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THE REACTIVE STROMA IN PANCREATIC DISEASES

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To all the victims of pancreatic cancer and their loved ones,

and to all of the struggling PhD students out there.

ABSTRACT

Stroma, or activated connective tissue, is a key component in both pancreatitis and pancreatic tumors. Acute pancreatitis (AP) is an inflammation of the pancreas which ranges from mild and local to systemic with severe complications and high mortality. **In paper I**, we sought out to identify new potential biomarkers of AP, by comparing gene expression in mice with caerulein induced AP and control mice injected with sodium chloride. Regulator of calcineurin 1 (Rcan1) arose as the most promising candidate for an early marker of AP and was found to be regulated by oxidative stress, as it was upregulated by caerulein and H_2O_2 and this upregulation was inhibited by the antioxidant N-acetylcystein. Rcan1 protein was also found upregulated in the blood of mice early on after AP induction, and in patients with AP, suggesting the potential use of RCAN1 as a marker of AP in *e.g.* post-ERCP-pancreatitis.

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and has a dismal prognosis, in part due to resistance to available treatments. A desmoplastic stroma has become one of the hallmarks of PDAC and is currently receiving more and more attention from researchers. Overexpression of High mobility group A2 (HMGA2) protein has been associated with many cancers as well as with epithelial mesenchymal transition (EMT) and cancer stem cell properties. **In paper II** HMGA2 was shown to be correlated with lower overall survival in a cohort of 253 PDAC patients, acting as an independent prognostic marker. Tumor cell HMGA2-positivity was also significantly correlated with the abundance of $PDGFRB^+$ stroma. Co-injections of Panc1 cells and pancreatic stellate cells (PSCs) *in vivo* increased tumor growth and also HMGA2 and PGDFRB expression. *In vitro*, HMGA2 expression was increased in Panc1 cells grown in 3D spheroids, both by co-culture with PSCs or TGFB1 stimulation.

In **paper III**, the 3D co-culture spheroid models of either Panc1 or HPAFII PDAC cells with human PSCs, were extensively characterized by immunohistochemistry (IHC) and real time PCR. CK19 staining was utilized to identify the tumor cells by IHC within co-cultures. A novel method of detecting cell type specific mRNA expression within the co-cultures without the need for physical separation of the cells was also developed, where cells of different species (human and mouse) were co-cultured and analyzed by real time PCR using species specific primers. Co-culturing of PDAC cells and PSCs was shown to activate the stellate cells and increase tumor cell proliferation.

As presented in the **additional preliminary data**, PDGFB did not induce TGFB1 expression by the PSCs as a possible source for the HMGA2 upregulation in cancer cells. HMGA2 protein expression in the 3D co-culture spheroid model was inconsistent and displayed some $HMGA2^+$ PSCs in addition to the positive tumor cells. HMGA2 was found to be differentially expressed also in 3 clinical PDAC samples and one duodenal cancer, where HMGA2-positivity was found in both stroma and tumor cells, close to each other as well as separated.

In conclusion, this thesis work identified RCAN1 as a novel marker for AP and HMGA2 as a prognostic marker of PDAC. A novel 3D co-culture spheroid model of PDAC as well as a novel method of identifying cell type specific gene expression in 3D cultures without the need for physical separation, were developed. In addition, a complex relationship between HMGA2 expression in tumor and stroma cells was revealed, although the mechanism is not yet clarified.

LIST OF SCIENTIFIC PAPERS

The dissertation includes the following papers, which will be referred to throughout the text by their Roman numerals.

I. **Norberg KJ**, Nania S, Li X, Gao H, Szatmary P, Segersvärd R, Haas S, Wagman A, Arnelo U, Sutton R, Heuchel RL[§], Löhr JM[§].
Rcan1 is a marker of oxidative stress, induced in acute pancreatitis.
Pancreatology 2018; 18: 734-741.

II. Strell C, **Norberg KJ**, Mezheyeuski A, Schnittert J, Kuninty PR, Moro CF, Paulsson J, Aagaard Schultz N, Calatayud D, Löhr JM, Frings O, Verbeke CS, Heuchel RL, Prakash J, Sidenius Johansen J, Östman A.
Stroma-regulated hmga2 is an independent prognostic marker in pdac and aac.
Br J Cancer 2017; 117: 65-77.

III. **Norberg KJ**, Fernandez Moro C, Strell C, Blümel M, Balboni A, Liu X, Bozóky B, Östman A, Heuchel R[§], Löhr JM[§].
A novel pancreatic tumor and stellate cell 3D co-culture spheroid model.
Manuscript.

[§] Equal contributors

LIST OF PAPERS NOT INCLUDED IN THE THESIS

I. **Norberg KJ**, Sells E, Chang HH, Alla SR, Zhang S, Meuillet EJ.
Targeting inflammation: multiple innovative ways to reduce prostaglandin E_2.
Pharm Pat Anal 2013; 2(2):265-88. *Review.*

II. Gerling M, Zhao Y, Nania S, **Norberg KJ**, Verbeke CS, Englert B, Kuiper RV, Bergström A, Hassan M, Neesse A, Löhr JM, Heuchel RL.
Real-time assessment of tissue hypoxia in vivo with combined photoacustics and high-frequency ultrasound.
Theranostics 2014; 4(6):604-13.

III. Begum A, Ewawich T, Jung C, Huang A, **Norberg KJ**, Marchionni L, McMillan R, Penchev V, Rajeshkumar NV, Maitra A, Wood L, Wang C, Wolfgang C, De-Jesus-Acosta A, Laheru D, Shapiro IM, Padval M, Pachter JA, Weaver DT, Rasheed ZA, Matsui W.
The extracellular matrix and focal adhesion kinase signaling regulate cancer stem cell function in pancreatic ductal adenocarcinoma.
PLoS One 2017; 12(7):e0180181.

IV. **Norberg KJ**, Gao H, Segersvärd R, Hagman B, Lu Y, Arnelo U, Heuchel RL[§], Löhr JM[§].
Islet Amyloid Polypeptide (IAPP) is not involved in acute pancreatitis induction by caerulein.
Manuscript.

V. **Norberg KJ**, Blümel M, Nania S, Heuchel RL[§], Löhr JM[§].
A fluorescent cell line panel for studies of pancreatic diseases.
Manuscript.

[§] Equal contributors

LIST OF ABBREVIATIONS

AP	Acute pancreatitis
α-SMA	Alpha smooth muscle actin
CAF	Cancer-associated fibroblast
CC	Co-culture
CCK	Cholecystokinin
CD10	Membrane metalloendopeptidase (MME)
CK19	Cytokeratin 19 (epithelial marker)
CN	Calcineurin
CP	Chronic pancreatitis
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
ERCP	Endoscopic retrograde cholangiopancreatography
FACS	Fluorescence activated cell sorting
H&E	Hematoxylin and eosin
HMGA2	High mobility group A2
HPDE	Human pancreatic ductal epithelial cells
Ki67	Marker of proliferation Ki67
KPC	Genetically engineered mouse model (GEMM) of PDAC ($\underline{K}ras^{LSL\text{-}G12D/+};Trp\underline{5}3^{LSL\text{-}R172H/+};Pdx\text{-}\underline{C}re$)
KPCT	KPC GEMM crossed with the tdTomato allele (B6.Cg-$Gt(ROSA)26Sor^{tm9(CAG\text{-}tdTomato)Hze}$/J)
MC	Mono-culture
MCTS	Multicellular tumor spheroid
NAC	N-acetylcystein
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa B
OMS	Organotypic multicellular spheroids

PanINs	Pancreatic intraepithelial neoplasias
PDAC	Pancreatic ductal adenocarcinoma
PDGFRB	Platelet derived growth factor receptor Beta
PDGFB	Ligand for PDGFRB
PEP	Post-ERCP-pancreatitis
PSCs	Pancreatic stellate cells
hPSC	Human PSC
mPSC	Mouse PSC
RCAN1	Regulator of calcineurin 1
ROS	Reactive oxygen species
Sesn2	Sestrin 2
TAMs	Tumor-associated macrophages
TGFB1	Transforming growth factor beta
TS	Tumorospheres
TTS	Tissue derived tumorospheres
WT1	Wilms tumor 1 (epithelial marker)
3D	Three-dimensional

TABLE OF CONTENTS

1. INTRODUCTION

Activated connective tissue, or stroma, is a key component in pancreatitis, and pancreatic tumors are typically surrounded by an intense fibrotic scar tissue. Acute pancreatitis (AP) is a reversible inflammatory process of the pancreas, which may present with severe complications and high mortality [1, 2]. AP can also develop into a chronic state, *i.e.* chronic pancreatitis (CP), in which case normal pancreas structure and function is irreversibly altered. CP is the most common disease of the pancreas, characterized by progressive inflammation and fibrosis [3]. To date, no curative treatment exists. Notably, patients with CP bear an increased risk for developing pancreatic ductal adenocarcinoma (PDAC). Although PDAC has become the subject to increasing research efforts over the past decades, poor response to therapy resulting in a dismal prognosis has become the hallmark of this disease. PDAC mortality rate has remained high and pancreatic cancer still is the fourth leading cause of cancer related death in the world. It is expected to shortly climb to number two on the list, unless a remarkable break-through is soon attained [4]. At least in part, this is due to an almost complete resistance against both conventional and targeted chemotherapy. With the present standard of care, conventional chemotherapy results in a median life expectancy around 6 months [5]. As a result, pancreatic cancer cannot be survived as compared with colorectal or breast cancer [6, 7].

2. BACKGROUND

2.1 The pancreas

The pancreas is a glandular organ in the digestive system, located in the abdominal cavity posterior to the stomach. Anatomically the pancreas can be divided into the head (in close proximity to the duodenum), the body (lying behind the stomach) and the tail (ending adjacent to the spleen). The pancreatic duct (*Ductus pancreaticus*) stretches from the tail all the way through-out the organ to the head, where it merges with the common bile duct, passes through the papilla of Vater and terminates in the duodenum. The pancreas is made up of two major tissue compartments, the endocrine and the exocrine pancreas [8]. All cell types of the pancreas, endocrine, exocrine and ductal, are of endodermal origin [9]. The exocrine and endocrine pancreas arise from a common progenitor cell population expressing *Pdx1*, *Ptf1a*, and *Sox9*. In the presence of other factors, like *Ngn3, Pax4, NeuroD*, and *Hnf6*, these cells contribute to the proliferation and differentiation of the endocrine pancreas. In the absence of pro-endocrine factors and transcription factors like *Ptf1a* and *Mist1*, the exocrine pancreas is developed [8].

The endocrine part of the pancreas constitutes about 2% of the organ mass. It is composed by islands of Langerhans, small islet-like structures spread throughout the pancreas. Three major cell types makes up the islets; the alpha, the beta, and the delta cells. All cell types secrete hormones, important to metabolic regulation, directly to the blood stream (portal vein). The alpha cells produce and secrete glucagon, the beta cells insulin and amylin (Islet amyloid polypeptide, IAPP) and the delta cells somatostatin [8]. Deficiency of the endocrine part of the pancreas is involved in diabetes mellitus and pancreatic neuroendocrine tumors originate from the endocrine cells [8].

Exocrine pancreas produces, secretes and transports several enzymes essential for the digestive process to the intestines, and makes up about 90% of the organ mass. The remaining mass of the organ, in addition to the endocrine and exocrine parts, are made up of blood vessels, lymphatics, nerves and fibrous connective tissue stroma [8]. One type of cell of great importance for the reactive stroma in pancreatic diseases, which will be described in more detail later on, is the pancreatic stellate cell (PSC) [10]. The exocrine pancreatic secretion is tightly regulated by the neuroendocrine system. The endocrine pancreas is closely integrated anatomically and physiologically with the exocrine pancreas and regulates its function. Endocrine and exocrine pancreas are richly innervated with central and autonomic nerves, and intra-pancreatic postganglionic neurons are activated by efferents arising from the duodenal mucosa. When activated, these nerves release acetylcholine, which binds on muscarinic receptors on the acinar cells and stimulates secretion. Cholecystokinin (CCK), secreted by brain neurons and small intestinal cells, is also a very important mediator of pancreatic exocrine secretion [8].

The exocrine pancreas is made up of acinar, centro-acinar and ductal cells. The gland is divided into lobules containing numerous acini, each made up of a single layer of pyramidal acinar cells arranged concentrically around a lumen. The acinar cells are polarized in a basolateral and apical axis, and are the cells which are producing the digestive enzymes. The broader base of

the pyramidal cells is basophilic and contains the nucleus. The narrow apices of the pyramidal cells are facing the lumen and contain eosinophilic zymogen granules filled with precursors of pancreatic digestive enzymes, such as lipases (fat digestion), alpha-amylases (carbohydrate digestion) and trypsin (protein digestion). As the name denotes, centro-acinar cells are located centrally within the acinus, where they form an interface between the acinus and the intercalated duct. The intercalated duct continues into intra-lobular ducts made up by the ductular cells. The centro-acinar cells and the duct cells secrete bicarbonate and water, resulting in acinar secretions being flushed into the pancreatic ducts. The intra-lobular ducts fuse to form the interlobular ducts that then open into the pancreato-hepatic (biliary) duct (and to a lesser extent directly into the duodenal lumen). Each pancreatic acinus is surrounded by a thin basal lamina, scarce stroma and pancreatic stellate cells (similar to the hepatic stellate/Ito cells) [11]. The quiescent pancreatic stellate cells stain positive for GFAP, nestin, desmin and vimentin. Once activated, they express α-smooth muscle actin. The pancreatic stellate cells play important roles in the pathogenesis of both chronic pancreatitis and pancreatic cancer [8].

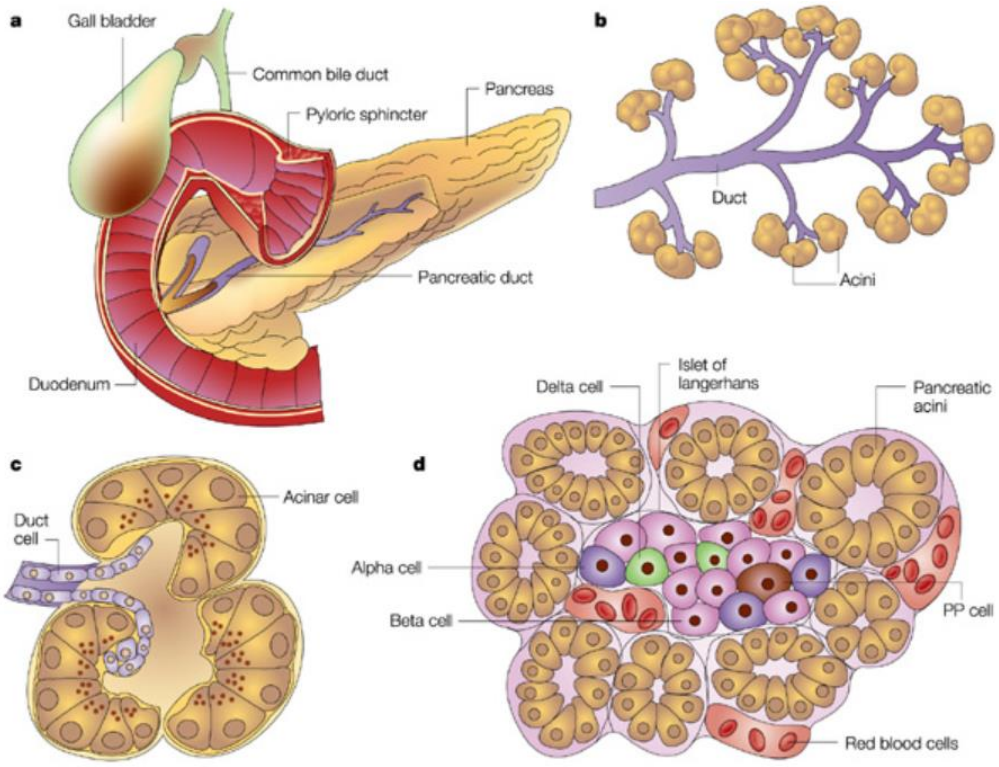

Figure 1. Anatomy of the pancreas. Republished with permission, from[12]. **a**) Gross anatomy of the pancreas displaying the pancreatic duct, the common bile duct and the gall bladder, as well as the duodenum. **b**) The exocrine pancreas with the pancreatic duct and the acini. **c**) A single acinus. **d**) The endocrine pancreas; a pancreatic islet embedded in exocrine tissue.

2.2. Acute pancreatitis

Acute pancreatitis (AP) is an inflammation of the pancreas, which ranges from a mild localized disease to a severe systemic inflammatory disease with high mortality. Mild AP is characterized by inflammation, edema and damage of the pancreatic parenchyma. Severe AP can induce multi-organ failure and extensive pancreatic necrosis, leading to sepsis [13, 14]. The two most common causes of AP are prolonged excessive alcohol abuse and bile acid reflux due to gallstone obstruction [15, 16].

Under physiological conditions the exocrine pancreas stores inactive precursors of digestive enzymes in zymogen granules. The zymogen granules are released from pancreatic acinar cells into the pancreatic duct, upon endocrine and neuronal stimuli and the precursors are flushed out into the duodenum where they after activation participate in digestion [13]. In AP however, the general theory is that digestive enzymes are prematurely activated, leading to auto-digestion of acinar cells and subsequent inflammation [15, 17]. Active digestive enzymes co-localize with lysosomal enzymes and form large intracellular vacuoles, inducing the production of pro-inflammatory cytokines by stimulation of macrophages and acinar cells. [13]. Cytokines are important players in driving the inflammatory response and hence in the pathogenesis of the disease. Tumor necrosis factor alpha (TNF-α) [18, 19] as well as interleukins (IL) such as IL-6 [20-22] are key mediators of inflammation in AP.

Inflammation leads to production of reactive oxygen species (ROS) and ROS contribute to worsening the inflammatory response [23, 24]. This complex relationship makes it difficult to distinguish if oxidative stress is an initiating event in the pathogenesis of acute pancreatitis, or a mediator amplifying the disease. In order to understand the mechanisms of disease development, various rodent models of experimental AP have been established. One of the most commonly used models is the caerulein induction model. Caerulein is an analogue of CCK, as already mentioned an important hormone inducing secretion of digestive enzymes from the pancreas. CCK induces ROS production and AP in animals and cells [25, 26].

