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The role of G protein-coupled receptor 35 in pancreatic cancer

Minkyoung Kim

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Declaration

To the best of my knowledge and belief, this thesis titled 'The role of GPR35 in pancreatic cancer', contains no material previously published by any other person except where referenced and due recognition has been made. This thesis includes no material that has been accepted for the award for any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th Edition (2013). The proposed research study received animal research ethics approval from Harry Perkins institute of medical research animal ethics committee (AEC), permit number AE184.

Signature:

Date: 25-August-2020

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the highest aggressive cancers. Most PDAC patients are diagnosed at a late stage because symptoms are rare; thereby, the majority of patients rely on chemotherapy. Even when patients can have surgery, which is the only curative treatment for this disease, recurrences after surgical resection of PDAC are still high, occurring in 80% of cases with a low three-year survival rate (less than 30%). Five-year survival for this disease is only less than 10% and PDAC is predicted to be the second leading cause of cancer deaths by the year 2030 in the US. Current standard chemotherapies, including gemcitabine, FOLFIRINOX, cisplatin and nab-paclitaxel (Abraxane), have shown poor efficacy. Diagnostic markers, such as CA19-9, have insufficient sensitivity and specificity and are very limited. Despite scientists' efforts in the last decades to develop new therapeutic targets for PDAC, the treatment has not been essentially changed. Therefore, continuing research on novel therapeutic targets for PDAC, especially focusing on its malignancy, is urgent and important.

Here, we suggest GPR35 as a novel therapeutic target for PDAC. GPR35, which endogenous ligand is still controversial, has been recently spotlighted thanks to the research carried on in the last 10 years which opened the door to this receptor's understanding. Few previous GPR35 studies have shown its high potential as a new drug target in cancer by providing experimental evidence related to key physiological characteristics of cancer, such as inflammation, pain, metabolic changes and hypoxia. Even though GPR35 expression in diverse human body parts, especially gastrointestinal tissues, had been reported, a comparison of expression patterns between pancreatic cancer and normal tissues had not been attempted. Therefore, to evaluate GPR35 as a therapeutic target for PDAC, we have performed the screening of GPR35 expression in PDAC compared to normal pancreas. As a result, high expression of this molecule in human pancreatic cancer compared to the normal pancreas was observed by data analysis, WB and IHC. Our continued studies show that the pharmacological or genetical inhibition of this receptor has significant effects on the proliferation and survival of PDAC cells. Two different caspase assays demonstrated that GPR35 is related to the regulation of apoptosis. Moreover, the screening of autophagy-related proteins, such as p62 and LC3B, in the genetically modified MIA PaCa-2 cells expressing lower GPR35, indicated that GPR35 has a role in autophagy in human PDAC. Furthermore, the decreased expression of GPR35 observed in p53 mutated PDAC cells supports its high relevance in autophagy. Both pharmacological and genetical inhibition of GPR35 reduced the expression of phosphorylation of AKT at S473 and HIF-1α, suggesting that the regulation of PDAC cell proliferation and survival occurs either via AKT pathways or via HIF-1α expression. Additionally, the pivotal

metastatic role of GPR35 in PDAC was identified by migration and invasion assays. In addition to testing PDAC adherent cell types, PDAC tumorspheres, mimicking cancer stem cell-like cells, showed a more mesenchymal state in MIA PaCa-2 with deficiency of GPR35. All these remarkable findings indicate that GPR35 has diverse roles in PDAC progression, especially regulating PDAC malignancy. To assess the safety of commercially available synthetic antagonists of GPR35 (ML145 and CID2745687) as new anti-cancer reagents, a toxicological zebrafish model was used and showed low toxicity of ML145. Lastly, the low relevance of CXCL17 with GPR35 as a potential endogenous ligand was identified, even though CXCL17 still provided its important potential as another therapeutic target for PDAC.

The pivotal roles of GPR35 in PDAC, as a cellular mediator regulating pancreatic cancer proliferation and progression, especially its malignancy, have been verified from our outstanding work. This study is the first report to describe GPR35 as the novel therapeutic target of PDAC. Moreover, its metastatic role in PDAC may suggest a new therapeutic strategy to inhibit pancreatic cancer metastasis and recurrence.

This thesis consists of the following chapters;

Chapter 1: includes 2 review articles that I published during my PhD and literature reviews providing an in-depth background of pancreatic cancer, GPCRs, GPR35 and CXCL17. Also, the main goals and objectives of this study will be presented in this chapter.

Chapter 2: provides the materials and methods of all experiments presented in this study.

Chapter 3 to 7: contain all results that I obtained for understanding the role of GPR35 in PDAC. Each chapter will serve diverse experimental evidence and brief discussions.

Chapter 8: explains the role of CXCL17 in PDAC and its potential as an endogenous ligand for GPR35 will be evaluated.

Chapter 9: discusses the findings and their significances that I presented in this thesis. I will provide the combined data of all chapters in a coherent story and suggest future studies.

Chapter 10: serves all references in this thesis.

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Table of contents

List of Abbreviations

ABC	transporters ATP-binding cassette transporters
AC	adenylyl cyclase
ACC	pancreatic acinar cell carcinoma
AKT	Protein kinase B
ALDH	aldehyde dehydrogenase
ANOVA	one-way analysis of variance
AP2	adaptor protein 2
ARHGAP4	Rho GTPase-activating protein 4
CAFs	cancer-associated fibroblasts
cAMP	cyclic AMP
COX-2	cyclooxygenase-2
CSCs	cancer stem cells
CTCs	circulating tumour cells
CTGF	connective tissue growth factor
CTL	cytotoxic T lymphocyte
CXCL17	C-X-C motif chemokine 17
CXCR4	C-X-C motif chemokine 4
DAG	diacylglycerol
DCLK1	doublecortin like kinase 1
DMSO	dimethyl sulfoxide
E-cadherin	E-calcium-dependent adhesion
ECAR	extracellular acidification rate
ECM	extracellular matrix
EGF	epidermal growth factor
EL	extracellular loop
EMT	epithelial mesenchymal transition
EPs	E prostanoid receptors
ERK	extracellular signal-regulated kinase
ESA	epithelial-specific antigen
ETC	electron transport chain
FBS	foetal bovine serum

FCCP	carbonyl cyanide-4 phenylhydrazone
FCM	flow cytometry
FDA	Food and Drug Administration
FGF-2	fibroblast growth factor-2
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GLUT	glucose transporter
GPCR	G protein-coupled receptor
GRAFS	glutamate, rhodopsin, adhesion, frizzled /taste2 and secretin
GRKs	GPCR-regulating kinases
HDR	homology-directed repair
Hh	Hedgehog
HIF-1	hypoxia inducible factor-1
HK2	hexokinase 2
hpf	hours postfertilization
IF	immunofluorescence
IHC	immunohistochemistry
IL	intracellular loop
IP3	inositol (1, 4, 5)-trisphosphate
IPMN	intraductal papillary mucinous neoplasm
KD	knock down
KO	knock out
KYNA	kynurenic acid
LC3B	light chain 3 isoform B
LPA	lysophosphatidic acid
LPARs	lysophosphatidic acid receptors
LPI	lysophosphatidylinositol
MAPK	mitogen activated protein kinases
MCN	adenosquamous carcinoma and mucinous cystic neoplasms
MDSCs	myeloid derived suppressor cells
MET	mesenchymal-to-epithelial transition
MMPs	matrix metalloproteinases
Mucin1	a type 1 transmembrane protein
NSCLC	non-small-cell lung cancer

NTS	neurotensin
OCR	oxygen consumption rate
OCT4	octamer-binding transcription factor 4
PanIN	pancreatic intraepithelial neoplasia
PanNETs	pancreatic neuroendocrine tumours
PARs	proteinase-activated receptors
PDAC	pancreatic ductal adenocarcinoma
PDE	phosphodiesterase
PDK	pyruvate dehydrogenase kinase
PE	phosphatidylethanolamine
PGE2	prostaglandin E2
PHDs	prolyl hydroxylases
PI3Ks	phosphoinositide 3 kinase
PIP3	phosphatidyl inositol (3, 4, 5)-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA	phospholipase A
PLC	phospholipase C
PS	penicillin/streptomycin
PSCs	pancreatic stellate cells
RGS	regulators of G protein signalling
RIPA	radioimmunoprecipitation
SHH	sonic hedgehog
TAMs	tumour-associated macrophages
TCA	tricarboxylic acid
TCGA	the cancer genome atlas
TFG-2	fibroblast growth factor-2
TGF-β1	transforming growth factors-β1
Tregs	regulatory T cells
TXNIP	thioredoxin-interacting protein
VCC-1	VEGF co-regulated chemokine 1
VEGF	vascular endothelial growth factor
VHL	von Hippel Lindau protein
WB	Western blot

List of Publications

This PhD thesis includes 2 review publications to directly describe some parts of chapter 1. Contributions consents and copyright for each publication are attached in the appendix.

In the following publication, I contributed as the second co-author.

1. Falasca, Marco, Minkyoung Kim, and Ilaria Casari. "Pancreatic cancer: Current research and future directions." *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 1865.2 (2016): 123-132. doi.org/10.1016/j.bbcan.2016.01.001

In the following manuscripts, I shared the first name authorship for equal contribution with Aikaterini Emmanouilidi, Omar Elaskalani, Aleksandra Adamska, Norbaini Abdol and Dr Pat Metharom.

2. Adamska, Aleksandra, et al. "Molecular and cellular mechanisms of chemoresistance in pancreatic cancer." *Advances in biological regulation* 68 (2018): 77-87. doi.org/10.1016/j.jbior.2017.11.007

Chapter 1

Chapter 1: Literature review

1.1 Pancreatic cancer

Pancreatic cancer is one of the deadliest human malignancies having a poor prognosis because there are no distinct symptoms at an early stage. Due to this difficulty in early diagnosis, pancreatic cancer is notorious for its high mortality rate and low survival rate. According to a recent document from the Australian Institute of Health and Welfare, 3,599 new diagnosed cases of pancreatic cancer, which is ranked top 11 in the most commonly diagnosed cancers were estimated in 2019. However, the estimated mortality was ranked top 4 in all cancers, with 3,051 mortality cases in 2019 (AIHW, 2019). Within this report, as regard gender, a higher incidence was estimated in female patients (AIHW, 2019). Moreover, the five-year survival rate for Australian pancreatic cancer patients in 2011-2015 was 9.8% which represents one of the lowest rates compared to other cancers. This low survival rate has been very little improved in the last decades, while most cancers have a relatively improved 5-year survival in Australia. This trend has been seen not only in Australia but also in the United States (US). The American cancer society pointed out the slow advances in the 5-year survival rate for pancreatic cancer over the past decades in the US (Siegel, Miller, & Jemal, 2019). The 5-year survival rate between 2009 and 2015 was 9% in the US, which is the lowest rate among all cancer types (Siegel, Miller, & Jemal, 2020). Worldwide, total death from pancreatic cancer accounts for above 200,000 cases every year. Pancreatic cancer is predicted to be the second leading cancer-related cause of death in the US by 2030 (Rahib et al., 2014). Because of the limited therapeutic advances, pancreatic cancer has been extremely challenging for cancer researchers.

Pancreatic cancer is originated from two different functional areas, exocrine and endocrine. The majority of carcinomas occur in the exocrine area of the pancreas, which has roles in the production and secretion of digestive enzymes. Pancreatic exocrine tumours are typically very aggressive and invasive, unlikely pancreatic neuroendocrine tumours (PanNETs) generated from the hormone-producing cells in the endocrine area of the pancreas. Among these exocrine tumours, pancreatic ductal adenocarcinoma (PDAC), originated from the ducts of the pancreas, occupies 85-90% of pancreatic cancer cases, and is commonly referred to as pancreatic cancer. The other minor exocrine tumours are pancreatic acinar cell carcinoma (ACC), intraductal papillary mucinous neoplasm (IPMN), adenosquamous carcinoma and mucinous cystic neoplasms (MCN).

Several risk factors for developing pancreatic adenocarcinoma have been reported, such as age, chronic pancreatitis, diabetes mellitus, obesity, tobacco smoking, excessive consumption of

alcohol and family history, as well as the mutations in *KRAS, BRCA1/2, ATM, MLH1, MSH2, PMS2, MSH6, STK11, PALB2, TP53* or *CDKN2A* genes (Grant et al., 2015; C. Hu et al., 2018; Kastrinos et al., 2009; Paternoster & Falasca, 2020; Petersen et al., 2010; Pihlak, Valle, & McNamara, 2017; Roberts et al., 2016; Zhen et al., 2015).

1.1.1 Staging system for pancreatic cancer

Pancreatic cancer is staged generally based on abdominal radiographic images. The American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) have designed the cancer staging system based on TNM classification which includes three key components: primary tumour (T), lymph nodes (N) and metastasis (M). Table 1.1 contains the definition according to the TNM classification. This AJCC/UICC cancer staging system has been widely used for pancreatic cancer to develop a stage-specific treatment plan. The AJCC/UICC staging system has been revised over decades to provide a better applicable staging system for patients and researchers; currently, the eighth edition is available (summarized in Table 1.2). Stage 1 includes tumours that have no metastasis on regional lymph nodes and distant organs. Depending on the size of the primary tumour, it is divided into two subgroups: stage 1A (maximum tumour diameter is less than 2cm) and 1B (maximum tumour diameter is between 2cm and 4cm). Stage 2 includes either bigger size tumours (maximum tumour diameter is bigger than 4cm) without any metastasis, called stage 2A, or primary tumours (any sizes) with metastasis in 1-3 regional lymph nodes, called stage 2B. Stage 3 refers to tumours containing metastasis on more than 4 regional lymph nodes or primary tumours involved in the tumour-artery interface. Stage 4 includes tumours with metastasis on distant organs and regional lymph nodes.

TABLE 1.1 TNM CLASSIFICATION

		Definitions
Primary Tumour (T)	TX	Primary tumour cannot be assessed
	T0	No evidence of primary tumour
	Tis	Carcinoma in situ
	T1	Tumour diameter ≤ 2cm
	T2	Tumour diameter > 2cm and ≤ 4cm
	T3	Tumour diameter > 4cm
	T4	Tumour involves the celiac axis or the superior mesenteric artery
Lymph Nodes (N)	NX	Lymph node cannot be assessed
	N0	No regional lymph node metastasis
	N1	Metastasis in 1-3 regional lymph nodes
	N2	Metastasis in more than 4 regional lymph nodes
Metastasis (M)	M0	No distant metastases
	M1	Distant metastases

TABLE 1.2 THE 8TH EDITION OF AJCC/UICC CANCER STAGING SYSTEM FOR PANCREATIC CANCER (2018)

Stage groups	**TNM**
1A	T1 N0 M0
1B	T2 N0 M0
2A	T3 N0 M0
2B	T1, T2, T3 N1 M0
3	T1, T2, T3 N2 M0
	T4 any N M0
4	Any T any N M1

Pancreatic tumours can be also categorized into three groups in terms of surgical resectability, namely resectable, borderline resectable and unresectable. Typically, pancreatic tumours on stage 1 and 2 are resectable. Thanks to the advancement of surgical techniques, stage 3 tumours with limited involvement of the celiac axis or the superior mesenteric artery, have been

considered as resectable tumours, called borderline resectable. Stage 4 tumours still remain in the unresectable group.

Unfortunately, most patients present advanced PDAC; thereby, only 15-20% of patients receive surgical resection, regarded as the only potentially effective treatment for curing PDAC. Even when patients are eligible to have a surgical resection, the 5-year survival rate after surgery is still very low, about 20-25%, due to PDAC recurrence.

1.1.2 Development of PDAC

Pancreatic intraepithelial neoplasia (PanIN), where most invasive PDAC arises from, is histologically defined as a microscopic non-invasive precursor lesion of the pancreas. Less commonly PDAC can develop from visible non-invasive cystic precursors including IPMNs and MCNs. PanINs can be anywhere in the pancreas, even in the heterotopic pancreas. Usually, they are found in branch ducts of less than 0.5mm diameter, rather than in main pancreatic ducts. These tumours, are too small to be detected by radiologic imaging. According to one clinicopathological study, PanINs were observed in 82% of the pancreas with PDAC, in 60% of those with chronic pancreatitis and even in 16% of the normal pancreas (Andea, Sarkar, & Adsay, 2003).

PanINs used to be divided into three classes, PanIN1, PanIN2 and PanIN3. However, in the Baltimore consensus meeting in 2014, international experts recommended replacing this conventional classification with a two-tiered system (low grade vs. high grade) for all precursor lesions and invasive carcinomas including PanIN, IPMN and MCN. The major change regards PanIN2, categorized as low grade, and PanIN3 only regarded as high grade. The characterized PanINs are detailed in Table 1.3

TABLE 1.3 CLASSIFICATION OF PANIN

Definition	WHO classification 2010	Baltimore consensus meeting 2014
Mucinous metaplasia (goblet cell) and hypertrophy or nonpapillary (flat duct lesion) hyperplasia	PanIN1A	Low grade PanIN
Micropapillary hyperplasia without atypia	PanIN1B	
Papillary dysplasia with mild cytological atypia	PanIN2	
Carcinoma in situ with frequent most significant atypia or severe dysplasia	PanIN3	High-grade PanIN

Numerous studies have identified the correlation between genetic alterations accumulation and increasing grade of dysplasia. For example, mutation of *KRAS* and inactivation of cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16)* are detectable on early pancreatic neoplasms. Tumour suppressor protein 53 (*TP53)* mutations and mothers against decapentaplegic homologue 4 (*SMAD4)* inactivation are observed in late pancreatic neoplasms with genetic alterations of *KRAS* and *CDKN2A/p16*. Not only these four genes but also PanIN3 shares several other same gene expression changes with invasive PDAC, as has been reviewed. (Yonezawa, Higashi, Yamada, & Goto, 2008).

1.1.3 PDAC Metastasis

More than 80% of PDAC patients have unresectable pancreatic cancer at the stage of diagnosis due to vascular involvement and/or metastasis (Siegel et al., 2020). The majority of cases in PDAC patients have widespread metastasis most commonly in the liver (76-94%), lung (45-48%), peritoneum (41-56%) and/or abdominal lymph node (41%) (Le Large et al., 2017).

Metastasis is a very complex process enabling cells from the primary tumour to move, via either lymphatic or vascular system, to distant organs and establish other tumours in the target organs. Metastasis itself can be a huge challenge for tumour cells due to the complicated steps in the whole journey and low survival potential in the bloodstream caused by being non-attached single cells (anoikis). The journey starts from the activation of the epithelial-to-mesenchymal transition (EMT). Once EMT is activated, epithelial phenotype cells, characterized by adherent cells contacting other cells, become mesenchymal phenotype cells, defined as detached cells with enhanced cell motility. The dissociated cells from the primary tumour can now invade the special extracellular matrix (ECM) and the walls of blood vessels to enter the bloodstream, a process called intravasation. The malignant cells entered in the bloodstream, called circulating tumour cells (CTCs), commonly die by anoikis; thereby, survival in the bloodstream is crucial for successful metastasis. Animal models show that only less than 0.01% of the CTCs in the vascular system survived and then formed the secondary tumour (Fidler, 1970). Once CTCs meet their favourite environment, they are entering the target organs through extravasation and mesenchymal-to-epithelial transition (MET) by passing through the wall of blood vessels and the ECM of target organs. At this stage, the malignant cells from the distant primary tumour can finally establish and grow the secondary tumour in the target organs. Each of these steps of the metastatic cascade requires massive support from multicomponent to ensure a successful metastasis.

1.1.3.1 EMT in PDAC

EMT in cancer is an essential step for metastasis, categorized as EMT type III. Low expression of epithelial markers, mainly E-cadherin (calcium-dependent adhesion) which is a cell adhesion molecule able to form the tight cell-cell bond, is commonly observed in tumour cells during the EMT. Meanwhile, the expression of cytoskeletal components of mesenchymal cells, such as vimentin, is increased. Transcription factors including TWIST1, SNAIL and SLUG are involved in these phenotype changes. Numerous EMT inducers such as transforming growth factor-β (TGF-β), matrix metalloproteinases (MMPs), Wnt, Hedgehog (Hh) and epidermal growth factors (EGFs) also have been suggested.

The downregulation of E-cadherin has been accepted as a requirement in metastatic PDAC tumours. It is confirmed that highly metastatic PDAC cells selected from *in vivo* express reduced E-cadherin (von Burstin et al., 2009). 42-60% of human PDAC tumours specimens from the patients having distant metastasis have shown negative expression of E-cadherin (Joo, Rew, Park, & Kim, 2002; Pignatelli et al., 1994). Moreover, PDAC patients who have low E-cadherin expression show a poor prognosis (Shin et al., 2005).

The overexpression of vimentin has been accepted as an indicator of rapid tumour progression in PDAC cells. The expression of vimentin has been observed in 52.8% of human pancreatic cancer cases with poor clinical outcomes (Myoteri et al., 2017) and the author has suggested vimentin as a potential adverse prognostic marker for PDAC. Similarly, a higher expression of vimentin was detected in PDAC patients with shorter overall survival (Maehira et al., 2019). In addition, 76% of CTCs with vimentin expression were observed in PDAC patients (T. Wei et al., 2019).

Moreover, it is well known that EMT in cancer occurs in response to signalling molecules secreted by stromal cells in the tumour microenvironment. PDAC is surrounded by a specific environment consisting of abundant tumour stroma and extracellular components such as collagens, fibronectin, laminin, and their secretion molecules, MMPs and TGF-β1. ECM components, especially collagen and its secretion of MMPS, are involved in PDAC metastasis and invasion (Procacci, Moscheni, Sartori, Sommariva, & Gagliano, 2018). Many other studies have suggested that TGF-β from the microenvironment can induce EMT followed by apoptosis in PDAC (David et al., 2016; Mohd Faheem et al., 2020; Ripka et al., 2007).

EMT can be regulated by serine/threonine-protein kinase (AKT). Apoptosis is inhibited during EMT by several transcription factors through mitogen-activated protein kinases (MAPK) and PI3K/AKT pathways (Kajita, McClinic, & Wade, 2004). A recent study shows that the

interaction between vimentin and Girdin, an actin-binding protein also known as an AKT-phosphorylation enhancer (APE), affects EMT in PDAC (W. Wang et al., 2020).

1.1.3.2 Cancer stem cells in PDAC

The term cancer stem-like cells (CSCs) refers to subpopulations of cancer cells that have stemness features such as self-renewal, differentiation and extensive proliferation. These three stemness characteristics can also cover the basis of metastasis in malignant tumours. Primary epithelial tumour cells which exhibit tumorigenic potential and invasiveness without EMT, are often detectable, especially at the boundary of the tumour mass. Moreover, it is well known that EMT can be controlled by basic developmental signalling pathways including Wnt and TGF-β pathways, suggesting that metastasis can be fully explained with both EMT and stem cell concepts rather than with each one alone.

A minority of cancer cells, less than 5% of total tumour cells, have shown their proliferative potential *in vitro* or *in vivo* assays (Reya, Morrison, Clarke, & Weissman, 2001). It is believed that the majority of CSCs originate from any cells with proliferative ability during primary tumour progression by an accumulation of genetic mutations and epigenetic modifications, while the minority of CSCs are from normal stem cells with oncogenic transformation (Krivtsov et al., 2006). The first presence of CSCs in human acute myeloid leukaemia has been reported by Bonnet and Dick (Bonnet & Dick, 1997). These authors verified the cancer initiation of acute myeloid leukaemia from malignant cells with the expression of cell surface marker CD34 and lack of expression of CD38 in an immunocompromised mouse model. Since this discovery, CSCs have been observed in other diverse malignancies including breast (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003), brain (Singh et al., 2004) and pancreatic cancer (C. Li et al., 2007).

The first study on pancreatic CSCs revealed that pancreatic cancer cells with the expression of CD44, CD24 and epithelial-specific antigen (ESA) have a 100-fold higher tumorigenic potential compared to non-tumorigenic cancer cells. The authors also described that the sonic hedgehog (SHh) signalling pathway is involved in these stem cell properties on pancreatic CSCs. In the same year, other experimental evidence has suggested that pancreatic CSCs are defined by the expression of CD133 (Hermann et al., 2007). Moreover, this publication has emphasized the metastatic role of pancreatic CSCs which predominantly express the CXCR4 receptor (also known as CD184 or fusin). The subpopulation of $CD133^{+}/CXCR4^{+}$ has shown significantly higher migratory activity in the L3.6pl cell line, which has high metastatic

capacity compared to FG cells. In addition, ATP-binding cassette transporters (ABC transporters), including ABCB1 and ABCG2, have been suggested as a marker of pancreatic CSC involved in malignant characteristics (Domenichini et al., 2019; Moitra, Lou, & Dean, 2011; Sasaki et al., 2018; Van den Broeck et al., 2013; J. Zhou et al., 2008).

Functional markers for CSCs allowed to overcome false conclusions from studies only focusing on cell surface markers of CSCs as metastatic cells originated from CSCs do not need only stemness features to adapt to new environments. In pancreatic cancer, aldehyde dehydrogenase (ALDH), octamer-binding transcription factor 4 (OCT4) and doublecortin like kinase 1 (DCLK1) have been suggested as a functional marker for CSCs (Bailey et al., 2014; Kim et al., 2011; Lu et al., 2013; Rasheed et al., 2010). The expression of ALDH, specifically ALDH1A, has been verified in the pancreatic cancer patient group with worse survival, especially in metastatic lesions (Rasheed et al., 2010). In the latter study, the ALDH-positive pancreatic tumour cells have shown more stem cell and mesenchymal features. The higher tumorigenic potential has been detected in ALDH-positive pancreatic tumour cells without expressing CD133, leading to identify a new distinct population of tumour initiating cells in the pancreas (Kim et al., 2011). OCT4 is a transcription factor regulating gene expression involved in embryonic development. The strong expression of OCT4 has been verified in human pancreatic carcinogenesis, especially in metaplastic ducts, and the authors have identified that the expression of OCT4 is associated with KRAS mutations, indicated the relevance of the early stage of pancreatic cancer progression (J. Wen et al., 2010). The loss of stemness in pancreatic CSCs isolated from PANC-1 cell line by knockdown of OCT4 and Nanog, another homeobox transcription factor, has been reported and it has demonstrated that those altered genes are involved in pancreatic metastasis, EMT, carcinogenesis and drug resistance (Lu et al., 2013). A similar study has found that knockdown of OCT4 inhibits pancreatic cell proliferation and invasion with downregulation of AKT (Lin et al., 2014). DCLK1, a serine/threonine-protein kinase that acts as a microtubule regulator, has been reported as a marker of CSCs in the intestine and pancreas. The upregulation of DCLK1 has been detected in a subpopulation of distinct pancreatic tumour cells, which enable to initiate tumorigenesis mainly with mutant KRAS (Qiu et al., 2018). Moreover, it has been suggested that DCLK1 expression is pivotal not only in CSCs but also in EMT and metastasis (Bailey et al., 2014).

1.1.4 Tumour plasticity and heterogeneity of PDAC

Acinar cells have the highest degree of plasticity among pancreas cells and are known as the main driver for pancreas homeostasis and regeneration. Acinar cells often transdifferentiate to ductal cells (epithelial phenotype), in a process called acinar-to-ductal metaplasia (ADM), when they are stimulated by signals from microenvironment, inflammation or, tissue damage generated by diverse stress conditions (Sandgren, Luetteke, Palmiter, Brinster, & Lee, 1990). During ADM, acinar cells have embryonic progenitor cell properties with concomitant oncogenic activation, such as proto-oncogene KRAS, eventually leading them to transform to PanINs (Liou et al., 2013; Logsdon & Ji, 2009). The ADM process is generally considered the first step in PDAC development.

One of the difficulties facing PDAC treatment is the great heterogeneity observed frequently in PDAC tumours. The heterogeneity can be generated by somatic mutations and/or epigenetic alterations. Next-generation sequencing approaches have revealed several gene alterations involved in PDAC progression. The most commonly mutated gene in PDAC is the proto-oncogenic GTPase *KRAS* (in > 90% of PDAC tumours), followed by tumour suppressor genes such as *TP53*, *p16/CDKN2A* and *SMAD4*. Furthermore, mutations of RAC-beta serine/threonine-protein kinase (*AKT2*) and breast cancer early onset genes 2 (*BRCA2*) in PDAC have been reported. Beyond the transcriptomic landscape, the epigenetic landscape also uncovers the mechanisms of this PDAC heterogeneity. Epigenetic alterations including histone modifications, DNA methylation, chromatin remodelling and non-coding RNA molecules (miRNAs) cause gene variation by generally silencing specific genes, which contribute to the great heterogeneity of PDAC. The repression of key tumour suppressor genes and upregulation of oncogenes caused by epigenetic alterations have been studied (Buchholz et al., 2006; W. Gao et al., 2015; Mazur et al., 2015; Nones et al., 2014). Moreover, this epigenetic regulation affects tumour progression leading to a malignant state (Roe et al., 2017).

1.1.4.1 PDA tumour subtypes

PDAC is a very heterogeneous and genetically unstable disease, and therefore, patients have different responses toward each chemotherapy. To provide the most effective treatment strategies to each patient, scientists have done diverse attempts to define tumour subtypes (summarized in Table 1.4). The widely known classification of these PDAC tumours, including “basal-like” and “classical” subtypes by the transcriptome of PDAC, was reported in 2011 (Collisson et al., 2011). The “basal-like (quasi-mesenchymal and exocrine-like)”

subtype has the highest expression of mesenchymal-associated genes with worse prognosis, while the "classical" subtype has the highest expression of adhesion-associated and epithelial genes. Identification of stroma-specific subtypes added details to this molecular landscape of PDAC in 2015 (Moffitt et al., 2015). The subsequent year, another classification of PDAC subtypes, squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX), was suggested (P. Bailey et al., 2016). More recently, a xenotransplantation model of PDAC using patient-derived organoids enabled us to distinguish two main subtypes, the "fast progressors" and "slow progressors" subtypes (Miyabayashi et al., 2020). Thus, studies on the classification of PDAC tumours considering transcription factors and/or epigenetic regulators are expected to improve all personalized treatment strategies as well as providing new potential biomarkers.

TABLE 1.4 CLASSIFICATION OF PANCREATIC TUMOUR SUBTYPES

PDA Tumour subtypes	**References**
1) Classical, 2) quasi-mesenchymal, and 3) exocrine-like	(Collisson et al., 2011)
1) Classical, and 2) basal-like (tumour subtype)/ 1) activated, and 2) normal (stromal subtype)	(Moffitt et al., 2015)
1) Squamous, 2) pancreatic progenitor, 3) immunogenic, and 4) ADEX	(P. Bailey et al., 2016)
1) Fast progressors, 2) slow-progressors	(Miyabayashi et al., 2020)

1.1.5 Pancreatic cancer microenvironment

An important hallmark of PDAC is its unique microenvironment consisting of extensive desmoplastic stroma including connective tissues, blood vessels and diverse inflammatory cells. The majority (up to 90%) of the tumour volume is occupied by a redundant amount of ECM proteins such as collagens, laminin, hyaluronan, and fibronectin produced mainly by pancreatic stellate cells (PSCs), which eventually generate a hypovascular microenvironment impacting multidrug resistance. Pancreatic cancer behaves like a chronic wound because of the high infiltration by diverse surrounding immunosuppressive cells such as tumour-associated macrophages (TAMs), regulatory T cells (Tregs) and myeloid-derived suppressor

cells (MDSCs), which negatively impact the effector function of immune cells such as cytotoxic T lymphocyte (CTL). These stromal cells and cancer cells along with endothelial cells and neuronal elements, maintain the PDAC microenvironment by secreting MMPs, fibroblast growth factor-2 (FGF-2), connective tissue growth factor (CTGF) and TGF-β. Accumulating evidence has demonstrated that this unique microenvironment is highly related to PDAC progression and metastasis and is associated with a poor prognosis (Carstens et al., 2017; C. Li et al., 2020).

1.1.5.1 The role of HIF-1 in PDAC

Cells within the PDAC microenvironment are easily exposed to hypoxic conditions. Oxygen delivery in PDAC is usually compromised due to the hypovascular microenvironment, established by both stromal cells and cancer cells, which activates antiangiogenic factors such as angiostatin, endostatin, and pigment epithelium-derived factors. Moreover, both stromal cells and cancer cells in PDAC tumours require high oxygen consumption for rapid proliferation and infiltration contributing to severe hypoxic conditions. Therefore, an optimal cellular adaptation to this low oxygen condition is highly required for PDAC and it is known that hypoxia-inducible factor-1 (HIF-1) commonly provides PDAC cancer cells with a unique regulation of multiple cellular signalling.

Hypoxia-inducible factors (HIFs), which are transcription factors responding to oxygen level, exist as a heterodimer form composed of an alpha and a beta subunit. In the presence of oxygen (normoxia), α subunit of HIF-1 (HIF-1α) is hydroxylated at its conserved proline residues by prolyl hydroxylases (PHDs). This hydroxyl α subunit, allows the conjugation with von Hippel-Lindau protein (VHL), providing the sites to be marked by ubiquitin (ubiquitination), eventually leading to rapid degradation of HIF-1α by proteasomes. In the absence of oxygen, HIF-1α is unable to be hydroxylated and degraded, leading to the stabilization of HIF-1α. The stabilized HIF-1α then is able to form a heterodimer with a beta subunit, finally inducing the transcription of over 200 genes related to biological processes, encouraging oxygen delivery to hypoxic areas.

