins, or due to the metabolic inhibition by free adenosine [292] which could explain the increased exhaustion. This hypothesis is explored further in *paper III*.

Another possibility is that the same number of exhausted T-cells were similar in both types of tumors but the continuous expression of gal 4 in control tumors induced apoptosis of the exhausted T-cells thus reducing their population.

High Gal 4 expression reduce survival in immunocompetent mice models

To evaluate the effect of gal 4 on survival, we performed transplantations studies using relatively slow-growing pancreatic organoid cell lines with either high gal 4 expression (scramble control), or reduced levels of gal 4 (gal 4-kd). The survival experiments in immunocompetent B6J mice showed that animals transplanted with gal 4-kd organoids had a significant survival benefit over animals transplanted with gal 4 expressing scramble control organoids. However, there was no survival benefit when the organoids were transplanted into Rag1ko transgenic

mice, lacking mature B- and T-cells (Paper II, Fig.4). The difference in survival outcome between the immunocompetent and immunocompromised mice indicated that the effect of gal 4 on survival is immune cell dependent. Early in the formation of the transplanted tumors, especially ≤14 days post-transplantation, we could detect a higher density of CD8+ T cells/mm^2 of tumor, and on average smaller tumors in the gal 4-kd transplants (Paper II, Fig. 4).

Interestingly, we also observed a rescue of gal 4 expression, albeit slow, in gal 4-kd tumors. We believe that this phenotype was due to a selective immune escape of the few gal 4-expressing cells at the onset of the experiment, and the subsequent clonal expansion of these cells while the cells with low expression of gal 4 were eliminated (Paper II, Fig.4 S3).

Single cell Sequencing reveals differences in stroma cell subpopulations

To investigate the influence of gal 4 on the immune cell populations and subtypes of cancer-associated fibroblasts, we performed single cell sequencing on dissociated and sorted CD45+ (immune cells) and PDPN+ cells (fibroblasts) from tumors with high or low gal 4 expression (Paper II, Fig.5). The data indicated that M1 macrophages, described in the literature as pro-inflammatory and a beneficial prognostic factor in PDAC, were upregulated in gal 4-kd tumors. On the other hand, suppressive M2 macrophages, that are linked to poor survival in PDAC and can suppress the activity of CD8+ T -cells, were downregulated in gal 4-kdtumors.

We also observed differences in the CAF populations which showed a decrease in the tumor promoting inflammatory CAFs (iCAFs) and an increase in the proportion of myofibroblastic CAFs (myCAFs) (Paper II, Fig.5) in gal 4-kd tumors. Cancer-associated fibroblasts have been described as having varying effects on tumor progression. Historically, all CAFs were described as tumor promoting, though later studies have found that the contractile myCAF subtype is linked to tumor restrictive properties [78, 84, 85], while the IL-6 producing iCAFs have been linked to poor prognosis [311]. Our observations on increase in the number of myCAFs and immunogenicity in gal 4-kd tumors conform results reported by *Lee et al.* where oncostatin M receptor deficient mice transplanted with PDAC tumor showed increased αSMA positive myofibroblasts and a more immunogenic environment [312].

Collectively, our data show that many immunological factors with prognostic potential are upregulated in gal 4-kd tumors, and that intrinsic

CAF subpopulation tends to shift towards a more tumor restrictive phenotype.

Gal 4 induces apoptosis in T cells through interaction with CD3

To model the interaction between pancreatic cancer cells and immune cells, we developed model systems including KPC derived cell lines or organoids, co-cultured with isolated immune cells.

With organoid models, we could observe an increase in apoptosis in gal 4-kd epithelial cells after 120 h. This result was consistent with the organoid *ex vivo* assays determining apoptosis in organoids with activated naïve CD8+ T cells performed by *Koikawa et al. [313].* In addition, when gal 4 producing cancer cell lines were grown together with isolated splenocytes, an increase in the apoptosis was observed in CD8+ T-cells (Paper II, Fig.6). Both results supported the hypothesis that while CD8+ T-cells target and eliminate cancer cells, gal 4 induces apoptosis in T-cells, thereby affecting the CD8+ T cell mediated removal of cancer cells.