ROS [26, 27], as well as the pro-inflammatory transcription factor nuclear factor kappa B (NFκB) [28, 29], are important mediators of inflammation in AP. Both non-oxidative alcohol metabolites and bile acids mediate AP development through prolonged increase of intracellular calcium levels in the acinar cells [30]. At least in part, the excess of intracellular calcium then acts by activating calcium-dependent calcineurin (CN), which dephosphorylates nuclear factor of activated T-cells (NFAT). This triggers translocation of NFAT proteins from the cytoplasm to the nucleus. There, NFAT then controls the expression of target genes involved in many biological functions [31], including zymogen activation. In AP this contributes to damage of the acinar cell compartment [32, 33]. Bile acids have been shown to activate both NFκB and the pre-digestive enzyme trypsinogen through induction of CN-NFAT signaling in acinar cells, thus inducing acinar cell injury [34]. CN-NFAT signaling also upregulates its own endogenous feed-back inhibitor, Regulator of calcineurin 1 (Rcan1) [35].

2.3. Regulator of calcineurin 1

Rcan1 does not only play a role in AP, but also in multiple other diseases, such as atherosclerosis and cardiovascular disease [36, 37], Alzheimer´s disease [38, 39], Down syndrome [40, 41], various cancers [42-46] and others [47]. RCAN1 is also known as modulatory calcineurin-interacting protein 1 (MCP1) and Down syndrome critical region 1 (DCSR1), as it was first identified as involved in the pathogenesis of Down syndrome. In addition, a higher expression of Rcan1 in brains of young rats compared to adults suggests a role for Rcan1 in the central nervous system development [40] and it has also been suggested to play a role in cardio protection in the heart [48].

In neuronal cells, Rcan1 has been shown to be regulated by both CN-NFAT signaling, NFκB [49] and oxidative stress [50]. Knock-down of endogenous Rcan1 in endothelial cells increased NFAT activity and stimulated expression of inflammatory genes [51]. The specific role of Rcan1 in the pancreas is not well studied, although Rcan1 overexpression in pancreatic beta-cells was shown to cause hypoinsulinemia, beta-cell dysfunction and diabetes [52]. Also, CN-NFAT signaling induced Rcan1 expression in acinar cells, following damage-induced regeneration [35].

Over the years, numerous diagnostic and prognostic markers for AP have been evaluated, including both single markers as well as more complex multifactorial prognostic systems. In spite the many currently available prognosis systems [53], there still is a lack of specific AP markers, specifically an early marker that reliably can predict mortality or disease severity.

2.4 Chronic pancreatitis

Repetitive AP may lead to CP [54], a chronic inflammation of the pancreas and a progressive fibrotic destruction of the pancreatic parenchyma [3, 55]. CP is mainly characterized by interstitial fibrosis and acinar cell atrophy [56]. Eventually, the disease leads to a progressive loss of the lobular morphology and structure of the pancreas, severe changes in the arrangements and composition of the islets, and deformation of the large ducts. This leads to irreversible damage resulting in the impairment of both pancreatic endocrine and exocrine function, eventually resulting in malnutrition and/or diabetes [55]. Increasing evidence indicates that the pancreatic stellates cells (PSCs) are major mediators of fibrosis, involved in the production of the fibrotic extracellular matrix (ECM) in the interstitial spaces responsible for the morphological changes of the pancreas. The pancreatic destruction in CP is associated with the activation of the PSCs into myofibroblast-like, α-SMA expressing cells, producing high levels of fibrotic ECM proteins, such as collagens I and III and fibronectin [55, 57]. Activation of the stellate cells is increased by cytokines released by injured acinar cells and infiltrating leucocytes.

A total loss of secretory tissue, disappearance of immune cells and an intense fibrosis denotes the final stages of CP [57]. Transforming growth factor beta (TGFB), which is upregulated and activated in fibrotic diseases including CP, is the most potent fibrogenic factor known to date [58].

2.5 Pancreatic cancer

Pancreatic tumors may originate in different cell types of the pancreas and are named thereafter. The most common tumors of the pancreas are of ductal, acinar and endocrine cell lineage. Still, neuroendocrine tumors only account for 1-2% of pancreatic tumors. The most common pancreatic endocrine tumor is the pancreatic endocrine neoplasms (PENs). Acinar cell carcinomas (ACCs) are also very rare, accounting for less than 2% of all pancreatic malignancies. In ACCs, pancreatic enzymes such as trypsin and lipase are produced by the tumor cells and can be detected by immunohistochemistry [59].

Pancreatic ductal adenocarcinoma (PDAC) accounts for up 85-90% of pancreatic cancers, making it by far the most common of the pancreatic neoplasms. For this reason, the term pancreatic cancer is often used synonymous with PDAC. PDACs are solid, poorly defined tumors, predominantly occurring in the pancreatic head and measuring about 2.5-3 cm when diagnosed. Due to their predominant location to the head, PDACs often cause cholestasis as a consequence of narrowing the common bile duct and the pancreatic duct. PDACs also cause dense fibrosis of the pancreatic parenchyma. Histologically, the tumors are often well-differentiated with duct-like structures surrounded by a desmoplastic stroma. The fibrotic stroma is characteristic for PDAC and is further discussed in the section on the tumor associated stroma (section 2.6). The cancer cells in PDAC commonly express mucins (MUC1, MUC5AC), cytokeratins (7, 8, 18, and 19), and p53 protein. The most common genetic mutations in PDAC are mutations in the KRAS gene, followed by mutations in TP53, CDKN2A/p16 and SMAD4, the latter commonly deleted in PDAC. These mutations are generally regarded as "driver mutations", necessary for the neoplasms to develop [59].

PDACs are believed to commonly develop through precursor lesions, mainly pancreatic intraepithelial neoplasias (PanINs) but also intraductal papillary mucinous neoplasms (IPMNs), and mucinous cystic neoplasms (MCNs) [59]. The PanINs have been subcategorized into different stages, PanIN-1A to PanIN-3. Genetic alterations in the different PanIN stages have been identified through tissue microarrays [60]. It was revealed that mutations in the PanIN stages occur in a specific order during their development, and this order is thought to be necessary for malignant transformation analogous to the situation in colorectal cancer [61]. Activating KRAS-mutations and telomere shortening are early events, p16 and MUC1 mutations show up in PanIN-2 lesions, whereas mutations in p53 is a later event which generally is detected first in PanIN-3 lesions.

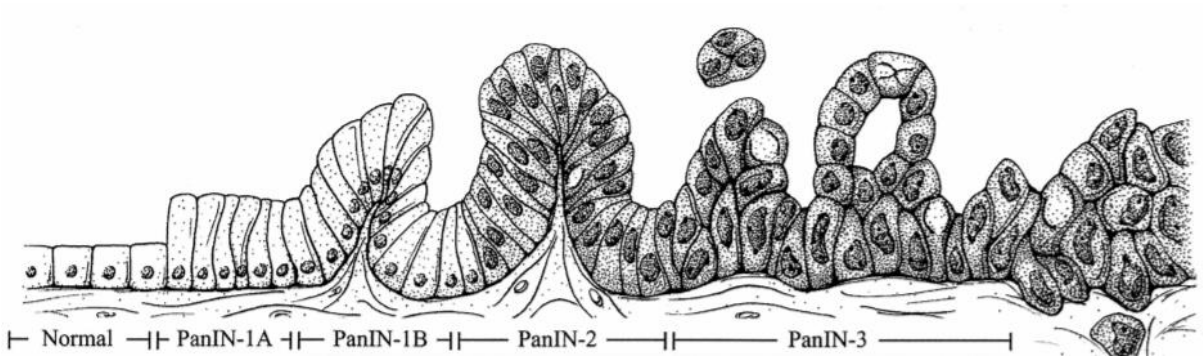

Figure 2. PanIN development in PDAC. Illustration of our current understanding of multistep progression of PDACs. Republished with permission from [60].

2.6 The tumor associated stroma

The stroma is defined as the supportive framework of an organ, usually composed of connective tissue cells, as distinguished from the parenchyma (tissues or cells performing the special function of the organ). The stroma is made up of vascular, epithelial and inflammatory cells within an extracellular matrix (ECM) rich environment containing different ECM proteins (collagens fibronectin, glycosaminoglycans and proteoglycans). Interestingly, only a small part of a tumor is actually made up of cancer cells, and the rest, in extreme cases up to 90%, is made up by the tumor stroma (often referred to as the desmoplastic or reactive stroma). Interactions between the cancer cells and surrounding cells are gradually getting more in focus, as it is becoming increasingly evident that the tumor associated stroma plays a major role in the development and progression of cancer. Cancer-associated stromal fibroblasts (CAFs) have been shown to promote pancreatic tumor progression [62, 63], and cancer-stroma interactions have been shown to be important in promoting tumorigenesis, angiogenesis, therapy resistance as well as metastatic spread [64]. Tumor-associated macrophages (TAMs, M1 and M2 type), are involved in promoting angiogenesis by regulating the angiogenic switch [65, 66], and in promoting proliferation and metastasis [66]. The production of the tumor associated stroma is promoted by growth factors such as fibroblast growth factor (FGF-2), platelet derived growth factor (PDGF), and transforming growth factor beta (TGFB1), which are produced by the cancer cells and activate fibroblasts to produce more ECM [67].

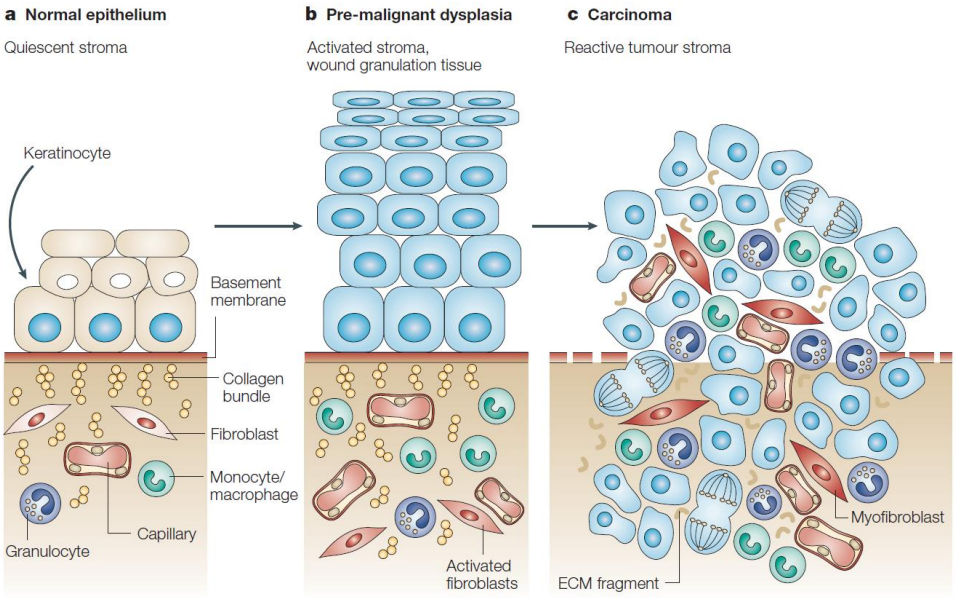

Figure 3. Stromal activation. Quiescent stroma in normal epithelium (**a**), activated stroma in pre-malignant dysplasia (**b**) and reactive tumor stroma in a carcinoma (**c**). Image re-published with permission from [68].

2.7 PDAC stroma

PDAC is generally characterized by a particularly dense and fibrotic stroma [69]. As mentioned, the organ residing pancreatic stellate cells (PSCs) are fibroblast-like mesenchymal cells which are involved in producing the fibrotic stroma in both pancreatitis [70] and pancreatic cancer [71]. The role of PSCs in fibrosis and cancer have recently been reviewed [72]. The PSCs, similar to the hepatic stellate cells responsible for fibrosis in the liver, were first identified and isolated in 1998 by two independent groups [10, 73]. PSCs can be identified by their expression of desmin, GFAP and vimentin. Freshly isolated cells have abundant lipid droplets stored in their cytoplasm, and a characteristic blue-green fading fluorescence of cytoplasmic vitamin A. Initially primary PSCs are negative for α-SMA but after being cultured they stain positive, meanwhile the number of cytoplasmic lipid droplets decrease. Culture activated PSCs [73] and PSCs activated by pro-inflammatory cytokines [74] produce ECM proteins such as collagen I and III, fibronectin and laminin. TGFB1 induces ECM production in cultured PSCs, and PDGF increases stellate cell proliferation. TGFB is known to moderate fibroblast phenotype and function, inducing myofibroblast transdifferentiation while promoting preservation of the ECM. The expression of TGFB is leading to increased stromal stimulation and increased ECM production, both in the tumor cells and in the surrounding fibroblasts; *in vivo*, desmoplasia is developing [75]. TGFB pro-fibrotic actions are, at least in part, mediated through its downstream effector, connective tissue growth factor (CTGF) [58]. To facilitate studies of PSC function in pancreatic fibrosis, immortalized rodent [76, 77] and human [78, 79] PSC lines have been established.

The function of the PDAC stroma has been debated and proposed as a double-edged sword. It is unclear whether it is a host defence against the cancer, a tumor driven process as to facilitate the tumor growth and spread, or possibly context dependent. Up until recently most evidence suggested that the role of the stroma was in promoting tumor progression [80]. For example, pancreatic cancer cells stimulate proliferation and matrix synthesis of PSCs [71] and PSCs promote epithelial to mesenchymal transition (EMT) in pancreatic cancer cells [81]. Also, as mentioned, cancer associated fibroblasts (CAFs) have been shown to promote pancreatic tumor progression [62]. The standard of care for pancreatic cancer patients, gemcitabine, only modestly extends survival for a small subset of patients [82]. PDAC response to chemotherapy is generally very poor and it was shown that gemcitabine and other drugs have problems to penetrate the tumor stroma [83]. This could at least in part explain why many drugs showing promising results in preclinical *in vitro* and *in vivo* models failing to recapitulate the tumor stroma, later on fail in the clinic. However, clinical trials targeting the PDAC stroma surprisingly revealed that the complete depletion of the stroma is associated with decreased patient survival. Two recent papers support this notion. Özdemir et al showed that depletion of CAFs and fibrosis induced immunosuppression and accelerated pancreatic cancer growth, reducing survival. This in a mouse model with the ability to delete α-SMA$^+$ myofibroblasts in mice with already established tumors [84]. Rhim et al showed that mice deficient in sonic hedgehog (Shh), had tumors with reduced stromal content but that these tumors were more aggressive than tumors with a higher content of tumor stroma [85]. More recently, a re-education of the stroma rather than a complete ablation, has been suggested [86, 87]. It is becoming more and more evident that the PDAC stroma is much more complex than initially proposed. Öhlund et al. presented two separate but co-

existing CAF populations, one α-SMA$^+$ CAF population adjacent to the tumor cells and another α-SMA$^-$/IL-6$^+$ CAF population, located further away from the tumor cells [88]. A recently published paper demonstrated an even more complex CAF profile, with four distinct subtypes of primary derived CAFs, each with distinct markers and correlations to overall survival as well as tumor promoting abilities [89].

2.8 3D cell culture models of PDAC

Solid tumors *in vivo* grow in a three-dimensional (3D) conformation with cell-cell interactions in all directions, and a heterogeneous exposure to oxygen, nutrients and other chemical and physical pressures. This is very different from the monolayer growth of cells in traditional cell culture. It is now generally accepted that cell-cell interactions in 3D influence not only cell structure and adhesion, but also cell signaling in response to soluble factors, greatly affecting cell function [90]. 3D culture of tumor cells was introduced already in the early 70's. Early interest was related to the morphology and (ultra-) structure of the tumor cells [91] and later tumor cell-cell interactions [92]. Today, there are various 3D models of human cancer reviewed in *e.g.* [93, 94]. 3D culture models include multilayered tumor cell cultures, tumor slices, organoids, 3D cultures with reconstituted basement membranes and spherical cancer models. Weiswald et al recently elegantly reviewed the different variants of spherical tumor models, and proposed a rational classification and nomenclature [90]. The four suggested classes of spheroid tumor models are: the multicellular tumor spheroid (MCTS) model, obtained by culturing cancer cell lines under nonadherent conditions; tumorospheres (TS), a model of cancer stem cell growth in serum free media supplemented with growth factors; tissue-derived tumorospheres (TTS), generated by partial dissociation of cancer tissue and containing only tumor cells; and organotypic multicellular spheroids (OMS), obtained by mechanical cutting and dissociation of tumor tissue [90]. To date, there are various 3D-culture models of pancreatic cancer, counting numerous MCTS models [95-97], including our 3D spheroid culture model, characterized by a higher ECM expression and significantly increased chemo-resistance compared to cells cultured in monolayers [98]. There also are various 3D PDAC models of cells grown in/on different matrix substrates, *e.g.* [99-101], tumorosphere models [102-105], as well as mouse and human organoids [106-109]. Although organoids and OMS derive from and are closer to real tumors than other 3D tumor models, there are other qualities which favors the use of simpler models such as MCTS. For example, the ease of maintenance and the possibility for high through put drug screening and genetic manipulation of the cells [90]. Extending MCTS and other matrix 3D models to allow for studies of tumor-stromal cross-talk, there also are co-culture models adding a stromal compartment. There now are co-culture models including tumor spheroid models of cancer cells and fibroblasts in lung cancer and cervical carcinoma [110], melanoma[111] , breast [112, 113], colorectal [114, 115] liver cancer [116] and pancreatic cancer [117], and 3D co-culture models of colon cancer [118, 119] and breast cancer cells [113] with macrophages. Recently, also a triple cell co-culture model of pancreatic cancer was developed, containing tumor cells, fibroblasts and endothelial cells [120].

2.9 Mouse models of PDAC

Many genetically engineered mouse models of human pancreatic cancer have been developed, and were recently reviewed by Mazur and Siveke [121]. Currently, the *Kras*$^{LSL\text{-}G12D/+}$;*Trp53*$^{LSL\text{-}R172H/+}$;*Pdx-Cre* (KPC) genetically engineered mouse model (GEMM) [122] is the most accurate and validated preclinical *in vivo* model of human pancreatic cancer, successfully recreating the dense stroma seen in the human disease which other *in vivo* models (subcutaneous, orthotopic) have failed to develop so far. In mice expressing the *Kras*$^{LSL\text{-}G12D/+}$ allele [123], PanIN lesions occur in pancreatic acinar cells developing into ductal cells through acinar-to-ductal metaplasia. These progressive lesions recapitulate all features of human PanIN stages 1, 2 and 3, and local invasive cancer develops in 9-12 months. In combination with the *Trp53*$^{LSL\text{-}R172H/+}$ allele [122], latency time is reduced to 3 to 5 months and 60% of the mice develop metastases to the same sites as in humans.