A consistent stabilization of HIF-1α is frequently observed in PDAC. The overexpression of HIF-1 was detected in PDAC tissues correlating with poor prognosis (Shibaji et al., 2003; L. Y. Ye et al., 2014). Around 60% of pancreatic carcinoma tissues and 76% of regional lymph node metastatic tissues showed overexpression of HIF-1, but the expression was almost absent in non-cancerous pancreatic tissues (Kitada et al., 2003). Pancreatic tumour tissues showing a high level of HIF-1α were associated with patients' shorter survival (Spivak-Kroizman et al.,

2013). A study of nuclear HIF-1 expression in resected pancreatic adenocarcinoma tissues only suggested that high expression of HIF-1α is significantly related to patients' distant recurrence versus local recurrence (Colbert et al., 2015).

Increasing evidence suggested that HIF-1 mediate diverse cellular mechanisms regarding PDAC progression such as proliferation, survival, angiogenesis, metabolic reprogramming, invasion and metastasis. The antitumor effects of a hypoxic cytotoxin, TX-2098, in pancreatic cancer *in vitro* and *in vivo* suggested that HIF-1α promotes PDAC proliferation (Miyake et al., 2012). Pancreatic cells with stabilized HIF-1α expression have a higher survival capability by inducing expression of anaerobic metabolism-associated genes, *Glut1* and *aldolase A*, versus cells without constitutive expression of them (Akakura et al., 2001). HIF-1 has also been demonstrated to mediate the secretion of vascular endothelial growth factor (VEGF) which is one of the angiogenic factors in PDAC cell lines (Buchler et al., 2003; Tanaka et al., 2015). Several studies confirmed that the activation of diverse metabolic reprogramming regulators such as Rho GTPase-activating protein 4 (ARHGAP4) and a type 1 transmembrane protein (Mucin 1), is altered in a HIF-1 dependent manner in pancreatic cancer (Y. Shen et al., 2019; Shukla et al., 2017). A large number of studies have focused on HIF-1α regulating pancreatic cancer metastatic mechanisms including EMT, migration and invasion. The upregulated TWIST expression under hypoxia with the expression of HIF-1α induced EMT (downregulation of E-cadherin and p16 protein) of pancreatic cancer cells *in vitro* and *in vivo* (S. Chen et al., 2016). The hypoxia-induced overexpression of fascin, a prometastatic actin-bundling protein, promoted PDAC cell migration and invasion by accelerating MMP-2 expression (X. Zhao et al., 2014). The higher expression of HIF-1α was detected in pancreatic CSCs marked with CD133 (a representative cancer stem cell marker) under hypoxic conditions compared to $CD133^-$ cells and they showed better migration ability in the presence of EMT gene expression (Maeda et al., 2016). Furthermore, recent studies suggested that long non-coding RNA (IncRNA-BX111887 and IncRNA-MTA2TR) and microRNAs (miR-142, miR-212 and miR-224) under the regulation of HIF-1α have a role in the regulation of PDAC metastasis by promoting pancreatic cancer cell migration and invasion (S. J. Deng et al., 2018; Lu et al., 2017; Yue, Liu, & Song, 2019; Zeng et al., 2019; G. Zhu, Zhou, Liu, Shan, & Zhang, 2018).

1.1.6 Current therapies for PDAC and their limitations

The content of this section is covered by the published review article:

Adamska, Aleksandra, et al. "Molecular and cellular mechanisms of chemoresistance in pancreatic cancer." *Advances in biological regulation* 68 (2018): 77-87. doi.org/10.1016/j.jbior.2017.11.007

Synopsis and significance of this review article

This review provides a detailed explanation of the molecular and cellular mechanisms of chemoresistance to current chemotherapy including 5-FU, gemcitabine, cisplatin, oxaliplatin, taxanes and FOLFIRINOX. In our comprehensive study of the mechanism of gemcitabine, which has been a reference first-line therapy for PDAC, we described the gemcitabine resistance pathway revealed from recent studies. Further, cellular factors contributing to pancreatic cancer chemoresistance such as pancreatic stellate cells, tumour-initiating cells, fibroblasts, macrovesicles, tumour-associated macrophages, tumour-associated neutrophils and platelets, are extensively described in this review, to provide a better understating of the current challenges and limitations of available therapeutics.

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Molecular and cellular mechanisms of chemoresistance in pancreatic cancer

Aleksandra Adamska[a,1], Omar Elaskalani[b,1], Aikaterini Emmanouilidi[a,1], Minkyoung Kim[a,1], Norbaini Binti Abdol Razak[b], Pat Metharom[b], Marco Falasca[a,*]

[a] Metabolic Signalling Group, School of Biomedical Sciences, Curtin Health Innovation Research Institute, Faculty of Health Sciences, Curtin University, Perth, Western Australia 6102, Australia

[b] Platelet Research Laboratory, Curtin Health Innovation and Research Institute, Faculty of Health Sciences, Curtin University, Perth, Western Australia 6102, Australia

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ABSTRACT

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the most chemoresistant cancers, and current therapies targeting cancer-associated molecular pathways have not given satisfactory results, owing in part to rapid upregulation of alternative compensatory pathways. Most of the available treatments are palliative, focussing on improving the quality of life. At present, available options are surgery, embolization, radiation, chemotherapy, immunotherapy and use of other more targeted drugs. In this review, we describe the cellular and molecular effects of current chemotherapy drugs such as gemcitabine, FOLFIRINOX (5-fluorouracil [5-FU], oxaliplatin, irinotecan, and leucovorin) and ABRAXANE (nab-Paclitaxel), which have shown a survival benefit, although modest, for pancreatic cancer patients. Nevertheless, gemcitabine remains the standard first-line option for advanced-stage pancreatic cancer patients and, as resistance to the drug has attracted an increasing scientific interest, we deliberate on the main intracellular processes and proteins vital in acquired chemoresistance to gemcitabine. Lastly, our review examines various microenvironmental factors capable of instigating PDAC to develop resistance to chemotherapeutic drugs.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most chemoresistant cancers due to the broad heterogeneity of genetic mutations and the dense stromal environment. Thus far, therapies targeting cancer-associated molecular pathways have not given satisfactory results (Adamska et al., 2017). Although tumour resection is the most realistic option for PDAC patients, less than 20% of them can undergo surgery at the time of diagnosis (Sohn et al., 2000) due to the fact that metastasis has already occurred. In case the tumour has only spread to the local blood vessels, then it is characterised as borderline resectable, and the patients are subjected to neoadjuvant therapy. The prognosis for metastasised cancer cases is poor, and the only treatment option is chemotherapy.

Since 1997, for nearly 15 years, gemcitabine has been a reference first-line therapy drug for patients with a good performance status (PS) (Burris et al., 1997). Therefore, gemcitabine in combination with different cytotoxic and biological agents is the predominant therapeutic option for patients. However, it only manages to diminish the symptoms of the disease and increase survival rate, and its toxicity limits the application to patients with good PS. Therefore, with the PS as a point of reference, patients are

* Corresponding author.
E-mail address: marco.falasca@curtin.edu.au (M. Falasca).
[1] Equally contributing authors.

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subjected to either single-agent treatment or combination therapy. A combination that exhibited a significant response is that of gemcitabine with albumin-bound paclitaxel, also known as ABRAXANE (Von Hoff et al., 2013), which increased the capacity of gemcitabine to penetrate the tumour (Frese et al., 2012). Due to the increased survival rate of the patients who responded to this combination, the FDA established it as a first-line therapy option, along with another multidrug combination called FOLFIRINOX (Conroy et al., 2011). However, their advantage over single-agent gemcitabine was quite moderate, with mostly palliative effects. Factors such as cell plasticity, heterogeneity of the tumour, composition of the tumour stroma, epithelial-to-mesenchymal transition (EMT), altered metabolism and cancer cells-derived vesicles, can highly impact treatment outcomes (Elaskalani et al., 2017c; Falasca et al., 2016). The development of drug resistance is the main cause of the lack of efficacy of current treatments for pancreatic cancer, and in this review, we will summarise the main mechanisms of chemoresistance in pancreatic cancer with emphasis on the emerging role played by the tumour microenvironment.

2. Chemotherapy in pancreatic cancer: mechanism of action and cellular metabolism

In this section, the mechanism of action of drugs such as gemcitabine, FOLFIRINOX (5-fluorouracil [5-FU], oxaliplatin, irinotecan, and leucovorin) and ABRAXANE (nab-Paclitaxel), which have shown a survival benefit for pancreatic cancer patients, will be described.

3. 5-FU (5-fluorouracil)

5-Fluorouracil (5-FU) has been widely used in cancer therapies for approximately 50 years and it still remains one of the main drugs for PDAC therapies (Manji et al., 2017). 5-FU is an S-phase-specific uracil analogue also known as a pyrimidine analogue by incorporating into DNA, RNA or both, leading to accumulation of 5-FU in cells which results in increased cytotoxicity, eventually causing cell death (Longley et al., 2003). 5-FU is converted intracellularly to fluorodeoxyuridine monophosphate (FdUMP), which then forms a complex with thymidylate synthase (TS), inhibiting the production of deoxythymidine monophosphate (dTMP), which is essential for DNA replication. 5-FU can be also converted to 5-fluorouridine 5′-triphosphate (FUTP) which is then incorporated into RNA transcribed by RNA polymerase, mainly causing interference of mRNA synthesis. The incorporation of 5-FU into RNA not only affects mRNA synthesis but also inhibits ribosomal RNA maturation, post-transcriptional modification of transfer RNAs, and pre-messenger RNA splicing. In addition, it has been reported that 5-FU induces apoptosis through activation of caspase-6, upregulation of phospho-Bcl-2, and generation of mitochondrial reactive oxygen species (ROS) (Chan et al., 2008; Hwang et al., 2001). However, 5-FU has marginal efficacy in PDAC therapies due to low stability inside the cells (Heggie et al., 1987). The majority of administered 5-FU tends to be easily broken down to *dihydrofluorouracil* (*DHFU*) by dihydrophyrimidine dehydrogenase (DPD) in the liver. Thus, DPD is a vital element for the activity of 5-FU, impacting on the sensitivity and resistance of the drug. DPD can be also an important molecule in terms of the toxicity of this drug. It has been reported that deficiency of DPD causes fluorouracil-related severe toxicity (Milano et al., 1999).

4. Gemcitabine

Research has focused on improving patient survival and, towards this direction, gemcitabine has proven to be a good candidate, showing a significantly higher level of efficacy, compared to 5-FU. More specifically, the comparative phase III studies of single agent gemcitabine and 5-FU revealed that 5-fold more patients experienced clinical benefit from gemcitabine over 5-FU, and the 1-year survival rate was 9-fold higher (Burris et al., 1997). Gemcitabine (2′, 2′-difluorodeoxycytidine, dFdC) is a nucleoside cytidine (pyrimidine) analogue that can be incorporated into replicating DNA, thereby inhibiting DNA synthesis, which results in premature chain termination (Fig. 1) (Hertel et al., 1990). Gemcitabine is a prodrug, and its cellular uptake is mediated by nucleoside transporters, molecules pivotal for cell sensitivity to drugs (Baldwin et al., 1999; Plunkett et al., 1995). Once inside, deoxycytidine kinase (dCK) mediates phospho-activation of gemcitabine into gemcitabine di- (dFdCDP) and triphosphate (dFdCTP), which is an essential step in gemcitabine-mediated cytotoxicity. These active drug metabolites have multiple inhibitory actions on DNA synthesis. As an inhibitor of ribonucleotide reductase, dFdCDP prevents the *de novo* synthesis of deoxyribonucleotides, while dFdCTP is perceived by the cell as a cystidine and is incorporated into the DNA chain during replication (Huang et al., 1991). Incorporation of the active drug forms into nucleic acids terminates DNA polymerase action, eventually leading to cell death (Huang and Plunkett, 1995). Moreover, gemcitabine metabolites can inhibit a plethora of other enzymes including cytidine triphosphate synthetase (CTP synthetase) (Heinemann et al., 1995) or deoxycytidine monophosphate deaminase (dCMP-deaminase) (Heinemann et al., 1992), which creates a positive feedback loop extending gemcitabine activity and efficacy by reducing dCTP pools and increasing the incorporation of dFdCTP into DNA.

5. Cisplatin & oxaliplatin

Cisplatin is a platinum complex surrounded by ammonia and chlorine atoms, incorporated into cells by the copper transporter (Ctr1) (Ishida et al., 2002). Intracellularly, chloride ions in cisplatin are displaced by water molecules converting it into a charged electrophilic compound (active form). This active form has the ability to bind to nucleophilic groups such as oxygen, nitrogen and sulphur atoms, which are present in amino acid side chains and purine bases of DNA or RNA, to form DNA adducts, such as intrastrand cross-linked d(GpG) and d(ApG). This leads to the bending of the DNA helix, followed by binding of the high-mobility-

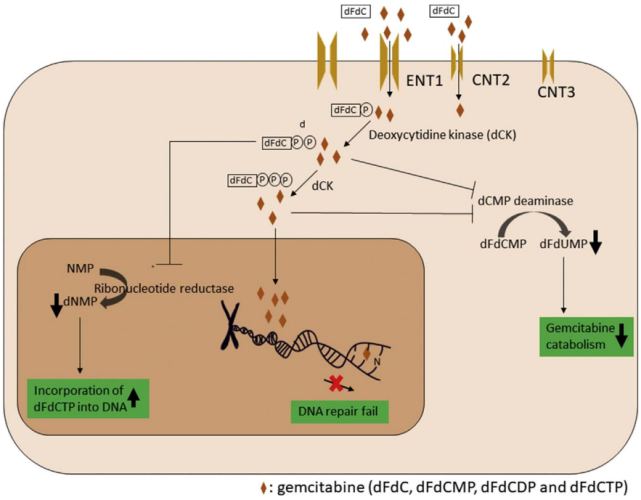

Fig. 1. Mechanistic representation of gemcitabine metabolism. Gemcitabine (dFdC) is transported by multiple active nucleoside transporters (e.g. ENT1, CNT2 and CNT3). The transported dFdC is consecutively phosphorylated and is converted to gemcitabine diphosphate and tri-phosphate (dFdCDP and dFdCTP). These two activated metabolites (dFdCDP and dFdCTP) disturb DNA synthesis. dFdCDP inhibits ribonucleotide reductase which catalyses the *de novo* synthesis of deoxyribonucleotides (dNMPs). Decreased dNMPs help dFdCTP to bind into DNA by reducing competing natural deoxyribonucleotide pools. The incorporation of gemcitabine to DNA by DNA polymerase terminates DNA chain elongation. Interestingly, exonuclease enzymes are not able to detect and repair this gemcitabine-incorporated DNA because DNA polymerase permits one more nucleotide to pair after binding gemcitabine to DNA chain. dFdCDP and dFdCTP also inhibit dCMP deaminase which converts dFdCMP to dFdUMP. This activity allows a decrease of gemcitabine catabolism and perpetuates gemcitabine's own activity and survival.

group (HMG) proteins to this changed structure. The binding of HMG proteins to cisplatin-DNA adducts is structurally similar to their natural binding formation and contributes to the cells' inability to fix the DNA lesions. A charged electrophilic cisplatin also induces cellular oxidative stress that can damage mitochondria (Saad et al., 2004). On the other hand, binding of cisplatin to other nucleophiles, e.g. glutathione or metallothionein, can contribute to the gain of cisplatin resistance.

Oxaliplatin is a platinum analogue and a component of the combination therapy, FOLFIRINOX. Unlike cisplatin, the platinum atom of oxaliplatin is surrounded by an oxalate group and 1,2-diaminocyclohexane (DACH) (Raymond et al., 1998). Oxaliplatin forms inter- and intra-strand DNA adducts similarly to cisplatin, but it remains active in cisplatin-resistant tumour by means of a mechanism that is not fully understood.

The application of gemcitabine and of the described platinum analogues combination therapy has given contradictory results since in some trials pancreatic cancer patients did not respond, whereas in others, the median overall survival (OS) was increased from 6 months to 7.5 (Heinemann et al., 2006; Louvet et al., 2005).

6. Taxanes

Taxanes, including paclitaxel and docetaxel, are anti-microtubule agents which suppress microtubule dynamics, eventually leading to the arrest of the cell cycle. Their mechanism of action includes prevention of microtubule rearrangement by binding to α-tubulin and formation of highly stable tubulin polymers that inhibit the function of the spindle during cell division, resulting in cell arrest (G2/M phase) and, ultimately, cell death (Fig. 2) (Abal et al., 2003). Until recently, delivery of these drugs had limitations associated with a poor solubility. Solvents such as Cremophor EL (CrEL) and polysorbate 80 (Tween 80) were utilised but exhibited drawbacks, due to a change of the disposition of intravenously administered drugs, hypersensitivity reactions and peripheral neurotoxicity (Gelderblom et al., 2001). Recently, a solvent-free nanoparticle albumin-bound (Nab) paclitaxel combined with gemcitabine (ABRAXANE) showed a significant increase in efficacy in the treatment of advanced PDAC patients (Hennenfent and Govindan, 2006; Von Hoff, Ervin, 2013).

In a phase III trial (n = 861), the ABRAXANE-gemcitabine group exhibited a median OS of 8.5 months compared to the 6.7 months of the gemcitabine group, and higher survival rates at all time points (Von Hoff et al., 2013). The superiority of this combination treatment to the single-agent therapy led to its approval by the FDA and its establishment as a first-line therapy for PDAC patients with dismal prognosis.

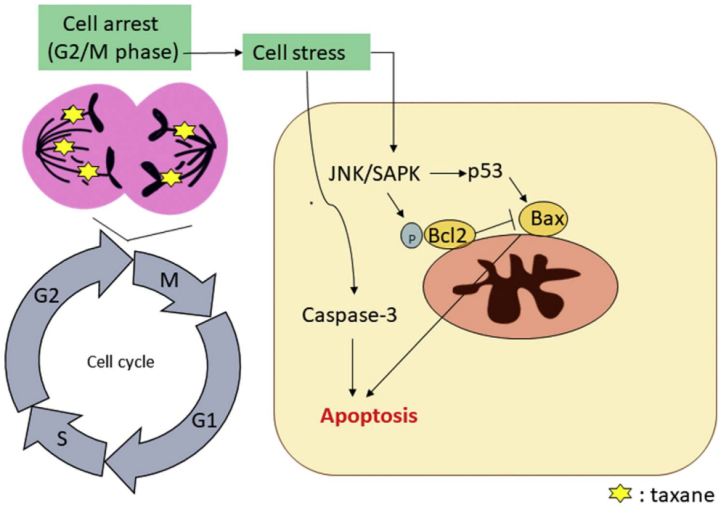

Fig. 2. Mechanistic representation of Taxane action. Taxane binds to α-tubulin and forms stable tubulin polymers during cell division. This activity results in inhibiting the function of the spindle and arresting G_2/M phase of cell cycle. The cell stress caused by the cell arrest allows JNK/SAPK phosphorates Bcl-2 which is an anti-apoptotic protein. The phosphorylated Bcl-2 is unlikely to bind to pro-apoptotic Bax protein, and then free Bax proteins can easily activate apoptosis. Moreover, cell stress induces high expression of p53 and caspase-3 which are related to apoptosis.

7. FOLFIRINOX

Recently, FOLFIRINOX has been used as an effective first-line therapy, especially for patients experiencing metastasis (Conroy et al., 2011). FOLFIRINOX is a combination of drugs that have proven their efficacy both individually and synergistically (Azrak et al., 2004; Ducreux et al., 2004; Ueno et al., 2007). It comprises of 5-Fluorouracil (a pyrimidine antagonist acting in a similar way with gemcitabine), Leucovorin (a folinic acid which acts to reduce its side effects), Oxaliplatin (a DNA repair inhibitor), and Irinotecan (a topoisomerase inhibitor which blocks DNA duplication).

In all three phases of clinical trials, FOLFIRINOX surpassed gemcitabine in all efficacy parameters, increasing OS of 1.6-fold, and the progression-free survival (PFS) and 1-year survival rate of almost 2-fold (Conroy et al., 2011; Ychou et al., 2003). However, due to the unfavourable safety profile of FOLFIRINOX (Gourgou-Bourgade et al., 2012), its use is constrained to patients under 75 years of age, and with a good PS; nevertheless, it remains a first-line option for advanced-stage patients.

8. Mechanism of acquired resistance to gemcitabine

High resistance to chemotherapy and delayed diagnosis make pancreatic cancer one of the most aggressive and malignant tumours. Mechanisms of acquired resistance can be attributed to genetic mutations arising from treatment, adaptive responses and natural selection of a drug-resistant tumour subpopulation. Gemcitabine is one of the most clinically utilised options for pancreatic cancer, and the acquired gemcitabine resistance has attracted recent interest in literature. An increased understanding of the molecular mechanisms involved in adaptive responses in pancreatic cancer during chemotherapy may facilitate the development of novel treatment strategies. In the sections below, we detail key intracellular processes and proteins involved in acquired chemoresistance to gemcitabine (Fig. 3).

9. Nucleoside transporters

Gemcitabine is a hydrophilic molecule, and its molecular targets are intracellular. Therefore, cellular uptake of gemcitabine requires specialized transporters to pass through the lipid bilayer of the plasma membrane.

Human equilibrative and concentrative nucleoside transporters (ENT, CNT) are two structurally unrelated families of proteins that mediate nucleoside transport through the plasma membrane (Griffiths et al., 1997a, 1997b). Several studies have examined the validity of using ENT1 as a biomarker to anticipate gemcitabine efficacy; however, the results are contradictory. Deficiency in hENT1 is associated with significant gemcitabine resistance in *vitro* (Mackey et al., 1998). *In vivo,* there have been reports indicating an association of better overall survival with the expression of hENT1 (Morinaga et al., 2012; Spratlin et al., 2004). On the contrary, in a

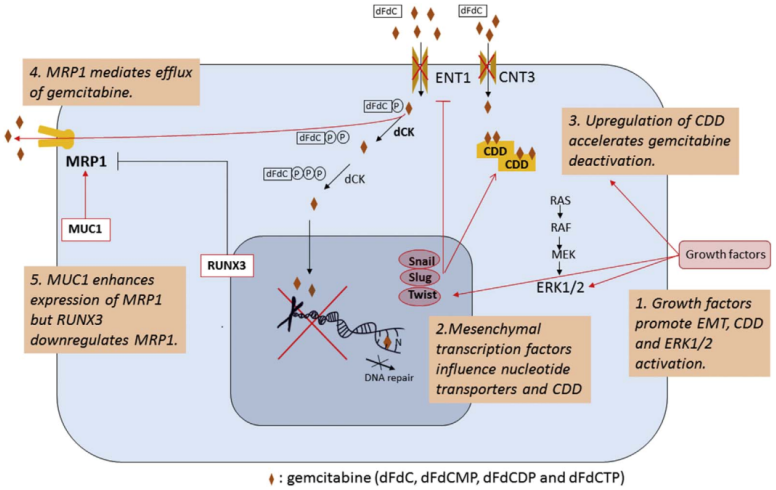

Fig. 3. Mechanistic representation of gemcitabine resistance pathway. Stroma or autocrine derived growth factors promote EMT, CDD upregulation and ERK1/2 activation. The mesenchymal transcription factors (e.g. snail, slug and twist) could influence resistance by targeting nucleotide transporters (e.g. ENT1 and CNT3) and CDD. CDD upregulation accelerates deactivation of gemcitabine. MRP1 mediates efflux of gemcitabine. MUC1 enhances gemcitabine resistance by bolstering expression of MRP1, while RUNX3 reduces resistance by downregulating MRP1 expression.

group of pancreatic cancer patients receiving neoadjuvant therapy including gemcitabine, a desirable prognosis appeared to be associated with hENT1 negative patients (Kawada et al., 2012). More recently, the results of the randomised clinical trial (CONKO-001) showed that high expression of ENT1 in the gemcitabine group was not associated with disease-free survival (Sinn et al., 2015).

10. Expression of ATP-binding cassette transporters

One of the principal mechanisms of chemoresistance in pancreatic cancer is the regulation of drug efflux. For instance, multidrug resistance-associated proteins (MRP)s are ATP-binding cassette (ABC) pumps that mediate the outflow of a range of chemotherapeutic agents, gemcitabine among others, thereby decreasing the intracellular drug concentration and contributing significantly to resistance. The normal function of these proteins is to mediate transport of amphiphilic anions which are overexpressed in some types of cancer (reviewed in (Borst et al., 2000; Deeley and Cole, 1997)), including pancreatic cancer (Miller et al., 1996). The discovery of MRP1-dependent chemotherapy resistance has resulted in the development of MRP1 as a drug target. Studies indicated inhibitors of MRP1 could significantly elevate the intracellular accumulation of chemotherapeutic drugs (Zhang et al., 2015).

The control of MRP1 expression in cancer has been examined in several basic studies with promising results. For instance, expression of runt-related transcription factor 3 (RUNX3), a tumour suppressor gene, in a group of pancreatic cancer cell lines resulted in enhanced gemcitabine sensitivity by reducing MRP1 expression (Horiguchi et al., 2013). Undoubtedly, further examination of the influx/efflux protein regulation pathway is required in order to better utilise them as anti-cancer targets.

11. Nucleoside enzymes

Different enzymes participating in the regulation of nucleosides metabolism are directly involved in gemcitabine resistance. For instance, deoxycytidine kinase (dCK) phosphorylates and activates gemcitabine once it reaches the cytoplasm, and low levels of this enzyme correlate with significantly decreased OS (Marechal et al., 2010). The levels of cytidine deaminase (CDD), the enzyme that catalyses the metabolic inactivation of gemcitabine, were also found to correlate with clinical outcome (ref PMID: 21652582). In addition, the enzyme ribonucleotide reductase (RRM1 and RRM2), that catalyses the conversion of ribonucleotides to deoxynucleosides and therefore has a key role in DNA synthesis, is also associated with gemcitabine resistance and its high levels correlate with worse prognosis (Aye et al., 2015).

12. Gemcitabine resistance mediated by epithelial-mesenchymal transition (EMT)

EMT is a stage of phenotypic alterations in cancer cells that favours a more mesenchymal phenotype with enhanced invasive properties. The process is characterised by a morphological transformation in cancer cells as well as by changes at the genomic and

protein level (Huber et al., 2005; Zavadil et al., 2008). However, a possible role of the mesenchymal transcriptional factors (e.g., Snail, Slug, and Zinc finger E-box-binding homeobox 1 - Zeb1) in chemotherapy resistance has recently attracted more attention. Inactivation of *Zeb1* results in cellular plasticity and stemness abrogation, reduction of the number of genes related to TGF-β signalling and metabolic changes which affect the capacity of cancer cells to colonize distant organs (Krebs et al., 2017). The knockdown of slug resulted in an enhanced sensitivity of $CD133^+$ pancreatic cancer cells to gemcitabine (Tsukasa et al., 2015). Interestingly, EMT has been found to impart gemcitabine resistance in pancreatic cancer mouse models (Zheng et al., 2015). Moreover, tumour microenvironment is enriched with mediators of EMT, including cytokines (e.g. TGF-β1), growth factors (e.g. EGF kinase receptor) and hypoxia (Halder et al., 2005; Jiang et al., 2011; Lo et al., 2007). Nevertheless, the molecular signalling that links mesenchymal transcriptional factors (e.g., snail and slug) to gemcitabine resistance in pancreatic cancer has yet to be fully identified.

13. Gemcitabine resistance mediated by ERK

Extracellular regulated kinase (Erk) is a critical effector of mitogen-activated protein kinases (MAPK) that transduces several cellular signalling stimuli. Tumour microenvironment contains a plethora of mediators that strengthen chemotherapy resistance by activating Erk-dependent pro-survival pathways in cancer cells. Therefore, Erk1/2 hyperactivity can contribute to gemcitabine resistance in pancreatic cancer. Activation of Erk1/2 triggers upregulation of the pro-survival Bcl2 proteins and downregulation of the pro-apoptotic Bax proteins, leading to resistance of gemcitabine-mediated apoptosis (Wang et al., 2015). The anti-proliferative activity of gemcitabine on a panel of pancreatic cancer cell lines was enhanced by the addition of Erk1/2 activation inhibitor (U0126) (Zheng et al., 2013). Moreover, gefitinib, a tyrosine kinase inhibitor, restored gemcitabine sensitivity by inhibiting Erk activation and reversed the gemcitabine enhanced expression of multidrug resistance proteins (e.g., MRP1) in BxPC3 pancreatic cancer cells (Xiao et al., 2012). On the contrary, CXCL-12, a chemokine released by pancreatic cancer stromal cells, has been shown to potentiate gemcitabine resistance via activation of several survival pathways including Erk (Singh et al., 2010).

14. Microenvironmental factors affecting chemoresistance

The tumour microenvironment also contributes significantly to the chemotherapy resistance process and cancer relapse. Desmoplasia is the main characteristic in PDAC, and its main components are cancer-associated fibroblasts (CAFs) which originate from pancreatic stellate cells (PSCs), as well as endothelial and inflammatory cells (Nielsen et al., 2016). Interestingly, the role of the desmoplastic stroma in PDAC is controversial. On the one hand, it prevents the chemotherapeutic drugs from being effective by reducing tumour perfusion, but on the other hand, it possibly averts PDAC cells from penetrating the surrounding tissue and thus metastasizing (Özdemir Berna et al., 2014).

14.1. Pancreatic stellate cells

Pancreatic stellate cells (PSCs) are an important stromal component in PDAC (Jaster, 2004), as well as a representative key player in chronic pancreatitis (Sönke et al., 2006). They are activated in the region surrounding defective cells, as a result of innate defence of the stromal cells to encapsulate the damage and restrain its spread. In turn, activated PSCs start expressing extracellular matrix (ECM) proteins which initiate hypoxia and fibrosis within the stroma (Fig. 4). The barrier that is gradually being created can affect the genetic stability of the neighbouring normal cells and, subsequently, aggressive and chemoresistant cells are positively selected (Erkan, 2013). Cancer initiation or tissue injury can activate quiescent PSCs through secreted factors such as the tumour necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), interleukins 1, 2, 10 (IL1, IL2, IL10), and the platelet-derived growth factor (PDGF). After activation, PSCs can develop autocrine signalling by producing PDGF and TGFβ themselves (Nielsen et al., 2016). Apart from conferring resistance, this cell type can also promote pre-metastatic niche formation, since it was found to co-metastasize with cancer cells. Interestingly, PSCs seem to play a role in immune surveillance escape by secreting galectin1, which can block T cell activation and induce their death (Tang et al., 2012). Moreover, higher deposition of gemcitabine within the tumour can be achieved by the Hedgehog pathway inhibition in PSCs (Olive et al., 2009).

14.2. Tumour-initiating cells

Within a heterogeneous tumour, the highly proliferative cell populations that can survive intense chemotherapy or radiation and give rise to the tumour from which they were derived are called tumour-initiating cells (TICs); a term sometimes used interchangeably with cancer stem cells (CSCs) (Amp et al., 2009). Pancreatic TICs show all the properties of normal stem cells and are also implicated in many aspects of pancreatic cancer, including drug resistance (Fitzgerald et al., 2015; Fitzgerald and McCubrey, 2014). The actual mechanism through which tumour-initiating cells support chemoresistance in PDAC has not been fully elucidated, however the existence of hypoxic regions enriched in $CD133^+$ cells expressing increased levels of ABC transporters and anti-apoptotic genes such as Bcl-2 has been recently revealed (Banerjee et al., 2014).