Gal 4 binds conserved glycosylation residues on CD3 delta

The mechanism by which gal 4 acts on T-cells has not been completely understood. *Paclik et al.* showed that gal 4 binds CD3 on activated T cells and causes calpain dependent apoptosis [134].

We proceed to explore the binding fidelity of gal 4 to its binding site on CD3[134] CD3 consists of three distinct protein chains, namely epsilon (ε), gamma (γ), and delta (δ), which form cell surface heterodimers. By examining the published biochemical and structural data, [314, 315], we identified a conserved amino acid residue in human, mouse, and sheep CD3. The residue was annotated to have a N-linked glycosylation on N38 of the CD3 delta chain. It has previously been described that gal 4 binds glycosylated residues on immune cells [316]. We surmised that this N-linked glycosylation (glycosylation of asparagine) on N38 of the CD3 delta chain could be the binding target of gal 4 on CD3. To determine how gal 4 binds CD3 and induces apoptosis in CD8+ T-cells, we performed a series of binding experiments where the affinity between recombinant CD3 heterodimers and recombinant human gal 4 was determined. We found that gal 4 binds CD3ε/δ heterodimer 10-times stronger than CD3ε/γ heterodimer. This binding could be sequestered by adding a galactoside, such as lactose which is a β-galactoside and is described as the main binding partner of gal 4 [317], but was unaffected by fructose, a non-galactoside monosaccharide. Interestingly, we were able to partly block binding between gal 4 and CD3 using a glycosidase treatment which

specifically removed N-linked glycosylation (Paper II, Fig.6). Since the location of N-linked glycosylation on CD3 was determined to be the conserved N38 residue on the delta chain, our results strongly indicated the role of conserved N38 residue N38 in binding of gal 4 and subsequent apoptosis of CD8+ T-cells.

Competitive inhibition of gal 4 with CD3 heterodimers

Finally, we proceeded to test if we could competitively inhibit gal 4–CD3 binding by saturating pancreatic cancer cell/immune cell co-culture model with soluble CD3 heterodimers. In accordance with our previous data, we observed increased apoptosis in CD8+ T-cell cocultured with gal 4 expressing cancer cells than those co-cultured with gal 4 deficient cancer cells.

Addition of CD3ε/δ and CD3 ε/γ heterodimers to the co-cultures inhibited the interaction between gal 4 and CD3 on CD8+ T-cells and the difference in apoptosis was lost (Paper II, Fig.6).

Galectin 4 expression levels co-vary with that of other galectin family members

The galectin family of proteins consists of many family members with similar immune cell binding partners, inducing many similar cellular processes. There is also a plethora of immunosuppressive mechanisms, including metabolic inhibition, chemokines repelling immune cells and surface bound proteins contributing to immune evasion in PDAC. To determine if there were correlations in the expression patterns of galectins or towards other immunosuppressive mechanisms we performed *in silico* analysis of two RNA sequencing datasets.

By analyzing the single cell RNA-seq dataset from *Peng et al.* [86], we identified the cellular sources of several immunosuppressive proteins (Paper III, Fig.1). *LGALS4, LGALS3, LGALS9* (Gal 4, 3 and 9) were expressed in epithelial cells, while *LGALS9* was also expressed in macrophages. We further found that *LGALS1* (gal 1), which previously has been described to be expressed in stromal cells, also was expressed in epithelial cancer cells, where its expression levels were inversely correlated to those of *LGALS4* (Gal 4). *TGFBI* and *CXCL12* were expressed in fibroblasts. In epithelial cells gal 4 is co-expressed with two other galectin protein family members, *LGALS3* (Gal 3) and *LGALS9* (gal 9). Interestingly, gal 1 also co-expressed with TGF beta inducible protein (*TGFBI*), a measure of TGFβ signaling (Paper III, Fig.1).

In the literature, gal 3 and gal 9 are known to inhibit T cell immunity similarly to gal 4 [162] [167]. This indicates that the immunosuppressive groups form based on similar mechanisms that could synergize to inhibit the function of intratumoral T cells.

The negative correlation between gal 1 and gal 4 might be an example of signaling between cancer cells and the stroma, similar to signaling between cancer cells and macrophages driving angiogenesis and metastasis [192, 203] . TGFβ produced by cancer cells have been shown to increase gal 1 expression in CAFs [163]. Gal 1 has been described as promoting metastasis [157] and immunosuppression [318], notably very similar to the reported function of gal 4 [134]. These correlation data between TGFB1 and Gal 1 corresponds with previous published data [163], in addition, our data show that gal 1 and TGFBI both negatively correlate with gal 4 expression.