Depending on the question asked simpler injection models may however be the best choice. Cells may be injected subcutaneously under the skin or orthotopically into the pancreas. Co-injection of PDAC cells and PSCs resulted in enhanced tumorigenicity [124, 125] and PSCs were shown to be involved in metastasis *in vivo* [125].

2.10 High mobility group A2

The HMGA2 protein belongs to the family of non-histone chromosomal high-mobility group proteins (reviewed in [126-129]). These proteins are known to alter DNA structure and consequently regulate DNA-dependent activities, like transcription, replication and repair [128]. The AT-hook is a structural DNA-binding motif in the HMGA2 protein, which enables binding to the minor groove of AT-rich DNA regions [130]. It also promotes the recruitment of additional transcriptional regulators and makes up part of the so called enhanceosome, by interaction with multiple protein complexes on promoter/enhancer sites [130, 131]. HMGA2 has been suggested to play a role in regulation of cell proliferation and differentiation, due to its high expression in the embryo and complete or close to absence in adult human tissue [128, 131, 132]. In neoplastic cells, HMGA2 expression is often re-induced and its involvement in the regulation of EMT and cancer stem cell properties has been demonstrated [133, 134]. This goes in line with HMGA2 over-expression being found associated with metastasis and poor prognosis *e.g.* in non-small cell lung cancer [135], gastric cancer [136, 137], colorectal cancer [138], triple negative breast cancer [139] and ovarian cancer [140]. In PDAC, HMGA2 expression was found associated with loss of tumoral E-Cadherin expression [131], lymph node metastases and high tumor grade [141, 142]. One study, limited to 91 cases of PDAC, also identified an association with poor prognosis in evaluations limited to univariate analysis [143]. Both epithelial growth factor (EGF) and TGFB have been shown to act as inducers of HMGA2 [134, 144].

2.11 Transforming growth factor beta signaling

There are three strongly conserved TGFB genes giving rise to three distinct isoforms known as B1, B2 and B3, TGFB1 being the one mainly involved in the immune system. When secreted *in vivo*, TGFB is incorporated as a part of an inactive complex in the extra-cellular matrix awaiting activation. Once activated, TGFB1 and B3 bind to TBRII with high affinity, whereas TGFB2 requires the presence of type III TGFB receptor (TBRIII) to efficiently bind TBRII. Ligand-bound TBRII then recruits and activates TBRI through phosphorylation, which in turn phosphorylates and activates downstream SMAD proteins. The SMAD proteins are transcription factors and SMAD2 and SMAD3 are directly activated by TBRI phosphorylation. This causes a conformational change, allowing SMAD2/SMAD3 to form a heterotrimeric complex with SMAD4 which then translocates to the nucleus and regulates gene transcription [145-148]. The involvement of the canonical activin receptor-like kinase 5/Smad3 pathway in fibrosis, has been demonstrated in a wide range of experimental models [58]. SMAD7 is also activated by TGFB but acts as the main negative feedback regulator of TGFB signaling [149]. TGFB downstream signalling can also be mediated through SMAD-independent pathways [147, 150].

TGFB is a pluripotent cytokine which is expressed in almost every cell type of the body. Its role in fibrosis has been subject to intensive investigation but is not yet fully understood, and the exact contribution of the different cellular compartments of the pancreas with respect to TGFB as source and effect in the role of fibrosis is not known. TGFB is also involved in cancer, through its role in immune function [151, 152] , cell growth, apoptosis, angiogenesis and metastasis, first acting as a tumor suppressor and later as a tumor promotor [147, 153-156].

3. AIMS

The overall aim of this thesis is to enhance the understanding of the role of the reactive stroma in pancreatic diseases, namely pancreatitis and pancreatic ductal adenocarcinoma.

Specific Aims

- To investigate the role of pancreatitis-related genes and identify potential biomarkers of acute pancreatitis.

- To establish, characterize and validate a 3D co-culture spheroid model of pancreatic cancer and stellate cells.

- To investigate the role of tumor cell to stromal cell crosstalk in promoting pancreatic cancer carcinogenesis, using the established 3D *in vitro* model.

4. PATIENTS, MATERIALS AND METHODS

Here I will briefly discuss patients, materials and methods, with a focus on the key methods used for my own contribution to the studies and related experiments. More comprehensive and detailed descriptions are available in respective paper.

4.1 Cell culture (Papers I-III + additional data)

The Panc1, HPAFII, AR42J, AsPC1 and CFPAC-I cell lines were purchased from ATCC. The immortalized pancreatic fibroblasts (PSCs/hPSCs) were derived from a patient with chronic pancreatitis as described in [78], and the KPCT 86-2 cell line was isolated in-house from a *Kras*$^{LSL\text{-}G12D/+}$*;Trp53*$^{LSL\text{-}R172H/+}$*;Pdx-Cre* (KPC) mouse [122] mated to the tdTomato allele (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J) [157]. The immortalized mouse pancreatic stellate cell line clone 3 (imPSCc3; in text and figures referred to as mPSC) was a kind gift from Dr. Raul Urrutia and Dr. Angela Mathison at the Mayo Clinic College of Medicine, Rochester, Minn, USA, the Panc1-H2B-mCherry cells from Dr. Maarten Bijlsma at the Academic Medical Center, Amsterdam, The Netherlands [77] and the PaCa44 from Günter Klöppel at the Technical University of Münich, Germany. Panc1-mock (Panc1-M) and Panc1 overexpressing TGFB1 (Panc1-T) cell lines were previously established by JML [75]. The HPDE cells were a kind gift from Patrick Michl (University Hospital Halle, Germany).

HPAFII and CFPAC-I cells were cultured in RPMI-1640 medium, AR42J cells in F12-K and all other cell lines in DMEM/F12. All cell lines were cultured under standard culture conditions (5% CO_2, at 37°C), supplemented with fetal bovine serum (FBS) (20% for AR42J cells and 10% for all other cell lines) and penicillin/streptomycin. All cell lines were tested negative for mycoplasma (MycoAlertTM PLUS Mycoplasma Detection Kit, LT07-705, Lonza, Switzerland) before initial use and regularly thereafter. Primary murine acinar cells were also isolated and cultured, as described previously in [158] as well as in paper I.

For more detailed information on culturing of the cells and specific cell culture experiments, see specific article or manuscript.

4.2 3D co-culture spheroid assay (Papers II and III + additional data)

Tumor cells and PSCs were seeded alone or in co-culture (1:1), at a total concentration of 2500 cells/well. Cells were seeded in culture media with a final concentration of 0.24% of the crowding agent methylcellulose, in non-cell culture treated round bottom 96-well plates (Falcon, BD NJ, USA). For spheroid preparations, all cells were seeded in DMEM/F12 media. A more thorough description of the 3D co-culturing method is described in [98], as well as in papers II and III, along with detailed information on further processing.

Shortly, for dissociation (paper II) spheroids were treated with trypsin/EDTA at 37ºC followed by trituration, in a series of steps. Trypsin was thereafter inactivated by the addition of FBS-containing media and cells were further processed for fluorescence activated cell sorting (FACS). To prepare spheroids for immunohistochemical analysis (paper III), the 3D cultures were fixed in 4% paraformaldehyde, dehydrated in 70% ethanol and then embedded in HistoGel, according to a protocol modified from [159], as described in paper III.

For specific information on antibodies, see the manuscript. For electron microscopy analysis, spheroids were fixed in 2.5% glutaraldehyde buffer.

Mono-cultured Panc1 tumor cells treated with TGFB1 (paper II + additional data) were seeded in the same way as other mono- and co-cultures, except in low serum media containing 0.5% FBS. The cells were seeded in the presence of 5 ng/ml TGFB1 or equal volume of vehicle control and then treated again on day 3 at double concentration, as to compensate for the media volume already present in the well.

4.3 Patient samples

4.3.1 AP patients (paper I)

Peripheral blood samples were collected from patients at the Royal Liverpool University Hospital (Liverpool, UK), as well as healthy controls at the Karolinska University Hospital (Stockholm, Sweden). AP patient samples were collected within 24 hours of admission (48 hours of onset of abdominal pain; NIHR Liverpool Pancreas Biomedical Research Unit Acute Pancreatitis Biobank), from patients with mild, moderate and severe AP.

All sample collections were done in accordance with local ethical guidelines (REC 15/YH/0193 for Liverpool Hospital, approved by NRES Committee Yorkshire & The Humber - Sheffield; EPN Dnr 2014/1155-31/4 and 2016/2090-32 for Karolinska Hospital approved by Regionala etikprövningsnämnden i Stockholm). Each volunteer agreed to sample collection and use through written consent.

4.3.2 PDAC patients (paper II)

In this study, four hundred and forty-five patients from the Herley Hospital (n=277) and Rigshospitalet (168), both University of Copenhagen, Denmark, were included. They all underwent pancreatic resection between 1976 and 2012. Due to an unclear cause of death, 37 patients were excluded from analyses. Out of the included patients, 253 had PDAC and will be the focus of this thesis, whereas 155 had ampullary adenocarcinoma (AAC). The study was approved by the local Ethical committee (H-KA-20060181 and VEK ref. KA-200601113) and the Danish Data Protection Agency (j.nr. 2006-41-6848).

4.4 Mouse models

4.4.1 Acute pancreatitis experimental mouse model (Paper I)

The caerulein induction model of acute experimental pancreatitis [160], was utilized in wild type C57Bl/6J mice (Scanbur-BK, Sollentuna, Sweden). Caerulein, at 50 µg/kg, was injected intraperitoneally (i.p) 9 times hourly. Control mice were injected with the corresponding volume of saline. One hour after the last injection, mice were anesthetized and cardiac blood and pancreas was collected. Blood samples were used for blood chemistry and ELISA analyses, pancreas for mRNA and tissue myeloperoxidase (MPO) activity evaluations.

A detailed description of the performance of the mouse experiments, collection and processing of samples, can be found in the article (ethical permission number: S176-08, ID 707 from Stockholms södra djurförsöksetiska nämnd).

4.4.2 KPC mouse model (Paper II)

The pancreas from *Kras*$^{LSL\text{-}G12D/+}$*; Trp53*$^{LSL\text{-}R172H;+}$*;Pdx-Cre* (KPC) mice [122] were collected at different time points. Tissues were fixed in 4% paraformaldehyde at room temperature for 24 hours and then transferred in 70% ethanol for at least another 24 hours at 4°C before further dehydration and paraffin embedding (ethical permission number from Stockholms södra djurförsöksetiska nämnd: S31-15).

4.4.3 Co-injection xenograft model (Paper II)

Panc1 cells alone or together with PSCs at a 1:1 ratio were injected subcutaneously into CB17 SCID mice (Janvier Labs, Le Genest-Saint-Isle, France, n = 5 per group). Tumor growth was monitored twice per week and tumor volumes calculated with the formula (V = length x height x width/2). When the tumor volume reached 500 mm^3 the mice were euthanized under anesthesia, tumors collected and immediately snap frozen in liquid nitrogen (ethical permission number 2014.III.02.022, Utrecht University animal ethical board).

All animal experiments were performed in accordance with the local ethical guidelines and with the approval of the local ethical committee.

4.5 Regulator of calcineurin 1 ELISA (Paper I)

Whole blood from mice and peripheral blood samples from AP patients and healthy controls were collected in EDTA tubes. The samples were processed within 30 minutes of arrival in the lab. The resulting plasma was aliquoted and stored at -80°C. Plasma was later diluted in assay diluent and assayed in duplicates according to manufacturer´s instructions, in either a mouse (CUSABIO, CSB-EL019500MO) or human (EIAab, E13874h) RCAN1 ELISA kit.

The patient samples were also divided into three groups of mild, moderate and severe AP based on the clinical diagnosis (n = 19, 13 and 8), according to the revised Atlanta criteria [161], and assayed against healthy controls (n = 8). A two-sided Student´s *t*-test for individual samples was used to evaluate the difference between AP patients or mice and respective controls. Differences between the three groups of mild, moderate and severe AP patients were evaluated by ANOVA.

4.6 Immunohistochemistry (Papers I-III + additional data)

Murine pancreas head samples (paper I) and human patient samples (paper II) were fixed in 4% paraformaldehyde and embedded in paraffin before being sectioned and stained with hematoxylin and eosin and antibodies as specified in each paper. Spheroid cultures (paper III), were treated the same way except for an intermediate step of HistoGel embedding, between fixation and paraffin embedding, as described above.

More detailed information on analysis can be found in respective paper.

4.7 Transcriptional profiling (Paper I)

Affymetrix mouse gene 1.0 ST arrays (Affymetrix, Inc., Santa Clara, CA) was used for gene expression profiling. Bioconductor packages (www.bioconductor.org) was used for analysis. Normalization and gene expression calculations were performed with the Robust Multichip Average expression measure, using oligo package [162]. Prior to further analysis, a non-specific filter was applied in order to include only genes with expression signal > 50 in at least 20% of all samples. Differentially expressed genes with an adjusted P value lower than 0.01, were identified using Limma package [163].

4.8 mRNA isolation and quantitative RT-PCR (Papers I-III + additional data)

Mouse pancreas samples and cells (papers I-II) and spheroids (papers II-III + additional data) were processed for total RNA extraction and reverse transcription. Thereafter, cDNA was subjected to real-time PCR. Detailed information on RNA/cDNA preparations, primers, housekeeping genes, cycling programs and analyses are found in the respective papers.

Briefly, delta Ct values for each sample were used for statistical analysis with a Students *t*-test for individual samples, and a p value below 0.05 was considered as statistically significant. Relative expression ($2^{\wedge-dCT}$) values were used to create boxplots (paper I) and mRNA expression values normalized to untreated control (papers I-II), corresponding mono-culture (paper II), day 3 mono-culture expression (paper III + additional data), or HPDE expression (additional data) were used to create staple graphs. Error bars in staple graphs are 95% confidence intervals.

4.9 Virtual sorting (Paper III + additional data)

A method for determination of cell type specific gene expression in 3D spheroid cultures, without the need for pre-dissociation and physical separation, was developed and named virtual sorting. Spheroid cultures pairing human tumor cells with mouse PSCs and mouse tumor cells with human PSCs, were prepared. Cell type specific gene expression was then determined by direct real time PCR, using species specific primers specifically developed in regions genetically diverse in between the mouse and human homologue genes.

As a pre-test of species specificity, real time PCR was performed with the developed primers, on a test panel including human tumor cells (Panc1), human PSCs (hPSC), mouse tumor cells (KPCT 86-2) and mouse PSCs (mPSC). The products from the real time PCR test were also run on a 2% agarose gel, in order to ensure species specificity, amplicon size and singularity.

5. RESULTS

In this chapter I will summarize the findings from each paper, with focus on the results derived from my own contributions to the studies and related results needed to be discussed for a more comprehensive picture. More detailed descriptions are available in respective paper. I will also present some additional, preliminary data not included in any of the papers.

5.1 Paper I

5.1.1 Rcan1 and Sesn2 are identified as candidate biomarkers of acute pancreatitis

Caerulein treatment was shown to induce AP in mice through histological signs of pancreatic inflammation, induction of inflammatory cytokines and an increase in serum amylase, the clinical standard of AP. Through a gene expression profiling on murine pancreatic tissue, mRNA samples from the caerulein treated AP mice and sodium-chloride treated controls (n = 5-6), 2038 genes were found to be differently expressed in between the groups. The variation within each group was very low, indicating high reproducibility of the experimental procedure. We screened the literature to find novel candidates for prognostic markers and confirmed the upregulation of these candidate genes by real-time PCR in two independent experiments. A selection of genes were then further investigated in freshly isolated primary murine acinar cells, and *Rcan1* and *Sesn2* were found to be induced at early time points upon caerulein stimulation.

5.1.2 Rcan1 is regulated by oxidative stress

Our results from the animal experiments were further verified by inducing AP-like stress in the AR42J cell line, as previously described, by exposure to caerulein [164] or H_2O_2 [165]. The AR42J cell line is often used as an alternative to primary acinar cells and display essential neuroendocrine features of normal acinar cells. Oxidative stress was induced upon caerulein exposure as measured by oxidized glutathione (GSSG). Both *Rcan1* and *Sesn2* expression increased rapidly but transiently upon exposure to both caerulein and H_2O_2. The antioxidant N-acetylcystein (NAC) dose dependently inhibited the increase of *Rcan1* upon caerulein exposure, confirming a dependency on oxidative stress for regulation of the gene. NAC alone did not induce cell death, indicated by viability measurements using the Acid Phosphatase (APH) Assay (as previously described [98]) at 1 and 4 hours.

5.1.3 RCAN1 is a potential marker of acute pancreatitis

Rcan1 protein could be detected by ELISA in the blood of caerulein treated mice compared to mock-treated controls. We also found significantly higher levels of RCAN1 protein in the blood of AP patients (n = 9, mix of mild, moderate and severe) compared to healthy controls (n = 7).

We were, however, not able to demonstrate any difference in RCAN1 levels between mild, moderate and severe AP.

5.2 Paper II

5.2.1 HMGA2 correlates with shorter overall survival and is a prognostic marker of PDAC

In pancreatic cancer tissue samples of patients, HMGA2 was found expressed in the nucleus of cancer cells, while completely absent in stroma cells, including fibroblasts, immune cells and vascular cells. The amount of HMGA2-positive tumor cells however varied between cases and both positive and negative tumors were found, consistent with previous data [141-143]. A connection was also found between HMGA2 expression and clinic-pathological parameters associated with worse prognosis, such as poor tumor differentiation and advanced stage group. Further analyses on potential associations between HMGA2 and EMT markers found no restriction of HMGA2 to cells with low expression of cytokeratin, as examined by IHC. Nor did we find any strong link between HMGA2 expression and investigated EMT-associated miRNAs. $HMGA2^+$ cells were however found to be associated with a significantly shorter OS and an increased hazard ratio for death in patients with PDAC, identifying HMGA2 as an independent prognostic marker.

5.2.2 HMGA2 is differently expressed in human and mouse precursor lesions

In the KPC mouse model of PDAC, very weak positive staining for Hmga2 was detected in the tumor cells already in pancreatic intraepithelial neoplasia (PanIN)-1 lesions, with an increasing frequency and intensity in PanIN-2 and PanIN-3 lesions. In the developed murine PDAC, heterogeneous staining was observed with positivity primarily in the more poorly differentiated part. This is in agreement with HMGA2 expression in established human PDACs, connected with poor tumor differentiation (*5.2.1*). Human PDAC samples contained few PanIN lesions and the detected PanIN-1 and PanIN-2 lesions were all negative for HMGA2 (n=18), while no PanIN-3 lesions were identified.