14.3. Fibroblasts

Cancer-related fibroblasts (CAFs) and PDAC are inextricably linked in aspects of differentiation and proliferation. Conditioned media from PDAC cells has been shown to trigger extracellular matrix (ECM proteins) production from PSCs, and conditioned media

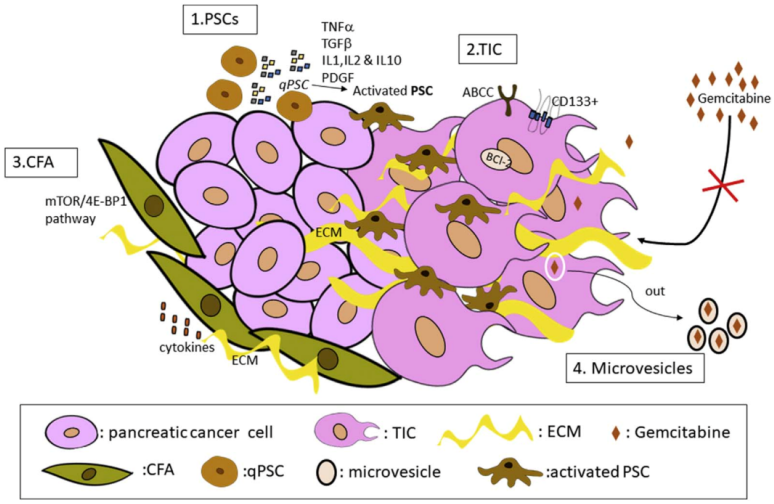

Fig. 4. Major factors contributing to the development of chemoresistance in pancreatic cancer. Pancreatic stellate cells (PSCs) are an important stromal component in PDAC with a possible dual role in neoplastic lesions. PSCs are initially activated in the region surrounding any defective cells in order to encapsulate them and restrain the damage from spreading. However, later on they initiate hypoxia and fibrosis within the stroma and they gradually create a barrier (desmoplasia), which at the end plays a key role in chemoresistance. When quiescent, they store vitamin A and produce matrix metalloproteinases (MMPs), and upon activation resulting from cancer initiation or tissue injury, they start expressing α-SMA, gain a myofibroblast-like phenotype (cancer-associated fibroblasts- CAFs) and lose the capacity of storing vitamin A. Factors contributing to qPSCs activation include tumour necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), interleukins 1, 6, 10 (IL1, IL2, IL10), and platelet-derived growth factor (PDGF). Moreover, a subpopulation of CD133 + cells within the tumour itself, with increased expression of ABC transporters and anti-apoptotic genes such as Bcl-2, have been shown to confer chemoresistance in PDAC. Last but not least, pancreatic cancer cells -derived microvesicles (MVs) are able to eliminate drugs such as gemcitabine (GEM) both from the intracellular space as well as the microenvironment. Based on their protein composition, MVs can either trap the drug or allow it to return to the microenvironment, depending on whether the cells of origin are GEM-resistant or GEM-sensitive, respectively.

from CAFs can promote PDAC cell proliferation, migration and chemo- and radiation-resistance (Bachem et al., 2005; Hwang et al., 2008). The alpha-smooth muscle actin (αSMA)-positive CAFs harbour a highly active mTOR/4E-BP1 pathway which, once inhibited, can reduce chemoresistance. The SOM230 analogue Pasireotide targets the sst1 somatostatin receptor expressed by CAFs and abrogates the mTOR pathway, while at the same time it has translational effects such as cessation of IL-6 protein synthesis. Co-administration of SOM230 and gemcitabine in mouse xenografts resulted in reduced tumour growth and chemoresistance, decreased fibrosis and increased gemcitabine-induced cell death (Duluc et al., 2015). This knowledge is of great importance since very little is known about translation and translational regulation in CAFs. The initiation and rate-limiting steps are known to be linked to elF2α, and PI3K/mTORC1 pathways (Baer et al., 2015; Martineau et al., 2012) and more than 50% of PDAC cases are non-responsive to mTOR inhibitors due to loss of 4E-BP1 expression, which negatively regulates translation (Martineau et al., 2013). IL-6 is identified as an important CAF-derived component as it is considered to be a chemoresistance mediator in pancreatic cancer cells (Lesina et al., 2014). In fact, its concentration in serum from patients is used as a marker for gemcitabine treatment efficacy (Mitsunaga et al., 2013). Another study revealed that when pancreatic ductal epithelial cells (H6c7) and chemosensitive adenocarcinoma cells (T3M4) were co-cultured with PDAC stroma-derived myofibroblasts, they acquired a chemoresistant phenotype and underwent a series of intracellular changes, including decreased levels of caspase expression and the transcription factor STAT1 (Müerköster et al., 2008). Following treatment with 5-azadeoxycytidine – a DNA methylation inhibitor, chemosensitivity was restored, and caspase and STAT1 levels were increased, pointing out a link between epigenetic modification, caspase apoptotic pathway and chemoresistance. Recently, an interesting study has given a different perspective in the field of CAFs research, with its novelty being CAF heterogeneity within the stroma. More specifically, two topologically distinct CAF subtypes, which exclude each other and are reversible, were identified and termed myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs). The first group is located in a site distant to the cancer cells, is triggered in a paracrine signalling manner and expresses low αSMA and high IL6 levels, whereas the second group is found juxtacrine to the cancer cells and contrary to the iCAFs, it expresses high αSMA and low IL6 levels. The detection of these populations might be the answer to conflicting studies on CAFs, and is certainly going to draw attention to the development of therapies that will take into account both the anti- and pro-tumorigenic capabilities of CAFs (Ohlund et al., 2017).

14.4. Microvesicles

There is an increasing amount of interest in regard to extracellular vesicles (EVs) and their role in tumour growth and metastasis. EVs are microparticles with a lipid bilayer membrane, secreted from all cell types in physiological as well as pathological conditions. It is known that microvesicles (MVs) promote chemoresistance by mediating the cross-talk between drug-resistant and drug-sensitive cancer cells, enabling the exchange of drug transporter proteins between them (Bebawy et al., 2009). Lately, it has been reported that pancreatic cancer cells use MVs to efflux gemcitabine and eliminate it from the microenvironment. More specifically, experiments carried out in a panel of human pancreatic cancer cell lines with different levels of gemcitabine revealed that the degree of resistance was proportionate to the amount of MV released, and retention of MV release in resistant cells resulted in them being re-sensitised both *in vitro* and *in vivo*. Interestingly, MVs were able to eliminate drugs both from the intracellular space as well as the microenvironment, and it was gemcitabine that regulated the influx (e.g. ENT1) and efflux (e.g. MRP5, P-gp) of the protein amount present in the vesicles. Based on their protein composition, the MVs could either trap the drug or allow it to return to the microenvironment, depending on whether the cells of origin were drug-resistant or sensitive, respectively (Muralidharan-Chari et al., 2016). According to the latest studies, CAFs that underwent chemotherapy released a high amount of exosomes containing Snail mRNA as well as miR-146a, which once transferred in the recipient cells, increased their chemoresistance as well as proliferation rate. Pharmacological inhibition of exosome release from CAFs reversed this phenomenon, underscoring the significance of these MVs in pancreatic cancer chemoresistance (Richards et al., 2017).

Another research group pointed out the significance of exosomal microRNAs in gemcitabine resistance in PDAC. Prolonged gemcitabine treatment was found to cause an increase in the intracellular levels of microRNA-155, which affected both the levels of exosomes shed by cancer cells as well as their anti-apoptotic activity. Exosomes would, in turn, deliver the specific microRNA in other cells, starting a new cycle. Nevertheless, chemosensitivity was restored by either attenuation of the exosome secretion or the targeting of microRNA-155 (Mikamori et al., 2017).

15. Immune cells and the microenvironment

The innate immune cell population is another significant component of the tumour microenvironment and there have been cases where rather than protecting the host with anti-tumour responses, innate cells promote tumour progression, metastasis and chemoresistance (Grivennikov et al., 2010). Infiltrating immune cells in the tumour microenvironment include macrophages, neutrophils, lymphocytes, and myeloid-derived suppressor cells and platelets. These immune cells may contribute to the various potential mechanisms of drug resistance such as induction of EMT (Zheng et al., 2015), deregulation of key pathways (e.g. apoptotic pathways) and the establishment of cancer stem cells (Long et al., 2011).

15.1. Tumour-associated macrophages

The most abundant immune cells in the tumour microenvironment of many solid tumours, including pancreatic tumours, are the differentiated tumour-associated macrophages (TAMs), which have acquired a tumour-promoting phenotype, augmenting tumour growth, angiogenesis, remodelling of the matrix and suppressing immune responses (Mantovani et al., 2006). In pancreatic cancer, macrophages are implicated in resistance to gemcitabine through the upregulation of cytidine deaminase, an enzyme that metabolises gemcitabine to an inactive form (Weizman et al., 2014). In addition, TAMs were found to suppress anti-tumour immune responses. During the administration of gemcitabine, increased infiltration of anti-tumour $CD8^+$ T cells was achieved by blocking the recruitment of TAMs to the tumour site by targeting CCR2 or CSF1R (Mitchem et al., 2013). Furthermore, TAMs also enhanced STAT3 activation, a mediator of immune suppression, which resulted in increased tumour-initiating capacity of pancreatic tumour cells (Mitchem et al., 2013). More recent studies have revealed that TAMs together with activated myofibroblasts are responsible for the production of most of the IGF in the pancreatic cancer microenvironment *in vivo*, whereas almost 25% of pancreatic cancer patients are found to have an activated insulin/IGF1R pathway. Inhibition of this pathway would promote chemosensitivity to gemcitabine in this type of cancer and in fact, specific IGF-blocking antibodies (BI 836845, MEDI-573) being tested in phase II clinical trials, have shown to successfully block proliferation via the insulin and IGF1 receptors. Nevertheless, it is important to mention that TAMs can polarize into either an M2 subtype which acts in favour of the tumour, or into an M1 subtype which exerts anti-tumorigenic properties. This “switching” ability can be exploited and applied in therapies targeting TAMs reprogramming or inhibition of the M2 subtype (Ireland et al., 2016).

15.2. Tumour-associated neutrophils

More recently, the polarisation of neutrophils in tumours has been described where, similarly to macrophages, they polarise to a pro-tumour or anti-tumour phenotype depending on the chemokine milieu of the microenvironment (Fridlender et al., 2009; Granot and Jablonska, 2015). In pancreatic cancer patients, a high ratio of infiltrating neutrophils to lymphocytes is associated with poor prognosis (Templeton et al., 2014). Tumour cells can also prime neutrophils to release their granules containing proteinases such as neutrophil elastase and metalloproteinase-9, to exert pro-tumorigenic effects. Neutrophil elastase induced degradation of E-cadherin *in vivo* in pancreatic cancer cell lines and upregulated EMT markers, ZEB1 expression and the translocation of β-catenin to the nucleus (Grosse-Steffen et al., 2012). Furthermore, neutrophil extracellular traps (NETs) released from activated neutrophils are implicated in cancer-associated thrombosis (Abdol Razak et al., 2017; Demers et al., 2012; Thomas et al., 2015).

15.3. Platelets

In recent years, the role of platelets in tumour development has expanded considerably. In 2011, Labelle and colleagues showed that the interaction between cancer cells and platelets led to tumour EMT and increased metastasis (Labelle et al., 2011). Thromboembolism is a major complication for pancreatic cancer sufferers, indicating the crucial role of platelets and the coagulation system. Although the exact part platelets play, if any, in pancreatic cancer chemoresistance is not well understood, certain means by which they can sustain it have been identified, and it is interesting that an over-the-counter drug, aspirin, has been shown to positively affect-to some extent-individuals suffering from familial adenomatous polyposis, gastrointestinal cancer, whereas platelet targeted therapy seems to be beneficial in cancer cases with high thrombotic risk (Elaskalani et al., 2017a). A study showed that pancreatic cancer cell lines PANC-1 and BxPC-3, had reduced sensitivity to cisplatin when treated with activated platelets. The chemoresistant effect was found to be mediated by platelet-derived TGF-β1 (Chen et al., 2013). We have recently identified a potential role played by platelets in the association between the development of chemoresistance and EMT (Elaskalani et al., 2017b). Indeed, we found that platelets regulate the expression of hENT1 and CDD in pancreatic cancer cells. In turn, the expression of hENT1 and CDD is regulated by Slug, a mesenchymal transcriptional factor known to be upregulated during EMT. Moreover, we demonstrated that platelet-derived ADP and ATP regulate Slug and CDD expression.

16. Conclusions and future directions

The grim fact about pancreatic cancer is that in a few years it is expected to become the second leading cause of cancer death in the Western world, and by 2030 it will surpass liver, colon, lung and prostate cancers. This is due to both the increase in the incidence of pancreatic cancer and the improvement in the outcomes of other cancers. The persistent intractability of this cancer is demonstrated by the fact that survival rates have not changed in the last 40 years. One of the major challenges that we face in pancreatic cancer is to overcome its resistance to drug treatments. During the last decade, a better comprehension of these mechanisms has promoted our understanding of this grievous disease. The development of treatments currently used in therapy, such as Abraxane, can be seen as the product of this effort. Yet, major improvements are needed, and the strategy to selectively target elements of the tumour microenvironment is promising and may lead to major advancements.

Conflict-of-interest statement

Authors declare no conflict of interests for this article.

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1.1.7 Current pancreatic cancer research and future directions

The content of this section includes the published review article:

Falasca, Marco, Minkyoung Kim, and Ilaria Casari. "Pancreatic cancer: Current research and future directions." *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 1865.2 (2016): 123-132. doi.org/10.1016/j.bbcan.2016.01.001

Synopsis and significance of this review article

Although diverse combination therapies have been tested, including gemcitabine in combination with erlotinib, or Abraxane and FOLFIRINOX, trying to improve the survival rate of pancreatic cancer patients, this disease remains a huge challenge for oncologists because of their limited efficacy. Therefore, a better understanding of pancreatic cancer biology will provide new insight for better PDAC therapeutic strategies. In this review, the major biological features of pancreatic cancer, which are the main cause of pancreatic tumour heterogeneity and pancreatic cell plasticity, are described: 1) pancreatic tumour stroma, 2) tumour metabolism, and 3) microRNAs and exosomes.

Stroma cells surrounding pancreatic cancer have been considered to have tumour-promoting roles, but recent studies revealed the existence of a tumour-suppressor role for PDAC stroma. Current research on the anti-tumour role of stromal cells, such as myofibroblast and immune cells, have indicated a protective role of the Hh signalling pathway using the ligand SHh, αSMA myofibroblasts and a Hh inhibitor, as reported in this publication. Therefore, this review emphasizes that targeting pancreatic stroma as a new therapeutic target of PDAC requires careful consideration of this paradoxical role of PDAC stroma. In addition, this review explains in detail the metabolic changes of PDAC, showing not only the increased glucose and glutamate metabolism but also the enhanced lipid metabolism. Important recent studies showing the overexpression of fatty acid synthase and the low-density lipoprotein receptor in pancreatic tumours, the elevated requirement of cholesterol and the utilizing circulating lipids by pancreatic cancer cells, were stressed in this publication. Lastly, we presented emerging opportunities using micro RNAs and pancreatic cancer-derived exosomes for the potential target of PDAC, especially for future diagnostic tools. Collectively, the latest discoveries and advancements in pancreatic cancer we have described in this review help understanding pancreatic cancer biology for the selection of better therapeutic targets in future research on PDAC.

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Review

Pancreatic cancer: Current research and future directions

Marco Falasca *, Minkyoung Kim, Ilaria Casari

Metabolic Signalling Group, School of Biomedical Sciences, CHIRI Biosciences, Curtin University, Perth, Western Australia, Australia

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ABSTRACT

Despite the survival rate advancements in different types of cancer in the last 40 years, the perspective for pancreatic cancer patients has seen no substantial changes. Indeed, the five year survival rate remains around 5%. Nevertheless, in the last decade we have witnessed an increased interest in pancreatic cancer biology and this has produced a substantial increment in our knowledge on pancreatic cancer progression. The big challenge is now to translate this knowledge in better outcomes for patients. The aim of this review is to describe the latest discoveries and advancements in pancreatic cancer research and to discuss future directions.

Contents

1. Introduction

Pancreatic cancer is a deadly disease, mainly because it is generally discovered very late and it is very resistant to chemotherapy and radiation therapy [1]. The most common type of pancreatic cancer (over 90%) develops from the exocrine cells of the pancreas and is named pancreatic ductal adenocarcinoma (PDAC) [2]. There are very few treatments currently available, mostly just palliative and with several side effects [3]. Since its first clinical demonstration of efficacy in 1997, gemcitabine represented for more than a decade the first-line PDAC treatment and drug of reference [1–3]. All attempts to increase the efficacy of gemcitabine with combination therapy have produced at best marginal improvements in survival, as it is the case of its combination with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib [4]. Nevertheless, very recent approaches such as polychemotherapy, or strategies leading to improved efficacy of gemcitabine, have produced some substantial improvements [5]. Indeed in 2011, phase III trial data concerning the use in advanced pancreatic cancer of a combination of folinic acid, fluorouracil, irinotecan and oxilplatin (FOLFIRINOX) showed the longest survival improvement, around 4 months, compared to gemcitabine used as a single agent (6 months survival) [5]. Consequently, despite the fact that the side effects of such an aggressive combination make this

* Corresponding author at: Metabolic Signalling Group, School of Biomedical Sciences, Faculty of Health Sciences, Curtin University, Australia.
E-mail address: marco.falasca@curtin.edu.au (M. Falasca).
URL: http://curtin.edu.au (M. Falasca).

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regimen impractical in the majority of PDAC patients, FOLFIRINOX is currently an accepted standard of care for approximately 30–40% of PDAC patients [3]. Subsequently in 2013, the use of protein-bound paclitaxel (nab-paclitaxel or Abraxane) has shown better survival rates compared to gemcitabine, suggesting that PDAC is not a chemo-resistant disease and can be effectively tackled by chemotherapy. [6]. However, although both FOLFIRINOX and Abraxane are the standards of care for metastatic disease, their efficacy is limited, often leading to an improvement of quality of life rather than an effective cure of the disease. Therefore, it is imperative to find new therapeutic strategies and valid pharmacological targets to improve the grim survival prospect that oncologists have to offer to pancreatic cancer patients. The identification of molecules and proteins involved in pancreatic cancer development and progression is critical to discover novel potential targets and to develop novel and more active drugs. Here, we provide an overview of recent advancement on pancreatic cancer research that gives us a better insight in the peculiar heterogeneity and cell plasticity of this type of cancer. In this paper, that is not a comprehensive study of current research on PDAC, we review the most salient biological features of pancreatic cancer with a focus on pancreatic tumour stroma, tumour metabolism, microRNAs and exosomes.

2. Tumour heterogeneity and cancer cell plasticity

Over the last few years, there has been an increasing number of studies focused on the understanding of the molecular and biological nature of pancreatic cancer. In particular, this increased interest has led to important advances in the understanding of the genomic complexity of the disease, the importance of the tumour microenvironment, and the peculiar metabolic adaptation of pancreatic cancer cells to obtain nutrients in a hostile environment. These studies have contributed to identify the characteristics of pancreatic cancer and underlined that its two main features, although not unique to this type of cancer, are high tumour heterogeneity and elevated cancer cell plasticity. Pancreatic cancer heterogeneity can be both phenotypic and functional and can arise either among cancer cells within the same tumour or among individual PDAC tumours, making difficult any classification and identification of common therapeutic strategies. This heterogeneity is a consequence of genetic changes, a different environment and changes in cell characteristics.

Several recent studies have provided an extensive and comprehensive genetic analysis of pancreatic cancer and contributed to design the genetic landscape of pancreatic disease substantiating the concept that this is a genetic disease [7–9]. Indeed, pancreatic cancer is characterized by the successive accumulation of mutations in key oncogenes and tumour suppressor genes [10]. Once established this heterogeneous and genetically unstable disease reveals the complexity of its nature [10]. The most common genetic alteration in PDAC is the oncogene Ras that is mutated in more than 90% of tumours [11]. Similarly, around 95% of tumours have inactivation of the CDKN2 gene that encodes the p16 protein, a regulator of G1-S transition of the cell cycle [12]. Another frequent genetic modification is reported on the p53 gene that is altered in around 75% of patients [13]. Other frequently mutated or lost genes include the *SMAD4* gene (DPC4 or SMaD4) which is deleted in pancreatic carcinoma [14].

Additional heterogeneity to pancreatic cancer is given by the presence of different cell compartments. Indeed, while the bulk of the tumour is formed by "normal" cancer cells, a minority of cells possess stem cell characteristics that make this cell type potentially resistant to chemo- and radiotherapy [15]. Additional complexity and heterogeneity are provided by a dense and desmoplastic stroma composed of fibrillar elements, such as collagen and activated fibroblasts [16].

Furthermore, the complex and heterogeneous nature of pancreatic cancer is confirmed by the fact that not all pancreatic tumours have alterations in all pathways, and the key mutations in each pathway appear to differ from one cancer to another. Indeed, a genomic analysis of 24 pancreatic cancers revealed the existence of 63 genetic alterations that affect at least 12 distinct signalling pathways [7]. More recent genomic analyses of PDAC have further increased the number of genomic alteration [8,9]. The complex heterogeneity of genetic alterations in PDAC may explain why the targeted therapy is failing in PDAC. Indeed, apart from a marginal increase in survival rate in the gemcitabine plus erlotinib association, all other combinations designed to target different key signalling pathways have failed [1–4]. In addition, recent work identified a by-pass mechanism of oncogene addiction in PDAC. Indeed, it has been shown that PDAC tumour cells can survive in the absence of oncogenic Kras, and acquire alternative growth mechanisms signals involving the Yap1 oncogene [17,18].

The definition "cancer cell plasticity" refers to the extreme ability possessed by cancer cell in adopting a cellular phenotype that better adapts to a hostile environment. An example of tumour plasticity is the ability of cancer cells to undergo an epithelial to mesenchymal transition (EMT) that confers to cells a phenotype characterized by an increased motile and invasive capacity as well as a higher resistance to apoptosis [19]. Similar to normal epithelial cells during embryonic development, cancer cells can revert back to an epithelial phenotype, in precise conditions, such as after the invasion of tissues and spread to a secondary site. This process is called the mesenchymal to epithelial transition (MET). According to a recent model proposed for pancreatic cancer progression, the seeding of distant organs is a very early event and it occurs in parallel to tumour formation at the primary site [20]. This is in agreement with clinical evidence since the majority of PDAC patients have metastatic disease at the time of diagnosis [21]. Recent work has shown that EMT and stemness acquisition are tightly regulated by a hierarchical signalling network involving two antagonistic pathways, NFATc1-Sox2 and p53-miRNA200c [22]. Therefore, the molecular decision between epithelial cell preservation and conversion into a dedifferentiated cancer stem cell-like phenotype is made at the level of p53 and NFATc1 signalling activity. The plasticity to switch from one phenotype to another is determined not only by the genetic features of the cancer cells but also by the local microenvironment in the secondary site. Another example of cancer adaptability is the metabolic plasticity, a feature that refers to the adaptation of cancer cells to different environmental conditions and their ability to switch from one metabolic phenotype to another depending on nutrient availability and hostile environments [23]. A growing evidence points to a more active function of tumour metabolism, according to which the metabolic reprogramming of cancer cells is not a simple consequence of neoplastic transformation but a key driver in cancer progression. Both concepts of tumour heterogeneity and cancer cell plasticity are quintessential characteristics of PDAC tumours and are responsible for the peculiar aggressive nature of this tumour type. In addition, heterogeneity and plasticity are traits applicable to stroma, metabolism and microRNA, underlining that all these processes are tightly interconnected and so contributing to the intrinsic aggressive nature of pancreatic cancer. Given the failure of targeted therapy in PDAC, the focus is now on targeting more broad critical players of physiological functions as a more effective therapeutic strategy. Therefore, any attempt to identify a particular target in the tumour stroma or metabolism should consider the concept of cancer plasticity and the changeable nature of PDAC cells.

3. Stroma in pancreatic cancer

The malignant progression of PDAC is characterized by its diffuse fibrotic stroma (desmoplasia) that is composed of activated fibroblast, also known as pancreatic stellate cells, infiltrating immune cells, blood vessels and extracellular proteins (Fig. 1) [24]. Together with matricellular proteins such as collagen and fibronectin, several growth factors are released in the tumour microenvironment, such as transforming growth factor-β (TGFβ-1), platelet derived growth

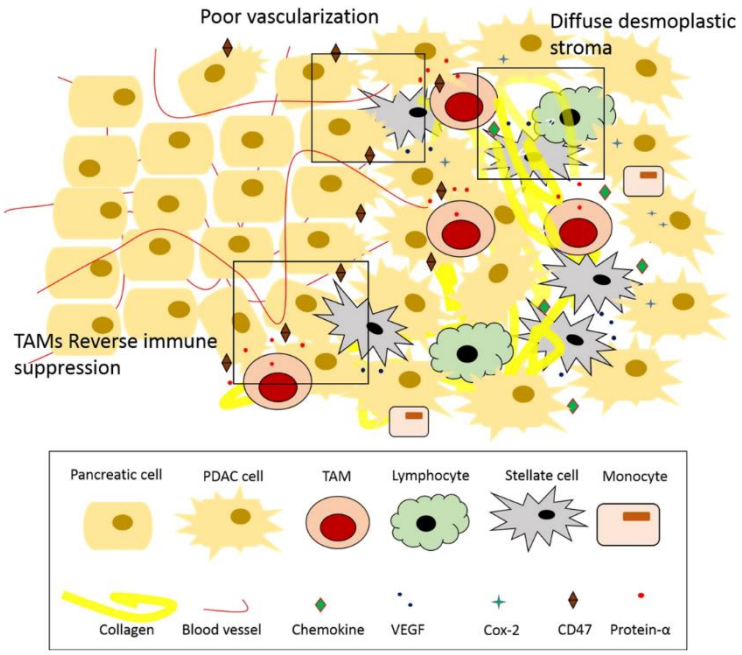

Fig. 1. Stroma in pancreatic cancer. Diffuse desmoplastic stroma in PDAC is composited by the activated stellate cells and lymphocytes. The stromal cells such as stellate cells, lymphocytes, macrophage and monocytes secrets multiple proteins including collagen, chemokine, vascular endothelial growth factors (VEGF), and cox-2. Poor vascularization which is one of characteristics of PDAC is derived by the stromal cells, especially the pancreatic stellate cells. Tumour-associated macrophages (TAM) express protein-α and cross-talk with the "don't eat me" signal CD47 on cancer cell.

factor (PDGF), fibroblast growth factor (FGF) vascular endothelial growth factor (VEGF) and connective tissue growth factor (CNGF).

The pancreatic stellate cells are activated by growth factors and play a critical role in the formation and turnover of the stroma [25]. Indeed, these specialized fibroblasts secrete both collagen and other components of the extracellular matrix and matrix-metalloproteinases. In addition, stellate cells also appear to be responsible for the hypovascularity that characterizes pancreatic cancer.

3.1. Tumour-promoting role of PDAC stroma

In the last 10 years the tumour microenvironment, for a long time considered a mechanical barrier or passive bystander, has been shown to be critically involved in tumour initiation, progression, invasion, and metastasis [26]. Stromal cells express multiple proteins such as Cox-2, PDGF receptor, vascular endothelial growth factors (VEGF), stromal derived factor (SDF), chemokines, integrins, SPaRC (secreted protein-acid rich in cysteine), and hedgehog (Hh) pathway elements, which have been associated with worse prognosis and resistance to treatment. Indeed, one of the main hypotheses proposed in the last few years is that the tumour microenvironment could play a role in therapeutic resistance. According to this hypothesis, the stiffness of the extracellular matrix impairs blood vessel perfusion and ultimately represents a barrier to drug delivery to cancer cells [27].

This hypothesis has been supported by preclinical studies using genetically engineered mouse models of PDAC that have shown that one of the characteristics of PDAC is its poor vascularization. Subsequent preclinical investigations introduced approaches to disrupt the stroma with agents such as inhibitors of the Hh. Work conducted by Olive et al. demonstrated that pharmacological inhibition of the Hh signalling resulted in increased vascular perfusion and elevated gemcitabine delivery [28]. Unfortunately, the clinical development of Hh inhibitors in PDAC has not been, for reasons that are not yet fully understood, too promising so far [29].

One of the characteristics of the pancreatic cancer stroma is that it contributes to create immunosuppressive tumour microenvironments that can restrain antitumour immunity. There has been an interest in reversing this phenomenon therapeutically. It is well known that CD40 activation can reverse immune suppression and drive antitumour T-cell responses. Clinical studies have tested the combination of an agonist CD40 antibody with gemcitabine chemotherapy in patients with surgically incurable PDAC and have shown antitumour activity [30]. Mechanistic studies showed that CD40 antibody works by stimulating the infiltration of tumour macrophages that deplete the cancer stroma [31]. It is well-known that PDAC is strongly promoted by tumour-associated macrophages (TAM) that are clinically associated with poor outcome. The "don't eat me" signal CD47 on cancer cells cross-talks with the signal regulatory protein-α expressed on macrophages, preventing their phagocytosis [32]. Therefore, CD47 inhibition has been proposed as a new therapeutic strategy able to induce TAMs action against PDAC cells, in particular cancer stem cells or tumour-initiating cells (TIC). Interestingly, CD47 was highly expressed on TICs compared to other non-malignant cells in the pancreas [32]. Indeed,

long-term inhibition of CD47 has been shown to induce not only phagocytosis of a set of primary human pancreatic TIC, but also direct induction of their apoptosis in the absence of macrophages. In patient-derived xenograft models, CD47 targeting alone did not result in a significant reduction of tumour growth, but the combination of chemotherapeutics such as gemcitabine and Abraxane resulted in a sustained tumour regression and in the prevention of disease relapse long after discontinuation of treatment.

3.2. Tumour-suppressor role of PDAC stroma

In contrast to the accumulating evidence suggesting that PDAC stroma acts as a tumour-promoting factor, other studies have supported a tumour-suppressor function for PDAC stroma in pancreatic cancer. Indeed, tumour stroma can have a dual nature and it may represent the host response to a tumour and a physical constrain to tumour spread. Several studies have shown that stromal cells, primarily myofibroblasts and immune cells, play a tumour-suppressor role in pancreatic cancer development and progression. For instance, activated proinflammatory macrophages (M1 phenotype) have been shown to possess an antitumour role and to contribute to the depletion of the tumour stroma. Similarly, stromal cells derived from human adipose tissue induce reduced pancreatic cancer growth *in vitro* and *in vivo*. Notably, two recent studies have shaken the general assumption that targeting tumour stroma is a valid strategy in PDAC therapy [33].

The Hh signalling pathway plays a key role in stromal desmoplasia in several tumours. The soluble ligand sonic hedgehog (Shh) is over-expressed in PDAC cells and drives the formation of a desmoplastic stroma enriched in fibroblasts. In order to define its role in stroma formation, Shh has been conditionally deleted in KPC mice, a well-defined pancreatic cancer model [34]. Surprisingly, while as expected the stromal content of Shh-deficient tumours was reduced, these tumours presented a more aggressive phenotype with increased proliferation rate, vascularity and undifferentiated histology. The same aggressive phenotype was recapitulated using a smoothened inhibitor in control mice. Noteworthy, treatment of Shh-deficient tumour with VEGFR blocking antibody improved survival of mice, suggesting that stroma may suppress tumour growth by restraining tumour angiogenesis.

A different study came to similar conclusions using a different approach. They used transgenic mice with the ability to delete αSMA (+) myofibroblasts in pancreatic cancer. What this study found was that myofibroblast loss impaired the ability of the immune system to control cancer progression and therefore that carcinoma-associated fibroblasts have a protective role [35].

More importantly, a recent trial using a Hh inhibitor (IPI-926) in metastatic patients was discontinued since patients on the gemcitabine + placebo arm were living longer (more than 6 months) than patients on the gemcitabine + IPI-926 arm (less than 6 months) [29]. A possible experimental explanation of this failure has been provided by a recent work where the effects of genetic or pharmacologic inhibition of Hh pathway activity was examined in three distinct genetically engineered mouse models [36]. The conclusions of this study were that Hh pathway inhibition accelerates rather than delay progression of oncogenic Kras-driven disease. In the same study it was found that Hh pathway activation using a small molecule agonist caused stromal hyperplasia and reduced epithelial proliferation. Taken together these studies suggest that strategies to target the tumour stroma or carcinoma-associated fibroblasts in PDAC should be carefully considered. Therefore, while past investigations have generated the paradigm that tumour stroma is a cancer promoting agent, it is now emerging as a different scenario whereby certain components of stroma may operate as tumour suppressor [33]. Further studies are required to assess if this is true only in certain tumour stages or context [33].

4. Cell metabolism in pancreatic cancer

Alteration of metabolic pathways is a key feature in cancer development and progression since reprogramming of metabolism is a strict requirement for cancer cells in order to support their elevated proliferation [37]. The importance of metabolism in cancer has been recently recognized as an emerging hallmark of cancer [38].

The profuse desmoplastic stroma forces proliferating tumour cells to adapt their metabolism to this new microenvironment. In recent years a renewed research interest on tumour metabolism has led to the identification of key signalling pathways controlling metabolic routes that fuel tumour progression [39]. Among these, Ras signalling, glutamine regulatory enzymes, lipid metabolism and autophagy have taken the centre stage [40–42]. Pancreatic cancer is characterized by a high increase in glucose uptake and metabolism [43] and high glycolytic rates (Warburg effect). In addition, cancer cells compared to normal cells have specific metabolic dependencies, such as the increased use of the amino acid glutamine [44]. The reprogramming of glutamine metabolism in PDAC is controlled by Kras that regulates key metabolic enzymes in this pathway [45]. On the other hand, therapeutic targeting of the Warburg effect has shown that the p53 status of PDAC determined the response to inhibitors of the enzyme lactate dehydrogenase-A [46]. However, although experimental evidence suggests that glucose and glutamine are the primary fuels used by cancer cells, recent studies have indicated that utilization of other amino acids as well as lipids and protein can also be important to cancer cells (Fig. 2) [47]. For instance, it has been recently shown that increased protein breakdown and subsequent increase in plasma levels of branched-chain amino acids are early events in pancreatic cancer progression [48]. Recent work suggests that mitochondrial respiration is another potential functional target to manage pancreatic cancer [49]. By using a mouse model of pancreatic cancer where expression of the Kras mutation can be activated in the pancreas through doxycycline administration, Viale et al. revealed that tumours can relapse 2–4 months after Kras mutation withdrawal. However, after a detailed analysis of tumours upon Kras mutation extinction, they found a sub-population of surviving cells that possesses stem cell characteristics. These resistant cells do not depend on Kras and anabolic glucose metabolism as the primary tumour but have a strong reliance on mitochondrial energy production [49]. These data suggests that to eliminate pancreatic tumours and prevent disease relapse we should target both populations, with inhibitors of glucose and mitochondrial metabolism. In other words, mitochondrial activity represents an additional functional target to prevent pancreatic cancer progression.