Galectin 4 expression correlates with different immunosuppressive proteins in the TCGA dataset

Analysis of the PDAC TCGA dataset showed that immunosuppressive proteins naturally group into three mutually exclusive groups; a “galectin” group, a “classical” group, and a “stromal” group (Paper III, Fig 1). In the galectin group, gal 4 clustered together with gal 3 and gal 9 in a hierarchal clustering of immunosuppressive factors. In contrast, the galectin group negatively co-regulated with gal 1 and TGFBI protein, constituting the stromal group. *NT5E* (CD73) and did not cluster into any group. *CXCL12* (CXCL12), a stromal derived factor of immunosuppression negatively co-expressed with the galectin group, and clustered together with *CD274* to form the “classical” immunosuppression group.

The natural clustering of factors in different patient tumors show an either/or mechanism of immunosuppression. Selection pressure applied to tumor cells cluster tumors into groups with different immunosuppressive mechanisms.

Pseudo-bulk analysis validates negative and positive co-expression

To validate the clustering seen in the bulk RNA-sequencing dataset we performed pseudo-bulk analysis on the Peng dataset [86]. Pseudo-bulk group single cell sequencing data into a single pseudo-bulk sample. Pseudo-bulk analysis validated negative co-expression between gal 4 and gal 1, notably the correlation was stronger than what was seen on single

cells. Gal 4 also negatively correlated with TGFBI in pseudo-bulk analysis. In the galectin group we could also validate co-expression of gal 4 with gal 3 but not with gal 9 (Paper III, Fig.2).

Taken together these results indicate that in single tumors dominant immunosuppressive mechanisms emerge. Certain mechanisms are dominant in certain tumors but not in others. In addition, some immunosuppressive mechanisms, notably, CD73, tend to vary between these groups, adding additional immunosuppression to some tumors.

These analyses indicate patient classification followed by combination therapy is a wise strategy in PDAC patient treatment.

Conclusion

CSTB, SERPINB5 and agrin regulate EMT, extravasation, and extracellular matrix degradation, features all important for the metastasis process in PDAC tumors. High levels of extracellular SERPINB5 correlates to shorter patient survival.

Gal 4 binds CD3 on activated T cells, inducing apoptosis, thereby preventing immune mediated destruction of tumors, which in turn leads to reduced patient survival.

Gal 4 positively and negatively correlates with other galectin family proteins with immunosuppressive function, and other known immunosuppressive proteins, forming distinct immunosuppressive groups.

The overall conclusion that can be drawn from results presented in this thesis is that cancer cell-derived matrisome proteins can be a source for novel therapeutic targets in PDAC.

Future Perspectives

Evaluating cancer cell derived matrisome proteins for therapy development

Cancer cell-derived ECM associated proteins discovered using mass spectrometry generated highly interesting candidates for therapy development. Early attempts to completely reduce or remove the PDAC stroma has failed and have resulted in a more nuanced view on the functional role on the stroma. A more targeted approach where specific ECM proteins are first identified and then functionally characterized is described in this thesis. The results presented herein indicate that individual components of the ECM in PDAC tumors can yield highly interesting targets for therapy development.

Therapeutic development targeting cancer cell derived matrisome proteins

SERPINB5
SERPINB5 promotes metastasis in pancreatic cancer. Since metastases are a major cause of morbidity and mortality in PDAC patients it qualifies as a candidate for therapy development. In data from PDAC patient, low levels of extracellular SERPINB5 correlates with better survival. This supports the idea of blocking the extracellular effect of SERPINB5.

However, the varying function of SERPINB5 described in the literature, both suppressing or promoting tumor progression in different types of cancer, warrants caution. More studies of the specific mechanism and function of extracellular SERPINB5 is needed before proceeding to preclinical therapy development. Premature therapy development could result in unintended side effects, like more aggressive cancer and shortened survival.