It therefore appears that HMGA2 seems to be differentially expressed within precursor lesions in mice and humans.

5.2.3 $HMGA2^+$ tumor cells correlate with $PDGFRB^+$ stroma

PDGFRB is an important signal transducer of chemotaxis and proliferation of mesenchymal cells, which is highly expressed on tumor stroma cells such as cancer-associated fibroblasts (CAFs) and pericytes [166, 167]. Double IHC staining of PDGFRB and HMGA2 on four different human PDAC specimens showed that $HMGA2^+$ tumor cells were more frequent in areas with higher abundancy of $PDGFRB^+$ fibroblast. One could however also detect $PDGFRB^+$ stroma not linked to high HMGA2 expression in the epithelium and vice versa, although these areas were less frequent.

These observations suggested a role of fibroblasts in the tumor cell HMGA2 induction.

5.2.4 PSCs increase HMGA2 expression and tumorigenic properties in PDAC cells

In order to test whether paracrine interaction is involved in regulation of HMGA2 expression and tumorigenic properties of pancreatic cancer cells, Panc1 tumor cells were injected into SCID mice, alone or in combination with PSCs. Co-injections resulted in faster growing tumors than those derived from Panc1 mono-injections. These faster growing tumors also had higher abundance of stroma cells positive for PDGFRB as well as an increased HMGA2 expression in tumor cells, both on an mRNA and protein level.

For further investigations of the impact of PSCs on tumor cell *HMGA2* expression, Panc1 cells were seeded under anchorage-independent conditions in either control medium, PSC conditioned medium or conditioned medium from PSCs pre-stimulated with PDGFB. PSC conditioned media increased the ability of Panc1 cells to form tumor cell spheroids under anchorage-independent conditions. Conditioned medium derived from PSCs pre-stimulated with PDGFB further enhanced this effect. Spheroids grown in either one of the conditioned media also showed a slight increase in *HMGA2* mRNA expression compared to controls. Using a 3D co-culture spheroid model with direct contact between Panc1 and PSCs (characterized and validated in paper III), *HMGA2* mRNA levels also increased. TGFB1 has previously been shown to induce HMGA2 [134], which we could confirm in our Panc1 mono-culture spheroids, where TGFB1-stimulation induced *HMGA2* mRNA expression.

Together these experiments demonstrate that fibroblasts are able to support HMGA2 expression in a manner associated with increased tumorigenic properties. As we did however find most PDAC cell lines as well as tissue specimen tumor cells to not express PDGFRs, PDGF ligands are unlikely to be part of the CAF secretome inducing HMGA2.

5.3 Paper III

5.3.1. 3D spheroid cultures are healthy and display distinct features

Electron microscopy as well as morphological analyses showed healthy spheroid cultures, with very little apoptosis and necrosis and moderate extracellular matrix, in both Panc1/PSC and HPAFII/PSC mono- and co-cultures.

Panc1 and PSC mono- and co-cultures displayed immature, desmosome-like contact surfaces, whereas HPAFII mono-cultures contained large amounts of well-developed desmosomes and HPAFII/PSC co-cultures both non-developed and developed desmosomes. The number of contact surfaces, immature and mature, increased generally from day 2 to 3 and then stabilized, with the exempt of HPAFII/PSC cultures, where desmosome-like structures increased steadily over time.

HPAFII mono-cultures were compact and contained epithelial cells with well-developed organelles and good ultrastructure at all time points, as well as fair amounts of microvilli with glycocalyx ("sugars") similar to those seen in the intestines, decreasing with time. HPAFII/PSC co-culture cells were round and displayed some microvilli with glycocalix, also here decreasing with time. Peripheral cells showed better ultrastructure than centrally located cells. The cell-cell contacts in the co-cultures were generally good.

5.3.2. PDAC/PSC co-culturing affects cell proliferation and PSC activation

Immunohistochemical staining with CK19 separated epithelial cells ($CK19^+$) and hPSCs ($CK19^-$). Panc1/hPSC co-cultures displayed a mixture of the two cell types throughout the cultures, whereas the spatial division between HPAFII cells and hPSCs was clear, as the HPAFII cells shaped a wreath around a compact core of stellate cells. CK19 staining of the co-culture spheroids also allowed to follow the cell type distribution in terms of cell numbers over time after initial seeding in a 1:1 ratio. In Panc1/hPSC cultures, the distribution was still equal at day 2, after which the hPSCs increased their proportion to around 60%, before stabilizing at slightly more than 50%. HPAFII cells on the contrary, made up a somewhat higher proportion of the co-cultures than the hPSCs at day 2, and this difference slightly increased over time. Staining of another epithelial marker (WT1) confirmed the results of the CK19 staining in the Panc1 cultures, but the marker was absent in HPAFII cells. Both Panc1 and hPSCs stained positive for vimentin, whereas HPAFII cells were negative. The hPSCs but none of the tumor cells were found positive for CD10.

Double staining with CK19 and the proliferation marker Ki67 revealed that basically all hPSCs were proliferating, independent of the presence of tumor cells. The percentage of proliferating Panc1 cells increased by co-culture with hPSCs, although the difference to the mono-cultured cells decreased over time. Proliferating cell numbers were higher in co-cultured HPAFII cells

as well, except on day 5. Real time PCR of spheroid preparations confirmed similar trends between mRNA and stainings for CK19, CD10, WT1 and Ki67.

Markers of activated stellate cells, *i.e.* α-SMA and the ECM protein fibronectin, were both expressed in hPSCs but not in Panc1 mono-cultures, on an mRNA level. Upon co-culture expression levels seemingly increased. Collagen type I mRNA expression increased over time in Panc1 mono-cultures, while expression remained stable in hPSC mono- and co-cultures. None of these genes were expressed in HPAFII mono-cultures. Co-culture of HPAFII and hPSCs seemingly increased the expression of fibronectin. TGFB1 mRNA expression suggested an increase in co- vs mono-cultured Panc1 cells. It was also found expressed in both HPAFII and hPSC mono-cultures, and clearly increased upon co-culture on day 7.

The protein epithelial marker E-cadherin was strongly expressed in HPAFII cells independent of the presence of hPSC, but as expected not at all in hPSCs. Positive staining was present in Panc1 mono-cultures and increased over time, however the occurrence was at a much lower percentage than in HPAFII cells. Interestingly, E-cadherin was almost non-existent in co-cultured Panc1 cells. Expression of *CDH1*, the gene encoding for E-cadherin, was consistent with protein data.

5.3.3. Virtual sorting confirms gene expression data from human-human co-cultures

In paper II, we determined cell type specific mRNA expression in the 3D co-culture spheroids by real time PCR upon cell sorting with fluorescence activated cell sorting (FACS) [168]. As this proved to be unsustainable due to an unreasonably large amount of spheroid cultures being required for preparation of a small amount of sample material, and as the prolonged preparation time possibly would induce cellular stress and affect gene expression in an unwanted way [169], we developed a novel method for determining cell type specific gene expression. This method uses normal real time PCR, but with especially designed species specific primers and co-cultures of human and murine cells. Thanks to the species specificity, intact spheroid co-cultures can be utilized and no dissociation step is needed. In order to validate the method and confirm species-specificity, we ran real time PCR products for each primer pair, from a panel of human and mouse tumor cells and stellate cells, on a 2% agarose gel.

Virtual sorting could confirm an upregulation of α-sma and fibronectin also in mouse PSCs (mPSC) co-cultured with Panc1 cells, and of α-sma, collagen type I and Tgfb1 upon co-culture with HPAFII cells. Further, the trends of an increase in proliferative ($Ki67^{+}$) Panc1 cells upon co-culture and lower expression of *CDH1* in co- vs mono-cultured Panc1 cells, were confirmed as well. The method also gave some more insight in gene expressions difficult to interpret in the human-human cultures, *e.g.* an increase of collagen type I and Tgfb1 in PSCs co-cultured with Panc1 was suggested. In addition, culture of mouse KPCT tumor cells and hPSCs presented the same trend as the other cell combinations when it came to PSC activation, *i.e.* an increased hPSC expression upon co-culture of α-sma, collagen type I, Tgfb1 and fibronectin.

5.4 Additional preliminary data

5.4.1 PDGFB does not induce TGFB1 expression in PSCs

In paper II, we discovered a correlation of PDGFRB-expressing stromal cells and $HMGA2^+$ tumor cells. We could also show that PSC conditioned media increased the anchorage-independent tumor cell spheroid forming ability of Panc1 cells, and that this effect was further increased when PSCs were pre-stimulated with PDGFB. As mentioned above in the results of paper II, TGFB1 has previously been shown to induce HMGA2 [134] and we could confirm an upregulation of *HMGA2* expression in TGFB1-stimulated Panc1 3D mono-cultures. In paper III, we also showed that expression of *TGFB1* was induced in co-cultured PSCs. Because of this and since PDGFRB-signaling can induce *TGFB1* expression [170], we hypothesized that PDGFB ligand may induce TGFB1 in the PSCs, ultimately leading to an increase in tumor cell HMGA2.

To test this hypothesis, we treated both regularly 2D cultured PSCs as well as PSC mono-culture spheroids with PDGFB, but were unable to detect any alteration of *TGFB1* mRNA expression (data not shown).

5.4.2 HMGA2 expression is inconsistent in the co-culture spheroid model

We performed double stainings of CK19 and HMGA2 on the 3D co-culture spheroid models using Panc1 or HPAFII PDAC cells and hPSCs, developed and characterized in paper III, as well as their mono-culture counterparts. As previously, CK19 stained epithelial tumor cells but not PSCs. HMGA2 staining was sparse in Panc1 mono-cultures although increasing over time, from day 2 to 7. There was however clearly a higher frequency of $HMGA2^+$ cells in co-cultured Panc1 cells (Fig.1A and C). HPAFII mono-cultures contained very few positive cells and co-cultures showed similar results, except on day 3 where there was a clear spike in $HMGA2^+$ cells (Fig.1.B and D). Surprisingly, since using PSCs from the same frozen batch and of similar passage numbers, the PSCs in the HPAFII experiment were negative for HMGA2, whereas a large percentage of $HMGA2^+$ PSCs were seen in the Panc1 experiment, both in mono- and co-cultured cells (Fig.1). As to exclude any technical errors, simultaneous staining was performed on PSC mono-cultures from both experiments, as well as from two additional replicate experiments, one with Panc1 and one with HPAFII cells, mono- and co-cultured with PSCs. These stainings showed that only one out of the four experiments (the first Panc1/PSC experiment) contained $HMGA2^+$ PSCs (data not shown), in accordance with previous data. All samples from the replicate Panc1 experiment, where PSCs were negative for HMGA2, were also stained with CK19/HMGA2 double staining and interestingly displayed completely different results compared to the Panc1 experiment containing $HMGA2^+$ PSCs. In this case, there were surprisingly more $HMGA2^+$ Panc1 cells in the mono-cultures compared to co-cultures (data not shown).

Taken together, this may indicate a strong variation of basal level HMGA2, and a possible connection between stromal and tumor cell HMGA2 expression, as HMGA2-positivity in co-cultured Panc1 cells was increased only in the presence of $HMGA2^+$ PSCs.

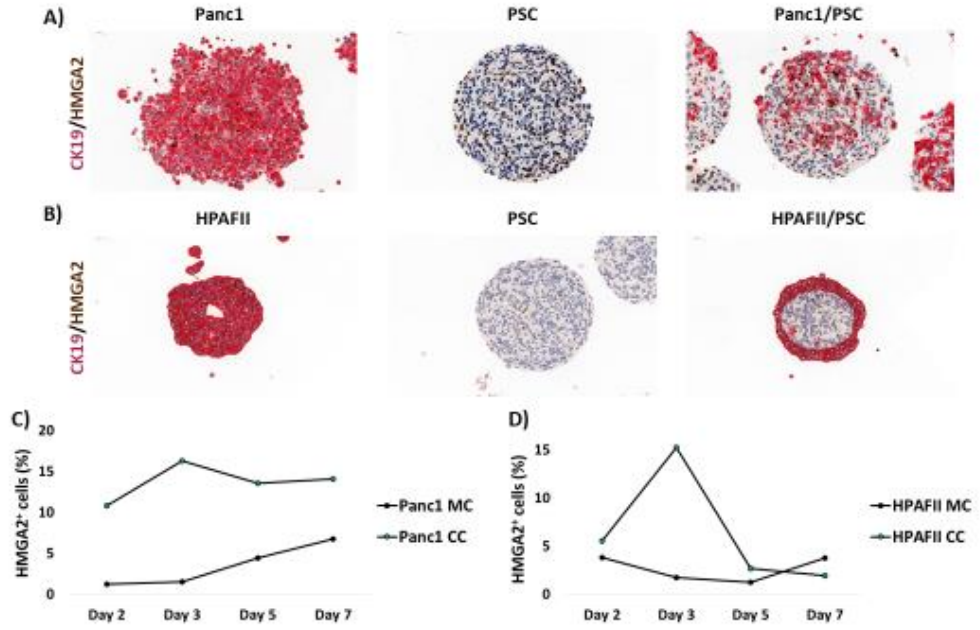

Figure 1. Panc1 and PSC (A) and HPAFII and PSC (B) mono- and co-culture spheroids stained with CK19/HMGA2. Quantification of $HMGA2^+$ cells in Panc1 (C) and HPAFII (D) mono- and co-cultures.

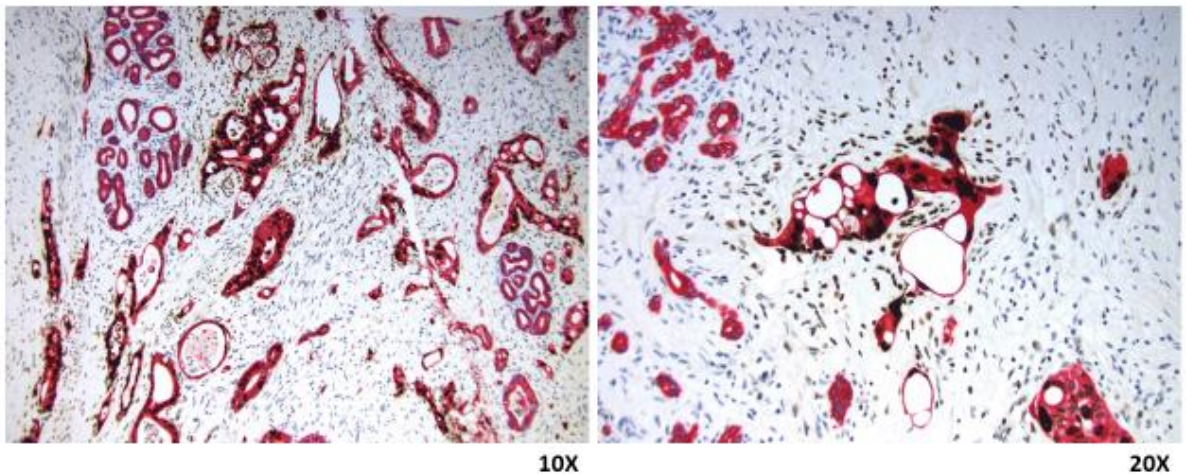

Figure 2. A clinical PDAC sample containing areas with strongly $HMGA2^+$ tumor cells in close proximity to strongly $HMGA2^+$ stroma, as well as areas completely negative for HMGA2.

5.4.3 HMGA2 is differentially expressed throughout clinical PDAC samples

To try to gain some clarity, we investigated clinical samples that had been stained during pathological diagnosis with HMGA2 and CK19, from patients operated for periampullary cancer at the Karolinska University Hospital. We found many to be negative for HMGA2, but also a few positive. In contrast to the patient cohort investigated in paper II (see section *5.2.1*), four cases (3 PDACs and one duodenal cancer) displayed $HMGA2^+$ stroma in addition to $HMGA2^+$ tumor cells. In all of the four cases both areas with strongly positive tumor cells in close proximity to strongly positive stroma, as well as areas completely negative for HMGA2 were identified (example of a PDAC sample is shown in Fig.2). In two of the PDAC samples and the duodenal cancer sample, there also were areas of $HMGA2^+$ tumor cells but a negative stroma. Stroma areas positive for HMGA2 without any $HMGA2^+$ tumor cells close by, also presented themselves in one of the PDAC samples and in the duodenal cancer sample. The PDAC sample containing all of these different areas, $tumor^+$ and $stroma^+$ cells, $tumor^+$ and $stroma^-$ cells as well as $stroma^+$ and $tumor^-$ cells, is shown in Fig.3.

This data in combination with the protein data from the 3D *in vitro* co-cultures, together suggest a complex relationship between HMGA2-positivity and the cross-talk between tumor cells and stroma cells.

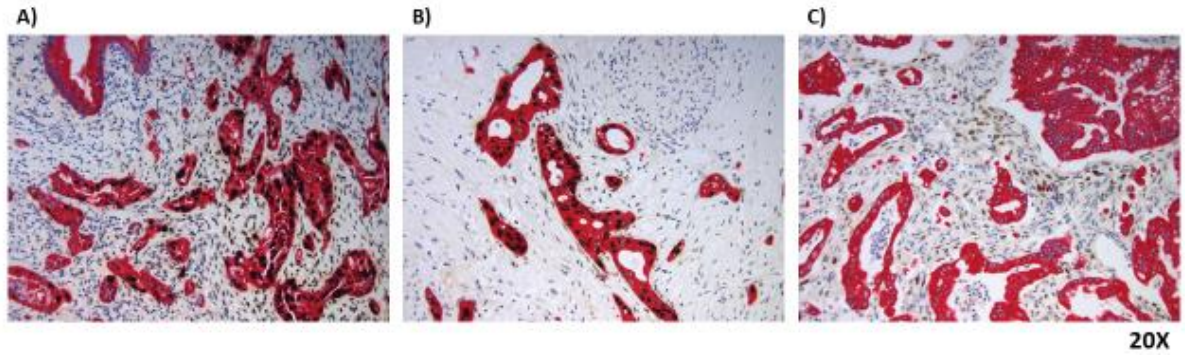

Figure 3. A clinical PDAC sample containing areas with strongly $HMGA2^+$ tumor cells in close proximity to strongly $HMGA2^+$ stroma and areas completely negative for HMGA2 (A), as well as with $HMGA2^+$ tumor cells but a negative stroma (B) and stroma areas positive for HMGA2 without any adjacent $HMGA2^+$ tumor cells (C).