PDAC is also associated with high levels of autophagy that occurs as a consequence of a transcriptional program that regulates lysosome biogenesis and nutrient scavenging [50,51]. Pharmacological or genetic ablation of autophagy has been shown to inhibit pancreatic cancer growth *in vitro* and *in vivo* [51,52]. However, autophagy can have a dual opposite role in different stages of cancer progression. Indeed, loss of autophagy in p53 deleted mouse models is accompanied with paradoxical increase in tumour progression suggesting that the role of autophagy in PDAC is genotype-dependent [53]. Nevertheless, the jury is still out on whether p53 status affects PDAC response to autophagy inhibition [54]. Autophagy in PDAC, especially in the first stages of cancer development, can be seen as an additional mechanism of cancer plasticity conferring to cancer cells the ability to adapt and survive in metabolic stress conditions characterized by hypoxia and nutrient deprivation. In addition to autophagy, other mechanisms of scavenging have been characterized in PDAC, such as micropinocytosis of protein as a source of amino acid and lysophospholipids as source of fatty acids. Macropinocytosis is an endocytic process characterized by internalization into cells of extracellular fluids through specialized vesicles named macropinosomes. Oncogenic Ras proteins have been shown to regulate this process by which cancer cells internalize extracellular proteins that represent a valuable source of amino acids including

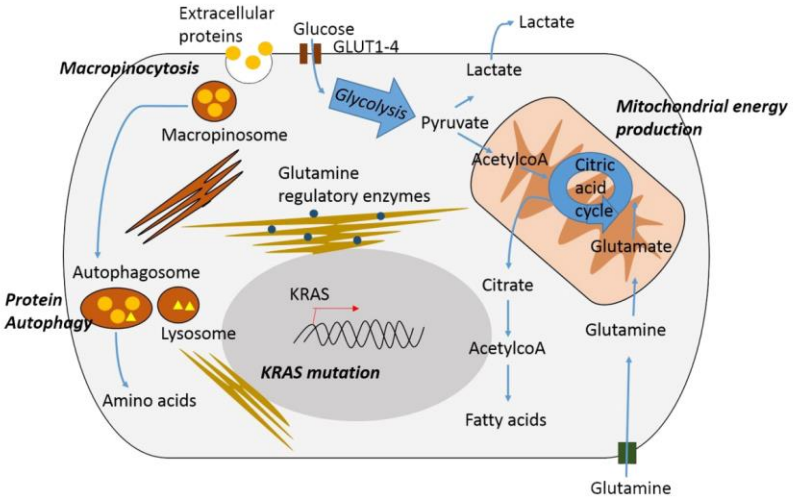

Fig. 2. Cell metabolism in pancreatic cancer. Reprogramming of metabolism in pancreatic cancer cell supports its elevated proliferation. KRAS mutation plays key role in the regulation of expressing metabolic enzymes in glutamine metabolism pathway. Pancreatic cancer is characterized by a high intake of glucose, extracellular proteins or amino acids including glutamine. Mitochondrial energy production and autophagy contribute to the development of primary tumour. Micropinocytosis in PDAC occurs to serve a valuable source of amino acids for the proliferation of cancer cell.

glutamine [55,56]. A similar oncogenic Ras-dependent mechanism of scavenging of fatty acid has been shown in PDAC cells [57]. Therefore, even though cancer cells are characterized by elevated levels of fatty acid synthesis, they need alternative mechanisms of fatty acid supply, such as the uptake of extracellular lipids, to face the massive demand of fatty acids that cancer growth requires.

There is currently an increasing interest in studying lipid metabolism in cancer cells and although many studies in the past have been focused on glucose metabolism and aerobic glycolysis, it is becoming clearly evident that the alteration of lipid metabolism is critical for cancer cell metabolism (Fig. 3) [58]. Enzymes involved in lipogenesis and lipolysis have been found overexpressed in PDAC, in particular fatty acid synthase (FASN) that catalyses the final steps of fatty acid synthesis and ATP citrate lyase [42]. Interestingly, elevated levels of FASN protein in cancer cells and in serum of PDAC patients are associated with poor prognosis [42]. The hyper activated lipogenesis in cancer cells is characterized by an increase in the degree of lipid saturation compared with non-lipogenic tumours [59]. Therefore, the rise in saturated and monounsaturated lipids in cancer cell membranes increases the resistance to oxidative stress, since polyunsaturated lipids are more susceptible to lipid peroxidation. As discussed above, pancreatic cancer cells can use alternative route to lipogenesis to obtain fatty acids, primarily through uptake of extracellular lipids derived from diet, liver synthesis or released by the adipose tissue. In addition, increasing evidences suggest that pancreatic cancer is highly dependent on cholesterol [60]. The elevated requirement of cholesterol by pancreatic cancer cells can be supplied by *de novo* synthesis, receptor-mediated uptake of cholesterol-rich low-density-lipoproteins (LDL) by the LDL receptor (LDLR), or by hydrolysis of cholesteryl ethers (CE) that accumulate in specific lipid droplets (LDs) that are thought to act as storage of triacylglycerol and CE [60].

Recent studies revealed that metabolic processes involving lipoproteins, in particular cholesterol uptake, are drastically activated in tumours [60]. Interestingly, it has shown an increase in the amount of cholesterol and an overexpression of the low-density lipoprotein receptor (LDLR) in pancreatic tumour cells [60]. Therefore, LDLR has been proposed as a novel metabolic target to limit PDAC progression. It has been demonstrated that in pancreatic tumour cells shRNA silencing of LDLR reduces considerably cholesterol uptake and alters its distribution, decreases tumour cell proliferation, and limits the activation of ERK1/2 survival pathway. Furthermore, cholesterol uptake blockage potentiates the effect of chemotherapy on PDAC regression by sensitizing cells to chemotherapeutic agents. Even though high LDLR expression in pancreatic cancer is not restricted to a specific tumour stage, it has been shown to correlate to a higher risk of disease recurrence. The implication of these studies is that pancreatic cancer cells are highly dependent on cholesterol uptake, and that either this process or LDLR is a promising metabolic target to use in combination with chemotherapy to limit PDAC progression and disease relapse in patients.

The emerging scenario of lipid metabolism in pancreatic cancer is that PDAC cells do not rely solely in *de novo* synthesis of lipids but also utilize circulating and diet derived lipids. This underlines the role of high dietary intake and obesity as a risk factor in pancreatic cancer.

5. MicroRNAs in pancreatic cancer

MicroRNAs (miRNAs) are small, around 19–25 nucleotides, non-coding RNAs that regulate gene expression post-transcriptionally. Recently, miRNAs have gained wide attention as molecules involved in cancer progression, including PDAC [61]. miRNAs have been found dysregulated in cancer and to be upregulated and downregulated. They can have oncogenic functions (oncomiRNA) or tumour suppressor activity (tsmiRNA). In recent years, several studies have assessed the miRNA profile in PDAC tumour tissue, compared to chronic pancreatitis and normal pancreas. These studies have underlined the great potential of miRNAs as therapeutic target and as diagnostic and prognostic biomarkers in PDAC [61]. Nevertheless, given the highly heterogeneous nature of PDAC, the pattern of expression of miRNAs is not uniform. miRNAs have been shown to influence different biological processes such as cell proliferation, apoptosis, invasion and metastasis, mainly

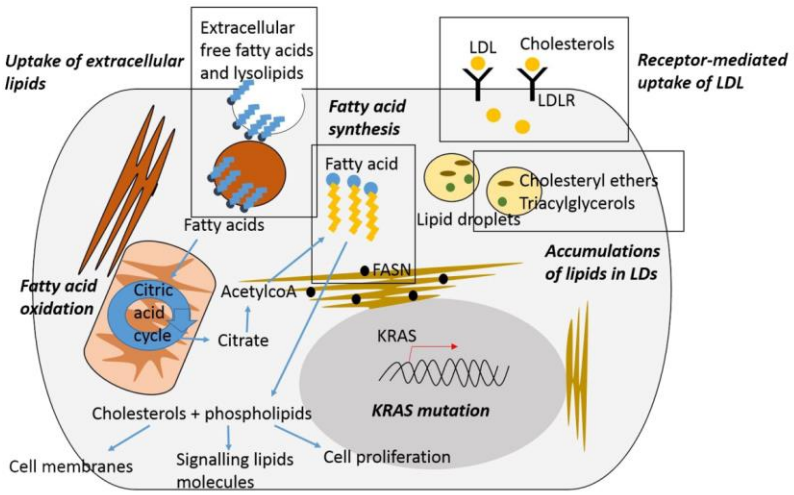

Fig. 3. Reprogramming of lipid metabolism in pancreatic cancer. Pancreatic cancer cells highly require lipids. Reprogrammed lipid metabolism is strongly related with cancer cell proliferation. Overexpression of fatty acid synthase (FASN) in PDAC reads to increase fatty acid synthesis. Cholesterol-rich low density lipoprotein (LDL) is up-taken through the LDL receptor (LDLR). Lipids such as cholesteryl ethers (CE) and triacylglycerols are accumulated in lipid droplets (LDs) in PDAC. Up-taken free fatty acids are oxidized in mitochondria.

through negative regulation of gene expression [62]. Among the down-regulated miRNAs in PDAC there are the *miR-34*, *mir-200*, *miR-375* and *Let-7* family members. Interestingly, the transcription factor and tumour suppressor p53 has been independently shown by several laboratories to directly transactivate the *miR-34* family of genes [63]. Expression of *miR-34* family members induces a G1 arrest, senescence, or apoptosis and inhibits migration and invasion [64]. These effects of *miR-34* expression are mediated by downregulation of important regulatory proteins such as Bcl2 and MYC. Therefore, *miR-34* genes may be important mediators of p53 tumour suppressor activity. Recent data indicate that gene deletion and lack of activation by p53 and, possibly, other mechanisms, contribute to underexpression of *miR-34* in human PDAC [65,66]). Furthermore, a miRNA microarray expression profiling of PDAC tissues found that reduced expression of *miR-34a* was associated with poor overall survival [67]. *miR-200* and *Let-7* families have been shown to regulate de-differentiation, EMT and PDAC progression [68]. *miR-200* members have been shown to suppress TICs by silencing key stem factors such as Sox2 and ZEB1, with consequent inhibition of EMT and PDAC invasiveness. Expression of miR-200 members has been found decreased in CD44+/CD24+/ESA+ cells derived from a pancreatic cancer cell line. Another interesting tumour suppressor is mir-375 that has been found downregulated in pancreatic cancer and to suppress key cancer functions. A recent study investigated the transcriptional regulation of miR-375 and has validated phosphoinositide-dependent kinase-1 (PDK1) as a miR-375 specific target in pancreatic carcinoma [69]. In addition, miR-375 was found to be downregulated in pancreatic tumours compared with non-tumour tissues [70] and to inhibit proliferation and promote apoptosis of pancreatic cancer cells [69]. We proposed that miR-375 may represent an additional link between Kras and PDK1, since Kras-induced downregulation of miR-375 results in increased PDK1 expression [71].

6. Pancreatic cancer-derived exosomes

Exosomes are small vesicles composed of a lipid bilayer membrane enclosing intracellular fluid. They originate inside cells from late endosomes whose membrane ingrowths englobing proteins, lipids, and nucleic acids, resulting in a dish or cup shaped formation with a size ranging from 30 to 150 nm and a density between 1.13 and 1.19 g/ml [72]. Afterwards, the newly formed membranes amalgamate with the cell membrane discharging the exosomes outside the cell and giving them access to the circulation. These extracellular vesicles have recently attracted abundant scientific interest after being discovered to be crucial information transporter between cells, both normal and malignant [73]. Once released into the intracellular space, exosomes transfer their biologically active content either by binding a receptor on the recipient cell membrane, by fusing with the membrane or by being phagocytized into the receiver cell. Lately, there has been growing evidence supporting the hypothesis of a crucial role played by exosomes in tumour development, progression and metastasis. For instance, exosomes have been found to have the potential to promote normal cell transformation into tumour cells in prostate and breast cancer cells [74,75]. Moreover, several studies have reported how exosomes contribute to tumour cell proliferation by suppling cancer cells with anti-apoptotic proteins, mRNAs, oncogenic proteins, cytokines, adhesion molecules and stimulating tumour growth [73]. Tumour angiogenesis has also be found to be directly correlated to exosomes' activity. Exosomes derived from mesothelioma, glioblastoma, breast cancer and multiple myeloma cells have all demonstrated to upregulate angiogenesis utilizing miRNAs or angiogenic proteins. Recently, new evidence has been gathered of exosomes' involvement in cancer proliferation. Exosomes derived from pancreatic ductal adenocarcinoma cells have been demonstrated to promote a favourable fibrotic microenvironment in the liver, a pre-metastatic niche, supporting metastasis formation in a mouse model [76]. This process is the result of a multistep mechanism, beginning with the release of exosomes containing macrophage migration inhibitory factor by pancreatic cancer cells that are subsequently picked up by liver Kuppler cells. These cells are stimulated to generate fibrotic cytokines which induce fibronectin production by hepatic stellate cells. As a consequence, a fibrotic microenvironment is established which stimulates the enrolment of bone marrow-derived cells, creating a favourable habitat for PDAC cells to grow and

metastasize. Exosomes released by pancreatic cancer cells in culture or in patients have been shown to induce endoplasmic reticulum stress in beta cell through the release of adrenomedullin that ultimately leads to beta cell dysfunction and death [77]. In addition, pancreatic cancer-derived exosomes have been found to induce lipolysis in adipose tissue and exosomal adrenomedullin is a candidate mediator of this effect [78]. Exosomes express specific cell surface markers and therefore may serve as diagnostic biomarkers [79]. Recently, pancreatic cancer-specific exosomes have been shown to express a cell surface proteoglycan, glypican-1. Interestingly, glypican-1 levels correlate with tumour burden and patient survival [80]. Therefore, glypican-1 represents and is an attractive candidate for detection and isolation of exosomes in the circulation of patients with pancreatic cancer, and hence a good candidate for early detection of pancreatic cancer. A recent study has demonstrated that a panel of discriminatory biomarkers appears in the saliva-contained exosomes as a direct consequence of PDAC development in a syngeneic mouse model [81]. Interestingly, a combined evaluation of serum exosomes expressing proteins and miRNA markers revealed that PDAC patients present a distinct pattern of exosomes and miRNA markers providing a novel diagnostic strategy [82].

7. Conclusion and future perspectives

Despite the fact that the increased interest on pancreatic cancer biology has provided a significant step forward in our understanding of this disease, it remains a pathology against which the oncologists' weapons are scarce to say the least. The major information that we obtained in the last decades from studies on this highly lethal malignancy is that it is extremely heterogeneous and complex and therefore targeted therapies have failed in the majority of patients. Only polychemotherapy or strategies to overcome gemcitabine resistance have recently provided significant improved survival, even though it is accompanied by a modest extension of the overall survival and severe side effects and therefore it is not well tolerated in the majority of patients. However, given the fact that for decades the survival rates for PDAC patients have seen no substantial improvements, this modest extension is extremely meaningful and above all demonstrates that this disease can be successfully tackled by chemotherapy. The way forward should be to exploit pancreatic cancer homogeneous features that occur despite the overall heterogeneity. For instance, the vast majority of PDAC presents mutations in few key genes such as Kras, p53 and CDKN2. In addition, despite its elevated cell plasticity, pancreatic cancer shows dependence and addiction to major cellular functions such as its abnormal metabolism. The constitutively active oncogene K-Ras is a key promoter of pancreatic cancer tumorigenicity and the Ras-driven signals act as a spur of metabolism reprogramming. Indeed, K-Ras signals regulate glycolysis, glutamine consumption, autophagy, macropinocytosis, protein and lipids scavenging, leading to a state of "metabolic addiction" in pancreatic tumour cells [83]. Therefore, the metabolic addiction of pancreatic cancer provides a valuable opportunity for future therapeutic strategy. A more intriguing opportunity would be to target tumour metabolism specifically in TICs cells, even though recent data suggest that TICs are characterized by very heterogeneous phenotypes [84]. Heterogeneity may play a more important role in the context of polychemotherapy and it has been hypothesized that genetically unstable PDAC cases (such as somatic or germline BRCA mutations) may be more sensitive to platinum compounds or poly (ADP-ribose) polymerase inhibitors (PARPi) [85]. The research in the field has abandoned the dismay of the past decades and showed a renewed resilience. For instance, a recent setback in strategies to target tumour microenvironment leads to a refinement of strategies rather than its discontinuation [86]. Discussion around the "good or bad" functions of tumour stroma has been replaced by the concept of stroma remodelling rather than stromal ablation as a desirable target. An example of stroma remodelling has been provided by the Vitamin D receptor stromal reprogramming that has been shown to enhance pancreatic cancer therapy [87]. Similarly, targeted inhibition of signal transducer and activator of transcription 3 (STAT3) combined with gemcitabine has been shown to enhance *in vivo* drug delivery and therapeutic response in a mouse model of pancreatic cancer [88]. Interestingly, these effects obtained by a combination of STAT3 inhibition and gemcitabine occur through tumour stroma remodelling without depletion of tumour stroma. In addition, this combination induces downregulation of the enzyme cytidine deaminase that is responsible for the metabolic inactivation of gemcitabine. The latter data, together with evidence showing that high levels of gemcitabine transporter human equilibrative nucleoside transporter 1 (hENT1) and deoxycytidine kinase are associated with better survival in patient treated with gemcitabine, encourage further strategies focusing on gemcitabine metabolism [89,90]. Another emerging opportunity to overcome the high chemoresistance of pancreatic cancer is to target EMT. Indeed, it has been recently shown by using mouse models of PDAC that EMT is dispensable for metastasis but plays a key role in pancreatic cancer chemoresistance [91]. This study demonstrates that EMT suppresses the expression of nucleotide transporter proteins, therefore protecting cancer cells from the antiproliferative effects of drugs such as gemcitabine. The classification of malignancies in distinct subtypes using histological and molecular information has been crucial for the understanding of cancer pathogenesis and as a guide for clinical practice. In contrast to other cancer types, PDAC classification has been very challenging and impractical. To address this issue a new study has used bioinformatics approaches and has identified distinct tumour- and stroma-specific PDAC subtypes [92]. This information can lead to better PDAC diagnosis and treatment. For instance, patients with PDAC subtype named basal-like had shorter overall survival but better responsiveness to chemotherapy compared to the classical subtype. In addition, it should be noted that another study showed that around 40% of patients with locally advanced disease never develop distant metastases, further suggesting the existence of PDAC subtypes [93]. Therefore, both tumour and stromal classification in subtypes may be instrumental in the identification of more tailored therapies.

Further momentum could be provided by the development of novel models, such as the organoid model of human and mouse ductal pancreatic cancer that provides a valuable platform for the identification of personalized treatments for pancreatic cancer patients [94].

Emerging opportunities to combat PDAC are represented by the identification of novel biomarkers for an early discovery of the disease. For instance, circulating microRNAs and exosomes are particularly promising diagnostic tools. In conclusion, PDAC remains a very aggressive and lethal disease and a promising therapeutic strategy is represented by the combined targeting of key cancer functions such as metabolism, mitochondrial activity, autophagy, and cell subpopulations, such as stroma components and cancer released exosomes and microRNAs.

Disclosure of potential conflicts of interest

The authors have declared no conflicts of interest exist.

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1.2 GPCR

1.2.1 Structure and mechanism of GPCRs

The main role of G protein-coupled receptors, also known as 7 transmembrane receptors, is essentially transducing signals into a cell. GPCRs consist of 7 transmembrane helices which are connected with an extracellular N terminus, an intracellular C terminus, three extracellular loops (EL-1, EL-2 and EL-3) and three intracellular loops (IL-1, IL-2 and IL-3). These 7 transmembrane helices surround a cavity in the plasma membrane. The cavity with extracellular loops and N terminus serves as a ligand-binding domain. The cysteine residues on the extracellular loops enable to keep a strong tertiary structure of a GPCR by forming disulphide bonds. GPCRs are associated with G proteins that consist of three different subunits: an alpha, beta and gamma subunit (Gα, Gβ and Gγ). They are coupled with GPCRs as a hetero-trimer form in the absence of a signal.

Once a ligand binds to a GPCR, the transmembrane helices change their conformation. This change then activates Gα by exchanging GDP to GTP on their guanine nucleotide exchange factor (GEF) domain. When GTP physically binds to Gα, the associated G protein subunits are dissociated into two parts: an alpha subunit and a beta-gamma dimer subunit. At the same time, the dissociated G proteins are detached from GPCRs allowing the G proteins to react with other effector molecules such as adenylyl cyclases (ACs), phosphodiesterases (PDEs), phospholipases (PLA_2 and PLC), and phosphoinositide 3-kinases (PI3Ks). In turn, the effector molecules can produce second messengers like cyclic AMP (cAMP), diacylglycerol (DAG), inositol (1, 4, 5)-trisphosphate (IP3), phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3), arachidonic acid and lysophospholipids.

GPCRs are eventually inactivated since Gα can hydrolyse GTP leading to signal self-termination. Once Gα hydrolyses GTP back to GDP, it allows the entire G proteins to be reassociated as a hetero-trimer. GTP hydrolysis is often accelerated up to more than 2,000-fold by regulators of G protein signalling (RGS proteins) which belongs to GTPase -activating protein (GAP) because the rate of GTP hydrolysis is relatively slow (with RGS: ~30 times/sec and without RGS: ~0.02 times/sec). This acceleration through RGS proteins allows GPCRs to have a fast reaction with external signals. Moreover, the self-termination of the activated receptors is an important feature that is able to stop possible adverse effects resulting from repeated signalling pathways.

There are other processes that can cause the termination of GPCRs. Once a ligand stimulates a GPCR, the agonist-binding GPCR recruits GPCR-regulating kinases (GRKs) that phosphorylate serine/threonine residues on IL-3 and C-terminal tail. This phosphorylation of

GPCR by GRKs allows increasing the affinity for β-arrestins to GPCR, hindering G proteins couplings sterically, and eventually leading to the desensitization of GPCR, called homologous desensitization. GRKs also have a GAP domain, so they can contribute to inactivate the receptor. Both a non-kinase mechanism and a kinase mechanism may occur at the same time causing the desensitization of GPCR. Either serine or threonine residues on IL-3 and C-terminal tail can be phosphorylated by protein kinase A (PKA) or protein kinase C (PKC). This PKA or PKC mediating phosphorylation also can increase the affinity of β-arrestins. PKA and PKC may also phosphorylate GRKs instead of GPCRs directly, which eventually enables to phosphorylate GPCR and bind β-arrestins to GPCRs. These latter two mechanisms involved in PKA and PKC also lead to the desensitization of GPCR, called heterologous desensitization.

Most, but not all GPCRs, bind to β-arrestin and undergo internalization, where GPCRs are physically moved from the cell membrane to the cytosol by endocytosis. Most of GPCR-internalization is initiated by the formation of clathrin-coated pits. Clathrin is an endocytic protein that plays an essential role in receptor endocytosis. However, GPCRs are not bound to clathrin directly, and therefore adaptor proteins are required, which are β-arrestin and adaptor protein 2 (AP2). Once β–arrestins bind to GPCRs, AP2 and clathrin are recruited and aggregated. They form a complex like a piece of endocytic machinery and these complexes surround a GPCR binding with β-arrestins, leading to the formation of clathrin-coated pits. The pits make eventually endocytic vesicles and GPCRs are removed from the cell surface. At this point, the fate of GPCRs is decided: they will be either directed to recycling, which is returning to the membrane, or trafficking, which is being degraded by lysosomes.

Signalling pathways activated by GPCRs are determined by their primary sequence, their tertiary structure, the GPCRs-specific ligands and/or the transducer molecules binding to GPCRs. The amino acid sequence contributes to defining the three-dimensional (3D) shape of GPCRs that can affect their binding ability to specific ligands in the extracellular space and transducer molecules in the intracellular space. These are connected in series, so the availability of transducer molecules in the cytoplasm ultimately contributes to change the conformation of GPCRs that decides particular signalling pathways. There are two representative primary types of transducer for GPCRs: G proteins and β-arrestins. Notably, it is well known that the G protein alpha subunit has a major role with regard to signalling activation compared to any other transducers whereas Gβγ proteins and β-arrestins have been demonstrated to have a major role in signalling termination. However, a growing number of studies demonstrate that not only Gα but also Gβ, Gγ and β–arrestins are involved in the regulation of cellular processes and they have specificity linked to their various genetic

combinations. There are 5 known human Gβ and 12 Gγ subunit genes providing numerous diversities of dimer combinations. The various $G\beta\gamma_2$ dimers activate or inhibit adenylyl cyclases (ACs) (Jones, Siderovski, & Hooks, 2004). β-arrestin has another role in signal transductions beyond the role in GPCR desensitization and internalization (Peterson & Luttrell, 2017). β-arrestin1 and β-arrestin2 act as signalling scaffold molecules for the MAPK pathway, including ERK1/2, p38 kinases and c-Jun N-terminal kinases (Tohgo et al., 2003). The MAPKs control diverse cellular functions such as apoptosis, cell cycle progression and transcriptional regulation. β-arrestins recruit c-SRC, a non-receptor tyrosine kinase family member, to GPCRs, resulting in ERK activation (Luttrell et al., 1999).

There are 15 Gα encoded by 16 individual genes. Gα proteins are broadly divided into four subclasses based on sequence homology and functional similarity, which are Gα (s), Gα (i/o), Gα (q/11) and Gα (12/13). Each subclass contains specific isotypes and targets on its own particular type of signalling cascade. Gα (s), containing another isotope which is Gα (olf), stimulates adenylyl cyclases. On the other hand, the Gα (i/o) family including Gα (i2), Gα (i3), Gα (oA), Gα (oB), Gα (z), Gα (t1), Gα (t2) and Gα (gust), inhibits adenylyl cyclases. Gα (q/11) family consisting of Gα (q), Gα (11), Gα (14) and Gα (16) is associated with the stimulation of phospholipase Cβ (PLC-β). Gα (12/13) is related to the stimulation of the low molecular weight G proteins of the Rho family. These many different subunits, generated by diverse genes combined, produce a wide variety of G protein families, eventually bringing enormous functional diversity and selectivity of GPCRs.

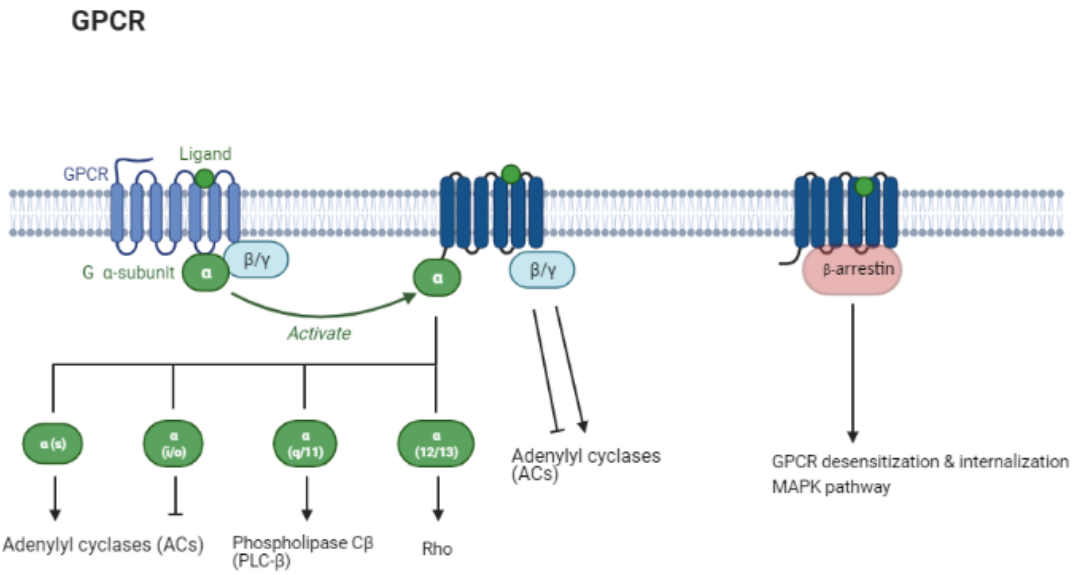


Figure 1.1 Mechanism of GPCR

1.2.2 Classification of GPCRs

The GPCR superfamily is known as one of the largest families of proteins and accounts for nearly 4% of the entire human genome. Genome sequence analysis suggests that the GPCR superfamily is encoded by nearly 800 different human genes. The A-F system is a widely used classification of GPCRs, even though many other classification systems have been suggested. The superfamily was classified based on their amino acid sequence homology and functional similarities. This A-F system is divided into 6 main classes: class A (rhodopsin-like receptors), class B (secretion and adhesion family), class C (metabotropic glutamate and pheromone receptors), class D (fungal mating pheromone receptors), class E (cyclic AMP receptors) and class F (frizzled and smoothened receptors) and it includes both vertebrates and invertebrates.

A GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled / Taste2, Secretin) classification system covering only mammalian GPCRs has been proposed more recently (Schioth & Fredriksson, 2005). The GRAFS categorizations include the glutamate receptor family, the rhodopsin receptor family, the adhesion receptor family, the frizzled/taste2 receptor family, and the secretin receptor family.

1.2.3 GPCRs in PDAC

Diverse GPCRs are expressed in PDAC. According to The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov) data analysis, the expression of more than a hundred of GPCRs was elevated in PDAC tumours compared to the normal pancreas (Sriram, Moyung, Corriden, Carter, & Insel, 2019). Above 80% of PDAC tumours examined by TCGA consistently express around 30 GPCRs, showing their potential to be a new therapeutic target for PDAC. These overexpressed GPCRs in PDAC include diverse members such as chemokine receptors, proteinase-activated receptors, lysophosphatidic acid receptors, neurotensin receptors, adrenoceptors, proteinoid receptors, and angiotensin receptors (summarised in Table 1.4).

The most expressed chemokine receptor member in PDAC is C-X-C motif chemokine receptor 4 (CXCR4) (Koshiba et al., 2000). Koshiba et al. demonstrated that CXCR4 and its ligand CXCL12 stimulated PDAC progression through the promotion of migration and angiogenesis. Further studies supported it by showing the overexpression of CXCR4 detected in malignant PDAC tumours, suggesting that CXCL12-CXCR4 signalling might be related to PDAC metastasis and angiogenesis (Cui et al., 2011; B. Shen et al., 2013; Wehler et al., 2006). The extracellular signal-regulated kinase (ERK) phosphorylation by CXCL12 is mediated by

KRAS mutations and was observed in diverse PDAC cell lines (Heinrich, Lee, Lu, Lowy, & Kim, 2012).

PAR2, also known as F2RL1, one of the proteinase-activated receptors (PAR) family, is highly expressed in PDAC (Versteeg et al., 2008). PDAC cells with PAR2 deficiency reduce the expression of TGF-β, which mostly affects tumorigenesis by controlling migration, invasion and metastasis of PDAC (Zeeh et al., 2016). A more recent study demonstrated that the expression of PAR1 is correlated with mesenchymal features in PDAC, and linked to PDAC progression (Tekin et al., 2018).

Several lysophosphatidic acid receptors (LPARs), which ligand is LPA that is an extracellular physiological lysolipid, are involved in the tumorigenesis of PDAC, as reviewed in (Lv et al., 2011). Among LPARs, LPAR1 expressed in PDAC shows the highest expression, especially in PDAC cancer-associated fibroblasts (CAFs) which are a major component of the PDAC tumour micro-environment, and it promotes PDAC cell migration through the ERK pathway (Stahle et al., 2003). Additionally, the role of LPAR1 in PDAC tumour progression was examined in a different study (Fukushima et al., 2017). The inhibition of cell motile and invasive activities was verified in LPA_1 and LPA_3 knockdown PANC-1 cells in this study.

The impact of β-adrenergic receptors, which are activated by adrenaline and noradrenaline, in PDAC has been also studied. The mRNA expression of β-adrenergic receptors, predominantly β_2-adrenergic receptor, in PANC-1 and BxPC-3 cells, was verified (Weddle, Tithoff, Williams, & Schuller, 2001). The authors suggested that β-adrenergic receptors regulate PDAC cell growth via the arachidonic acid-dependent regulatory pathway. More recent evidence has shown the important role of the β_2-adrenergic receptor in PDAC progression (Wan et al., 2016). The high expression level of this receptor was detected in PDAC cells; it promotes c-myc expression, leading to PDAC progression. Moreover, the authors indicated that the upregulated β_2-adrenergic receptor is highly related to poor prognosis in pancreatic patients.

It is well known that the overexpression of cyclooxygenase-2 (COX-2) and COX-2 derived prostaglandin E2 (PGE2) are commonly detected in PDAC. Consequently, the role of its receptors, E prostanoid receptors (EPs), in PDAC has been studied. The increases of VEGF production mediated by E-type prostanoid receptor 2 (EP2) in PDAC have been verified (Eibl et al., 2003). However, the EPs signalling positively related with PDAC progression is still controversial because their opposite effect has been reported. EP4 activated by PGE2 was demonstrated as a negative modulator of PDAC cell growth (Schmidt et al., 2017).

A large amount of evidence has shown the potential of neurotensin receptor 1 (NTR1) as a therapeutic target in PDAC. In addition, it has also been proposed as a predictive biomarker

since overexpression of NTR1 has been demonstrated in malignant PDAC, whereas a very low expression was detected in inflammatory areas and normal pancreas (Mijatovic et al., 2007; Reubi, Waser, Friess, Buchler, & Laissue, 1998; Tateishi, Funakoshi, Kitayama, & Matsuoka, 1993; J. G. Wang, Li, Li, Cui, & Wang, 2011; L. Wang et al., 2000; Yamada, Ohata, Momose, Yamada, & Richelson, 1995). Therefore, this biomarker is suggested as a diagnostic target by using radiolabelled neurotensin, that binds NTR1 with high affinity, analogues (Hodolic, Ambrosini, & Fanti, 2020; Renard et al., 2020; M. Wang et al., 2018).