Galectin 4
Galectin 4 is a highly interesting target for therapy development. In paper II we describe increased levels of gal 4 in the blood of pancreatic cancer patients. The potential use of gal 4 as a biomarker for pancreatic cancer or as a means of tracking treatment response is intriguing. Detection of gal 4 in healthy controls with inflammatory conditions in the GI canal, reduces the likelihood of this being an effective biomarker, since inflammation in the GI canal is common, especially in patients treated for cancer.

Pharmaceutical inhibition of extracellular gal 4 is highly promising. Induction of apoptosis and poor prognostic correlation of extracellular gal 4 described in paper II highlights the potential benefit of this treatment. However, combination therapies targeting multiple immunosuppressive proteins are preferable. Results from *paper III* show that there is co-expression of immunosuppressive proteins in PDAC tumors. This cooperation between different immunosuppressive proteins makes a single therapeutic agent less likely to succeed. Since PDAC tumors are made up of different clones, all potentially relying on different mechanisms to evade the immune system, the use of a single immunotherapy agent blocking one specific immunosuppressive mechanism, could just lead to expansion on certain clone of cancer cells through selection. There are also risks associated with treatment of extracellular gal 4 if the hypothesis of normal biological function of gal 4 in the GI canal is correct. If gal 4 prevents overreactions of the immune system to food antigens, treatment with gal 4 blocking therapeutics might induce colitis.

Targeting the extracellular gal 4 without affecting the intracellular function is crucial. Disrupting the intracellular fraction of gal 4 could disrupt beneficial effects of gal 4 on differentiation which could influence patient survival. Hence, development of an antibody targeting extracellular gal 4 is strategically sound since antibodies cannot pierce the cell membrane.

Our group is currently evaluating candidates of gal 4 blocking antibody candidates in collaboration with Sci-Life Lab's drug development division. We hope to have a candidate for preclinical testing in the near future.

Limitation of work included in thesis

Paper I and paper II are based on the same mass spectrometry dataset. In this dataset the ECM of well- moderate- and poorly differentiated PDAC tumors are analyzed together with healthy pancreatic tissue controls. The main limitation of this dataset is the number of samples.

The variation in differentiation between different tumors, and variation in stromal content may affect the tumor microenvironment and thereby metastatic potential and immunosuppression. The definition of what normal pancreatic tissue is, can also be discussed as a limitation. Healthy pancreatic tissue is sampled from patients with suspected pathological changes in the duodenum. Ethical limitations prevent collection of "true" healthy pancreatic tissue.

We observed that all proteins studied in paper I were overexpressed in PDAC tissue. To be able to study the effect of these proteins we performed experiments with cell lines overexpressing the proteins in questions. Overexpression studies have limitations since protein-overexpression cell lines typically show abnormal high expression of target proteins not reflective of expression levels found in tumors. Results from such experiments might therefore not accurately reflect the function of the protein in tumor progression.

Using the BxPC3 cell line, which is known for high frequency of lung metastases, could influence the results since the main metastasis site in patients with PDAC is the liver. Even though BxPC3 cell lines represent a good PDAC metastasis model, there are differences in the biology of this cell lines compared with the majority of PDAC tumor that preferentially home to the liver.

The number of serum samples could be increased, unfortunately our sample size was limited by the number of samples available in our biobank.

Gal-4ko and gal-4kd model systems might not accurately reflect the actual state of PDAC tumors. A model only restricting extracellular gal 4 would have more fully recapitulated the immunosuppressive effect. Cost and time constraint prevented sequencing of additional tumors which could have made cell population differences clearer. Performing additional studies on immunosuppressive cells, like macrophages, could have better recapitulated the shift in phenotype between control and gal4kd tumors.

Ex vivo modeling shares these limitations as well. *Ex vivo* models use collected splenocytes which are affected by stress when collected. In vitro conditions are also more oxygen rich than the environment inside tumors. Response might change in a hypoxic environment. However, ex vivo systems used in this context add understanding on the interaction between tumor and immune system which could not otherwise be studied.

Analysis of TCGA dataset which is based on bulk sequencing of PDAC tumors are limited by variable contributions of different cell types. More stromal cells in one tumor could dilute epithelial cells markers as an example. No mechanistic insight is provided into the correlations between immunosuppressive proteins. Correlations are not causal links. Models with combinational blocking of co-expressed immunosuppressive proteins could determine effectiveness of this approach in a better way. Despite these drawbacks TCGA dataset is an excellent resource for hypothesis generation.

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