5.4.4 HMGA2 mRNA expression in the co-culture spheroid model is difficult to interpret

mRNA levels of *HMGA2* in the Panc1/PSC mono- and co-culture spheroids indicated an increase of *HMGA2* in co-cultures (Fig.4A), consistent with the protein data from the first Panc1/hPSC staining experiment. This data was obtained using the same *HMGA2* primers as in paper II, recognizing different splice variants of the gene. In order to be able to use the virtual sorting approach also for investigating tumor cell specific *HMGA2* expression in co-cultures, human specific primers were designed recognizing either *HMGA2* transcript variant 1 (*HMGA2-1*) or transcript variant 3 (*HMGA2-3*), as it due to the similarities between the human and murine genome was not possible to compose a universal species specific primer not detecting mouse mRNA. Utilizing these species and transcript specific primers on human-human cultures, indicated an induction of mainly *HMGA2-1* in the co-cultures (Fig.4B), although *HMGA2-3* expression is difficult to interpret due to an interestingly high expression in the PSCs (Fig.4C). In HPAFII/PSC cultures, mRNA levels of *HMGA2* and seemingly mainly *HMGA2-1*, appeared to be increased in the co-cultures, especially on day 7 when the difference was clear (Fig.5A-B). *HMGA2-3* was also here more highly expressed in the hPSCs than the *HMGA2-1* transcript (Fig.5C), however not at the levels seen in the Panc1/PSC experiments.

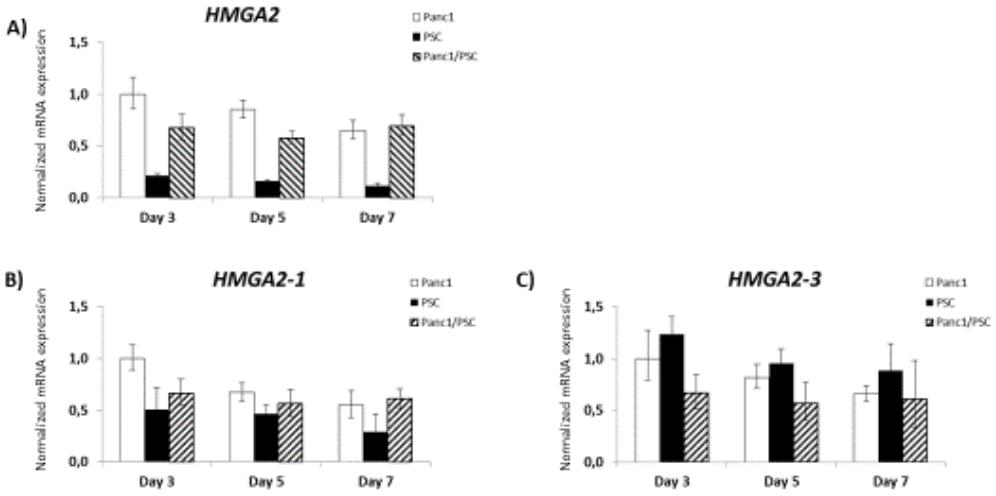

Figure 4. *HMGA2* mRNA expression for Panc1 and hPSC mono- and co-cultures normalized to Panc1 mono-culture spheroids from day 3. Total *HMGA2* (A), *HMGA2* transcript variant 1 (*HMGA2-1*) (B) and *HMGA2* transcript variant 3 (*HMGA2-3*) (C) expression, from indicated time points (n=3-5).

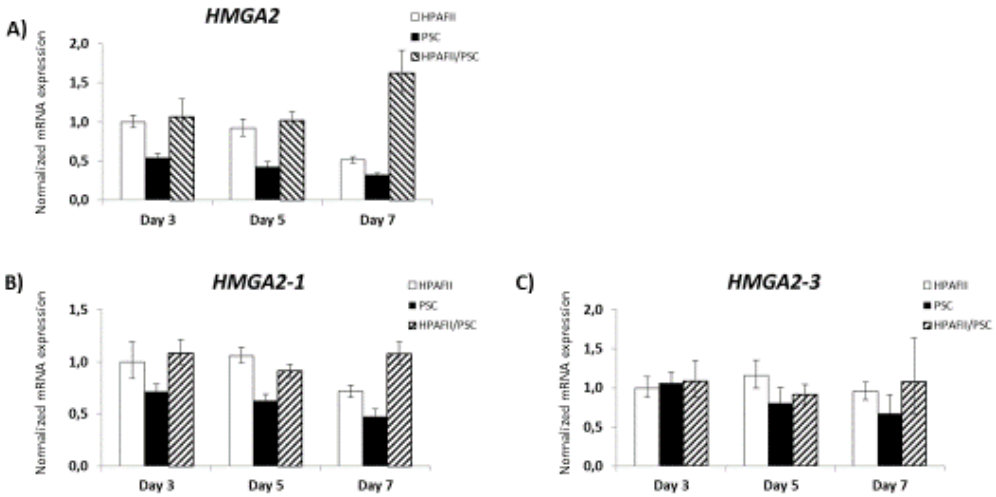

Figure 5. *HMGA2* mRNA expression for HPAFII and hPSC mono- and co-cultures normalized to HPAFII mono-culture spheroids from day 3. Total *HMGA2* (A), *HMGA2* transcript variant 1 (*HMGA2-1*) (B) and *HMGA2* transcript variant 3 (*HMGA2-3*) (C) expression, from indicated time points (n=3-5).

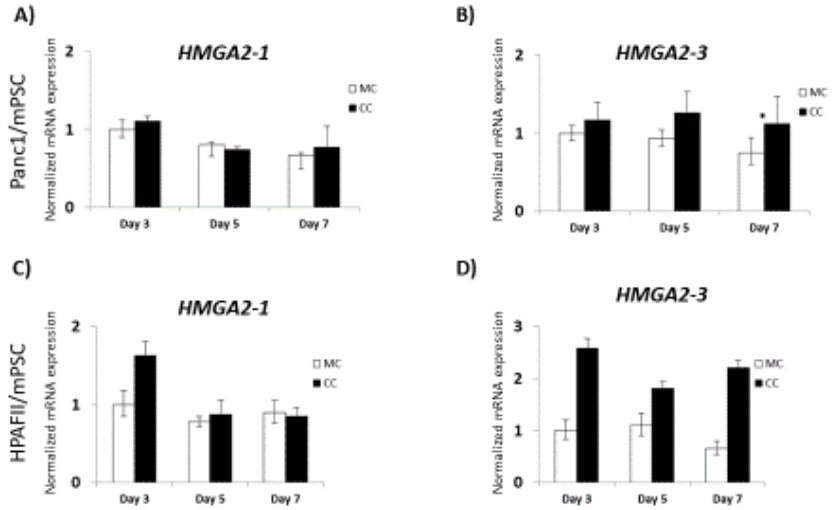

Figure 6. *HMGA2* mRNA expression for Panc1 and HPAFII mono- and co-cultures with mPSCs, normalized to either Panc1 (A-B) or HPAFII (C-D) mono-culture spheroids from day 3. *HMGA2* transcript variant 1 (*HMGA2-1*) (A, C) and *HMGA2* transcript variant 3 (*HMGA2-3*) (B, D) expression, from indicated time points in mono- and co-cultured tumor cells (n=3).

Using the virtual sorting approach on Panc1 and HPAFII cells cultured with mPSCs, could not confirm the data from the human-human cultures. When co-cultured with mPSCs, *HMGA2-3* was induced in the Panc1 cells and not *HMGA2-1* as indicated by the human-human cell data (Fig.6A-B). In the HPAFII cells, *HMGA2-1* was induced only on day 3 instead of also at day 7 as when co-cultured with the hPSCs (Fig.6C). In addition, *HMGA2-3* was clearly induced in HPAFII cells co-cultured with mPSCs (although not significantly) (Fig.6.D), something that was not to be expected based on the co-culture together with the hPSCs.

5.4.5 TGFB1 induces HMGA2 but the mechanism behind is unclear

As we in paper II could show that TGFB1 induced mRNA expression of *HMGA2* in the Panc1 mono-culture spheroids, we decided to further investigate the connection between TGFB1 and HMGA2. By preparing paraffin sections of mono-cultured Panc1 spheroids stimulated with TGFB1, we could confirm that HMGA2 was upregulated in the Panc1 cells also on a protein level (Fig.7.A-B). Not surprisingly by adding a growth factor to serum starved cells, there also was an increased number of proliferative ($Ki67^+$) cells (Fig.7.C-D) as well as a downregulation of apoptotic ($M30^+$) cells (Fig.7.E-F), upon TGFB1-stimulation.

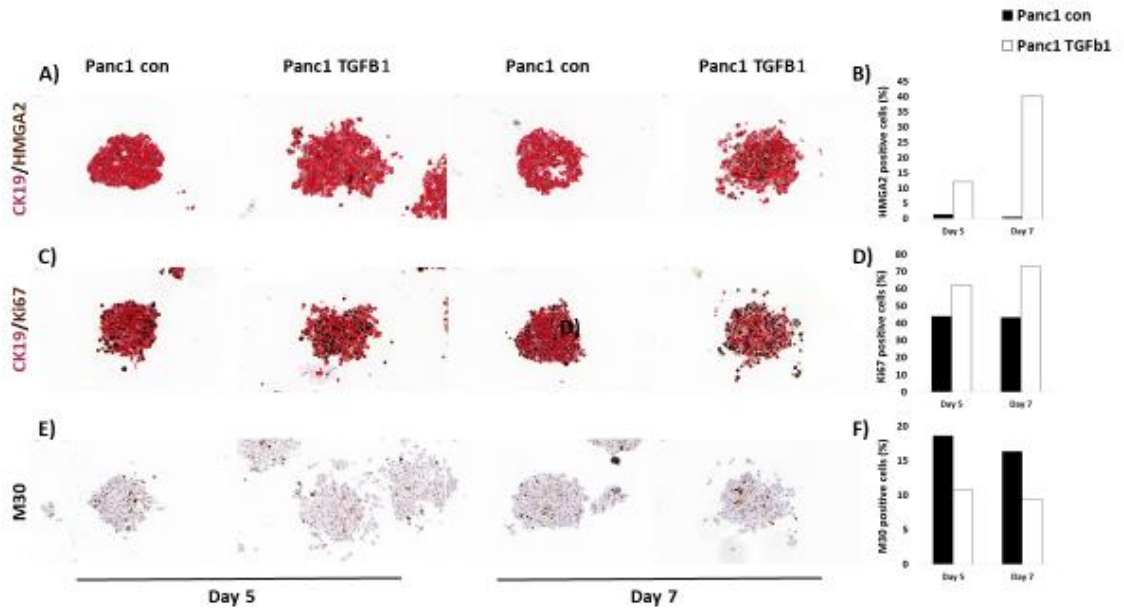

Figure 7. CK19/HMGA2 (A), CK19/Ki67 (C) and M30 (D) staining in Panc1 control and TGFB1-stimulated mono-cultures from days 5 and 7. Quantification of the stainings are seen in (B, D, and F).

In a cell panel of normal but immortalized human pancreatic ductal epithelial cells (HPDE), PSCs and a number of PDAC cell lines, *TGFB1* and *HMGA2* mRNA levels were shown to be in parallel for almost all cell lines in both 2D and 3D mono-cultures (Fig.8A-D). Internal controls with Panc1, Panc1-Mock (Panc1-M) and Panc1 TGFB-overexpressing (Panc1-T) cells, could further confirm this. Out of the three PDAC cell lines that did not have a clear synchronization between levels of *TGFB1* and *HMGA2*, one was Smad4-negative and one Smad4 mutated. All other cell lines in the panel were wild type for Smad4. Interestingly, PSCs did not display higher levels of *HMGA2* with higher levels of *TGFB1* in the 3D cultures. TGFB1-stimulation of mono-cultured HPAFII and PaCa44 cells, with low endogenous levels of *TGFB1*, did not induce *HMGA2* expression as seen in the Panc1 cells (data not shown).

In order to investigate the effects of TGFB1 on co-culture induced expression of *HMGA2*, we treated mono- and co-cultures with TGFB-inhibitors. This however gave inconclusive results,

due to inconsistency of both the inhibitor effects and the co-culture induced expression itself (data not shown). Preliminary data on mRNA expression of *HMGA2* and *TGFB1* in tumor cells, as well as *TGFB1* in PSCs, from 3D mono- and co-cultures, was in line with some of the previous data and indicated that *HMGA2* levels in the tumor cells mimicked *TGFB1* levels in the same, but were independent of highly increased *TGFB1* mRNA expression in the PSCs (data not shown). Interestingly, expression of *HMGA2-1* and *-3* transcripts in the 2D-culture cell panel showed that *HMGA2-1* but not *-3*, was synchronized with *TGFB1* expression (Fig.8E-F).

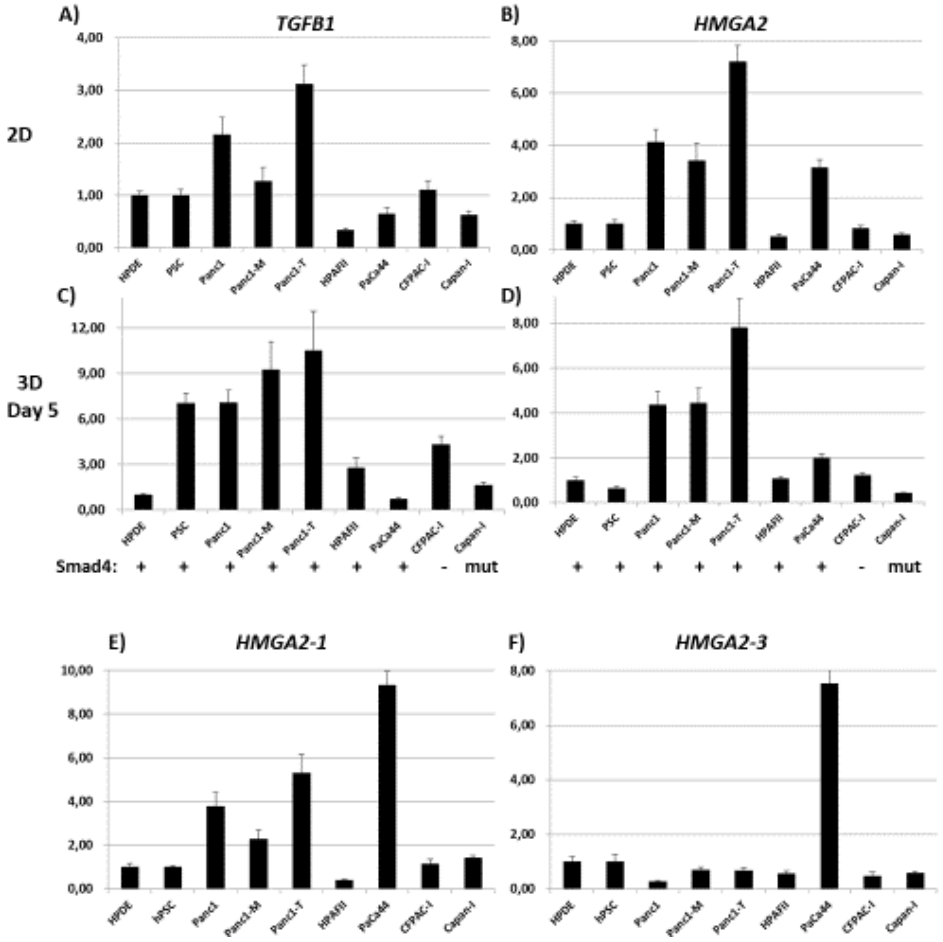

Figure 8. *HMGA2* (total, transcript variant -1 and -3, as indicated) and *TGFB1* mRNA expression normalized to human pancreatic ductal epithelial (HPDE) cell expression, in a cell line panel of HPDE cells, human pancreatic stellate cells (hPSC), Panc1 Mock control (Panc1-M) and TGFB1-overexpressing (Panc1-T) cells, and a number of pancreatic cancer cell lines (Panc1, HPAFII, PaCa44, CFPAC-I and Capan-I), in 2D (A-B and E-F) and 3D (C-D) cultures. Smad4 status of the cells is indicated below (C-D).

6. DISCUSSION

6.1 Paper I

6.1.1. The caerulein model

The validity of caerulein induced AP has been questioned, due to the potential lack of physiological relevance, as the existence of CCK receptors on human acinar cells still remain to be proven. Rodent pancreatic acini, in contrast to human acini, respond to physiological concentrations of CCK *in vitro*. CCK-A receptors are present on rodent but not on human acinar cells, and it is generally accepted that exocrine pancreatic stimulation in humans is almost exclusively mediated through the cholinergic stimulation, where as in rodents direct CCK-A stimulation plays a role in addition to cholinergic stimulation [52, 171]. Caerulein however, induces pancreatitis by increasing levels of intracellular calcium and thereby activating calcium dependent CN-NFAT signaling [30]. As does bile acid [34, 172], one of the two major clinical causes of AP [15]. This strongly supports the use of the caerulein induction model for studying the downstream effects of CN activation, independent of the true mechanism behind the initiation of the intracellular calcium induction.

6.1.2 AR42J cells as a substitute for primary acinar cells

AR42J cells are often referred to in the literature as rat acinar cells, although they are in fact derived from a transplantable tumor of rat exocrine pancreas. Caution should therefore be taken when using these cells for studying normal acinar cell function. The cells do however retain many characteristics of normal acinar cells. They are able to synthesize, store and secrete digestive enzymes (Jessop & Hay 1980) [173], and elicit normal receptor expression and signal transduction mechanisms [174]. Primary human acinar cells, due to their nature as specialized secretory cells, are still difficult to culture and they have not been possible to maintain long-term [175]. When acinar cells are taken out of their natural environment they lose their cellular identity and transdifferentiate into ductal cells. Transfection of these cells also has been challenging and no long-term cultures of normal acinar cells has yet been reported [176], although now both murine [158, 177] and human [178] primary acinar cells have been cultured for more than 1 week. Until primary acinar cell culture becomes more reliable, there still is a good reason for the use of the AR42J cell line, of course after validation of use and for appropriate scientific questions.

6.1.3 RCAN1 as a marker of AP and inflammation

As RCAN1 is inhibiting its own induction through binding and blocking calcineurin, high initial increase of RCAN1 should in theory inhibit further production. This is in line with our data showing an early peak of *Rcan1* mRNA expression, with a declination at later time points [179].