TABLE 1.5 THE EXPRESSED GPCRS IN PDAC

Classifications	Representative receptor (Corresponding reference)	Other receptors
Chemokine receptors	CXCR4 (Koshiba et al., 2000)	CXCR1, CXCR2, CXCR3, CXCR7, CCR7, CCR9, CCRL2, CCL21 etc.
Proteinase-activated receptors	PAR2 (F2RL1) (Zeeh et al., 2016)	PAR1 (F2R)
Lysophospholipid receptor	LPAR1(Stahle et al., 2003)	LPAR3, LPAR4, LPAR5 and LPAR6
Adrenergic receptors	β_2-adrenergic receptor (Weddle et al., 2001)	β_1-adrenergic receptor
E Prostanoid receptors	EP4 (Eibl et al., 2003)	IP, EP2 and EP3
Neurotensin receptors	NTS1 (L. Wang et al., 2000)	-

1.2.4 Significance of GPCRs in cancer study

GPCRs and GPCR-related molecules targeting drugs represent around 34% of total Food and Drug Administration (FDA)-approved drugs, and international sales are valued at over 180 billion US dollars annually (Hauser et al., 2018). GPCRs are the largest family of human membrane receptors targeted by currently marketed therapeutics. However, around 100 out of 360 human endo-GPCRs still remain orphan receptors (Sriram & Insel, 2018), indicating that GPCRs have huge potential to be a new therapeutic target. GPCRs as onco-targets have been spotlighted recently compared to many other different therapeutic areas such as allergy, cardiovascular disease and metabolic disease. Increasing GPCR related studies focusing on cancer have shown that diverse GPCRs regulate signalling pathways that mediate tumorigenesis, progression, invasion, angiogenesis and metastasis in multiple cancer types.

1.3 GPR35

GPR35 remains an orphan receptor since it was discovered in 1998. Few endogenous molecules have been suggested as the ligand for GPR35, but none of them has been clearly defined because they are either non-selective towards GPR35 or they lack high potency. For a better understanding of GPR35, several synthetic agonists and antagonists have been identified as strong pharmacological tools in the last 10 years. The expression of GPR35 has been detected in various organs and tissue systems including gastrointestinal tissues, liver, pancreas, spleen, nervous system, cardiovascular system and immune cells. This expression pattern suggests that GPR35 can be a new therapeutic target due to its relevance to diverse diseases. In particular, oncologists have recently raised their interest in GPR35 as a novel potential therapeutic target for improving current cancer chemotherapies and markers. Many physiological studies have determined the role of GPR35 in inflammation, pain, metabolic changes and hypoxia, all potential cancer features.

In this sub-chapter, I provide a broad overview of pharmacological, pathological and physiological research on GPR35, highlighting the recent cancer studies.

1.3.1 Pharmacological studies of GPR35

1.3.1.1 Structure of GPR35

GPR35 consists of 309 amino acids and its translational open reading frame is located on chromosome 2, region 37.3 in humans (O'Dowd et al., 1998). Two isoforms, GPR35a and GPR35b, are detected in humans; on the other hand, both rats and mice have only one isoform. Despite the fact that the distinctive functions of these isoforms it is still unclear, the expression of GPR35b in gastric and colon cancer tissues was reported in few studies, indicating its correlation with carcinogenesis (Ali et al., 2019; Okumura et al., 2004). GPR35b has a longer amino acid sequence than GPR35a containing an N-terminal extension of 31 amino acids which might contribute to the additional formation of an extracellular disulphide bond (Okumura et al., 2004). GPR35 belongs to rhodopsin-like GPCR, Class A, based on the phylogenetic analysis. Several G protein alpha subunits, such as Gαi/o(J. Guo, Williams, Puhl, & Ikeda, 2008; Ohshiro, Tonai-Kachi, & Ichikawa, 2008; J. Wang et al., 2006), Gα13(Jenkins et al., 2010), and Gq (J. Wang et al., 2006), are known to be coupled with GPR35. Internalization of GPR35 by β-arrestin-2 recruitment upon stimulation with agonists such as zaprinast and kynurenic acid (KYNA) has been verified (Jenkins et al., 2010; P. Zhao et al., 2010). GPR35 shares homology with several purinergic P2Y receptors such as GPR23/$P2Y_9$, $P2Y_1$ and $P2Y_4$, which are stimulated by ATP, ADP, UTP, UDP and UDP-glucose (O'Dowd et al., 1998). Also, HM74 (O'Dowd et al., 1998) which is activated by nicotinic acid, and GPR55, whose ligand is lysophosphatidylinositol (LPI) (Pineiro & Falasca, 2012; Sawzdargo et al., 1999), shares homology with GPR35.

1.3.1.2 Antagonists of GPR35

Few antagonists of GPR35 are commercially available to study the function of this receptor. ML145 (CID-2286812), ML144, ML194 and CID2745687 (SPB05142) have been discovered as antagonist compounds (Heynen-Genel et al., 2010; P. Zhao et al., 2010). However, their activity across species is still under evaluation. To date, CID2745687 and ML145 have shown their high affinity and selectivity toward human GPR35, providing a useful tool to understand GPR35 biology. One well-described GPR35 antagonist is CID2745687, methyl-5-[(*tert*-butylcarbamothioylhydrazinylidene)methyl]-1-(2,4-difluorophenyl)pyrazole-4-carbocylate. Several studies indicated that this compound at a dose range of 10-20nM showed its efficacy in a competitive manner with the agonists (either pamoic acid or zaprinast) towards human GPR35a, but its efficacy for mouse GPR35 is still controversial (Jenkins et al., 2012; P. Zhao

et al., 2010). Another widely studied GPR35 antagonist compound is ML145 (SPB05142), 2-hydroxy-4-[4-(5Z)-5-[(E)-2-methyl-3-phenylprop-2-enylidene]-4-oxo-2-sulfanylidene-1,3-thiazolidin-3-yl]butanoylamino]benzoic acid. This compound at a dose of 20nM has shown its high affinity to human GPR35, but a limited affinity to mouse and rat GPR35 has been reported (Heynen-Genel et al., 2010; Jenkins et al., 2012).

1.3.1.3 Agonists of GPR35

Synthetic agonists of GPR35

A large number of synthetic agonists of GPR35 (summarized in Table 1.6) have been suggested in the last 15 years, even though all of them are still under assessment. The majority of the suggested agonists were selected from the Prestwick Chemical Library containing above 1000 small molecule compounds, also commercially available drugs, with their chemical details. Once the candidate agonists for GPR35 were selected, the compounds were generally tested by diverse GPCR assays such as β-arrestin recruitment, receptor internalization, calcium mobilization, inositol phosphate accumulation and ERK phosphorylation, and DMR assays. In this subchapter, the agonists of human GPR35 will be described, despite the fact that a large variety of suggested agonists' potency between species has been shown, due to ~70% homology shared between rodents and human orthologues.

The agonist widely used in most GPR35 studies is zaprinast, which is a selective inhibitor of cGMP-dependent PDEs, showing moderate potency toward human, rat and mouse GPR35, and suggested first by Taniguchi (Taniguchi, Tonai-Kachi, & Shinjo, 2006). Many other studies have supported zaprinast as the standard GPR35 agonist by using mammalian cells expressing human GPR35 or HT-29 human colon cancer cells expressing endogenous GPR35 (Funke, Thimm, Schiedel, & Muller, 2013; Neetoo-Isseljee et al., 2013; L. Wei et al., 2017). One chloride channel blocker, 5-nitro-2-(3-phenylpropylamino)benzoic acid, also named NPPB, has been suggested as a human GPR35 agonist by calcium mobilization assay using HEK293 cells expressing GPR35 and promiscuous G proteins (Taniguchi, Tonai-Kachi, & Shinjo, 2008). A dose-dependent DMR assay using HT-29 cells with (or without) the treatment of zaprinast or CID2745687 supported its high potential as a GPR35 agonist (Sun et al., 2014). Pamoic acid, which is widely used as a counterion of many drug compounds to increase dissolvability, has been suggested as an agonist of both human and mouse GPR35 (P. Zhao et al., 2010) but other authors (Jenkins et al., 2010) noted that it shows weaker affinity with human GPR35 compared to zaprinast. Cromolyn disodium and nedocromil sodium, which are asthma drugs, have shown a potency similar to zaprinast toward human GPR35, but not at

mouse and rat orthologues (Yang et al., 2010). The authors used human GPR35 transfected CHO and HEK293 cells for calcium mobilization assay (aequorin) and inositol phosphate accumulation assay. Jenkins et al. also mentioned that cromolyn disodium acted as a full agonist for human GPR35 showing effects similar to zaprinast in their study (Jenkins et al., 2010). In addition to testing a wide range of agonists of GPR35, Jenkins et al. suggested other new drugs for potential human GPR35 agonists including Niflumic acid, dicumarol, quercetin, oxantel pamoate, pyvinium pamoate and luteolin. The same group continued revealing new agonists of this receptor, one of which is thiazolidinediones, (Z)-[4-(2,4-Dioxo-thiazolidin-5-ylidenemethyl)-phenoxy]acetic acid (named as compound 10), sharing an overlapping binding site in GPR35 with zaprinast and KYNA. Furthermore, the GPR35 agonist activity of tyrphostin analogues, 2-(3-cyano-5-(3,4-dichlorophenyl)-4,5-dimethylfuran-2(5H)-ylidene)malononitrile (YE120), 6-bromo-3-methylthieno[3,2-b]thiophene-2-carboxylic acid (YE210), aspirin metabolites, D-luciferin and coumarin derivatives were studied (H. Deng & Fang, 2012; H. Deng, Hu, & Fang, 2011; H. Deng, Hu, He, et al., 2011; H. Hu, Deng, & Fang, 2012; L. Wei et al., 2017). Chromen-4-one-2-carboxylic acid derivatives sharing a substructure with cromolyn were suggested as a result of screening their own compound library (Funke et al., 2013; Thimm, Funke, Meyer, & Muller, 2013). Other anti-allergic mast cell stabilizers, lodoxamide and bufrolin, were suggested as agonists of GPR35 by (MacKenzie et al., 2014).

TABLE 1.6 SYNTHETIC AGONISTS OF HUMAN GPR35

Name	Methods	Reference
Zaprinast	Calcium mobilization assay	(Taniguchi et al., 2006)
5-nitro-2-benzoic acid (NPPB)	Calcium mobilization assay	(Taniguchi et al., 2008)
Pamoic acid	β-arrestin recruitment assay, receptor internalization assay and ERK phosphorylation assay	(P. Zhao et al., 2010) also identified by (Jenkins et al., 2010)
Cromolyn disodium and Nedocromil sodium	Calcium mobilization assay and inositol phosphate accumulation assay	(Yang et al., 2010) also identified by (Jenkins et al., 2010)
Niflumic acid, dicumarol, quercetin, oxantel pamoate, pyvinium pamoate, luteolin	β-arrestin-2 interaction assay, GTP binding assay	(Jenkins et al., 2010)
Compound 10: (Z)-[4-(2,4-Dioxo-thiazolidin-5-ylidenemethyl)-phenoxy]acetic acid	β-arrestin-2 recruitment assay, β-arrestin-2 interaction assay, cell-surface biotinylation assay	(Jenkins et al., 2011)
Tyrphostin analogues	DMR assay, receptor internalization assay, ERK phosphorylation assay, β-arrestin translocation assay	(H. Deng, Hu, & Fang, 2011)
YE210 and YE120	DMR assay, β-arrestin translocation assay, GPR35 siRNA transfection and receptor internalization assay.	(H. Deng, Hu, He, et al., 2011)
Aspirin metabolites	DMR assay, β-arrestin translocation assay, qPCR	(H. Deng & Fang, 2012)
D-luciferin	DMR assay, β-arrestin translocation assay, ERK phosphorylation assay, receptor internalization assay	(H. Hu et al., 2012)
Chromen-4-one-2-carboxylic acid derivatives (8-Benzamidochromen-4-one-2-carboxylic acids, 6-Bromo-8-(4-H-3 methoxybenzamido))	β-arrestin recruitment assay	(Funke et al., 2013) (Thimm et al., 2013)
Lodoxamide and Bufrolin	β-arrestin-2 recruitment assay, GPR35 internalization assay, DMR assay, computational homology modelling and ligand docking studies.	(MacKenzie et al., 2014)
Coumarin derivatives	β-arrestin translocation assay, DMR assay	(L. Wei et al., 2017)

Potential endogenous ligands for GPR35

Several potential endogenous ligands have been suggested. KYNA, which is a tryptophan metabolite and also acts as an ionotropic glutamate receptor antagonist, is the first reported ligand of GPR35 (J. Wang et al., 2006). CHO and HeLa cells expressing GPR35 were used to identify KYNA as a ligand for GPR35 through aequorin assay, internalization assay, GTPγS binding assay, and inositol phosphate accumulation assay. However, in this study, the working concentration of KYNA was in the micromolar range, which is considered too high a concentration to be found in the human body. Therefore, KYNA remains unclear as a potential endogenous ligand for GPR35. Lysophosphatidic acid (LPA) is the second reported natural endogenous ligand of GPR35 (Oka, Ota, Shima, Yamashita, & Sugiura, 2010). The author contrived the idea from the sequence similarity (30% homology) with GPR55 whose ligand is LPI. HEK293 cells expressing GPR35 were used to test the activation of the receptor by several lipid molecules. Among those molecules, 2-acyl lysophosphatidic acid-activated cellular responses in calcium assay, RhoA and pERK activation assay and receptor internalization assay. However, Deng (H. Deng, Hu, & Fang, 2012) pointed out that lysophosphatidic acid's role still remains unclear because other LPA receptors can be co-expressed with GPR35 in native cells. In addition, tyrosine metabolites including DHICA, T3 and reverse T3 have been suggested as a potential endogenous ligand of GPR35 (H. Deng et al., 2012). DMR assay, internalization assay, and pERK activation assay were used to test these molecules at endogenous GPR35 in HT-29 cells, but supplemental evidence from individual studies has not been reported yet. In the following year, cyclic guanosine monophosphate has been suggested as a possible endogenous agonist for GPR35 by β-arrestin recruitment assay (Southern et al., 2013), but this candidate also required future studies. A recently suggested endogenous ligand of GPR35 is CXCL17 (Maravillas-Montero et al., 2015). Maravillas-Montero et al. performed calcium mobilization assay using GPR35-transfected BA/F3, GPR35-transfected HEK293 cells and human monocytes cell line THP-1 expressing endogenous GPR35. This study showed that CXCL17, at a dose of 100nM, induced a calcium flux, but some of the experiments were repeated less than three times, making it difficult to show significant value. Moreover, the potentials of binding with other endogenous receptors in the cells that they used cannot be excluded, as in other receptor-ligand pairing studies. Therefore, experiments designed to test the deficiency of GPR35 in human cells are required. Many other recent studies have failed to support CXCL17 as an endogenous ligand for GPR35 (Binti Mohd Amir et al., 2018; Park, Lee, Nam, & Im, 2018), even though several studies have attempted to use CXCL17 to identify the function of GPR35 (Y. J. Guo, Zhou, Yang, Shao, & Ou, 2017; Schneditz et al., 2019). Very recently, LPA has been re-examined as an endogenous

ligand of GPR35 (Kaya et al., 2020). They used genetically modified zebrafish and a mouse model with a deficiency of GPR35 expression to demonstrate LPA as a ligand for GPR35.

TABLE 1.7 POTENTIAL ENDOGENEIOUS AGONISTS OF HUMAN GPR35

Name	Methods	Reference
Kynurenic acid (KYNA)	Calcium mobilization and inositol phosphate production, internalization of GPR35	(J. Wang et al., 2006)
LPA (2-acyl-lysophosphatidic acid)	Calcium activation, ERK phosphorylation and GPR35 internalization assay.	(Oka et al., 2010) also suggested by (Kaya et al., 2020)
Tyrosine metabolites: DHICA, T3 and reverse T3	DMR assay, β-arrestin translocation, ERK phosphorylation and receptor internalization	(H. Deng et al., 2012)
Cyclic guanosine monophosphate (cGMP)	PathHunter-β-arrestin recruitment assay	(Southern et al., 2013)
CXCL17	Calcium assay and Chemotaxis assays	(Maravillas-Montero et al., 2015)

1.3.2 Pathological and physiological studies of GPR35

Cardiovascular diseases (heart diseases)

Overexpression of the GPR35 gene in myocardial samples from heart failure patients was observed, and a GPR35 KO mouse model showed higher blood pressure compared to the wild-type mouse model, suggesting GPR35 as a good candidate for new strategies to control high blood pressure (Min et al., 2010). Moreover, GPR35 transcripts in cardiac myocytes were upregulated by both oxygen-dependent and -independent HIF-1 expression and the mouse model used in this study that develops cardiac failure, showed the potential of GPR35 gene expression as an early marker of the progression of this disease (Ronkainen et al., 2014). The overexpression of GPR35 in human vascular smooth muscle cell (VSMC) was observed by real-time PCR (McCallum et al., 2015). Their additional *in vitro* studies indicated that activation of GPR35 by zaprinast and pamoic acid regulates vascular proliferation and migration. Another individual exome array analysis using samples from anthracycline-treated paediatric cancer patients showed that GPR35 has an important role in cardiac physiology and pathology (Ruiz-Pinto et al., 2017). Another report on a GPR35 KO mice study emphasized

GPR35 as a potential new drug target to control hypertension, despite the GPR35 KO mice showing no differences in blood pressure, cardiac function and cardiomyocyte morphology compared to wild type mice, which conflicts with the previous study (Divorty, Milligan, Graham, & Nicklin, 2018). Overall, the role of GPR35 in cardiovascular disease has shown its potential as a new drug target, although the physiological function of this receptor remains unclear.

Malfunction in the nervous system and pain model

Several studies have been focused on the analgesic role of GPR35 in the nervous system, especially paying attention to KYNA (reviewed in (Mackenzie & Milligan, 2017)). The mRNA GPR35 expression in rats was detected in both whole ganglion and cultured dorsal root ganglion neurons by RT-PCR and its anti-nociceptive effects with agonists of GPR35, KYNA and zaprinast, were demonstrated in rat nociceptive DRG neurons, together suggesting that GPR35 might provide a good target for modulating pain perception (Ohshiro et al., 2008). In addition, the expression of mouse GPR35 in dorsal root ganglion and the spinal cord of rodents was verified. The following Writhing test in a mouse model showed the consistent potential of GPR35 agonists, both KYNA and zaprinast, as a new pharmacological agent to control inflammatory pain (Cosi et al., 2011). Following researches on GPR35 in mice nervous system revealed that this receptor has an important role in the KYNA-dependent reduction of extracellular glutamate levels in the brain, eventually contributing to reduced excitatory transmission (Berlinguer-Palmini et al., 2013). A functional GPR35 expression was observed particularly in the CA1 field of rat hippocampus by fluorescence immunohistochemistry staining (Alkondon et al., 2015). Accumulating evidence demonstrated that the activation of GPR35 by both KYNA and zaprinast reduces adenylate cyclase activity and modulates HCN channels in dorsal root ganglion neurons (Resta et al., 2016). Both KYNA and zaprinast reduced neuropathic pain from sciatic nerve injury and increased the effectiveness of morphine uses in a neuropathic pain mouse model (Rojewska, Ciapala, & Mika, 2019).

Metabolic disorder

Genome-wide screens in particular of the regions located in type 2 diabetes genes (chromosome 2) identified the GPR35 gene as a putative diabetes-susceptibility gene (Horikawa et al., 2000). However, an additional study focusing on the evolution of type 2 diabetes variants including GPR35 and CAPN10 genes didn't show significant linkage evidence, even though the authors still indicated the relevance of GPR35 in this disease by

mentioning its abundant expression in pancreatic islets (Vander Molen et al., 2005). Activation of GPR35 by KYNA increased energy utilization together with enhanced lipid metabolism, and thermogenic and anti-inflammatory gene expression in adipose tissue. Interestingly, an *in vivo* study using the GPR35 KO mouse model suggested that GPR35 has an important role in regulating obesity (Agudelo et al., 2018). The expression of GPR35 (also co-expressed with CCK1R and GPR65, which are known as GI afferents markers) was detected by qPCR array, RNA sequencing and *in situ* hybridization studies, in the vagal afferents which innervate the gastrointestinal (GI) mucosa, suggesting a role of GPR35 in gut-brain communication (Egerod et al., 2018). Moreover, GPR35 promoted glucose uptake and oxidation in murine macrophages and human colorectal cancer cells (Caco2), leading to a contribution in energy homeostasis (Schneditz et al., 2019). Hepatic steatosis is generated by an accumulation of triacylglycerols in the liver, leading to liver metabolic dysfunction. Studies using lodoxamide, suggested as a potential agonist of GPR35, demonstrated that GPR35 has a protective role in liver steatosis, indicating GPR35 as a pivotal regulator of energy homeostasis (Nam, Park, & Im, 2019). A very recent publication also suggested GPR35 as a key modulator in intestinal homeostasis (Kaya et al., 2020).

Immune disease

Human and mouse GPR35 expression was detected in diverse immune cells including peripheral leukocytes, neutrophils, monocytes ($CD14^+$), dendritic cells and T cells ($CD3^+$) (J. Wang et al., 2006). The expression of GPR35 in human natural killer T (iNKT) cells ($CD56^+$) was reported, and the activation of this receptor by potential agonists (KYNA and zaprinast) reduced interleukin-4 release from iNKT cells (Fallarini, Magliulo, Paoletti, de Lalla, & Lombardi, 2010). An upregulated GPR35 mRNA was observed in human macrophages exposed to polycyclic aromatic hydrocarbons, also known as an immunotoxic and carcinogenic environmental contaminant, suggesting that GPR35 has a protective role in immune cells exposed in toxic environments (Sparfel et al., 2010). The GPR35 mRNA expression in human mast cells, human basophils and human eosinophils was verified by qPCR analysis and the upregulated GPR35 expression in mast cells induced by IgE antibodies, suggesting GPR35 as a potential target for asthma highly related to mast cell biology (Yang et al., 2010). In fact, several antiallergic mast cell stabilizers, including cromolyn disodium, nedocromil sodium, bufrolin and lodoxamide, have shown their affinity toward GPR35 (MacKenzie et al., 2014; Yang et al., 2010). Single nucleotide polymorphisms in GPR35 are highly associated with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Imielinski et al., 2009). A worsened colitis disease was observed in dextran sulphate

sodium (DSS)-induced experimental colitis GPR35$^{-/-}$ mice compared to GPR35$^{+/+}$ mice induced by DSS, indicating its protective role in colonic inflammation (Farooq et al., 2018). The T108M polymorphism of GPR35 in macrophages was associated with ulcerative colitis, cholangitis, and inflammatory disease with cancer risk by engaging with the sodium potassium-pump (Schneditz et al., 2019). Recent experimental evidence demonstrated that the activation of GPR35 by LPA controls intestinal immune homeostasis by maintaining TNF production in macrophages (Kaya et al., 2020).

Cancer

GPR35 transcripts were observed in gastric cancer tissues but not in non-cancerous mucosa (Okumura et al., 2004). However, other studies showing GPR35 protein expression in gastric cancer have not been performed.

The overexpression of GPR35 in non-small-cell lung cancer (NSCLC) tissues correlating with poor prognosis was demonstrated (W. Wang, Han, Tong, Zhao, & Qiu, 2018). Chemoresistant A549 (A549R) cells, established by gradual exposure to doxorubicin toxicity, showed upregulation of GPR35 with an expression of antiapoptotic proteins compared to A549 cells. A549R cells expressing low GPR35 by siRNA transfection had an increased apoptosis rate in response to cisplatin *in vitro*, indicating that GPR35 may regulate chemoresistance. Moreover, *in vivo* experimental evidence using a xenograft mouse model transplanted with A549R cells depleted of GPR35 by shRNA transfection showed consistent results suggesting GPR35 as a key regulator of chemoresistance in NSCLC. Furthermore, the authors suggested that the GPR35/β-arrestin-2/AKT loop might contribute to chemoresistance.

The expression of GPR35 was detected in most breast cancer tissues associating higher cell proliferation (showing high Ki-67 expression) compared to normal adjacent tissues (Y. J. Guo et al., 2017). Moreover, most breast cancer cell lines show GPR35 protein expression. Guo et al. have shown the translocation of GPR35 with the treatment of CXCL17 by IF in breast cancer cells. The activation of ERK was inhibited upon treatment of CXCL17 in MCF-7 cells expressing low GPR35, but the loading control, which analyses relative abundance, was not indicated in WB.

As I mentioned above, the HT-29 cell line, one of the human colorectal cancer cell lines, is widely used for validating the agonist activity of GPR35 because of its endogenous GPR35 expression. Three remarkable studies have shown the importance of GPR35 in colon cancer. Hu et al. screened GPR35 transcripts upon treatment with GPR35 agonists YE210 and pamoic acid, by label-free short hairpin RNA kinome screening and DNA microarray (H. H. Hu et al., 2017). As a result of these chemical genomic approaches, the gene ontology analysis indicated

that GPR35 activation by YE210 influences cellular stress responses, immune system, and cell morphogenesis in HT-29 cells. Additional validating experimental evidence verified that the activation of GPR35 particularly regulates cellular response under hypoxic stress in colorectal cancer cells (H. H. Hu et al., 2017). A recent publication has shown that GPR35 affects intestinal tumour development. Around 40% fewer intestinal tumours were observed in *Gpr35*$^{-/-}$;*Apc*^{min} mice compared to *Apc*^{min} mice which develop adenomatous polyposis coli driving spontaneous colorectal cancer. Not only the spontaneous tumour model but also inflammation-driven tumour mouse models showed that the deletion of GPR35 decreases intestinal tumour development (35% fewer). Moreover, reduced proliferation, detected by Ki-67 staining in IHC, was detected in intestinal tumour tissues of the GPR35 deficient mice compared to mice having wild-type GPR35. The authors reported that this proliferation and the oncogenic signalling promoted by GPR35 are highly associated with the sodium-potassium pump function and Src signalling activity (Schneditz et al., 2019). Concurrently, another study highlighted the importance of GPR35 expression in colon cancer for the prediction of prognosis (Ali et al., 2019). The mRNA expression of GPR35 extracted from primary tumour specimens, regional lymph nodes specimens and normal colon tissues specimens obtained from colon cancer patients was analysed by real-time qPCR. The high level of GPR35b mRNA, in particularly in regional lymph nodes, was related to poor prognosis, which includes increased recurrence rate and decreased survival time compared to patients having regional lymph nodes metastases expressing a low level of GPR35b mRNA.

1.4 CXCL17

1.4.1 Chemokines in PDAC

Chemokines are chemotactic cytokines, generally known as attractants for leukocytes from the circulation, mediating immune responses. Chemokines have a structural characteristic of four conserved cysteine residues and, based on their positions, chemokines are divided into four families: CXC, CX3C, CC and C family. Nearly 50 human chemokines and 20 corresponding receptors, which are seven-transmembrane G-protein-coupled receptors (GPCRs), have been revealed. The fact that there is a smaller number of receptors than the number of chemokines explains that multiple chemokines can bind to the same chemokine receptor. This redundancy is the main characteristic of chemokines and it often hampers the development of receptor-specific therapeutics. Most chemokines are secreted from a wide variety of cells in response to infections and/or diverse inflammatory stimuli. Their key role in chronic inflammation, which is a major cause of initiation and progression of cancer, has been identified, thereby

more recently, chemokines have been spotlighted on cancer research. Increasing evidence has shown the importance of chemokine in cancer study, not only for initiation and for progression of cancer, but also in metastasis.

Diverse chemokines in PDAC have been identified as key mediators of cancer progression (summarized in Table 1.5). The expression of CCL2 (monocyte chemotactic protein 1, MCP1), known as a proinflammatory chemokine, was observed under normal or Fas antigen stimulation conditions in PANC-1 or/and MIA PaCa-2 cells (Shimada, Andoh, Araki, Fujiyama, & Bamba, 2001; Takaya et al., 2000). Additionally, CCL2 has been shown to recruit immune cells to PDAC and promote cancer cell proliferation and angiogenesis (Sun et al., 2017). A recent publication has reported that CCL2 and CXCL8 axes have important roles in PDAC metastasis (Pausch et al., 2020). CXCL8, also known as IL-8, has been identified as a key mediator of aggressive pancreatic tumour progression and survival (Awaji, Futakuchi, Heavican, Iqbal, & Singh, 2019). CXCL12, which receptor is CXCR4, has been relatively well studied in a wide array of cancer types compared to other chemokines. Much experimental evidence shows the important role of the CXCL12/CXCR4 axis in PDAC. This axis has shown its positive correlation with chemoresistance (Khan et al., 2020), metastasis (B. Shen et al., 2013), and progression (Sleightholm et al., 2017) in PDAC.

TABLE 1.7 CHEMOKINES IN PDAC WITH THE CORRESPONDING LITERATURE

Chemokines	Alternative name	Corresponding receptor	Functions in PDAC	References
CCL2	MCP-1	CCR2/4/5	Poor patient survival Metastasis	(Pausch et al., 2020; Sanford et al., 2013)
CCL5	RANTES	CCR1/3/5	Migratory and invasiveness	(Singh et al., 2018)
CCL20	LARC, Exodus-1	CCR6	Growth and metastasis	(B. Liu et al., 2016; Rubie et al., 2010)
CCL21	SLC, 6Ckine, Exodus-2	CCR7	Migration, CSC metastasis	(Hirth et al., 2020; L. Zhang et al., 2016)
CCL28	MEC	CCR3/10	Increased expression	(Roy et al., 2017)
CXCL1	GRO1, NAP-3	CXCR2	Migration and invasion	(Z. Wen et al., 2019)
CXCL2	GRO2, MIP-2a	CXCR2	Survival in murine model	(Sano et al., 2019)
CXCL4	PF-4	CXCR3	Angiogenesis and invasion	(Quemener et al., 2016)
CXCL5	ENA-78	CXCR2	Poor prognosis and survival, Cell migration and invasion	(Ando et al., 2020; A. Li et al., 2011; R. Zhang et al., 2020)
CXCL8	IL-8, NAP-1, MDNCF, GCP-1	CXCR1/2	Angiogenesis, high expression, metastasis	(Awaji et al., 2019; Matsuo et al., 2009; Pausch et al., 2020)
CXCL9	Mig	CXCR3	Survival, progression, chemotherapeutic efficacy	(H. F. Gao et al., 2020; Qian et al., 2019)
CXCL10	IP-10, CRG-2	CXCR3	Progression and migration	(H. F. Gao et al., 2020; Hirth et al., 2020; Qian et al., 2019)
CXCL12	SDF-1, PBSF	CXCR4/7	Progression, high expression, metastasis	(Roy et al., 2014; B. Shen et al., 2013; Sleightholm et al., 2017; J. Zhang, Liu, Mo, Shi, & Li, 2018)
CXCL14	BRAK, bolekine	undefined	Invasiveness	(Wente, Mayer, et al., 2008)
CXCL16	SRPSOX	CXCR6	High expression	(Roy et al., 2017; Wente, Gaida, et al., 2008)
CX3CL1	Fractalkine, Neurotactin, ABCD-3	CX3CR1	Malignant behaviour, Poor prognosis	(Marchesi et al., 2008; Stout, Narayan, Pillet, Salvino, & Campbell, 2018; Xu et al., 2012)

1.4.2 CXCL17 studies

Chemokine 17 (CXCL17), known as a VEGF co-regulated chemokine 1 (VCC-1), is the newest chemokine of the CXCL family reported so far. The gene of CXCL17, located at human chromosome 19q13.2, was first described in 2003 by the Secreted Protein Discovery Initiative (SPDI) (Clark et al., 2003). Structural studies showed that its amino acid sequence has similarities with CCL16 and CCL17 (Weinstein et al., 2006). The folding structure of CXCL17 is similar to IL-8 and CXCL8 (Eigenbrot, Lowman, Chee, & Artis, 1997). The molecular weight of CXCL17 is about 13,819 Da before translational cleavage and about 11,418 Da post-translational cleavage. Precursor proteins of CXCL17 include six cysteine residues, but mature proteins consist of four cysteine residues resulted from the removal of 22 amino acid sequences including two cysteine residues. These findings identified CXCL17 as a chemokine of the CXCL family (Pisabarro et al., 2006). A chemoattractant activity of the four-cysteine mature peptide twice as high compared to one of the six-cysteine premature peptides has been elucidated, to recruit human THP-1 monocytes and mouse J774 macrophages (W. Y. Lee, Wang, Lin, Hsiao, & Luo, 2013). This maturation process may decide the affinity to the receptor of CXCL17, even though the specific receptor of this chemokine has not been identified.

GPR35 has been suggested as the receptor of CXCL17 (Maravillas-Montero et al., 2015). The CXCL17 mediated calcium flux in Ba/F3 and HEK293 cells expressing GPR35 by transfection has been measured by calcium-mobilization assay. The authors also have described that a downregulated GPR35 expression was detected in the lung tissues of Cxcl17-/- mice. Another experimental evidence has supported the existence of this CXCL17-GPR35 axis in breast cancer cells (Y. J. Guo et al., 2017). The activation of GPR35 induced by recombinant human CXCL17 (rhCXCL17) has been identified by immunofluorescence analysis showing receptor endocytosis in the cytoplasm of MCF-7 cells in response to CXCL17. The authors have also described that phosphorylation of ERK is induced by the CXCL17-GPR35 axis in breast cancer cells. However, the receptor of CXCL17 remains still unclear because other publications have provided experimental evidence showing a lack of GPR35 as the receptor of CXCL17 (Binti Mohd Amir et al., 2018; Park et al., 2018).

Recent cancer studies have suggested CXCL17 as a potential oncogene. Enhanced expression of CXCL17 has been demonstrated in a variety of cancer types including lung (Hsu et al., 2019), breast (Y. J. Guo et al., 2017), colon (Ohlsson, Hammarstrom, Lindmark, Hammarstrom, & Sitohy, 2016; Rashad et al., 2018), pancreatic (Hiraoka et al., 2011) and liver cancer (L. Li et al., 2014; L. Wang et al., 2019; Z. Zhou et al., 2012). The tumorigenic potential of CXCL17 has been examined using breast cancer cells (Y. J. Guo et al., 2017) and

hepatocellular carcinoma cells (Z. Zhou et al., 2012). Both *in vitro* and *in vivo* assay showed that CXCL17 promotes breast cancer cell proliferation and migration. HepG2 cells overexpressing CXCL17 increase their proliferation and inhibit cisplatin-induced apoptosis. A recent publication supports the functional mechanism of CXCL17 tumorigenic potential in hepatocellular carcinoma (L. Wang et al., 2019). The authors provide experimental evidence to prove that CXCL17 promotes cell invasion and suppresses autophagy in liver cancer.

1.5 Thesis objectives

Pancreatic cancer is one of the most lethal human cancers and it remains a major challenge of human health problems. Due to the lack of symptoms in the early stages of this cancer, most patients are diagnosed too late and miss the opportunity to undergo surgery which is the most effective treatment to date. The majority of patients have to rely on conventional chemotherapy which has not shown a huge improvement in recent decades. Despite all efforts on understanding oncogenes and tumour suppressor genes of pancreatic neoplasm, their molecular mechanisms corresponding to the genetic changes have been poorly researched. In addition, pancreatic cancer shows high aggressiveness and recurrence, which shows the limitation of current therapy targeting for general cell growth mechanisms. Therefore, the development of novel PDAC drug targeting on PDAC-specific targets must be required.

Research in Marco Falasca's group has identified an autocrine loop involving the ABC transporter ABCC1, the GPCR GPR55 and the release of LPI in ovarian and prostate cancer. Our initial hypothesis was that GPR35 could be activated by LPI due to its structural similarity with GPR55. To date, GPR35 appears to be quite promiscuous in its binding affinity but recent data show that GPR35 is modulated by inositol. Nevertheless, the preliminary data shows the high potential of GPR35 as a new therapeutic target for PDAC because of its cancer-specific expression pattern.

The main hypothesis of this project was 1) GPR35 mediates PDAC proliferation and progression. 2) GPR35 is a new therapeutic target for PDAC. 3) GPR35 and one of its putative ligands CXCL17 have an important role in PDAC.

To evaluate our hypothesis, the specific objectives of my project were to:

1) Identify the expression of GPR35 in PDAC

2) Investigate the influence on PDAC from the inhibition of GPR35 *in vitro* and *in vivo*

3) Suggest the role of GPR35 in PDAC

4) Evaluate CXCL17 as an endogenous ligand for GPR35 in PDAC

5) Verify the pharmacological potential of GPR35 *in vitro* and *in vivo*

Chapter 2

Chapter 2: Materials & Methods

2.1 Reagents and chemicals

All chemicals used in this study are on Table 2.1.

TABLE 2.1 LIST OF CHEMICALS

Name	Descriptions	Concentration	Manufacturer	Catalogue number
ML145	Synthetic GPR35 inhibitor	5-10μM	Tocris Bioscience	#4172
CID2745687	Synthetic GPR35 inhibitor	5-10μM	Tocris Bioscience	#4293
Compound 10	Synthetic GPR35 agonist	1-10μM	Sigma-Aldrich	#SML0174
Zaprinast	Synthetic GPR35 agonist	5μM	Sigma-Aldrich	#Z0878
Pamoic acid	Synthetic GPR35 agonist	10μM	Sigma-Aldrich	#45150
CXCL17	Proposed endogenous ligand for GPR35	50ng/mL	R&D systems	#4207DM
Puromycin	Used for selection of CRISPR/Cas9 transfection	1-2μg/mL	Sigma-Aldrich	#P8833
Hygromycin B	Used for selection of the Luc plasmid transfection	500μg/mL	AG Scientific	#H-1012-SOLID
D-Luciferin (potassium salt)	A chemiluminescent substrate of firefly luciferase	100μg/mL	Cayman	#14681

2.2 Antibodies

Antibodies and their conditions used for Western blot (WB), immunofluorescence (IF), flow cytometry (FCM) and immunohistochemistry (IHC) are listed in Table 2.2 (Primary antibody) and 2.3 (Secondary antibody).

TABLE 2.2 LIST OF PRIMARY ANTIBODIES

Antigen	Host species	Application conditions	Manufacturer	Catalogue number
α-actinin	Rabbit	WB (1:1000)	Cell signalling	#3134
ABCG2	Rabbit	WB (1:1000)	Cell signalling	#42078
GPR35	Rabbit	WB (1:1000)	Proteintech™	#552 48-1-AP
GPR35	Rabbit	WB (1:1000)	Cayman	#10007660
GPR35	Rabbit	WB (1:1000) IHC & FCM (1:50)	Origene	#TA 338287 #TA 340416
HIF-1α	Rabbit	WB (1:1000)	Novus Biologicals	#NB100-479
Tubulin	Rat	WB (1:1000)	Cell signalling	#2148
Cleaved caspase 3	Rabbit	WB (1:1000)	Cell signalling	#9664
pERK1/2	Rabbit	WB (1:2000)	Cell signalling	#4370
ERK	Rabbit	WB (1:2000)	Cell signalling	#4695
pAKT S473	Rabbit	WB (1:1000)	Cell signalling	#4060
AKT	Rabbit	WB (1:1000)	Cell signalling	#9272
SQSTM1/P62	Rabbit	WB (1:1000)	Cell signalling	#5114
LC3B	Rabbit	WB (1:1000)	Cell signalling	#3868
β-actin	Rabbit	WB (1:2000)	Cell signalling	#4970
Vimentin	Rabbit	WB (1:1000)	Cell signalling	#5741
CXCR4	Rabbit	WB (1:1000)	Novus Biologicals	#NB100-74396SS
CXCL17	Mouse	WB (1μg/mL)	R&D systems	#MAB4207

TABLE 2.3 LIST OF SECONDARY ANTIBODIES

Antigen	Application conditions	Manufacturer	Catalogue number
Rabbit-HRP	1:40000	Cell signalling	#7074
Rat-HRP	1:30000	Cell signalling	#7077
Rabbit-Alexa Fluor™ 488	1:1000	Cell signalling	#4412

2.3 Plasmids and constructs

TABLE 2.4 LIST OF SIRNA AND PLASMID FOR TRANSFECTION

Gene	Name on this study	Details	Manufacturer	Catalogue number
GPR35	siGPR35-Seq.2	CCGGCACAATTTCAACTCCAT	Qiagen	SI00075411
GPR35	siGPR35-Seq.3	CAGGACCATGAATGGCACCTA	Qiagen	SI00075418
Negative control	siControl	Non-targeting siRNA	Ambion	#4390843
GPR35	GPR35 CRISPR/Cas9 KO Plasmid (h)	Target three gRNA sequences Sense: 1.GGGCGCGACGGATCGTCTCC 2.TGTACTCACAGAGAGACGCA 3.CTGGCCCCCAGCGATCAAGC	Santa Cruz Biotechnology	#sc-416792
HDR	GPR35 HDR Plasmid (h)	A Puromycin resistance gene A RFP gene	Santa Cruz Biotechnology	#sc-416792-HDR
Negative control	Control CRISPR/Cas9 Plasmid	Non-coding scrambled control RNA sequence	Santa Cruz Biotechnology	#sc-4189922
Luciferase	Luc Plasmid	pGL4.50[luc2/CMV/Hygro] Vector	Promega	#E131A
miRNA-34b	miR-34b	CAAUCACUAACUCCACUGCCAU	Ambion	#MC12727

2.4 Cell culture

2.4.1 PDAC cell lines culture

All cell lines were cultured in a humidified 95% air and 5% CO_2 incubator at 37ºC, in conditions recommended by the manufacturer unless specific conditions indicated. For hypoxic incubation, cells were maintained in less than 2% O_2 within a modular incubator chamber (Billups-Rothenberg, Inc.) filled with 2% O_2, 2% CO_2, balanced with N_2 and humidified. All cell lines were cultured in their recommended media (Table 2.5) supplemented with 10% (v/v) foetal bovine serum (FBS, Bovogen #SFBS-F) with 1% (v/v) penicillin/streptomycin (PS, Sigma #P4333) and 2nM L-Glutamine (Sigma #G7513) unless specific conditions stated. Transfected cells by CRISPR/Cas9 cell lines were maintained with 1 μg/mL (for MIA PaCa-2) or 2μg/mL (for AsPC-1) concentration of puromycin in their complete media. All cell lines were cultured in *Mycoplasma*-free conditions and tested regularly every three months for *Mycoplasma* by PCR. 'The patient-derived cell lines were provided by the Australian Pancreatic Cancer Genome Initiative (APGI, www.pancreaticcancer.net.au) and the Garvan Institute of Medical Research (Sydney, Australia). The murine pancreatic cancer cell lines PZR1, PZPR1 and PZPflR were provided by Dr Owen Sansom (Beatson Institute, Glasgow, UK).

TABLE 2.5 LIST OF CELL LINES USED FOR THIS STUDY

Cell line	Source	Organ	Disease	Metastasis	Differentiation	Culture media
AsPC-1	ATCC/CRL-1682™	Pancreas	PDAC	Ascites	Poor	RPMI-1640 media/Sigma #R8758
HPAF-II	ATCC/CRL-1997™	Pancreas	PDAC	Ascites	Well	MEM/EBSS media/Hyclone-GE #SH3024401
CFPAC-1	ATCC/CRL-1918™	Pancreas	PDAC	Liver	Well	IMDM media /Hyclone-GE #HYCSH3025902
MIA PaCa-2	ATCC/CRM-CRL-1420™	Pancreas	PDAC	Primary tumour	Poor	DMEM-high glucose media/Sigma #D6429 2.5% horse serum/Sigma #1138
Capan-2	ATCC/HTB-80™	Pancreas	PDAC	Primary tumour	Well	McCoy 5A media/Hyclone #SH30200.01
BxPC-3	ATCC/CRL-1687™	Pancreas	PDAC	Liver	Moderate to poor	RPMI-1640 media/Sigma #R8758
PANC-1	ATCC/CRL-1469™	Pancreas	PDAC	Primary tumour	Poor	DMEM-high glucose media/Sigma #D6429
hTERT-HPNE	ATCC/CRL-4023™	Pancreas	-	-	-	70% DMEM no glucose media /Sigma # D-5030 25% M3 Base /Incell Corp. #M300F-500
HPDE	Prof. H Kocher	Pancreas	-	-	-	Keratinocyte-SFM culture media/Gibco EGF/Life Technologies Bovine pituitary extract/Life Technologies

2.4.2 Tumorspheres culture

The AsPC-1 cell line and the selected four clones of transfected MIA PaCa-2 cell lines with CRISPR/Cas9 for GPR35 gene modification were cultured in described conditions above. Once cells reached 80% confluency, the medium was replaced with FBS free medium and cells were incubated in serum-free condition for 7 days allowing the cells to start floating and forming spheroid-like structures. The supernatant and detached cells by trypsinization were collected and centrifuged at 200 × g rcf for 5 min at RT. Pelleted cells were washed twice with HBSS and resuspended in 500µl of Accutase™ (STEMCELL™ Technologies) to disaggregate cells. The cells in Accutase were disaggregated one more time by passing through a 25g needle. Then, the enough suspended cells were moved to a new ultra-low attachment flask (Corning) containing stem cell growth media (DMEM/F12 supplemented with enriched N2 and B27/R&D Systems) with growth factors (1µM EGF and 2µM FGF-2). The media was changed every three days and cells were passaged at a 1/10 dilution.

For the tumorsphere proliferation assay, the disaggregated cells in Accutase were stained with trypan blue and only alive cells were counted by using Neubauer chamber. The same number of cells (25,000 cells/a well) was seeded in 24-well low attachment plates with stem cell culture media mentioned above adjoined with ML145 and/or Gemcitabine (concentrations are indicated in Figure 6.2). Cells were incubated for 5 days and then the treated tumorspheres from each well were collected in different Eppendorf tubes. The cells were disaggregated with 50µl of Accutase and the number of cells (each concentration in duplicate) was counted with trypan blue exclusion.

2.4.3 Passaging and preservation of cells

Cells were typically passaged at a 1/15 dilution when cells reached 80% confluency. Cells were washed with PBS and trypsin-EDTA (0.25%) was added (1ml per a T75 flask). The cells were incubated with trypsin for 5-10 min at 37^{o}C and the detached cells were collected with a complete medium to neutralize the reaction. The cells were centrifuged at 1200 rpm for 5 min at room temperature. After centrifugation, the supernatant was removed and the cell pellet was resuspended in complete media. Cells were seeded into new culture plates. Cells were cultured for no more than ten passages after which fresh cells were thawed. For the preservation of cells, freezing media containing 5% DMSO was used. Cells were gradually frozen by using an isopropanol-filled freezing container at -80^{o}C overnight and then the vials were moved to

the liquid nitrogen tank (vapour phase). Cryovials (Thermo Scientific) were used for the storage of cells.

2.5 Transfection of PDAC cells

2.5.1 siRNA transient transfection

Cells were seeded in a 6-well plate with cell growth media that doesn't contain PS. Typically 3×10^5 cells per well were seeded except for MIA PaCa-2 and CFPAC-1 (2×10^5 cells per well) and incubated overnight. Once cell density between 50-60% is verified the next day, transfection was performed. Dharmafect 1 transfection reagent (Dharmacon®) was used for all siRNA transfection in this study. The procedure of transfection was performed according to the protocol provided by Dharmacon with a final siRNA concentration of 75nM. Two different sequences were used for each target gene and the details are indicated in Table 8. The optimal time showing high efficiency of transfection was 72 hours post-transfection.

For the miR-34b transfection, the used miRNA details are provided in Table 2.4. 75μM of working concentration was used in this transfection. The transfected AsPC-1 cells were harvested 72 hours post-transfection.

2.5.2 Development of a stable GPR35 or ABCG2 KD cell lines by CRISPR/Cas9 system

Cell growth media without PS was used for this transfection. Cells were seeded with cell density 4×10^5 cells per well for AsPC-1 and 2.5×10^5 cells per well for MIA PaCa-2 in a 6-well dish 24 hours prior to transfection to achieve 70-80% confluence. Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) was used for CRISPR/Cas9 transfection in this study. The lipofectamine 3000 reagent (7.5μl per well) was diluted in Opti-MEM medium (117.5 μl per well), called solution A and, at the same time, 1μg DNA (0.5μg of target gene KO/control plasmid and 0.5μg of HDR plasmid) was diluted with P3000 reagent (2μl per well) in Opti-MEM (113μl per well) medium, called solution B. Each solution was mixed well and incubated for 5min at RT. Subsequently, solutions A and B were mixed well (1:1 ratio) and incubated for 15 min at RT. The DNA-lipid complex (250μl of mixture) was applied into cells drop-wise and the total volume of growth medium without PS and the mixed solution was 2ml

per well. The following day, the medium was changed to a complete medium. Used DNA details are indicated in Table 2.4.

Transfection efficiency was recorded through IncuCyte Life Cell Analysis Imaging System (Sartorius) from 48 hours to 72 hours post-transfection. Figure 2.1 shows an example of ABCG2 KO CRISPR/Cas9 transfection efficiency. Co-expression of HDR (RFP) and control (GFP) can be shown in AsPC-1 control cells. In AsPC-1 ABCG2 KD cells, co-transfection of ABCG2 KO plasmid (GFP) and HDR (RFP) plasmid was shown. Cells were treated with puromycin (working concentration is 1μg/mL for AsPC-1 and 2μg/mL for MIA PaCa-2) for selection on the third day post-transfection. From then, selection media was replaced every 3 days until colonies of transfected cells were formed. Once the formation of a colony derived from a single cell was verified visually, each colony was picked up and reseeded to a well of a new 6-well plate containing selection media. When the clones were grown enough to make lysates, cells were harvested for Western blot in order to screen the expression of the gene of interest. Based on the western blot analysis, four clones which are two controls and two KD cell lines were selected and preserved for future study.

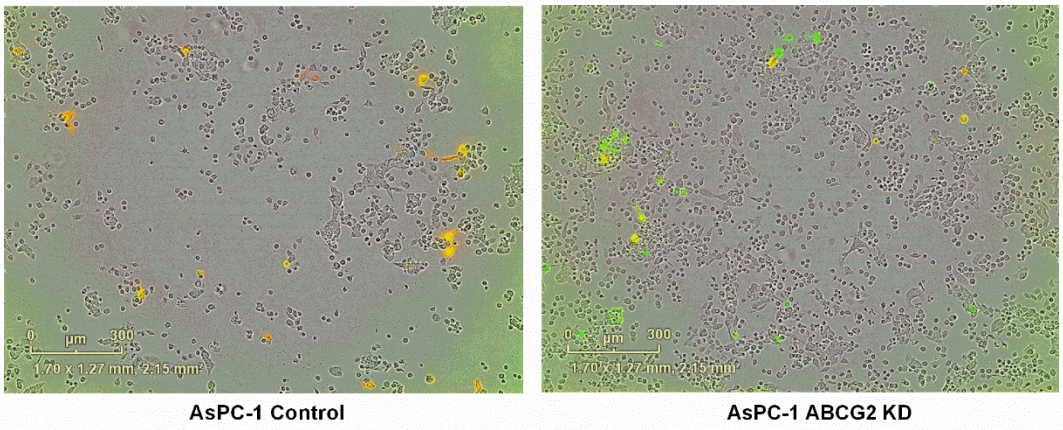


Figure 2.1 ABCG2 KO CRISPR/Cas9 transfection efficiency recorded by IncuCyte. *(Left) Co-expression of control (GFP) and HDR (RFP) can be shown in AsPC-1 control cells. (Right) Co-expression of ABCG2 KO (GFP) and HDR (RFP) was shown in AsPC-1 ABCG2 KD cells.*

2.5.3 Development of MIA PaCa-2_Luc

Transfection was performed as described in section 2.5.2. The DNA used for this transfection is indicated in Table 2.4. For selection, hygromycin B with 500μg/mL of concentration was used. After two weeks post-selection, the transfected cells were seeded to a flat bottom clear and black 96-well plate (Corning) with cell density 2×10^4 and 4×10^4 per well for screening luciferase activity. The next day, the cells were treated D-luciferin (100μg/mL) and the

quantitation of bioluminescent sources was analysed by EnSight (PerkinElmer) with spectral response from 450nm up to 645nm.

2.6 Western blotting

2.6.1 Western blot sample preparation

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150nM NaCl, 50mM Tris pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate (SDC), 1%Triton X-100) including protease inhibitor cocktail (Sigma Aldrich) and phosphoSTOP phosphatase inhibitor (Sigma Aldrich). Cells were harvested using a cold plastic cell scraper or trypsin. Lysates were sonicated in a cold-water bath sonicator and then centrifuged for 5 min at 25,000g. The supernatant excluding insoluble material was transferred to a new Eppendorf tube and protein concentrations were determined by Millipore protein quantification cards. LDS sample buffer was added and boiled at 95^{o}C for 5 min. Samples were stored at -20^{o}C.

2.6.2 SDS-PAGE electrophoresis and Western blotting

Samples were loaded on 10% (For most proteins) or 12% (For LC3B isoforms) acrylamide gel by SDS-PAGE at a constant 120V for 2 hours. The separated proteins were transferred to 0.45μm nitrocellulose membranes by using a semi-dry Trans-Blot Turbo Transfer System (BioRad) at a constant 25V for 10 min. The membrane was incubated in 3% BSA/TBS-T (20mM Tris-buffered saline, pH 7.6, 0.05% Tween 20) for 1 hour at RT for blocking followed by primary antibody incubation overnight at 4^{o}C.

The next day, the membrane was washed three times with TBS-T and secondary antibody incubation was performed at RT for 1 hour. Another washing of the membrane was performed with TBS-T three times and PBS once. Enhanced chemiluminescence (ECL) Start Western Blotting Detection Reagent (GE Healthcare) was added on the membrane and left for 5 min at RT. The membrane was developed by BioRad ChemiDoc Imaging system. Membranes were stripped at 50^{o}C for 15 min in stripping buffer and re-probed when necessary. The antibodies used for this study are indicated in Tables 2.2 and 2.3.

2.7 Flow cytometric analysis

2.7.1 Morphology study

AsPC-1 cells transfected by siRNA were harvested 72 hours post-transfection. Cells were washed with ice-cold PBS once. Cold fixation buffer (2% paraformaldehyde in PBS) was applied and cells were fixed on ice for 10min. Fixed cells were washed with cold PBS once and resuspended with permeabilization buffer (0.1% Triton X-100 in PBS) for 5 min on ice. After washing with cold PBS, cells were incubated with blocking buffer (1% BSA in PBS) for 30 min on ice. Blocking buffer was removed after centrifugation at 300 g for 5 min and then cell pellet was resuspended with 100ml of FACS buffer (0.5% BSA and 0.02% Sodium Azide in PBS) containing the primary antibody. GPR35 primary antibody incubation was performed for 30 min. The used antibodies and dilution details are indicated in Table 2.2. Cells were rinsed with FACS buffer once and incubated with secondary antibody conjugated with Alexa Fluor^{TM} 488 (1:500) for 10 min in the dark. Cells were rinsed again with FACS buffer and ready in PBS in the dark. Stained cells were analysed by FACS Canto II immediately.

2.7.2 Annexin V assay (Apoptosis assay)

AsPC-1 cells from siRNA transfection for targeting GPR35 knockdown were harvested 72 hours post-transfection. The same number of cells (1×10^6 cells for each sequence) was collected and washed with PBS once following another wash with Annexin-V binding buffer (MACS Miltenyi Biotec). Cells were stained with Annexin-V binding buffer containing Annexin-V stain (MACS Miltenyi Biotec) in the dark for 20min, accordingly to the manufacturer's instruction. Cells were centrifuged and washed with Annexin-V binding buffer at 300 g for 10min. Propidium iodide (PI) staining was continued by adding 5 μg/ml of PI reagent in order to exclude necrotic cells. Stained cells were analysed by FACS Canto II immediately.

2.7.3 Cell cycle analysis

AsPC-1 cells were harvested after 72 hours of siRNA transfection and washed with cold PBS twice. Cells were fixed and permeabilized with ice-cold 70% ethanol for 30 min. The fixed

cells were washed with cold PBS twice and then cells were incubated with cell cycle solution (0.5mg/ml PI, 0.1% Triton X-100, 100mg/ml RNAase A) for 30 min on ice. Stained cells were analysed by FACS Canto II.

2.8 Metabolic flux analysis: Seahorse assay

Extracellular flux assay has been performed using the Agilent Seahorse XFe96 Analyzer according to the manufactures' guidelines. The Seahorse XF96 Sensor Cartridge was hydrated and equilibrated with 200µl/well of XF Calibrant solution overnight at 37°C in a properly humidified non-CO^2 incubator on the day prior to the assay. The four selected clones from genetically modified AsPC-1 (at a density of 4×10^4 / a well) or MIA PaCa-2 (at density of 2.5×10^4 / well) cells to adjust GPR35 expression were seeded onto a Seahorse XF96 Cell Culture Microplate on the previous day of the assay. Cells were incubated overnight to adhere at 37°C in a 5% CO^2 incubator. On the day of the assay, the cells were washed with unbuffered media (pH7.4) three times, and 175µl of unbuffered media was added into each well. The cells were then kept in a non-CO^2 incubator for 90 minutes prior to the assay. The assay medium with substrates including Glucose (final concentration: 25mM), Oligomycin (2µM), FCCP (0.5µM), Rotenone and Antimycin (1µM) were prepared with the warm Agilent Seahorse XF Media. Prepared assay solutions will be loaded into each port (25µl) on the probe cartridge and placed onto the machine. The results were recorded in real-time. BCA assay for protein quantification was used for normalization. Wave Desktop software and Microsoft Excel Seahorse XF Cell Energy Phenotype Report Generator were used for analysing results. Three independent assays were performed for each cell line.

2.9 Immunohistochemistry (IHC)

The pancreatic tumour or normal pancreas tissues were resected from sacrificed mice. The IHC sample tissues are prepared using either frozen or paraffin sections. For frozen sectioning (cryostat sectioning), tissue slices were covered with the optimal cutting temperature (O.C.T) embedding medium (Tissue-Tek® O.C.T Compound, Sakura Finetek) and snap-frozen using liquid nitrogen. The frozen tissues were sliced as 5µm thickness using a Cryostat microtome and fixed with ice-cold fixation buffer and then 4% paraformaldehyde. For paraffin sectioning, tissues were fixed in 10% of formalin for 24 h and then stored at RT in 70% ethanol. Formalin-fixed tissues were embedded in paraffin and cut as 5µm thickness using a Leica microtome.

For deparaffinization, the tissue mounted slides were washed the following steps:1) xylene for 5 minutes three times, 2) 100% ethanol for 5 minutes three times, 3) 95% ethanol for 3 minutes, 4) 70% ethanol for 3 minutes, 5) 50% ethanol for 3 minutes and 6) final wash with running cold tap water. For antigen retrieval, slides were heated with appropriate antigen retrieval buffer by microwave for 20 minutes at temperatures just below boiling. The slides were cooled down and washed three times with TBS. To reduce non-specific background signals, endogenous peroxidase activity was blocked by incubating the slides in 0.3% of H_2O_2 for 20 minutes followed by three times washing with TBS. Additionally, blocking of avidin and biotin was performed. For blocking the proteins, a 5% BSA blocking solution was used and samples were incubated for 30 minutes. Primary antibody staining (dilution conditions are indicated in Table 6) was performed overnight at 4^{o}C. After that, the slides were washed with TBS three times for 20 minutes each at RT. Biotinylated secondary antibody staining was performed for an hour at RT. Negative controls were made by the omission of primary antibodies. The slides were incubated in diluted Avidin-HRP staining solution (Molecular Probes) for 30 minutes followed by three times washing with PBS. DAB chromogen reagent (Sigma Aldrich) was added onto the slides and incubated in dark for 30 minutes at RT. The tissue staining was monitored under a bright-field microscope. Once appropriate staining was verified, the slides were washed three times with distilled water. The samples were counterstained with haematoxylin and mounted with xylene-based mounting media.

2.10 Colony formation soft agar assay

To verify cell proliferation in a 3 dimensions environment representing real cancer, colony formation soft agar assay was performed. Two different concentrations of noble agar (Sigma-Aldrich) were prepared, 1.2% and 0.6% (w/v) in MilliQ ddH_2O. A 2-fold dilution of DMEM media supplemented with 20% FBS, 2% (v/v) PS and 4nM L-Glutamine was mixed with warm 1.2% noble agar solution (1:1 ratio) so that total concentration became 0.6%, of which 1.5 ml were immediately added to each well in a 6-well plate. The first layer in a 6 well dish was solidified in RT for 30 min. 1×10^4 cells were counted and resuspended in 750 µl of a 2-fold dilution of completed DMEM. For pharmacological inhibition of GPR35, 10µM of ML145 or DMSO was added. The medium containing cells was mixed with warm 0.6% noble agar solution at 1:1 ratio so that total concentration became 0.3%. This mixed solution was applied on top of the first layer. The dish was placed at RT to solidify for 30 min and 1 ml of their appropriate media (Table 2.5) was added. The dish was incubated for 4 weeks in a humidified

95% air and 5% CO_2 incubator at 37ºC. The colonies were fixed with 10% Acetone/Methanol and stained with 0.05% crystal violet. The stained colonies were counted by Image J.

2.11 Immunofluorescence

2.11.1 Caspase 3/7 probe assay (Apoptosis assay)

Transfected cells were reseeded on the second day post-transfection with 1×10^4 cell density into a 96-well dish and incubated overnight. The next day, the medium was replaced with a new medium containing Caspase 3/7 reagent as 1:1000 ratio (Essen Bioscience) in the dark, according to the manufacturer's instructions. Cells were incubated in the IncuCyte® ZOOM Live-Cell Analysis Imaging System (Sartorius) installed in a 37ºC incubator and were real-time monitored. Each experiment is representative of three independent experiments.

2.12 Migration and invasion assay

2.12.1 Migration assay

The selected clones from transfected cells by CRISPR/Cas9 targeting GPR35 were seeded at the density of 8×10^4 per well for MIA PaCa-2 and 10×10^4 per well for AsPC-1 in a 96-well dish. Cells were incubated overnight to reach 95-100% confluence the next day. The following day, a scratch wound was made by the IncuCyte® ZOOM Scratch WoundMaker (Sartorius). Then the dish was placed in the IncuCyte® ZOOM Live-Cell Analysis Imaging System (Sartorius) and monitored for 24 hours. Photos were analysed by Image J.

2.12.2 Invasion assay

The scratch wound was performed as described in section 2.12.1. After the scratch wound was made, the dish was incubated at 4ºC for 5 min to cool down. 50µl of cold matrigel (5mg/mL) per well were applied on top of cells and the dish was placed at RT for 15 min to solidify. The appropriate medium, as indicated in section 2.4.1, was added and the dish was placed in the IncuCyte® ZOOM Live-Cell Analysis Imaging System (Sartorius) and monitored for up to 72 hours. Photos were analysed by Image J.

2.13 In vivo assay

All animal experiments were carried out in compliance with the standards of Australian and institutional guidelines. Zebrafish-based toxicological experiments were performed with the approval of the Harry Perkins Animal Ethics Committee. The used methods in this study were in accordance with the approved guidelines. Zebrafish (*Danio rerio*) and embryos were kept at 28°C; pH 7.2-7.4 and 14h on and 10h off light cycle. Xenograft mouse experiments were performed with the approval of the Curtin Animal Ethics Committee. All mice were kept in their ventilated cages and monitored in the care of Curtin University. The mice were maintained under the standard conditions (12:12 light-dark cycle, 21°C room temperature and 55% humidity).

2.13.1 Zebrafish

Wild type Tubingen (TU) zebrafish were bred and maintained in aquaria systems (Techniplast) as guidelines described in the zebrafish book (Monte, 1995). Zebrafish embryos at 24 hours post fertilization (hpf) were removed from their chorion and distributed in separate wells in a 24-well plate (10 embryos/well). DMSO, ML145 and CID2745687 with a final concentration of 25, 50, 100, 150 and 200 μM in embryo medium (E2) were applied in each well. Embryos were observed daily and hatching and mortality scores were recorded for 5 days (120 hpf). For heart rate assessment, embryos at 48 hpf were anesthetized with tricaine (ethyl 3-aminobenzoate methoanesulfonate) and the heart beats per minute were counted under the stereomicroscope. The morphological changes from the toxicological effect of the drugs were documented using a digital camera (Nikon, Tokyo, Japan).

2.13.2 Xenograft mouse model

Six to 7-week-old NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice were purchased from the Animal Resources Centre (ARC, Murdoch, Western Australia). All mice were kept under pathogen-free conditions with ad libitum food and water supply. Mice were monitored daily and the cages were replaced twice weekly.

HPAF-II xenograft: HPAF-II cells were collected by trypsin and counted using a hemocytometer. The counted 3.5 $\times 10^6$ cells were resuspended in 200 μl of cold PBS and placed on ice. Mice were randomly divided into two groups (control group, n=9; ML145

treatment group, n=9). Cells were injected subcutaneously in the right flank of mice. When the size of a tumour from a group of 18 animals reached a volume of 50 mm^3, a treatment of ML145 by intraperitoneal injection was started. 50mg/Kg of ML145 or vehicle was applied daily for two weeks. The tumour size was monitored using a surgical calliper and the tumour volumes were calculated based on the formula: tumour volume=$(width)^2 \times length/2$. Mice were sacrificed when the drug treatment was terminated and tumour tissues were isolated and weighed. ML145 (Tocris Bioscience #4172) was prepared in 0.5% carboxymethyl cellulase/ 0.4% Tween-80.

MIA PaCa-2 xenograft: The four selected clones from genetically modified MIA PaCa-2 by CRISPR/Cas9 to adjust GPR35 gene expression were prepared as described above. 5×10^6 cells resuspended in 100μL of PBS were injected subcutaneously into either right or left flank of the randomly selected mice. The total number of mice used in this experiment was 20 instead of 40 because two injection sites per mouse were used. The size of tumours was monitored using a surgical calliper three times a week until a tumour size reached the critical limit of 1000mm^3. The calculated volume as described above was recorded and mice were sacrificed when the experiment was terminated. The tumour tissues were isolated and weighed.

2.14 Statistics

The Mean ± SEM is presented in all results. The significance analysis was performed by a two-tailed t-test, multiple t-tests and one-way analysis of variance (ANOVA) followed by Dunnett's test. A p-value less than 0.05 is considered significant in this study. All statistical analyses were carried out using GraphPad PRISM V6.0 software.