As illustrated in Suppl. Fig.2 in paper I, CCK increases intracellular Ca^{2+} levels, thus activating the CN-NFAT axis and inducing *RCAN1*. CN activity and the CN-NFAT axis is then blocked, once there is enough RCAN1 protein. This leads to a reduction of *RCAN1* mRNA and degradation of RCAN1 protein, thus causing oscillation.

Rcan1 protein in murine blood was also detected early upon onset of experimental AP, indicating the potential of RCAN1 as an early marker of AP, a quality desirable for clinical use. One potential specific use could, as mentioned in the discussion of paper I, be in the case of Endoscopic Retrograde Cholangiopancreatography (ERCP). Post-ERCP-pancreatitis, or PEP, develop in up to 15% of patients [180] and ERCP has been shown to cause acinar cell inflammation and injury through induction of CN signaling [181]. Preventative treatment of PEP with CN-inhibitors [180] has potential, and RCAN1 as a marker of the success of such treatment could be a good complement.

A fast decline of RCAN1 protein due to aforementioned oscillation, could on the contrary be potentially problematic as it would limit the window of detection. A continuous stimuli in the clinical situation would however likely override the auto-regulation and maintain an induction of RCAN1. Protein levels may also persevere for extended time independent of inhibition of further mRNA production, something that requires further investigations.

In paper I, we demonstrated that RCAN1 was regulated by oxidative stress. *RCAN1* mRNA was induced by caerulein and H_2O_2 and this induction was inhibited by the antioxidant N-acetylcystein (NAC) (Fig.2 in paper I). A major limitation of RCAN1 as a diagnostic marker would be the plausible lack of specificity, as an indirect marker of oxidative stress merely is a marker of inflammation and not a specific marker for AP. As ROS is correlated with AP severity, it could however prove to be of clinical value as a prognostic marker. As mentioned in the discussion of paper I, measuring ROS directly or indirectly through metabolites still remains challenging and it has therefore not been implemented into a clinical setting [53]. In our studies, we were however not able to stratify the RCAN1 levels in patients with mild, moderate and severe AP. In the discussion of the paper, we mention that one possibility is that this could be due to a short-lived transient induction of RCAN1 protein, which would coincide with oscillation of RCAN1 protein due to the CN-NFAT – RCAN1 loop described above, and our mRNA data. The above reasoning with continuous stimuli in the clinical setting likely overriding this auto-regulation, would however on the contrary argue for the continuous potential of RCAN1 as a prognostic marker, once better tools for RCAN1 detection will be available.

Another possible application for RCAN1 is as an alternative endpoint in anti-oxidant trials. As there are no evidence of RCAN1 enhancing pancreatic inflammation, it is not itself a feasible therapeutic target at the moment. On the contrary, treatment with RCAN1 or rather other synthetic inhibitors of calcineurin, would be the more prominent strategy for inhibiting the inflammatory response. As mentioned, CN-inhibitors already have been successfully tested for prevention of PEP. The potential of RCAN1 as an end-point for anti-oxidant trials however extends way beyond the use of CN-inhibitors in ERCP patients. Antioxidants have been thoroughly tested as a treatment for AP, mainly pointing at no benefit when used as

monotherapy [179], but the potential of combination therapies including antioxidants still needs to be further explored.

As suggested in paper I, RCAN1 may also prove useful as a marker of AP patients who might benefit from a specific therapy, as opposite results of CN-inhibitors have been demonstrated using different AP models, indicating that different treatment approaches may be necessary depending on the etiology of the disease. In addition, as a marker of oxidative stress and thereby inflammation, there also is a possibility that RCAN1 may prove useful beyond the scope of AP.

6.2 Paper II

This study demonstrated a negative connection between PDAC survival and HMGA2 IHC expression in tumor cells, and linked PDGFRB^{+} fibroblasts to tumor cell HMGA2 expression. Our findings strongly advocate a contribution of paracrine stroma-epithelial signaling to HMGA2 expression, encouraging further studies investigating the cross-talk mechanisms behind HMGA2 induction. As some studies were already carried out in this direction, presented in section 5.4, this is further discussed along with the discussion of this additional data, in section 6.4.

6.3 Paper III

6.3.1 Advantages and limitations of the 3D co-culture spheroid model

Our novel established 3D PDAC and PSC co-culture spheroid model has been very well characterized, as has each of the separate cell lines used [182]. Important advantages of this type of co-culturing model is that genes of interest involved in PDAC-PSC crosstalk can be overexpressed and knocked out. Thus, co-cultures with a specific genetic alteration can be compared to controls, eliminating difficulties in comparing co-cultures to mono-culture counterparts, where cell numbers as well as physical location of a cell type within the spheroid may vary and affect the outcome. Well characterized fluorescent cell lines may also be used as a substitute for the parental lines, expanding further the capabilities of the model. With fluorescent cell lines more scientific questions may be addressed using intact spheroid cultures in *e.g.* confocal microscopy. When suitable, cells can also be dissociated and used for flow cytometry and fluorescent activated cell sorting (FACS). Preparations of fluorescent cell lines and their characterization (Panc1-mCherry, HPAFII-mCherry, HPDE-mCherry, hPSC-eGFP and mPSC-eGFP), is currently ongoing in the laboratory (data not shown).

Using any model of immortalized cell lines as in our model may of course limit and or change certain characteristics and cell behaviors in comparison to primary cells, something that needs to be taken into consideration. Primary cells however sometimes do not survive at all *ex vivo* and if they do they may not be grown for the same amount of time and cannot be manipulated as described above. In order to be able to address various scientific questions, a variety of experimental models is needed and the appropriate model should be chosen depending on the question asked.

The lack of a rich ECM might be considered a disadvantage of the model, as the desmoplastic, fibrotic stroma has become the general picture of a PDAC, and for some studies that is certainly true. It has to be remembered however, that tumors are heterogenic and that the common notion of stroma rich PDACs composed to a large extent of ECM is not the whole truth. There are great variations in the amount of stroma in between different PDACs. Importantly, there are also different regions of tumors that are more and less composed of ECM [183], something that might depend on local variations in the microenvironment. As we have learned, there are various types of CAFs and PSCs with different markers and behaviors [89, 184]. With such a complex reality, we cannot limit ourselves to study only a certain type of PDAC.

As compared to traditional 2D cell culture, 3D cultures offer an environment more closely resembling the reality, although for the same reasons cause more technical difficulties and challenges than 2D culturing [185]. They *e.g.* give rise to different populations of cells exposed to various amounts of nutrients, oxygen and experimental treatments etc., depending on their localization within the spheroid. This is desirable but often makes it more difficult to interpret experimental outcomes. Additional issues are introduced with 3D co-culturing, such as which appropriate controls may be used. Accurately comparing co-culture spheroids to the mono-culture equivalents is not straight forward, as the cell numbers of any given cell type, spheroid

size etc. may differ. Keeping one parameter constant will alter another, and we need to find new ways of designing experiments in a meaningful way as to be able to answer our question at hand and provide easily interpretable results.

As an example, imagine investigating migratory properties of the tumor cells in our co-culture model compared to the mono-culture counterpart. If one keeps tumor cell numbers in the two cultures the same, the spheroid sizes will differ due to the addition of another cell type to the co-cultures. In a model such as our Panc1/PSC model where the tumor cells and the PSCs are mixed throughout the spheroid, the average distance for the tumor cells within the co-cultures to migrate before reaching the surface, would be longer. In order to have the same number of cells with the same distance to migrate, cells would need to first be dissociated and sorted. This would instead however, as previously discussed in paper III, increase preparation time and induce cellular stress, possibly affecting the outcome. In order to not dissociate the spheroids, a 3D sprouting assay, where intact spheroids are placed in a gel or ECM allowing cells to migrate out from the spheroid and into the matrix, is a valid option. The difference in average distance for cells to migrate would however still remain, and interpretation of the results would still be difficult. A better way may therefore be to first identify a potential genetic target that is likely involved in migration, manipulate the tumor cells to overexpress the gene of interest or knock it out, and then compare these co-cultured tumor cells to co-cultured appropriate tumor control cells.

6.3.2 Advantages and limitations of the virtual sorting method

The main advantage of the virtual sorting method, and the reason for us developing it, is the elimination of the need to dissociate and sort the cells composing the spheroids. As previously discussed in paper III, this does not only save valuable time and resources, but more importantly eradicates added cellular stress.

A major disadvantage is that cells of different species needs to be used. If initial studies have been performed in human cells, one cell line has to be exchanged for *e.g.* a murine cell line in order to use the method. This may in itself alter the experimental response as different cell lines of the same cell type can greatly differ. It is therefore crucial to first confirm that the parameters important to the specific scientific question are similar in the two cell lines.

6.4 Additional preliminary data

6.4.1 PDGFRB – HMGA2 link

Obtained data show that PDGFRB is not involved in induction of HMGA2 through TGFB1 induction in the PSCs. PDGFRB-signaling might induce other factors involved in the induction of HMGA2, or PDGFRB may be a marker of a CAF-subpopulation exhibiting a certain secretome inducing tumoral HMGA2 expression.

6.4.2 HMGA2 expression

HMGA2 expression was found in PDAC to be associated with loss of tumoral E-cadherin expression [131], much like our observations in the Panc1/PSC co-culture spheroids. In paper II, we found that HMGA2 was correlated to overall survival in PDAC, but could be detected in tumor cells independent of their epithelial cell differentiation status. The stroma in the clinical samples in paper II was negative for HMGA2, but we did identify a few cases with $HMGA2^+$ stroma (additional preliminary data, section *5.4.3*), both adjacent to $HMGA2^+$ tumor cells as well as isolated. Of note, the scoring of HMGA2 in the patient cohort from paper II was done without the additional CK19-staining used for the clinical samples and the spheroids in the additional data, and the staining protocols somewhat differ in terms of antibody incubation time, potentially causing discrepancy.

Preliminary results from the spheroid model indicated a potential connection between $HMGA2^+$ stroma and $HMGA2^+$ tumor cells (*5.4.2*), and more strongly positive tumor cells were found adjacent to strongly positive stroma cells in the desmoplastic stroma positive clinical samples (*5.4.3*). Interestingly, overexpression of stromal Hmga2 in prostate cells, lead to the formation of multifocal prostatic precancerous lesions in neighboring epithelium, in a paracrine Wnt-dependent manner [186].

Our inconsistent results of HMGA2 expression in both tumor cells and PSCs in the 3D co-culture spheroid model might reflect a complex reality, but makes our results difficult to interpret at the moment and does not explain the observed differences using the same model with no apparent experimental differences. It however encourages further studies and suggests our Panc1/PSC co-culture model as an appropriate model for continued investigations.

6.4.3 HMGA2 transcripts

As explained above, *HMGA2-1* was increased at an mRNA level in the HPAFII cells and also in the Panc1 co- vs mono-cultured cells seemingly mainly *HMGA2-1* transcript expression was induced. In the Panc1/hPSC co-cultures, HMGA2 protein was induced in co- vs mono-cultured Panc1 cells whereas no protein induction was seen in the HPAFII co-cultured cells. *HMGA2-3* was highly expressed in the hPSCs (also mono-cultures) in the Panc1/hPSC experiment but not

as clearly in the HPAFII/hPSC experiment. HMGA2 protein was found expressed in the hPSCs only in the Panc1/hPSC experiment. HMGA2 protein was also induced by TGFB1 stimulation in the mono-cultured Panc1 spheroids, and according to our preliminary data *HMGA2-1* but not *HMGA2-3* expression is influenced by *TGFB1* levels in the tumor cells themselves.

The HMGA2 antibody used recognizes the N terminus of the protein, which is conserved in all the transcript variants. Taken together, the *HMGA2* mRNA transcript data creates more questions than answers. The inductions seen in mRNA expression do not always translate into an increase of HMGA2 protein. The mRNA data is difficult to interpret with both the tumor cells and hPSCs expressing HMGA2. The mPSC cell line used may not adequately reflect similar *HMGA2* expression as the hPSCs, and the virtual sorting experiments do not clarify the situation in this case. Another mPSC cell line more similar to the hPSCs in terms of *HMGA2* expression may be considered for further experiments, but the relevance of various transcript variant expression may be limited and focus will likely be better used elsewhere.

6.4.4 TGFB1 induced induction of HMGA2

As mentioned, hPSCs did not display higher levels of *HMGA2* with higher levels of *TGFB1* in 3D mono-cultures. Preliminary data also indicated that induction of *HMGA2* expression in the Panc1/PSC co-cultures followed *TGFB1* expression in the Panc1 cells and not in the PSCs. Taken together, this indicates that expression of HMGA2 in the stroma seen in the clinical samples and in the PSCs in the 3D mono- and co-cultures, are induced by other means than PSC-produced TGFB1. The PSCs may have low levels of or not at all express TGFBRs, therefore not upregulating HMGA2 themselves. There also is a possibility that the receptors are there but that the PSCs are not able to induce HMGA2 through autocrine signaling. There might not be any translation of the *TGFB1* mRNA in to protein in the stellate cells, or the protein may be instantly degraded in these cells. It is however possible that the presence of PSCs might induce *TGFB1* expression in the tumor cells themselves, ultimately leading to an increase in HMGA2.

The fact that TGFB1 stimulation did not induce *HMGA2* expression in the HPAFII and PaCa44 cells might also be due to a lack of TGFBRs in these cell lines. HMGA2 is not expressed in all PDACs, although PaCa44 cells do express rather high levels of *HMGA2* mRNA in 2D culture. It is however plausible to believe that HMGA2 is induced by multiple pathways and not only by TGFB1.

Clearly, the relationship between HMGA2-positivity in PDAC cells and PSCs, as well as the induction of HMGA2, is complex. Our preliminary data opens up for many questions and a deeper investigation is needed to elucidate the mechanism behind.

7. CONCLUSIONS

Paper I:

- *RCAN1* is regulated by oxidative stress and is an early marker of the same.
- RCAN1 has the potential to be used as a surrogate endpoint in anti-oxidant therapy trials, as a marker of AP patients who might benefit from a specific therapy, a diagnostic marker for ERCP-induced pancreatitis and possibly as a prognostic marker of AP severity.
- As a marker of oxidative stress and thereby inflammation, RCAN1 may also prove useful beyond the scope of AP.

Paper II:

- HMGA2-positivity is a strong prognostic factor for shorter overall survival in PDAC.
- HMGA2 IHC can identify patients with a worse prognosis.
- Continued efforts are warranted to define novel fibroblast subsets for further exploration of their regulation, function and clinical importance.

Paper III:

- Our novel 3D co-culture spheroid model of PDAC cells and PSCs, can be used to study tumor cell – PSC cross-talk.
- Virtual sorting may be used to determine cell type specific gene expression in direct cellular co-cultures of mixed species, without previous cell separation.

Additional preliminary data:

- PSC PDGFRB-signaling is not inducing TGFB in the PSCs and may not be involved in HMGA2 induction, but rather a marker of a CAF-subpopulation inducing tumor HMGA2 expression.
- HMGA2 expression in tumor cells and tumor stroma and the relationship in between is complex and requires further investigations.
- Our Panc1/PSC co-culture model is likely an appropriate model for studying tumor HMGA2 induction.

8. FUTURE PERSPECTIVES

In paper I, we found RCAN1 to be an early marker of AP and suggested its use as a marker of post-ERCP-pancreatitis (PEP). A study further investigating this potential is already planned for. The ethical application has already been approved by Regionala etikprövningsnämnden in Stockholm and patient samples are currently being collected.

Once better tools for RCAN1 detection are available and/or the oscillation of RCAN1 has been better understood, a bigger study on correlation between RCAN1 expression and AP severity would be interesting to see. It would also be exciting to see the use of RCAN1 evaluated in other inflammatory diseases than AP, as well as one of the endpoints in antioxidant therapeutic studies, for AP as well as other diseases.

Regarding our findings in paper II, I foresee that HMGA2 IHC in the future will be part of the standard clinical evaluations of PDAC patients as a prognostic marker. More mechanistic studies are however needed to understand how HMGA2 is contributing to lower survival and how it is induced in the tumor cells. Of special interest to me would be to explore the relationship between HMGA2 in the stroma and the tumor cells and how the stroma is contributing to increased tumor cell expression. Involvement of *e.g.* the Wnt pathway has as mentioned been suggested in other cancers and it would be interesting to explore its role in pancreatic cancer, alongside further clarification of the involvement of TGFB1. Our 3D co-culture human-human cell spheroid model characterized in paper III is ready to be used for further mechanistic studies, and as suggested from our preliminary data could be a good model for mechanistic studies of HMGA2.

PDGFRB stainings of Panc1/PSC mono- and co-culture spheroids are ongoing, in order to explore if there is any correlation between $PDGFRB^+$ stroma and $HMGA2^+$ tumor cells in this model, as seen in the clinical samples in paper II. It would also be interesting to further study $PDGFRB^+$ stroma cells and explore if anything in this population of CAFs can contribute to HMGA2 induction in the cancer cells. Isolating $PDGFRB^+$ CAFs and studying their secretome might identify new potential pathways in the stroma and tumor cell communication, important for HMGA2 induction and the correlated lower survival of PDAC patients.

The virtual sorting method developed in paper III has the potential to be implemented in many studies far beyond the scope of pancreatic diseases. I believe that this model will find its place as an additional scientific tool where applicable. For our HMGA2 mechanistic studies I however believe that we need to focus elsewhere due to the lack of universal species specific primers.

Generally, I expect to see a continued increase of studies in regard to cancer and the tumor microenvironment. I anticipate an even more complex image to emerge, revealing many more different subtypes of CAFs and PSCs than known today, influencing tumor progression in different ways depending on the context. Revelations of intricate communication involving also additional cell types, such as TAMs and other immune cells, endothelial cells etc. is to be expected, as we will learn how to understand their conversations.

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10. REFERENCES

1 Carroll JK, Herrick B, Gipson T, Lee SP: Acute pancreatitis: Diagnosis, prognosis, and treatment. Am Fam Physician 2007; 75: 1513-1520.
2 Kahl S, Mayer JM: Update on experimental acute pancreatitis. Minerva Gastroenterol Dietol 2012; 58: 355-363.
3 Kleeff J, Whitcomb DC, Shimosegawa T, Esposito I, Lerch MM, Gress T et al.: Chronic pancreatitis. Nature reviews Disease primers 2017; 3: 17060.
4 Aier I, Semwal R, Sharma A, Varadwaj PK: A systematic assessment of statistics, risk factors, and underlying features involved in pancreatic cancer. Cancer Epidemiol 2019; 58: 104-110.
5 Vincent A, Herman J, Schulick R, Hruban RH, Goggins M: Pancreatic cancer. Lancet 2011; 378: 607-620.
6 Lohr M: Is it possible to survive pancreatic cancer? Nat Clin Pract Gastroenterol Hepatol 2006; 3: 236-237.
7 Lohr JM: Pancreatic cancer should be treated as a medical emergency. BMJ 2014; 349: g5261.
8 Pandiri AR: Overview of exocrine pancreatic pathobiology. Toxicol Pathol 2014; 42: 207-216.
9 Edlund H: Developmental biology of the pancreas. Diabetes 2001; 50 Suppl 1: S5-9.
10 Apte MV, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA et al.: Periacinar stellate shaped cells in rat pancreas: Identification, isolation, and culture. Gut 1998; 43: 128-133.
11 Buchholz M, Kestler HA, Holzmann K, Ellenrieder V, Schneiderhan W, Siech M et al.: Transcriptome analysis of human hepatic and pancreatic stellate cells: Organ-specific variations of a common transcriptional phenotype. J Mol Med (Berl) 2005; 83: 795-805.
12 Bardeesy N, DePinho RA: Pancreatic cancer biology and genetics. Nat Rev Cancer 2002; 2: 897-909.
13 Raraty MG, Connor S, Criddle DN, Sutton R, Neoptolemos JP: Acute pancreatitis and organ failure: Pathophysiology, natural history, and management strategies. Current gastroenterology reports 2004; 6: 99-103.
14 Portelli M, Jones CD: Severe acute pancreatitis: Pathogenesis, diagnosis and surgical management. Hepatobiliary Pancreat Dis Int 2017; 16: 155-159.
15 Forsmark CE, Vege SS, Wilcox CM: Acute pancreatitis. N Engl J Med 2016; 375: 1972-1981.
16 Gerasimenko JV, Peng S, Tsugorka T, Gerasimenko OV: Ca2+ signalling underlying pancreatitis. Cell Calcium 2017.
17 Lankisch PG, Apte M, Banks PA: Acute pancreatitis. Lancet 2015; 386: 85-96.
18 Norman JG, Fink GW, Franz MG: Acute pancreatitis induces intrapancreatic tumor necrosis factor gene expression. Arch Surg 1995; 130: 966-970.
19 Hughes CB, el-Din AB, Kotb M, Gaber LW, Gaber AO: Calcium channel blockade inhibits release of tnf alpha and improves survival in a rat model of acute pancreatitis. Pancreas 1996; 13: 22-28.
20 Leser HG, Gross V, Scheibenbogen C, Heinisch A, Salm R, Lausen M et al.: Elevation of serum interleukin-6 concentration precedes acute-phase response and reflects severity in acute pancreatitis. Gastroenterology 1991; 101: 782-785.
21 Lesina M, Wormann SM, Neuhofer P, Song L, Algul H: Interleukin-6 in inflammatory and malignant diseases of the pancreas. Semin Immunol 2014; 26: 80-87.
22 Stoelben E, Nagel M, Ockert D, Quintel M, Scheibenbogen C, Klein B et al.: Clinical relevance of cytokines il-6, il-8, and c-reactive protein in the blood of patients with acute pancreatitis. Chirurg 1996; 67: 1231-1236.

23 Closa D: Free radicals and acute pancreatitis: Much ado about ... Something. Free Radic Res 2013; 47: 934-940.
24 Rau B, Poch B, Gansauge F, Bauer A, Nussler AK, Nevalainen T et al.: Pathophysiologic role of oxygen free radicals in acute pancreatitis: Initiating event or mediator of tissue damage? Ann Surg 2000; 231: 352-360.
25 Kim H: Cerulein pancreatitis: Oxidative stress, inflammation, and apoptosis. Gut and liver 2008; 2: 74-80.
26 Yu JH, Kim H: Oxidative stress and inflammatory signaling in cerulein pancreatitis. World J Gastroenterol 2014; 20: 17324-17329.
27 Criddle DN: Reactive oxygen species, ca(2+) stores and acute pancreatitis; a step closer to therapy? Cell Calcium 2016; 60: 180-189.
28 Manohar M, Verma AK, Venkateshaiah SU, Sanders NL, Mishra A: Pathogenic mechanisms of pancreatitis. World J Gastrointest Pharmacol Ther 2017; 8: 10-25.
29 Sah RP, Dawra RK, Saluja AK: New insights into the pathogenesis of pancreatitis. Current opinion in gastroenterology 2013; 29: 523-530.
30 Petersen OH, Sutton R: Ca2+ signalling and pancreatitis: Effects of alcohol, bile and coffee. Trends Pharmacol Sci 2006; 27: 113-120.
31 Pan MG, Xiong Y, Chen F: Nfat gene family in inflammation and cancer. Curr Mol Med 2013; 13: 543-554.
32 Awla D, Zetterqvist AV, Abdulla A, Camello C, Berglund LM, Spegel P et al.: Nfatc3 regulates trypsinogen activation, neutrophil recruitment, and tissue damage in acute pancreatitis in mice. Gastroenterology 2012; 143: 1352-1360.e1351-1357.
33 Muili KA, Ahmad M, Orabi AI, Mahmood SM, Shah AU, Molkentin JD et al.: Pharmacological and genetic inhibition of calcineurin protects against carbachol-induced pathological zymogen activation and acinar cell injury. Am J Physiol Gastrointest Liver Physiol 2012; 302: G898-905.
34 Muili KA, Wang D, Orabi AI, Sarwar S, Luo Y, Javed TA et al.: Bile acids induce pancreatic acinar cell injury and pancreatitis by activating calcineurin. J Biol Chem 2013; 288: 570-580.
35 Gurda GT, Crozier SJ, Ji B, Ernst SA, Logsdon CD, Rothermel BA et al.: Regulator of calcineurin 1 controls growth plasticity of adult pancreas. Gastroenterology 2010; 139: 609-619, 619.e601-606.
36 Mendez-Barbero N, Esteban V, Villahoz S, Escolano A, Urso K, Alfranca A et al.: A major role for rcan1 in atherosclerosis progression. EMBO Mol Med 2013; 5: 1901-1917.
37 Torac E, Gaman L, Atanasiu V: The regulator of calcineurin (rcan1) an important factor involved in atherosclerosis and cardiovascular diseases development. J Med Life 2014; 7: 481-487.
38 Lloret A, Badia MC, Giraldo E, Ermak G, Alonso MD, Pallardo FV et al.: Amyloid-beta toxicity and tau hyperphosphorylation are linked via rcan1 in alzheimer's disease. J Alzheimers Dis 2011; 27: 701-709.
39 Ermak G, Davies KJ: Chronic high levels of the rcan1-1 protein may promote neurodegeneration and alzheimer disease. Free Radic Biol Med 2013; 62: 47-51.
40 Fuentes JJ, Pritchard MA, Planas AM, Bosch A, Ferrer I, Estivill X: A new human gene from the down syndrome critical region encodes a proline-rich protein highly expressed in fetal brain and heart. Hum Mol Genet 1995; 4: 1935-1944.
41 Fuentes JJ, Genesca L, Kingsbury TJ, Cunningham KW, Perez-Riba M, Estivill X et al.: Dscr1, overexpressed in down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. Hum Mol Genet 2000; 9: 1681-1690.
42 Liu C, Zheng L, Wang H, Ran X, Liu H, Sun X: The rcan1 inhibits nf-kappab and suppresses lymphoma growth in mice. Cell Death Dis 2015; 6: e1929.
43 Wang C, Saji M, Justiniano SE, Yusof AM, Zhang X, Yu L et al.: Rcan1-4 is a thyroid cancer growth and metastasis suppressor. JCI insight 2017; 2: e90651.
44 Jin H, Wang C, Jin G, Ruan H, Gu D, Wei L et al.: Regulator of calcineurin 1 gene isoform 4, downregulated in hepatocellular carcinoma, prevents proliferation, migration,

and invasive activity of cancer cells and growth of orthotopic tumors by inhibiting nuclear translocation of nfat1. Gastroenterology 2017.
45 Chen X, Hu Y, Wang S, Sun X: The regulator of calcineurin 1 (rcan1) inhibits nuclear factor kappab signaling pathway and suppresses human malignant glioma cells growth. Oncotarget 2017; 8: 12003-12012.
46 Ma N, Shen W, Pang H, Zhang N, Shi H, Wang J et al.: The effect of rcan1 on the biological behaviors of small cell lung cancer. Tumour Biol 2017; 39: 1010428317700405.
47 Harris CD, Ermak G, Davies KJ: Multiple roles of the dscr1 (adapt78 or rcan1) gene and its protein product calcipressin 1 (or rcan1) in disease. Cell Mol Life Sci 2005; 62: 2477-2486.
48 Sun L, Hao Y, An R, Li H, Xi C, Shen G: Overexpression of rcan1-1l inhibits hypoxia-induced cell apoptosis through induction of mitophagy. Mol Cells 2014; 37: 785-794.
49 Raghupathi R, Muyderman H, Zanin MP, Mackenzie K, Pritchard MA, Keating DJ et al.: Regulator of calcineurin 1 gene transcription is regulated by nuclear factor-kappab. Oxid Med Cell Longev 2014; 11: 156-164.
50 Lin HY, Michtalik HJ, Zhang S, Andersen TT, Van Riper DA, Davies KK et al.: Oxidative and calcium stress regulate dscr1 (adapt78/mcip1) protein. Free Radic Biol Med 2003; 35: 528-539.
51 Hesser BA, Liang XH, Camenisch G, Yang S, Lewin DA, Scheller R et al.: Down syndrome critical region protein 1 (dscr1), a novel vegf target gene that regulates expression of inflammatory markers on activated endothelial cells. Blood 2004; 104: 149-158.
52 Peiris H, Raghupathi R, Jessup CF, Zanin MP, Mohanasundaram D, Mackenzie KD et al.: Increased expression of the glucose-responsive gene, rcan1, causes hypoinsulinemia, beta-cell dysfunction, and diabetes. Endocrinology 2012; 153: 5212-5221.
53 Gomatos IP, Xiaodong X, Ghaneh P, Halloran C, Raraty M, Lane B et al.: Prognostic markers in acute pancreatitis. Expert Rev Mol Diagn 2014; 14: 333-346.
54 Kloppel G: Progression from acute to chronic pancreatitis. A pathologist's view. Surg Clin North Am 1999; 79: 801-814.
55 Brock C, Nielsen LM, Lelic D, Drewes AM: Pathophysiology of chronic pancreatitis. World J Gastroenterol 2013; 19: 7231-7240.
56 Hyun JJ, Lee HS: Experimental models of pancreatitis. Clin Endosc 2014; 47: 212-216.
57 Braganza JM, Lee SH, McCloy RF, McMahon MJ: Chronic pancreatitis. Lancet 2011; 377: 1184-1197.
58 Biernacka A, Dobaczewski M, Frangogiannis NG: Tgf-beta signaling in fibrosis. Growth Factors 2011; 29: 196-202.
59 Esposito I, Segler A, Steiger K, Kloppel G: Pathology, genetics and precursors of human and experimental pancreatic neoplasms: An update. Pancreatology 2015.
60 Maitra A, Adsay NV, Argani P, Iacobuzio-Donahue C, De Marzo A, Cameron JL et al.: Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. Mod Pathol 2003; 16: 902-912.
61 Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990; 61: 759-767.
62 Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD, Rivera A et al.: Cancer-associated stromal fibroblasts promote pancreatic tumor progression. Cancer Res 2008; 68: 918-926.
63 Augsten M, Hagglof C, Pena C, Ostman A: A digest on the role of the tumor microenvironment in gastrointestinal cancers. Cancer Microenviron 2010; 3: 167-176.
64 Hausmann S, Kong B, Michalski C, Erkan M, Friess H: The role of inflammation in pancreatic cancer. Adv Exp Med Biol 2014; 816: 129-151.
65 Schouppe E, De Baetselier P, Van Ginderachter JA, Sarukhan A: Instruction of myeloid cells by the tumor microenvironment: Open questions on the dynamics and plasticity of different tumor-associated myeloid cell populations. Oncoimmunology 2012; 1: 1135-1145.

66 Kruse J, von Bernstorff W, Evert K, Albers N, Hadlich S, Hagemann S et al.: Macrophages promote tumour growth and liver metastasis in an orthotopic syngeneic mouse model of colon cancer. Int J Colorectal Dis 2013; 28: 1337-1349.
67 Elenbaas B, Weinberg RA: Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. Exp Cell Res 2001; 264: 169-184.
68 Mueller MM, Fusenig NE: Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 2004; 4: 839-849.
69 Apte MV, Xu Z, Pothula S, Goldstein D, Pirola RC, Wilson JS: Pancreatic cancer: The microenvironment needs attention too! Pancreatology 2015; 15: S32-38.
70 Haber PS, Keogh GW, Apte MV, Moran CS, Stewart NL, Crawford DH et al.: Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis. Am J Pathol 1999; 155: 1087-1095.
71 Bachem MG, Schunemann M, Ramadani M, Siech M, Beger H, Buck A et al.: Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. Gastroenterology 2005; 128: 907-921.
72 Apte M, Pirola RC, Wilson JS: Pancreatic stellate cell: Physiologic role, role in fibrosis and cancer. Curr Opin Gastroenterol 2015; 31: 416-423.
73 Bachem MG, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A et al.: Identification, culture, and characterization of pancreatic stellate cells in rats and humans. Gastroenterology 1998; 115: 421-432.
74 Apte MV, Haber PS, Darby SJ, Rodgers SC, McCaughan GW, Korsten MA et al.: Pancreatic stellate cells are activated by proinflammatory cytokines: Implications for pancreatic fibrogenesis. Gut 1999; 44: 534-541.
75 Lohr M, Schmidt C, Ringel J, Kluth M, Muller P, Nizze H et al.: Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. Cancer Res 2001; 61: 550-555.
76 Masamune A, Satoh M, Kikuta K, Suzuki N, Shimosegawa T: Establishment and characterization of a rat pancreatic stellate cell line by spontaneous immortalization. World J Gastroenterol 2003; 9: 2751-2758.
77 Mathison A, Liebl A, Bharucha J, Mukhopadhyay D, Lomberk G, Shah V et al.: Pancreatic stellate cell models for transcriptional studies of desmoplasia-associated genes. Pancreatology 2010; 10: 505-516.
78 Jesnowski R, Furst D, Ringel J, Chen Y, Schrodel A, Kleeff J et al.: Immortalization of pancreatic stellate cells as an in vitro model of pancreatic fibrosis: Deactivation is induced by matrigel and n-acetylcysteine. Lab Invest 2005; 85: 1276-1291.
79 Vonlaufen A, Phillips PA, Yang L, Xu Z, Fiala-Beer E, Zhang X et al.: Isolation of quiescent human pancreatic stellate cells: A promising in vitro tool for studies of human pancreatic stellate cell biology. Pancreatology 2010; 10: 434-443.
80 Omary MB, Lugea A, Lowe AW, Pandol SJ: The pancreatic stellate cell: A star on the rise in pancreatic diseases. J Clin Invest 2007; 117: 50-59.
81 Kikuta K, Masamune A, Watanabe T, Ariga H, Itoh H, Hamada S et al.: Pancreatic stellate cells promote epithelial-mesenchymal transition in pancreatic cancer cells. Biochem Biophys Res Commun 2010; 403: 380-384.
82 Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y et al.: Folfirinox versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 2011; 364: 1817-1825.
83 Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D et al.: Inhibition of hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 2009; 324: 1457-1461.
84 Ozdemir BC, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu CC, Simpson TR et al.: Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. Cancer Cell 2015; 28: 831-833.

85 Rhim AD, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA et al.: Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. Cancer Cell 2014; 25: 735-747.
86 Thomas D, Radhakrishnan P: Tumor-stromal crosstalk in pancreatic cancer and tissue fibrosis. 2019; 18: 14.
87 Vennin C, Murphy KJ, Morton JP, Cox TR, Pajic M, Timpson P: Reshaping the tumor stroma for treatment of pancreatic cancer. Gastroenterology 2018; 154: 820-838.
88 Ohlund D, Handly-Santana A, Biffi G, Elyada E, Almeida AS: Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. 2017; 214: 579-596.
89 Neuzillet C, Tijeras-Raballand A, Ragulan C, Cros J: Inter- and intra-tumoural heterogeneity in cancer-associated fibroblasts of human pancreatic ductal adenocarcinoma. 2018.
90 Weiswald LB, Bellet D, Dangles-Marie V: Spherical cancer models in tumor biology. Neoplasia (New York, NY) 2015; 17: 1-15.
91 Lees RK, Sordat B, MacDonald HR: Multicellular tumor spheroids of human colon carcinoma origin. Kinetic analysis of infiltration and in situ destruction in a xenogeneic (murine) host. Exp Cell Biol 1981; 49: 207-219.
92 Mueller-Klieser W: Multicellular spheroids. A review on cellular aggregates in cancer research. J Cancer Res Clin Oncol 1987; 113: 101-122.
93 Nath S, Devi GR: Three-dimensional culture systems in cancer research: Focus on tumor spheroid model. Pharmacol Ther 2016; 163: 94-108.
94 Penfornis P, Vallabhaneni KC, Janorkar AV, Pochampally RR: Three dimensional tumor models for cancer studies. Frontiers in bioscience (Elite edition) 2017; 9: 162-173.
95 Tai J, Cheung SS, Ou D, Warnock GL, Hasman D: Antiproliferation activity of devil's club (oplopanax horridus) and anticancer agents on human pancreatic cancer multicellular spheroids. Phytomedicine 2014; 21: 506-514.
96 Matsuda Y, Ishiwata T, Kawamoto Y, Kawahara K, Peng WX, Yamamoto T et al.: Morphological and cytoskeletal changes of pancreatic cancer cells in three-dimensional spheroidal culture. Med Mol Morphol 2010; 43: 211-217.
97 Laurent J, Frongia C, Cazales M, Mondesert O, Ducommun B, Lobjois V: Multicellular tumor spheroid models to explore cell cycle checkpoints in 3d. BMC Cancer 2013; 13: 73.
98 Longati P, Jia X, Eimer J, Wagman A, Witt MR, Rehnmark S et al.: 3d pancreatic carcinoma spheroids induce a matrix-rich, chemoresistant phenotype offering a better model for drug testing. BMC Cancer 2013; 13: 95.
99 Grzesiak JJ, Bouvet M: Determination of the ligand-binding specificities of the alpha2beta1 and alpha1beta1 integrins in a novel 3-dimensional in vitro model of pancreatic cancer. Pancreas 2007; 34: 220-228.
100 Gutierrez-Barrera AM, Menter DG, Abbruzzese JL, Reddy SA: Establishment of three-dimensional cultures of human pancreatic duct epithelial cells. Biochem Biophys Res Commun 2007; 358: 698-703.
101 Sempere LF, Gunn JR, Korc M: A novel 3-dimensional culture system uncovers growth stimulatory actions by tgfbeta in pancreatic cancer cells. Cancer Biol Ther 2011; 12: 198-207.
102 Huang FT, Zhuan-Sun YX, Zhuang YY, Wei SL, Tang J, Chen WB et al.: Inhibition of hedgehog signaling depresses self-renewal of pancreatic cancer stem cells and reverses chemoresistance. Int J Oncol 2012; 41: 1707-1714.
103 Proctor E, Waghray M, Lee CJ, Heidt DG, Yalamanchili M, Li C et al.: Bmi1 enhances tumorigenicity and cancer stem cell function in pancreatic adenocarcinoma. PLoS One 2013; 8: e55820.
104 Sobrevals L, Mato-Berciano A, Urtasun N, Mazo A, Fillat C: Upar-controlled oncolytic adenoviruses eliminate cancer stem cells in human pancreatic tumors. Stem Cell Res 2014; 12: 1-10.