Chapter 3

Chapter 3: The expression of GPR35 in PDAC

3.1 Rationale

The mRNA expression of GPR35 has been reported in diverse parts of the human body including the small intestine, colon, spleen, stomach and various immune cells (J. Wang et al., 2006). Since the expression pattern of GPR35 was reported, several pathological studies have been performed, such as pain & inflammation (Cosi et al., 2011), metabolic disease (Vander Molen et al., 2005), cardiovascular disease (Divorty et al., 2018) and cancer study (Y. J. Guo et al., 2017; Okumura et al., 2004; Ruiz-Pinto et al., 2017; Sowers, Johnson, Conrad, Patterson, & Sowers, 2014). However, the role of GPR35 still remains unclear. This receptor started to be investigated only recently and it is a relatively new target compared to any other GPCRs, especially in cancer research. Only a few publications about GPR35 in cancer research can be found currently. The expression of GPR35 in gastric cancer (Okumura et al., 2004), non-small-cell lung cancer (NSCLC) (W. Wang et al., 2018) and colorectal cancer (Ali et al., 2019) was studied. Higher mRNA expression of GPR35 was detected on gastric cancer cells compared to normal gastric cells and overexpression of GPR35 in NSCLC tissue was identified compared to normal lung tissue. The high level of GPR35 mRNA expression in regional lymph nodes was correlated with a poor prognosis. However, no publication regarding GPR35 in pancreatic cancer was founded.

Thus, to verify the potential of GPR35 as a novel therapeutic target for pancreatic cancer, we analysed the expression pattern of GPR35 in the pancreas and pancreatic cancer by currently available databases. Based on the data analysis, we have performed further experiments to verify the expression of GPR35 in PDAC cell lines, patient-derived xenograft (PDX) cell lines, KPC mouse model tissues and human pancreatic cancer tissues.

3.2 Data analysis

Enhanced expression of GPR35 was reported by a large-scale cancer genomics dataset from Oncomine (Pei Pancreas microarray database, Cancer Cell 2009/09/08). More than 5-fold higher mRNA level of GPR35 in pancreatic cancer (36 samples) was recorded compared to the normal pancreas (16 samples) in humans (Pei et al., 2009) (Figure 3.1.A).

Moreover, the Oncomine dataset shows that GPR35 is highly expressed in pancreatic cancer compared to any other cancer types. Figure 3.1.B shows that pancreatic cancer is the top fourth cancer on the list of all cancer types that express GPR35.

Another largely used database, Cbioportal, was utilized for the GPR35 gene alteration study. Figure 3.1.C reveals that there are more GPR35 gene alterations in pancreatic cancer than in any other cancer, including neuroendocrine prostate cancer (NEPC), sarcoma, bladder, cervical and glioma cancer. In 109 cases of pancreatic cancer, there is more than 14.7% of GPR35 gene alteration frequency including amplification, deep deletion and mutation. Amplification of the GPR35 gene, which is the most common gene alteration, was found in 13 cases out of 109 total cases (11.9%). Deep deletion and mutation of the GPR35 gene were found in 2 cases and 1 case respectively out of 109 total cases.

The GEPIA database shows an interactive body map based on the expression of GPR35 (Figure 3.1.D). The median expression of GPR35 is 21.07 $\log_2$ scale in pancreatic tumour versus 0.55 $\log_2$ scale in normal pancreas. This data confirmed the enhanced expression of GPR35 in pancreatic cancer consistent with the Pei Pancreas Oncomine dataset (Figure 3.1.A).

Figure 3.1.E presents a plot correlating pancreatic ductal adenocarcinoma patient's survival with GPR35 expression by using a Kaplan-Meier Plotter database. Patients possessing higher expression of GPR35 have a lower survival rate compared to the patients having a low expression of GPR35. The median survival is 22.8 months for the low expression cohort and 17.27 months for the high expression cohort. Although the P-value is 0.058 this correlation is worth further consideration.

Indeed, we could find survival plots showing statistically significant data in another database, The Human Protein Atlas. Figure 3.2 presents three survival plots of pancreatic cancer patients divided by sex. Interestingly, the P-value is 0.04 in the plot of female patients' samples indicating that higher expression of GPR35 decreases the survival rate in pancreatic cancer (Figure 3.2.B). These data imply the potential of correlation between GPR35 and female hormones for future study.

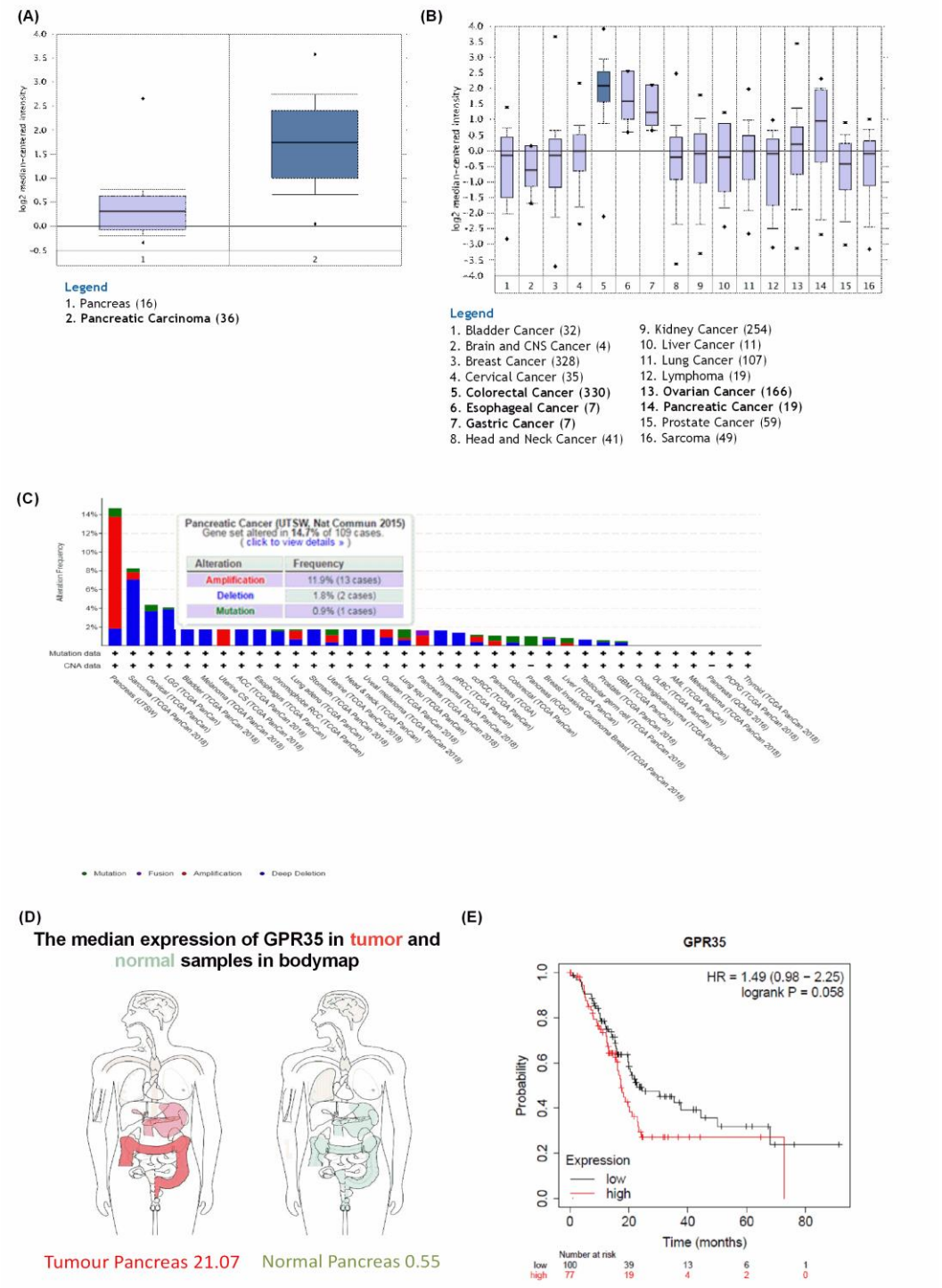


Figure 3.1. The expression of GPR35 by data analysis. *(A) Higher mRNA expression of GPR35 in human pancreatic cancer and (2) compared to the normal pancreas (1). Data from Oncomine. https://www.oncomine.org/ (B) Among all cancer types, pancreatic cancer is top 4 on the list of the overexpression of GPR35. Data from Oncomine. https://www.oncomine.org/ (C) Cross-cancer alteration summary of GPR35. Data from Cbioportal. https://www.cbioportal.org/ (D) Interactive body-map of expression of GPR35. Log$_2$ (TPM+1) scale. Data from GEPIA. http://gepia.cancer-pku.cn/detail.php?gene=GPR35 (E) Survival plot of GPR35 high expression group vs low expression group. Log rank test P-value is 0.058. Data from Kaplan-Meier Plotter. http://kmplot.com/*

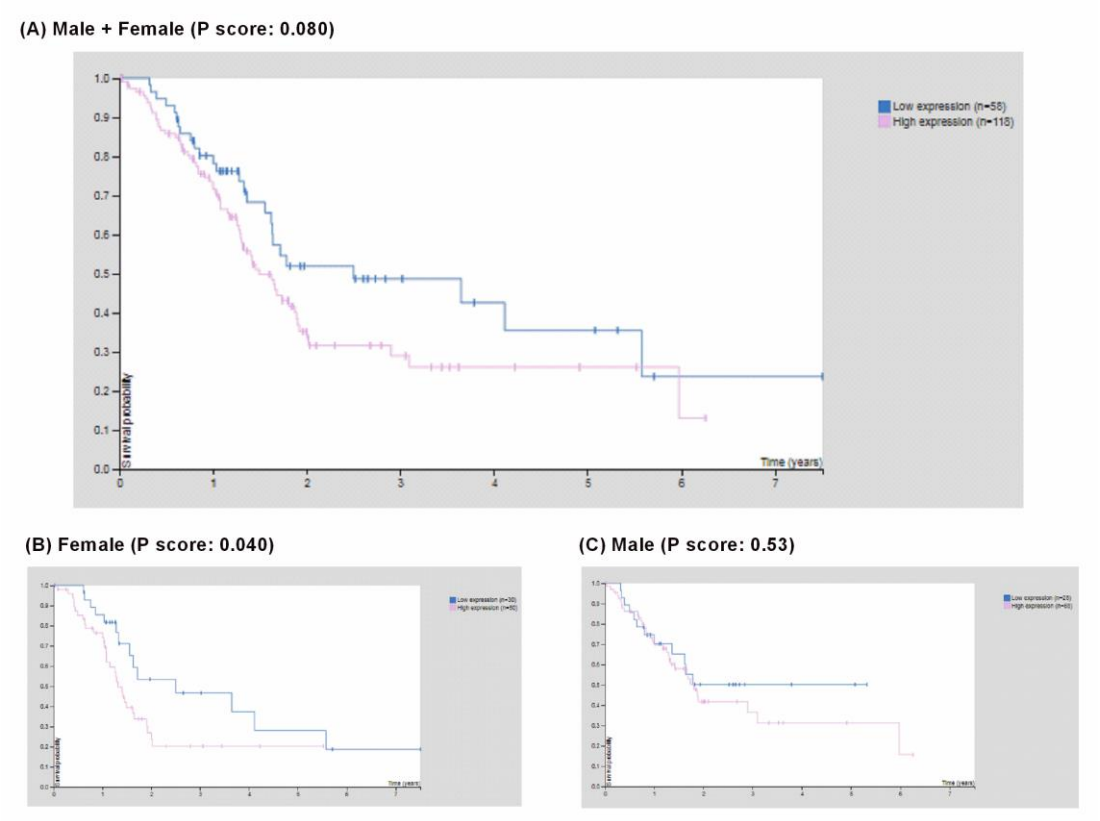


Figure 3.2 Survival plot of GPR35 high expression cohort vs low expression cohort in male and female (A), female only (B), and male only (C). *Data from the Human Protein Atlas https://www.proteinatlas.org/*

3.3 Overexpression of GPR35 in Pancreatic cancer

3.3.1 Overexpression of GPR35 in PDAC, PDX, KPC mouse tissue and human pancreatic cancer tissue

Based on previous data analysis, we investigated the GPR35 protein expression in PDAC. Overexpression of GPR35 was clearly detected in most PDAC cell lines such as AsPC-1, MIA PaCa-2, PANC-1, CFPAC-1, Capan-2, HPAF-II and BxPC-3 compared to a non-neoplastic pancreatic cell line such as hTERT-HPNE (hTERT-immortalized human pancreatic epithelial nestin-expressing cell line), suggesting that GPR35 could be a good candidate as a future therapeutic target for PDAC (Figure 3.3.A). However, we could still detect the moderate expression of GPR35 in HPDE, human papillomavirus (HPV) E6E7-immortalized human pancreatic ductal epithelial cell line, which is another non-tumorigenic pancreatic epithelial cell line. Moreover, most PDAC cell lines showed two different sized bands at around 34kDa.

Especially, Capan-2 containing wild type functional p53 show a strong signal band slightly under 34kDa.

In addition, we could detect overexpression of GPR35 in patient-derived xenograft (PDX) cell lines kindly provided by the Australian Pancreatic Cancer Genome Initiative (APGI, www.pancreaticcancer.net.au) and the Garvan Institute of Medical Research (Sydney, Australia). (Figure 3.3.B). The profile of PDX cell lines (Chou et al., 2018) is presented in Table 3.1 (https://www.pancreaticcancer.net.au/bioresource-pdcls/). Lysate from TKCC-05 cell lines shows the strongest signal of GPR35 compared to other PDX cell lines. TKCC-05 cell line has mesenchymal morphology while other cell lines have epithelial morphology (https://www.pancreaticcancer.net.au/wp-content/uploads/2017/02/TKCC-05-Profile.pdf). Moreover, the metastatic capacity of TKCC-05 cells on the liver and lung has been confirmed *in vivo*. This result implies that GPR35 might be involved with pancreatic cancer metastasis. Less expression of GPR35 was detected in TKCC-07 and TKCC-09 cell lines. According to profiles of PDX cell lines, both TKCC-07 and TKCC-09 cell lines are from a poorly differentiated adenocarcinoma biopsy (https://www.pancreaticcancer.net.au/bioresource-pdcls/).

IHC analysis supports GPR35 as a potential novel target for pancreatic cancer. Figure 3.4.A shows overexpression of GPR35 in KPC mouse tissues. The expression was detected mainly in pancreatic ducts in tumour tissues. Consistent with this, higher brown staining representing GPR35 expression was detected in human PDAC tissues compared to human normal pancreas tissue (Figure 3.4.B).

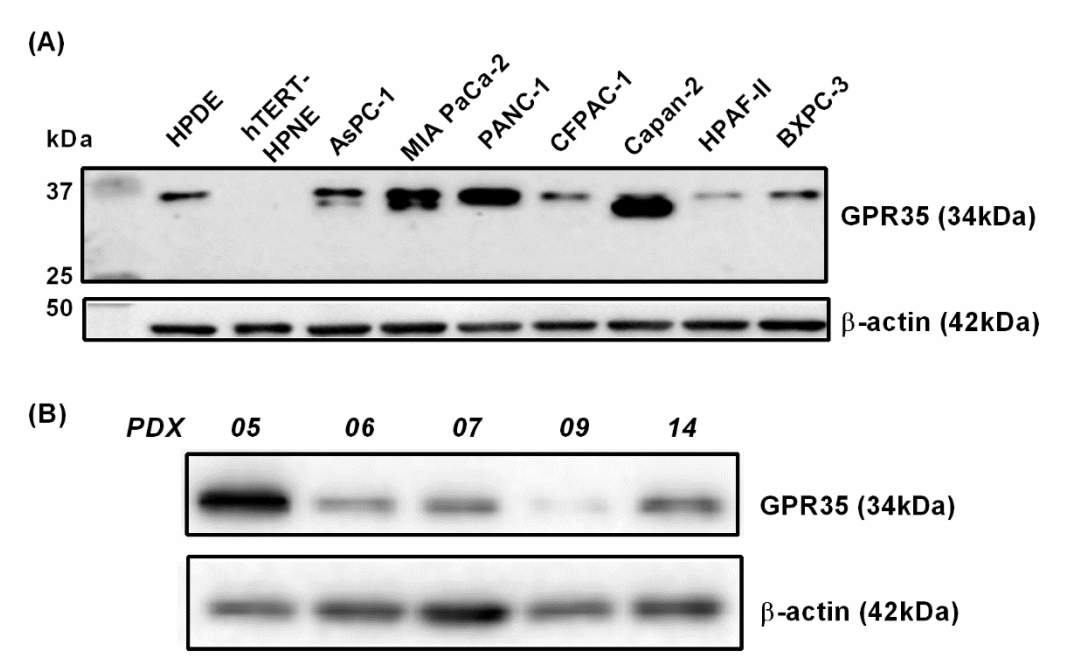


Figure 3.3. Overexpression of GPR35 in PDAC cell lines and PDX by Western Blot. *(A) Western blot analysis shows that the high expression of GPR35 was detected in most PDAC cell lines compared to HPDE and hTERT-HPNE cell lines. GPR35 antibody from Proteintechnology was used. (B) All PDX cell lines show the expression of GPR35 by Western blot analysis. Especially TKCC-05 has a stronger signal of GPR35. GPR35 antibody from Cayman was used.*

TABLE 10 PROFILES OF PDX CELL LINES. FROM AUSTRALIAN PANCREATIC CANCER GENOME INITIATIVE (APGI). HTTP://PANCREATICCANCER.NET.AU/

Name of PDX	Differentiation	Sex of patient	Growth property	Morphology	Metastasis	Structural variant	Gemcitabine cell viability response
TKCC-05	undifferentiated	Male	Adherent	Mesenchymal	Liver and lungs	Scattered	Moderate
TKCC-06	-	Male	Adherent	Epithelial	Untested	stable	resistant
TKCC-07	a poorly differentiated	Male	Adherent	Epithelial	Untested	Focal	Resistant
TKCC-09	a poorly differentiated	Female	Adherent	Epithelial	Untested	Unstable	Moderate
TKCC-14	moderately differentiated	Male	Adherent	Epithelial	-	-	-

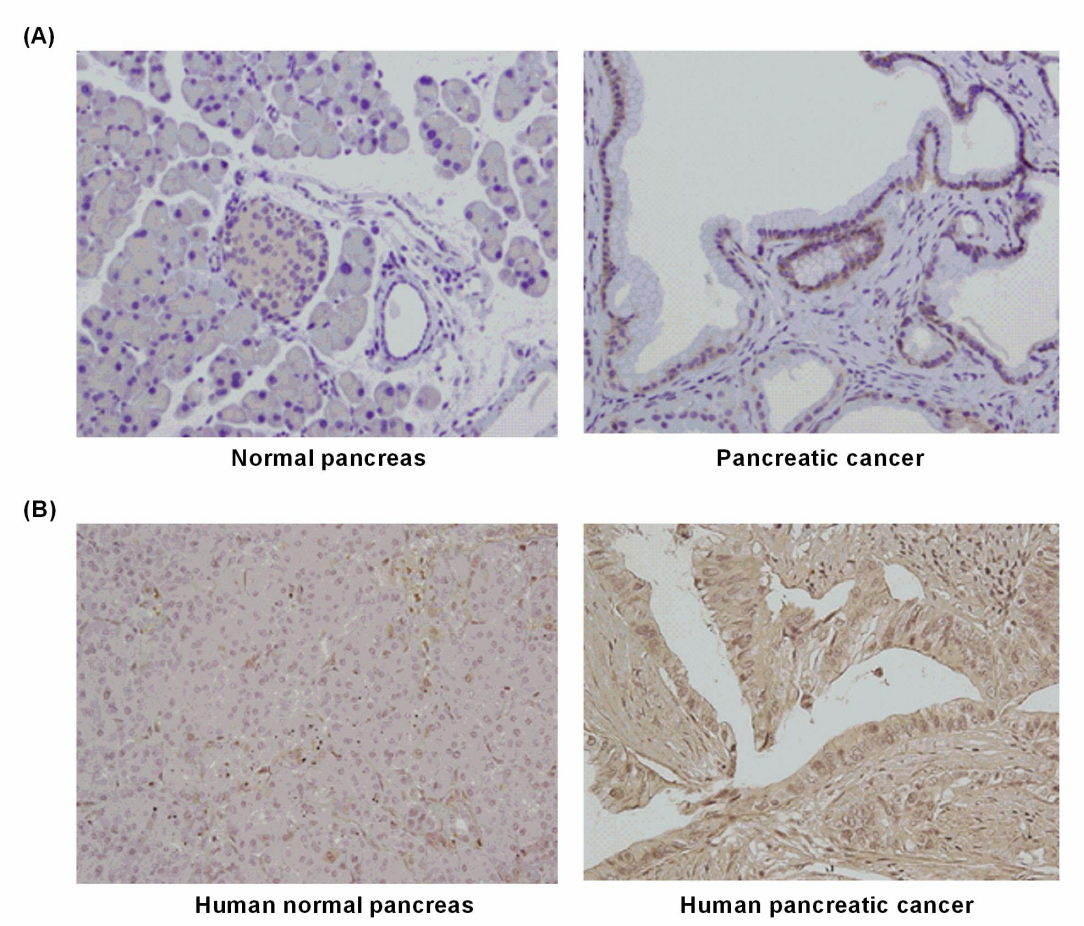


Figure 3.4. Representative images showing overexpression of GPR35 in mouse (A) and Human (B) tissues by IHC analysis. *(A) Stronger signals of GPR35 were detected in pancreatic cancer tissues (Pdx1-Cre$^{+/+}$/KRas$^{wt/G12D}$/p53$^{wt/R172H}$ mice) but less in normal pancreas tissues (control, Pdx1-Cre$^{+/+}$ mice) Magnification: 20X (B) Higher expression of GPR35 was shown in human pancreatic tissues than human pancreas tissues. Total 12 mice (6 KPC and 6 control) were assessed for GPR35 expression. For human tissues, 20 cancer and 4 normal tissues (from BioChain, catalogue number 27020090) were examined. Magnification: 20X. These experiments were performed with Dr Alice Domenichini.*

3.4 Chapter summary and discussion

This chapter demonstrated the following:

- Overexpression of GPR35 mRNA in pancreatic cancer compared to the normal pancreas was verified by data analysis.
- Overexpression of GPR35 in pancreatic cancer is ranked top 4 in the list of all cancers expressing GPR35 according to the Oncomine database.
- More alterations (mainly amplification) of the GPR35 gene are detected in pancreatic cancer compared to other cancers, as stated by the Cbioportal database analysis.
- In the survival plot from the pancreatic patients' group, the patients having high expression of GPR35 have a lower survival rate compared to the patients having a low expression of GPR35. Only the female group of patients shows a statistically significant result.
- Overexpression of the GPR35 protein in PDAC cell lines, PDX cell lines, KPC mouse tissues and human pancreatic cancer tissues was observed compared to normal pancreatic cell lines and tissues.
- A PDX cell line characterising mesenchymal morphology and metastasis shows higher protein expression of GPR35.

Our WB analysis using diverse pancreatic cancer cell lines, including ATCC cell lines and PDX cell lines, showed a consistent result suggesting that GPR35 is overexpressed in pancreatic cancer. Most PDAC cell lines from ATCC expressed a high level of the GPR35 protein compared to non-malignant HPNE, even though HPDE showed a moderate expression of GPR35 (Figure 3.3.A). Another interesting piece of data from Figure 3.3.A shows two distinct bands of GPR35 at around 34 kDa in most PDAC cell lines. These two bands might present two different isoforms of GPR35: GPR35a and GPR35b. Indeed, a high mRNA expression level of GPR35b was detected in most colorectal cancer cell lines but almost an absence of GPR35a was observed (Ali et al., 2019). Equivalent amounts of both GPR35a and GPR35b genes were expressed in cardiac myocytes (Ronkainen et al., 2014). Moreover, THP-1 cells expressed mainly GPR35a (Park et al., 2018). However, the difference in their functional roles has not been studied yet. We could estimate that the bands at a slightly heavier size represent GPR35b, which has 32 extra amino acid residues at the N-terminal. As the average mass of one amino acid is 110 Da, the total around 3.5 kDa is heavier in GPR35b. Based on our data (Figure 3.3.A), almost all PDAC cell lines express more GPR35b than GPR35a, with the exception of Capan-2 showing higher expression of GPR35a than GPR35b.

For future studies, it should be taken into consideration the functional p53 expression of Capan-2 (p53wt), a characteristic that distinguishes this cell line from others. Moreover, AsPC-1, MIA PaCa-2 and PANC-1 show higher expression of GPR35a compared to CFPAC-1, Capan-2, HPAF-II and BXPC-3. However, we could not find the correlation of high GPR35a staining with the four main mutations, which are KRAS, TP53, CDKN2A/p16 and SMAD/DPC4, among these cell lines. Therefore, further investigations involving isoforms of GPR35 in PDAC is required for future study. Another interesting piece of data from Figure 3.3.B shows only one clear band of GPR35 in PDX cell lines unlikely PDAC cell lines showing two distinct bands. This discrepancy can be explained by the difference of characteristics between PDX cell lines and PDAC cell lines. PDX cell lines are often mixed with contaminating host cells, such as fibroblasts, and PDX cell lines are more heterogeneous than PDAC cell lines. We could estimate that these differences can affect the expression of GPR35 isoforms.

The distinct expression pattern of GPR35 in pancreatic tissues and normal pancreas tissues resected from both humans and mice in our IHC data also indicated the potentials of GPR35 as a new therapeutic target for PDAC. Further investigation including morphometric analysis and statistical analysis to assess the correlations between GPR35 expression and cancer prognosis is required for future study based on our preliminary IHC data outlining optimized concentration of antibodies. Not many publications have focused on the importance of GPR35 in cancer. Only a few publications have shown the screening of GPR35 protein levels in cancer versus normal tissues in colon, breast and lung cancer research. In fact, the expression of GPR35 in the human colon has been widely reported and HT-29 cell line has been used as a standard cell line showing endogenous GPR35 in many other receptor-ligands paring studies. However, a similar protein expression of GPR35 in both primary colorectal tumours and normal colon tissues was reported, showing the limitation of this receptor for clinical use in colon cancer. Overexpression of GPR35 in breast and lung cancer compared to their normal tissues has been reported, also suggesting this receptor as a new target for breast and lung cancer. The survival plot, together with other results, indicates that the high expression of GPR35 might be associated with tumour progression and poor prognosis in PDAC. These significant findings will contribute to suggest GPR35 as a new therapeutic target for pancreatic cancer.

Chapter 4

Chapter 4: The important role of GPR35 in PDAC cell growth, apoptosis and autophagy

4.1 Rationale

Following the promising data showing the overexpression of GPR35 in PDAC, we tested GPR35 pharmacological inhibition, pharmacological activation and genetic inhibition to understand the role of GPR35 in PDAC. We hypothesized that the inhibition of GPR35 might reduce pancreatic cancer proliferation and the activation of GPR35 might increase cancer cell survival. Thus, in this chapter, we aim to verify the inhibition and activation of GPR35 in order to successfully target GPR35 as new therapeutic molecules for pancreatic cancer.

4.2 Inhibition by synthetic antagonists

Two selected commercially available synthetic antagonists of GPR35, ML145 and CID2745687, were tested for demonstrating the role of GPR35 in PDAC cell growth. Four PDAC cell lines, AsPC-1, MIA PaCa-2, HPAF-II and PANC-1 were incubated with five different concentrations of the synthetic antagonists of GPR35 for 72 hours and the number of cells was counted. Figure 4.1 shows changes in cell proliferation in PDAC cell lines under the treatment of ML145. AsPC-1, PANC-1 and HPAF-II cell growth was decreased particularly when cells were incubated with 5 and 10 μM of ML145 in normal media containing 10% FBS. We repeated the ML145 dose response experiment in serum-free conditions as this mimics the real human cancer environment in which cancer cells are suffering a shortage of glucose. Additionally, the serum free conditions enable avoidance of confounding effects of diverse growth factors in the serum. In serum-free media incubation, AsPC-1, MIA PaCa-2 and HPAF-II showed significantly less proliferation when treated with 2.5 μM to 10 μM of ML145 compared to PANC-1. Following this, we examined only hTERT-HPNE cells treated with ML145 and CID2745687 because HPDE showed moderate expression of GPR35 (Figure 3.3.A). Graphs C and D in Figure 4.1 show that there were no changes or even increases in cell number upon treatment with GPR35 inhibitors, suggesting that both GPR35 inhibitors showed no effects on cell proliferation in normal pancreas cells. To provide conditions more similar to those experienced by cancer cells growth in the body, we performed 3D soft agar colony assay with ML145 which shows dramatic effects on PDAC cell proliferation in 2D conditions. Figure 4.2 shows a decrease in the number of colonies in AsPC-1 and MIA PaCa-2 compared to the control, treated with only DMSO.

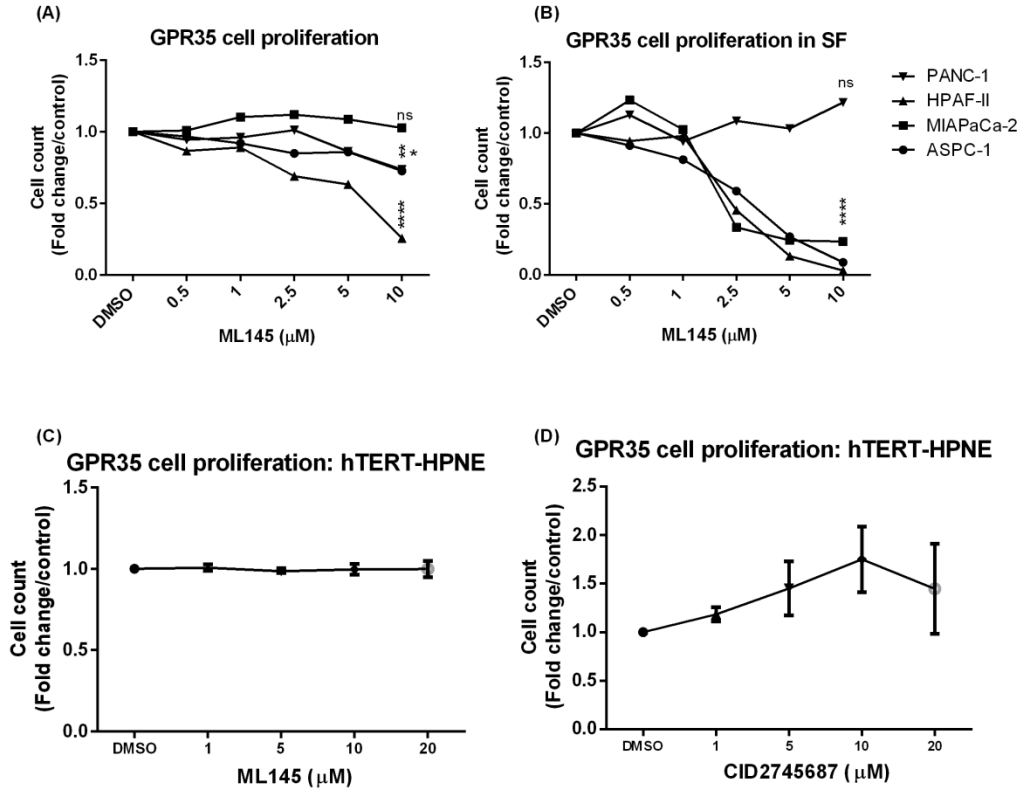


Figure 4.1. PDAC cell proliferation upon pharmacological inhibition of GPR35. *Four PDAC cell lines, AsPC-1, MIA PaCa-2, PANC-1 and HPAF-*II *were tested using complete media (A) and serum-free media (B) containing different concentrations of ML145. The same number of cells was incubated for 72 hours and the number of cells was counted manually. hTERT-HPNE were also tested with ML145 (C) and CID2745687 (D) for 72 hours and the number of cells was recorded. Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. *p-value is < 0.05, **p-value is <0.01, and ****p-value is <0.0001(t-test).*

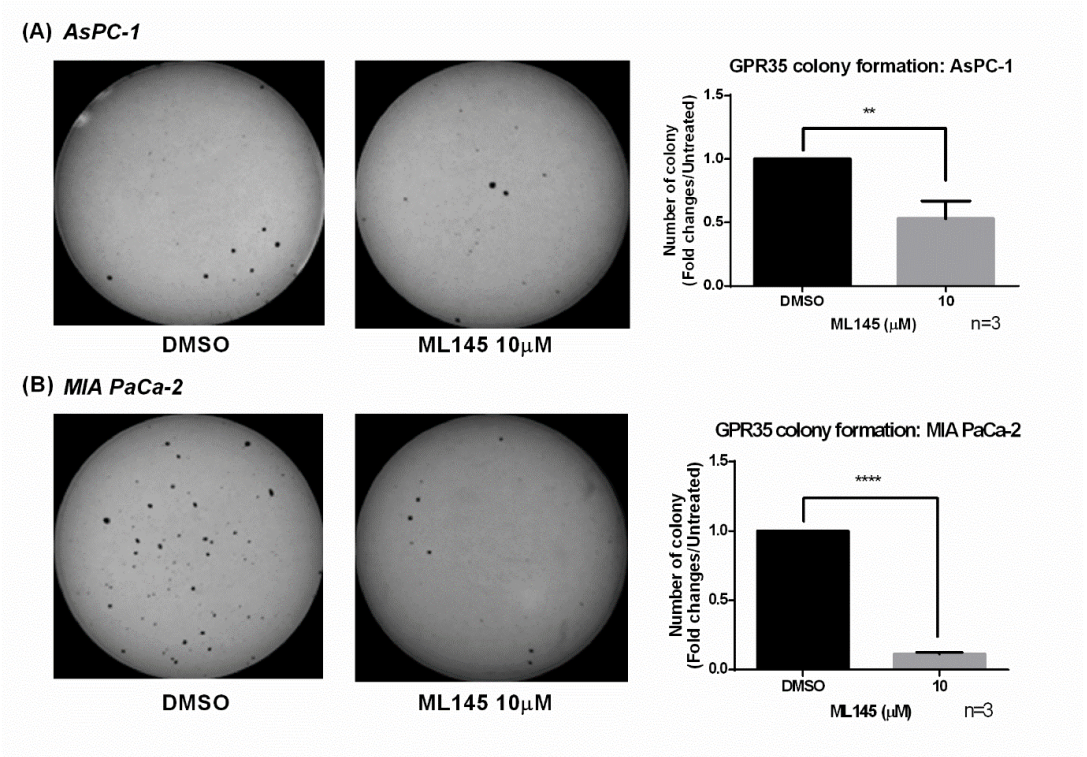


Figure 4.2. Representative images of colony formation assay with ML145 for PDAC cell proliferation. *AsPC-1 (A) and MIA PaCa-2 (B) were treated with ML145 (Concentration=10μM) in soft agar. The same number of cells was incubated for four weeks and the number of colonies was counted manually. Both cell lines show that the number of colonies was significantly less with ML145 compared to control (only with DMSO). Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. **p-value is <0.01 and ****p-value is <0.0001.*

4.3 Inhibition by gene silences

4.3.1 Small interference RNA

To show consistent results in the role of GPR35 in cell proliferation, we transiently silenced the GPR35 expression by small interference RNA (siRNA) transfection. Figure 4.3.A and B show that the transfection efficiency is high by IF and IHC. After 72 hours of transfection, we calculated the cell number and Figure 4.3.C shows the results. The cell number of AsPC-1, MIA PaCa-2, HPAF-II and PANC-1 transfected with two targeting GPR35 siRNA sequences was counted as significantly less compared to the cells transfected with a non-targeting siRNA (siControl).