105 Urtasun N, Vidal-Pla A, Perez-Torras S, Mazo A: Human pancreatic cancer stem cells are sensitive to dual inhibition of igf-ir and erbb receptors. BMC Cancer 2015; 15: 223.
106 Boj SF, Hwang CI, Baker LA, Chio, II, Engle DD, Corbo V et al.: Organoid models of human and mouse ductal pancreatic cancer. Cell 2015; 160: 324-338.
107 Hwang CI, Boj SF, Clevers H, Tuveson DA: Pre-clinical models of pancreatic ductal adenocarcinoma. J Pathol 2015.
108 Cruz-Acuna R, Garcia AJ: Engineered materials to model human intestinal development and cancer using organoids. Exp Cell Res 2019; 377: 109-114.
109 Dzobo K, Rowe A, Senthebane DA, AlMazyadi MAM, Patten V, Parker MI: Three-dimensional organoids in cancer research: The search for the holy grail of preclinical cancer modeling. OMICS 2018; 22: 733-748.
110 Osterholm C, Lu N, Liden A, Karlsen TV, Gullberg D, Reed RK et al.: Fibroblast ext1-levels influence tumor cell proliferation and migration in composite spheroids. PLoS One 2012; 7: e41334.
111 Carver K, Ming X, Juliano RL: Tumor cell-targeted delivery of nanoconjugated oligonucleotides in composite spheroids. Nucleic Acid Ther 2014; 24: 413-419.
112 Kunz-Schughart LA, Heyder P, Schroeder J, Knuechel R: A heterologous 3-d coculture model of breast tumor cells and fibroblasts to study tumor-associated fibroblast differentiation. Exp Cell Res 2001; 266: 74-86.
113 Rama-Esendagli D, Esendagli G, Yilmaz G, Guc D: Spheroid formation and invasion capacity are differentially influenced by co-cultures of fibroblast and macrophage cells in breast cancer. Mol Biol Rep 2014; 41: 2885-2892.
114 Dolznig H, Rupp C, Puri C, Haslinger C, Schweifer N, Wieser E et al.: Modeling colon adenocarcinomas in vitro a 3d co-culture system induces cancer-relevant pathways upon tumor cell and stromal fibroblast interaction. Am J Pathol 2011; 179: 487-501.
115 Kim SA, Lee EK, Kuh HJ: Co-culture of 3d tumor spheroids with fibroblasts as a model for epithelial-mesenchymal transition in vitro. Exp Cell Res 2015; 335: 187-196.
116 Yip D, Cho CH: A multicellular 3d heterospheroid model of liver tumor and stromal cells in collagen gel for anti-cancer drug testing. Biochem Biophys Res Commun 2013; 433: 327-332.
117 Ware MJ, Keshishian V, Law JJ, Ho JC, Favela CA, Rees P et al.: Generation of an in vitro 3d pdac stroma rich spheroid model. Biomaterials 2016; 108: 129-142.
118 Hauptmann S, Zwadlo-Klarwasser G, Jansen M, Klosterhalfen B, Kirkpatrick CJ: Macrophages and multicellular tumor spheroids in co-culture: A three-dimensional model to study tumor-host interactions. Evidence for macrophage-mediated tumor cell proliferation and migration. Am J Pathol 1993; 143: 1406-1415.
119 Devi KS, Mishra D, Roy B, Ghosh SK, Maiti TK: Assessing the immunomodulatory role of heteroglycan in a tumor spheroid and macrophage co-culture model system. Carbohydr Polym 2015; 127: 1-10.
120 Lazzari G, Nicolas V, Matsusaki M, Akashi M, Couvreur P, Mura S: Multicellular spheroid based on a triple co-culture: A novel 3d model to mimic pancreatic tumor complexity. Acta biomaterialia 2018; 78: 296-307.
121 Mazur PK, Siveke JT: Genetically engineered mouse models of pancreatic cancer: Unravelling tumour biology and progressing translational oncology. Gut 2012; 61: 1488-1500.
122 Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH et al.: Trp53r172h and krasg12d cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 2005; 7: 469-483.
123 Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA et al.: Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003; 4: 437-450.
124 Hamada S, Masamune A, Takikawa T, Suzuki N, Kikuta K, Hirota M et al.: Pancreatic stellate cells enhance stem cell-like phenotypes in pancreatic cancer cells. Biochem Biophys Res Commun 2012; 421: 349-354.

125 Xu Z, Vonlaufen A, Phillips PA, Fiala-Beer E, Zhang X, Yang L et al.: Role of pancreatic stellate cells in pancreatic cancer metastasis. Am J Pathol 2010; 177: 2585-2596.
126 Bustin M, Reeves R: High-mobility-group chromosomal proteins: Architectural components that facilitate chromatin function. Prog Nucleic Acid Res Mol Biol 1996; 54: 35-100.
127 Bustin M: Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. Mol Cell Biol 1999; 19: 5237-5246.
128 Cleynen I, Van de Ven WJ: The hmga proteins: A myriad of functions (review). Int J Oncol 2008; 32: 289-305.
129 Reeves R: High mobility group (hmg) proteins: Modulators of chromatin structure and DNA repair in mammalian cells. DNA repair 2015; 36: 122-136.
130 Reeves R: Molecular biology of hmga proteins: Hubs of nuclear function. Gene 2001; 277: 63-81.
131 Watanabe S, Ueda Y, Akaboshi S, Hino Y, Sekita Y, Nakao M: Hmga2 maintains oncogenic ras-induced epithelial-mesenchymal transition in human pancreatic cancer cells. Am J Pathol 2009; 174: 854-868.
132 Abe N, Watanabe T, Suzuki Y, Matsumoto N, Masaki T, Mori T et al.: An increased high-mobility group a2 expression level is associated with malignant phenotype in pancreatic exocrine tissue. Br J Cancer 2003; 89: 2104-2109.
133 Madison BB, Jeganathan AN, Mizuno R, Winslow MM, Castells A, Cuatrecasas M et al.: Let-7 represses carcinogenesis and a stem cell phenotype in the intestine via regulation of hmga2. PLoS genetics 2015; 11: e1005408.
134 Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH, Moustakas A: Transforming growth factor-beta employs hmga2 to elicit epithelial-mesenchymal transition. J Cell Biol 2006; 174: 175-183.
135 Sarhadi VK, Wikman H, Salmenkivi K, Kuosma E, Sioris T, Salo J et al.: Increased expression of high mobility group a proteins in lung cancer. J Pathol 2006; 209: 206-212.
136 Motoyama K, Inoue H, Nakamura Y, Uetake H, Sugihara K, Mori M: Clinical significance of high mobility group a2 in human gastric cancer and its relationship to let-7 microrna family. Clin Cancer Res 2008; 14: 2334-2340.
137 Lee J, Ha S, Jung CK, Lee HH: High-mobility-group a2 overexpression provokes a poor prognosis of gastric cancer through the epithelial-mesenchymal transition. Int J Oncol 2015; 46: 2431-2438.
138 Rizzi C, Cataldi P, Iop A, Isola M, Sgarra R, Manfioletti G et al.: The expression of the high-mobility group a2 protein in colorectal cancer and surrounding fibroblasts is linked to tumor invasiveness. Hum Pathol 2013; 44: 122-132.
139 Wend P, Runke S, Wend K, Anchondo B, Yesayan M, Jardon M et al.: Wnt10b/beta-catenin signalling induces hmga2 and proliferation in metastatic triple-negative breast cancer. EMBO Mol Med 2013; 5: 264-279.
140 Califano D, Pignata S, Losito NS, Ottaiano A, Greggi S, De Simone V et al.: High hmga2 expression and high body mass index negatively affect the prognosis of patients with ovarian cancer. J Cell Physiol 2014; 229: 53-59.
141 Piscuoglio S, Zlobec I, Pallante P, Sepe R, Esposito F, Zimmermann A et al.: Hmga1 and hmga2 protein expression correlates with advanced tumour grade and lymph node metastasis in pancreatic adenocarcinoma. Histopathology 2012; 60: 397-404.
142 Hristov AC, Cope L, Reyes MD, Singh M, Iacobuzio-Donahue C, Maitra A et al.: Hmga2 protein expression correlates with lymph node metastasis and increased tumor grade in pancreatic ductal adenocarcinoma. Mod Pathol 2009; 22: 43-49.
143 Haselmann V, Kurz A, Bertsch U, Hubner S, Olempska-Muller M, Fritsch J et al.: Nuclear death receptor trail-r2 inhibits maturation of let-7 and promotes proliferation of pancreatic and other tumor cells. Gastroenterology 2014; 146: 278-290.
144 Voon DC, Wang H, Koo JK, Chai JH, Hor YT, Tan TZ et al.: Emt-induced stemness and tumorigenicity are fueled by the egfr/ras pathway. PLoS One 2013; 8: e70427.

145 Moustakas A, Lin HY, Henis YI, Plamondon J, O'Connor-McCourt MD, Lodish HF: The transforming growth factor beta receptors types i, ii, and iii form hetero-oligomeric complexes in the presence of ligand. J Biol Chem 1993; 268: 22215-22218.
146 Brown KA, Pietenpol JA, Moses HL: A tale of two proteins: Differential roles and regulation of smad2 and smad3 in tgf-beta signaling. J Cell Biochem 2007; 101: 9-33.
147 Weiss A, Attisano L: The tgfbeta superfamily signaling pathway. Wiley interdisciplinary reviews Developmental biology 2013; 2: 47-63.
148 Lin HY, Moustakas A, Knaus P, Wells RG, Henis YI, Lodish HF: The soluble exoplasmic domain of the type ii transforming growth factor (tgf)-beta receptor. A heterogeneously glycosylated protein with high affinity and selectivity for tgf-beta ligands. J Biol Chem 1995; 270: 2747-2754.
149 Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R et al.: Identification of smad7, a tgfbeta-inducible antagonist of tgf-beta signalling. Nature 1997; 389: 631-635.
150 Yu L, Hebert MC, Zhang YE: Tgf-beta receptor-activated p38 map kinase mediates smad-independent tgf-beta responses. Embo j 2002; 21: 3749-3759.
151 Park HY, Wakefield LM, Mamura M: Regulation of tumor immune surveillance and tumor immune subversion by tgf-beta. Immune Netw 2009; 9: 122-126.
152 Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA: Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol 2006; 24: 99-146.
153 Bachman KE, Park BH: Duel nature of tgf-beta signaling: Tumor suppressor vs. Tumor promoter. Curr Opin Oncol 2005; 17: 49-54.
154 Bierie B, Moses HL: Tumour microenvironment: Tgfbeta: The molecular jekyll and hyde of cancer. Nat Rev Cancer 2006; 6: 506-520.
155 Jakowlew SB: Transforming growth factor-beta in cancer and metastasis. Cancer Metastasis Rev 2006; 25: 435-457.
156 Bierie B, Moses HL: Transforming growth factor beta (tgf-beta) and inflammation in cancer. Cytokine Growth Factor Rev 2010; 21: 49-59.
157 Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H et al.: A robust and high-throughput cre reporting and characterization system for the whole mouse brain. Nat Neurosci 2010; 13: 133-140.
158 Gout J, Pommier RM, Vincent DF, Kaniewski B, Martel S, Valcourt U et al.: Isolation and culture of mouse primary pancreatic acinar cells. Journal of visualized experiments : JoVE 2013.
159 Wong C, Vosburgh E, Levine AJ, Cong L, Xu EY: Human neuroendocrine tumor cell lines as a three-dimensional model for the study of human neuroendocrine tumor therapy. Journal of visualized experiments : JoVE 2012: e4218.
160 Niederau C, Ferrell LD, Grendell JH: Caerulein-induced acute necrotizing pancreatitis in mice: Protective effects of proglumide, benzotript, and secretin. Gastroenterology 1985; 88: 1192-1204.
161 Banks PA, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG et al.: Classification of acute pancreatitis--2012: Revision of the atlanta classification and definitions by international consensus. Gut 2013; 62: 102-111.
162 Carvalho BS, Irizarry RA: A framework for oligonucleotide microarray preprocessing. Bioinformatics 2010; 26: 2363-2367.
163 Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W et al.: Limma powers differential expression analyses for rna-sequencing and microarray studies. Nucleic Acids Res 2015; 43: e47.
164 Sata N, Klonowski-Stumpe H, Han B, Luthen R, Haussinger D, Niederau C: Supraphysiologic concentrations of cerulein induce apoptosis in the rat pancreatic acinar cell line ar4-2j. Pancreas 1999; 19: 76-82.
165 Weber H, Huhns S, Jonas L, Sparmann G, Bastian M, Schuff-Werner P: Hydrogen peroxide-induced activation of defense mechanisms against oxidative stress in rat pancreatic acinar ar42j cells. Free Radic Biol Med 2007; 42: 830-841.

166 Pietras K, Rubin K, Sjoblom T, Buchdunger E, Sjoquist M, Heldin CH et al.: Inhibition of pdgf receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. Cancer Res 2002; 62: 5476-5484.
167 Paulsson J, Sjoblom T, Micke P, Ponten F, Landberg G, Heldin CH et al.: Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer. Am J Pathol 2009; 175: 334-341.
168 Strell C, Norberg KJ, Mezheyeuski A, Schnittert J, Kuninty PR, Moro CF et al.: Stroma-regulated hmga2 is an independent prognostic marker in pdac and aac. Br J Cancer 2017; 117: 65-77.
169 van den Brink SC, Sage F, Vertesy A: Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations. 2017; 14: 935-936.
170 Pierce GF, Mustoe TA, Lingelbach J, Masakowski VR, Griffin GL, Senior RM et al.: Platelet-derived growth factor and transforming growth factor-beta enhance tissue repair activities by unique mechanisms. J Cell Biol 1989; 109: 429-440.
171 Peng M, Yin N, Li MO: Sestrins function as guanine nucleotide dissociation inhibitors for rag gtpases to control mtorc1 signaling. Cell 2014; 159: 122-133.
172 Muili KA, Jin S, Orabi AI, Eisses JF, Javed TA, Le T et al.: Pancreatic acinar cell nuclear factor kappab activation because of bile acid exposure is dependent on calcineurin. J Biol Chem 2013; 288: 21065-21073.
173 Christophe J: Pancreatic tumoral cell line ar42j: An amphicrine model. Am J Physiol 1994; 266: G963-971.
174 Yu JH, Lim JW, Kim KH, Morio T, Kim H: Nadph oxidase and apoptosis in cerulein-stimulated pancreatic acinar ar42j cells. Free Radic Biol Med 2005; 39: 590-602.
175 Singh L, Bakshi DK, Vasishta RK, Arora SK, Majumdar S, Wig JD: Primary culture of pancreatic (human) acinar cells. Dig Dis Sci 2008; 53: 2569-2575.
176 Armstrong JA, Cash N, Soares PM, Souza MH, Sutton R, Criddle DN: Oxidative stress in acute pancreatitis: Lost in translation? Free Radic Res 2013; 47: 917-933.
177 Blauer M, Nordback I, Sand J, Laukkarinen J: A novel explant outgrowth culture model for mouse pancreatic acinar cells with long-term maintenance of secretory phenotype. Eur J Cell Biol 2011; 90: 1052-1060.
178 Blauer M, Sand J, Nordback I, Laukkarinen J: A novel 2-step culture model for long-term in vitro maintenance of human pancreatic acinar cells. Pancreas 2014; 43: 762-767.
179 Norberg KJ, Nania S, Li X, Gao H, Szatmary P, Segersvard R et al.: Rcan1 is a marker of oxidative stress, induced in acute pancreatitis. Pancreatology 2018; 18: 734-741.
180 Orabi AI, Wen L, Javed TA, Le T, Guo P, Sanker S et al.: Targeted inhibition of pancreatic acinar cell calcineurin is a novel strategy to prevent post-ercp pancreatitis. Cellular and molecular gastroenterology and hepatology 2017; 3: 119-128.
181 Jin S, Orabi AI, Le T, Javed TA, Sah S, Eisses JF et al.: Exposure to radiocontrast agents induces pancreatic inflammation by activation of nuclear factor-kappab, calcium signaling, and calcineurin. Gastroenterology 2015; 149: 753-764.e711.
182 Sipos B, Moser S, Kalthoff H, Torok V, Lohr M, Kloppel G: A comprehensive characterization of pancreatic ductal carcinoma cell lines: Towards the establishment of an in vitro research platform. Virchows Arch 2003; 442: 444-452.
183 Campbell F, Verbeke, C.: Pathology of the pancreas: A practical approach, Springer London Ltd., 2013.
184 Lenggenhager D, Amrutkar M: Commonly used pancreatic stellate cell cultures differ phenotypically and in their interactions with pancreatic cancer cells. 2019; 8.
185 Duval K, Grover H, Han LH, Mou Y, Pegoraro AF, Fredberg J et al.: Modeling physiological events in 2d vs. 3d cell culture. 2017; 32: 266-277.
186 Zong Y, Huang J, Sankarasharma D, Morikawa T, Fukayama M, Epstein JI et al.: Stromal epigenetic dysregulation is sufficient to initiate mouse prostate cancer via paracrine wnt signaling. Proc Natl Acad Sci U S A 2012; 109: E3395-3404.