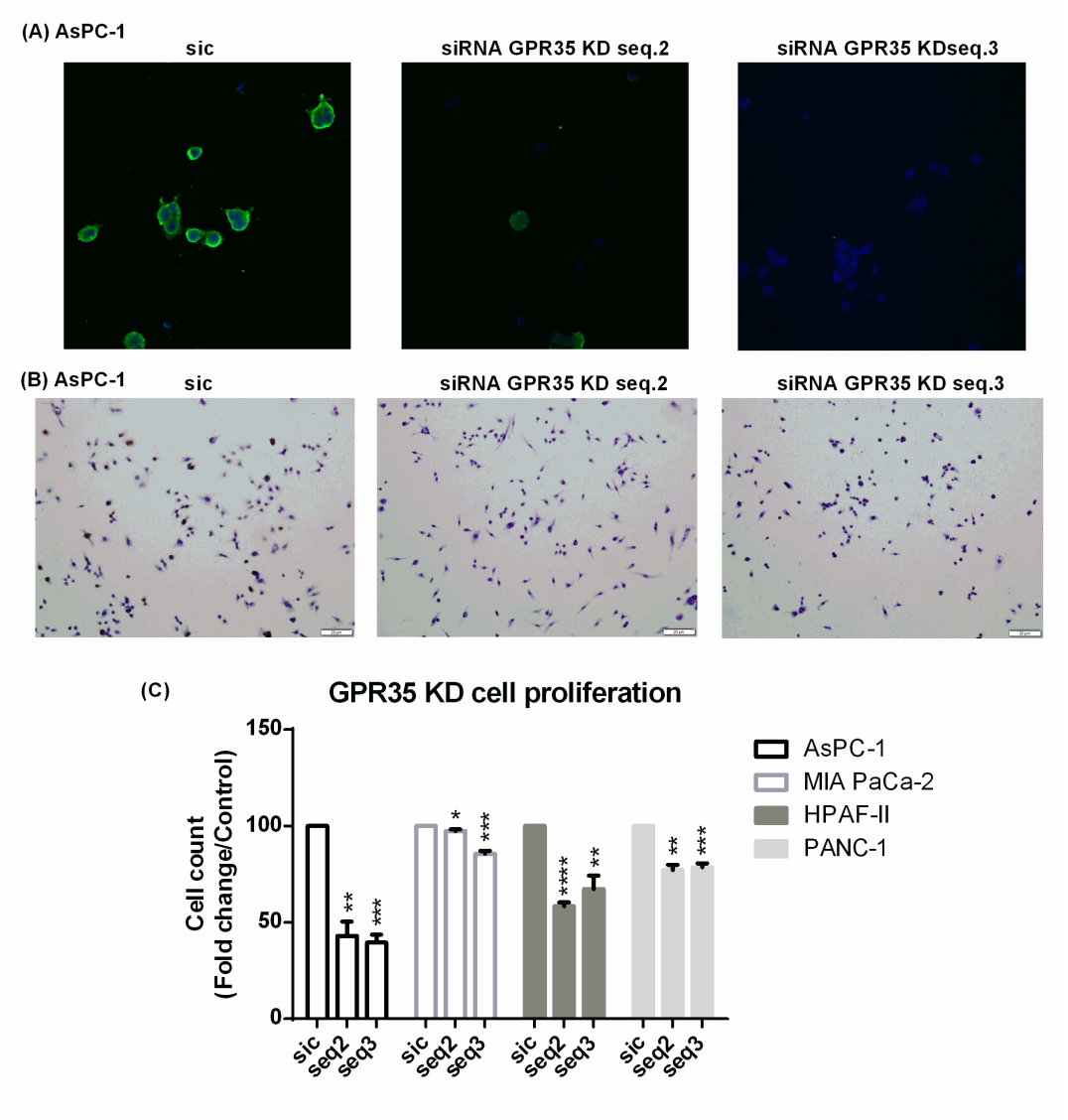


Figure 4.3. PDAC cell proliferation under the inhibition by siRNA. *AsPC-1, MIA PaCa-2, HPAF-*II *and PANC-1 were transfected to downregulate GPR35 expression by siRNA. (A) The immunofluorescence staining photos show the high efficiency of siRNA transfection of GPR35 in AsPC-1 cell line. Cells were stained with GPR35 antibody from Origene. Magnification: 20X (B) The immunohistochemistry staining photos show the high efficiency of siRNA transfection of GPR35 in AsPC-1 cell line. Cells were stained with GPR35 antibody from Origene. Magnification: 10X (C) The cells were counted after 72 hours of siRNA transfection. Four cell lines which are AsPC-1, MIA PaCa-2, HPAF-II and PANC-1 show that the cell number was significantly less in the cell lines expressing fewer GPR35 compared to control. Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. *p-value is < 0.05, **p-value is <0.01, ***p-value is <0.001 and ****p-value is <0.0001(t-test).*

4.3.2 CRISPR/Cas9 system

We modified the AsPC-1 and MIA PaCa-2 cell lines using the CRISPR/Cas9 system so that they were expressing stably a low GPR35 gene, for tracking the cell growth in 3D condition and *in vivo* experiments. CRISPR/Cas9 transfection was performed as described in chapter 2.5.2 and the transfection was visually confirmed by detecting GFP and RFP through IncuCyte (Figure 4.4.A). Co-transfection with a GPR35 HDR plasmid enabled to have a puromycin-resistant gene for a specific selection marker by homology-directed repair (HDR) pathway. After selection with puromycin (2μg/ml for AsPC-1 and 1μg/ml for MIA PaCa-2), expression of GPR35 in clones derived from a single cell were screened by Western blot analysis (Figure 4.4.B & C). Control 21 (NC21) and GPR35 KD clone 11 (KO11) for AsPC-1 were selected based on this blot. Control 1, 5 (NC1 and NC5), GPR35 KD clone 4 and 7 (KO4 and KO7) were selected for MIA PaCa-2 to continue long-term experiments.

Figure 4.5 shows the high efficiency of the CRISPR/Cas9 transfection analysed by Western blot (Figure 4.5.A and B). The selected clones of MIA PaCa-2 were grown for 5 days and the number of cells was manually counted every day (Figure 4.5.C). Less number of cells for both clones, KO4 and KO7, was recorded from day 3 to day 5 compared to controls, NC1 and NC5. The selected clone of AsPC-1, KO11, also showed less proliferation compared to NC21 at the end of 7 days incubation in a 2D environment (Figure 4.5.D). Following this data, we performed the soft agar colony formation assay to demonstrate the impacts of GPR35 on cell growth in a 3D environment. Figure 4.5. E and F are the representative images and G and H are the relative graphs. The number of colonies is significantly less in GPR35 KO cell lines compared to controls.

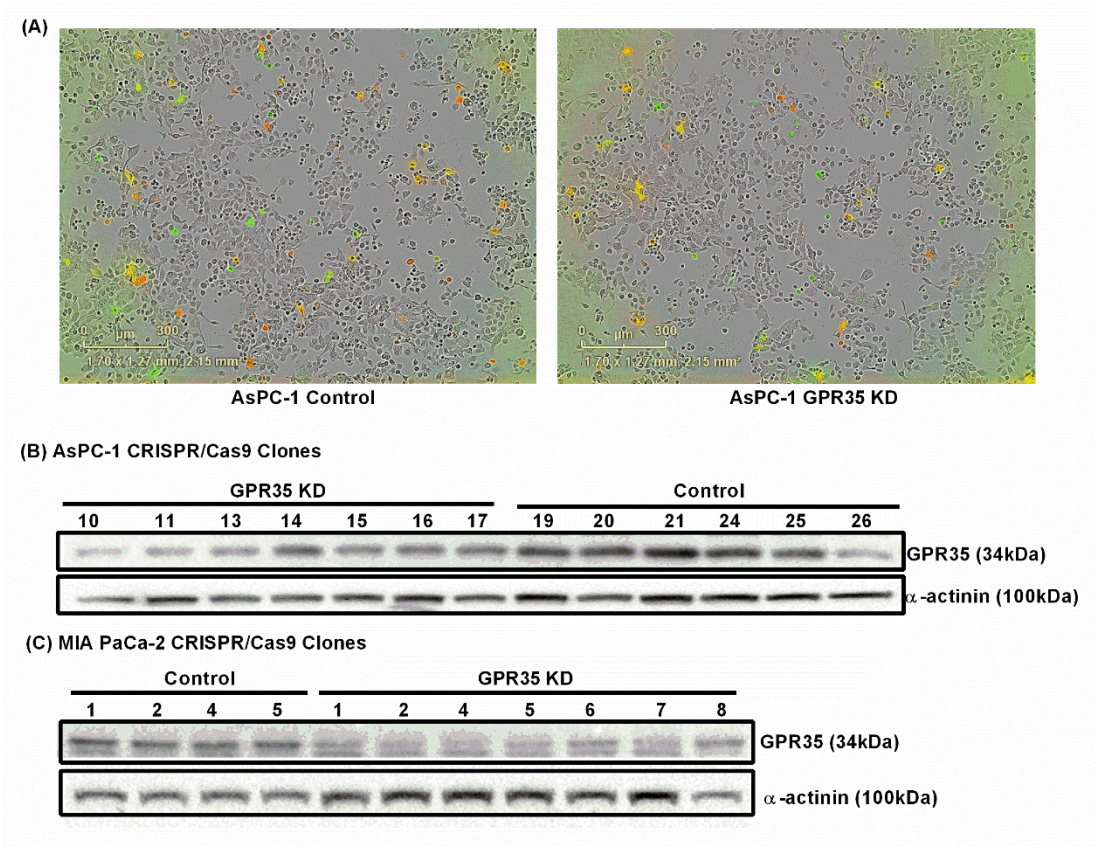


Figure 4.4 Stable AsPC-1 and MIA PaCa-2 cell lines silencing GPR35 gene established by CRISPR/Cas9 system. *(A) GFP (Control or GPR35 KO) and RFP (HDR insert) expression were detected in the transfected AsPC-1 cell line by IncuCyte at 72 hours post-transfection. (B) The protein expression of GPR35 was screened on clones of transfected AsPC-1 cell lines. Clone 11 shows less expression of GPR35 compared to control clone 21. (C) The clones of MIA PaCa-2 transfected by CRISPR/Cas9 system were screened by WB. The expression of GPR35 was verified. Based on the blot, clone 4 and 7 was selected toward control clone 1 and 5.*

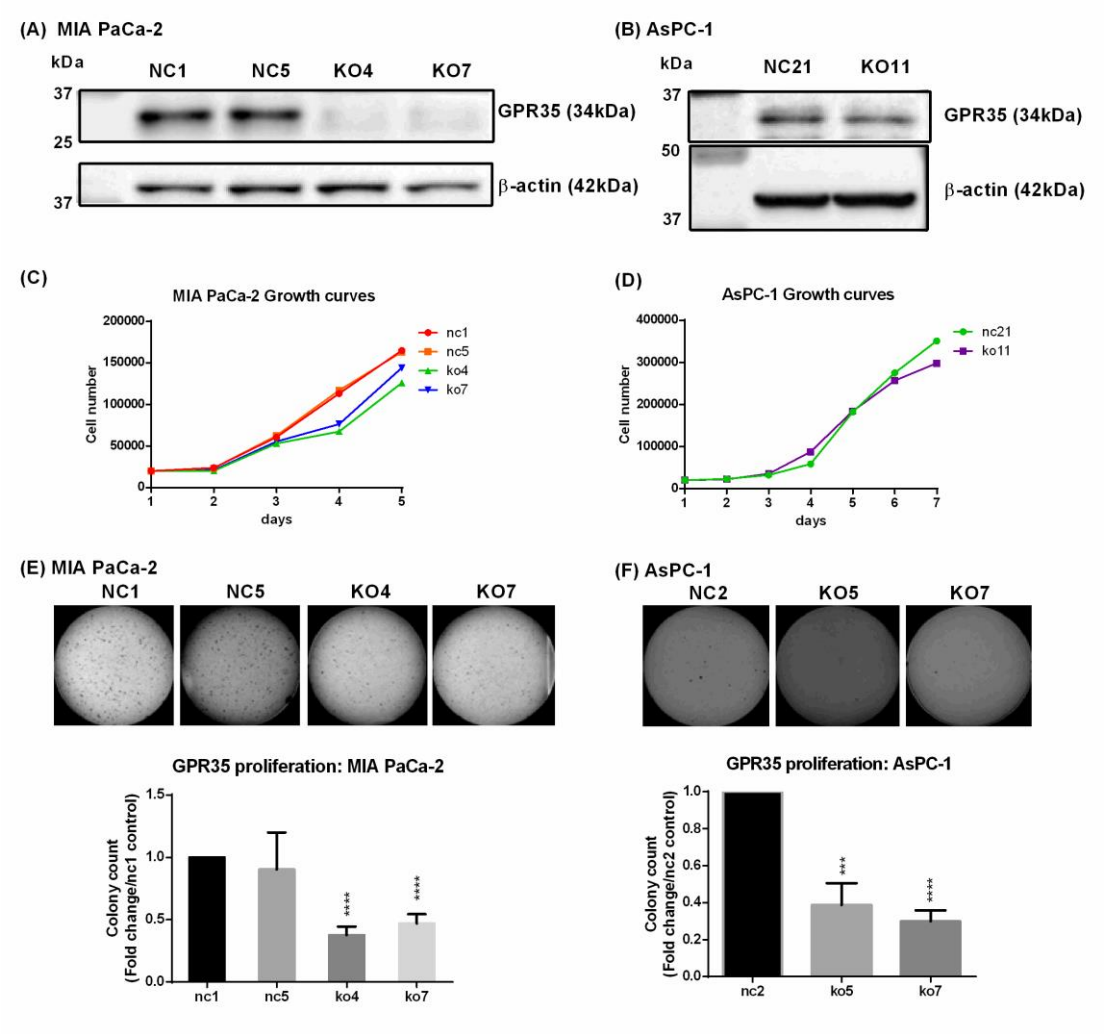


Figure 4.5. PDAC cell proliferation under the inhibition by CRISPR/Cas9. (A)(B) *The expression of GPR35 is identified by Western Blot. On the transfected cells of MIA PaCa-2 (A) and AsPC-1 (B), a lower level of GPR35 protein was detected compared to controls.* ***(C)*** *Long-term growth curves of MIA PaCa-2_CRISPR/Cas9_GPR35KO cells (KO4 and KO7) and MIA PaCa-2_ CRISPR/Cas9_vehicle cells (NC1 and NC5) were recorded. Both knocked down MIA PaCa-2 cell lines, which are KO4 and KO7, show a smaller number of cells from day 3 to day 5 compared to controls which are NC1 and NC5.* ***(D)*** *Long-term growth curves of AsPC-1_ CRISPR/Cas9_GPR35KO cells (KO11) and AsPC-1_ CRISPR/Cas9_vehicle cells (NC21) were recorded. AsPC-1_NC21 shows a lower growth pattern on day 4, 6 and 7.* ***(E)*** *Soft agar colony formation assay shows that MIA PaCa-2 cell lines expressing fewer GPR35 (KO4 and KO7) form a lower number of colony compared to controls, assessed by crystal violet uptake assay* ***(F)*** *Soft agar colony formation assay shows that AsPC-1_NC2 has more colonies than AsPC-1_ CRISPR/Cas9_GPR35KO cells (KO5 and KO7), assessed by crystal violet uptake assay. Three independent repeats of colony formation experiments were performed. Results are represented as mean ± SEM. ***p-value is <0.001 and ****p-value is <0.0001 (t-test).*

4.4 Agonist of GPR35 induces cell survival

Compound 10 and zaprinast, known as synthetic agonists of GPR35, were tested in this study. AsPC-1 and HPAF-II cell lines were incubated with both synthetic agonists in serum-free conditions for 72 hours. The number of cells was counted manually and Figure 4.6 shows the result. Compared to control, a higher number of AsPC-1 cells treated with 10 and 20 μM of compound 10 survived in serum starvation. HPAF-II cell line also shows similar results. However, zaprinast did not show any survival effects on both cell lines (data is not shown).

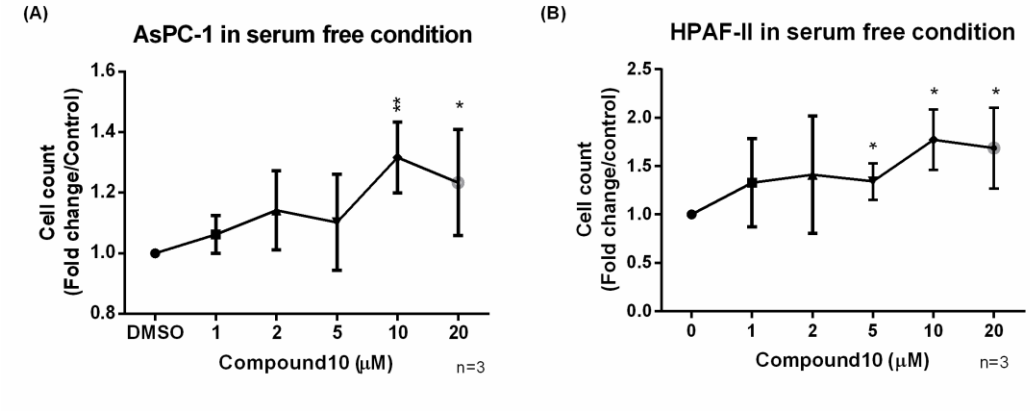


Figure 4.6. Compound 10 induces AsPC-1 and HPAF-II cell survival in serum starvation conditions. *AsPC-1 and HPAF-II cells were incubated with compound 10, which is one of the synthetic agonists of GPR35, for 72 hours and the number of cells was counted manually. A higher number of cells for the concentrations 10 and 20 μM of compound 10 was recorded compared to control. Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. *p-value is < 0.05 and **p-value is <0.01(t-test).*

4.5 The inhibition of GPR35 change PDAC cell morphology

Changes in cell morphology upon the inhibition of GPR35 were verified. AsPC-1 and HPAF-II were incubated with ML145 for 72 hours and images were taken under a light microscope. The morphology of AsPC-1 treated with 10μM of ML145 shows an elongated appearance and pseudopodia-like shape (Figure 4.7.A). HPAF-II with ML145 treatment also shows morphological changes (Figure 4.7.B): not only a pseudopodia-like shape but also vacuoles in 10 and 20 μM range of concentrations. Moreover, the inhibition of GPR35 by siRNA gene silence the altered morphology of AsPC-1 cells. Figure 4.7.C shows distinctive changes of cell shape such as pseudopodia-like shape and vacuoles, in both AsPC-1 and HPAF-II cells with ML145 incubation, in both sequences targeting GPR35 siRNA 72 hours post-transfection.

These morphological changes of AsPC-1 cells transfected by siRNA targeting GPR35 were consistently verified by FCM. The relative size or internal complexity of cells can be determined by measuring forward scatter (FSC) and side scatter (SSC) parameters. Higher intensity of FSC measurement indicates bigger cell size whereas higher SSC signal represents more internal complexity (i.e. granularity) of cell structure. Figure 4.7.D shows that the transfected cells by sequence 2 exhibit higher intensity of both FSC and SSC in comparison with siControl, but the cells by sequence 3 exhibit higher intensity of SSC only. These results demonstrate that the inhibition of GPR35 by siRNA transfection can induce cells to have a bigger size and more complex cellular components. In addition, the high complexity of internal cell structure might be related to nuclear condensation or vacuolization.

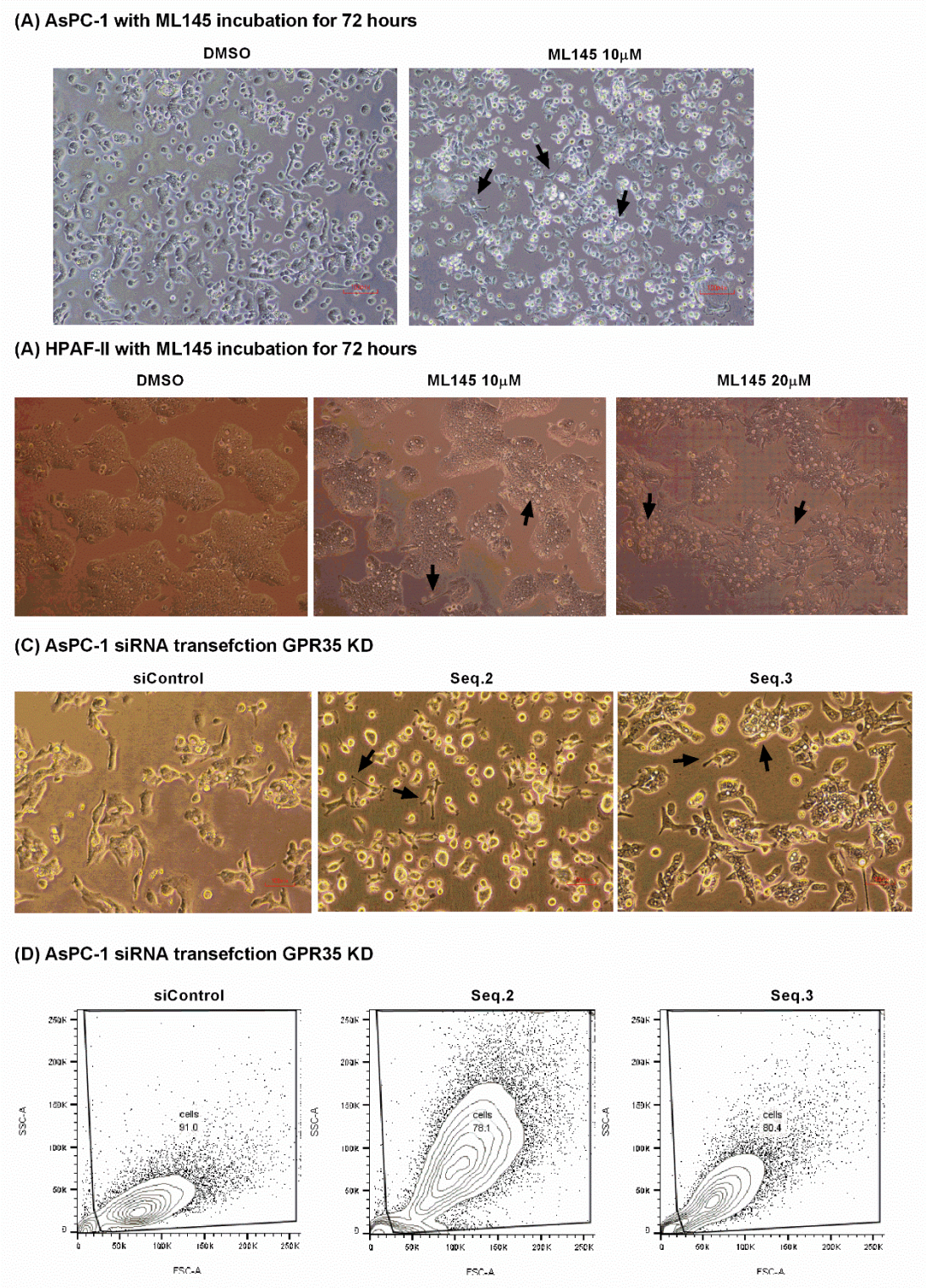


Figure 4.7. The inhibition of GPR35 alters morphology of PDAC cell lines. *AsPC-1 (A) and HPAF-II (B) were incubated with ML145 for 72 hours. The brightfield/phase-contrast images were taken under a light microscope. Magnification: 10×. (C) The brightfield/phase-contrast images of the transfected AsPC-1 cells were taken. Magnification: 10×. (D) The transfected AsPC-1 targeting GPR35 siRNA were analysed by FCM.*

4.6 The inhibition of GPR35 by siRNA gene silencing arrests G0/G1 cell cycle

To better understand the role of GPR35 in cell proliferation, we have performed cell cycle analysis. Proliferation is the main characteristic of cancer. Unstoppable cell growth without general control of the cell cycle forms tumours. The cell cycle consists of four phases: synthesis (S), gap2 (G2), mitosis (M) and gap1 (G1). Replication of DNA occurs in the S phase. Afterwards, cells are grown and double-checked for DNA damage at G2 phase in order to prepare for mitosis. Then cells are divided into two daughter cells in phase M when no error is founded at G2 phase. Phase G1 is the time interval between phase M and S, also called the growth phase. In phase G1, cells are either stopped or allowed to continue the cell cycle at the restriction point. Normal fully differentiated cells generally remain in G0 phase for resting, in what is called a "quiescence state". Some cells are due to leave the cell cycle in response to DNA damage caused by internal errors or external stresses and remain also in G0 phase; this is called a "senescence state".

AsPC-1 cells were transfected by siRNA and analysed by FCM. The data showed that the cells expressing less GPR35 by siRNA transfection occupied a higher proportion at G0/G1 phase of cell-cycle compared to siControl, indicating that the significant cell growth inhibition shown in previous data might occur through the induction of G0/G1 phase cell-cycle arrest.

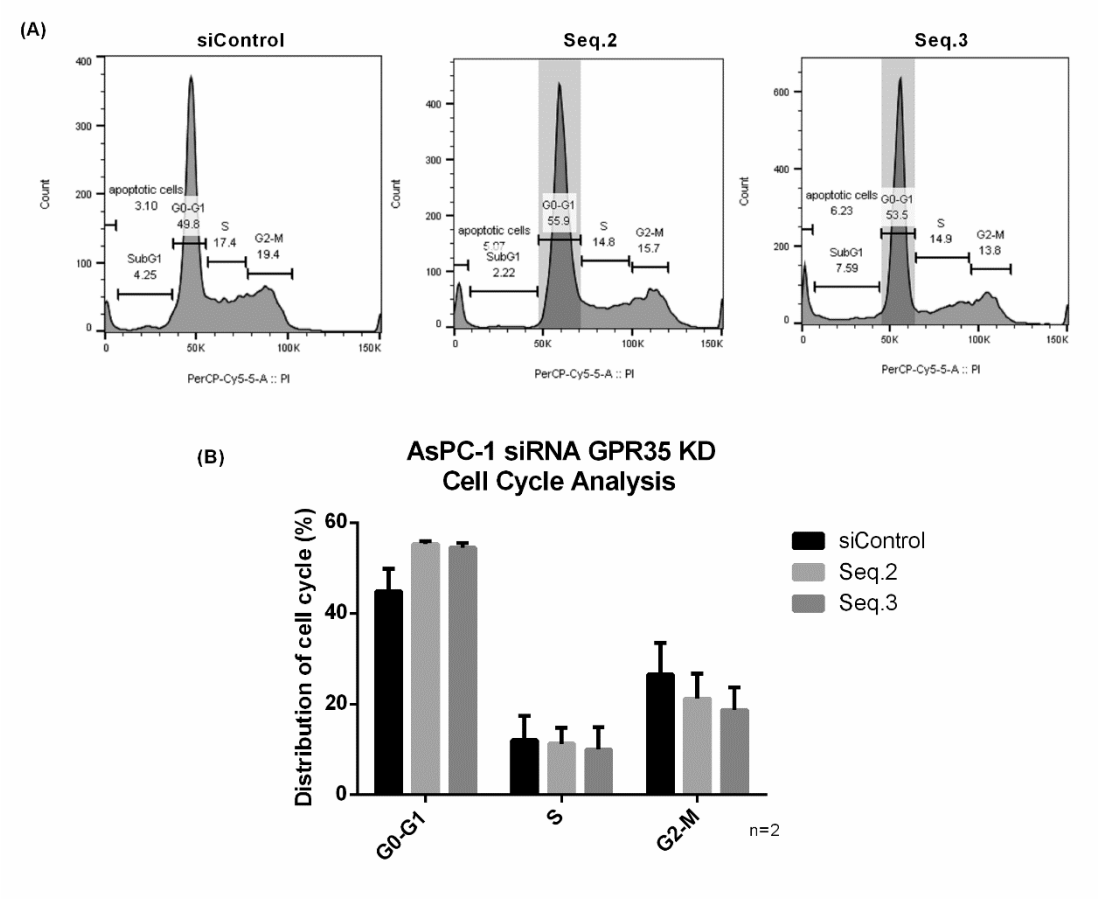


Figure 4.8. G0/G1 phase arrest was observed in the transfected AsPC-1 cells targeting GPR35 siRNA. *(A) Cell cycle analysis by staining PI and (B) quantification of cell cycle populations demonstrated that the cells expressing less GPR35 by siRNA transfection occupied a higher proportion of the G0/G1 phase compared to cells targeting scrambled control. Two independent repeats of the experiments were performed. Results are represented as mean ± SEM.*

4.7 The role of GPR35 in apoptosis

Cell death comprises two major forms named necrosis and programmed cell death. Necrosis is a passive and accidental form of cell death caused by external sources, such as physical damages including toxic chemicals and radiation. Necrosis is un-programmed cellular death that results in mostly detrimental effects to the organism. However, programmed cell death such as apoptosis and necroptosis are active forms of cell death mediated by intracellular programs. Programmed cell death is highly regulated and controlled to provide beneficial effects to the organism, unlike necrosis. In particular, apoptosis is well known as a key factor limiting cancer proliferation. A hallmark of cancer is unstoppable cell growth by avoiding

apoptosis. Therefore, it is an essential step to verify whether the decreased proliferation induced by the inhibition of GPR35 in PDAC occurs due to apoptosis.

To understand the mechanism of the decrease in PDAC cell growth upon inhibition of GPR35, we performed three different apoptosis assays: Annexin-V/-FITC/-PI assay by FCM analysis, Caspase3/7 probe activity assay by immunofluorescence analysis and cleaved caspase 3 protein detection assay by Western blot analysis.

Annexin V (or Annexin A5), conjugated to green-fluorescent FITC dye, is a commonly used indicator of apoptosis, which has a high affinity with the anionic phospholipid phosphatidylserine (PS) that is represented on the external cell surface when apoptosis occurs. Propidium iodide (PI) stains necrotic cells by labelling the cellular DNA of dead cells where the cell membrane has been totally broken. The combination of annexin V and PI enables to distinguish cell status among necrotic cells (+annexin V & +PI), apoptotic cells (+annexin V & -PI) and viable cells (-annexin V & -PI).

Apoptosis is coordinated by diverse caspases, a family of cysteine proteases. Caspase 3 and 7 are critical executioners of apoptosis, regulating the degradation of multiple cytoskeletal proteins, which are important for cell survival and maintenance. Therefore, detecting an active form of caspase 3 and 7 provides great markers of apoptosis.

AsPC-1 cells silencing the GPR35 expression by siRNA transfection were stained with annexin-V-FITC/PI at 72 hours post-transfection. The apoptotic population in AsPC-1 cells transfected with a non-targeting siRNA (siControl) was around less than 20% (the basal apoptotic population) while around 30% of the apoptotic population in AsPC-1 cells expressing a lower GPR35 (sequence.2) was observed (Figure 4.9). This significant strong binding affinity toward annexin-V of AsPC-1 cells expressing a lower GPR35 compared to control cells indicates that GPR35 might be highly involved in the inhibition of apoptosis.

Another apoptosis assay, which is a caspase3/7 probe activity assay by immunofluorescence analysis, also showed a consistent result. We incubated the same number of the transfected AsPC-1 and PANC-1 cells silencing GPR35 expression at 72 hours post-transfection with the treatment of caspase3/7 probe conjugated with a green fluorescent protein. Figure 4.10 shows the higher green fluorescent expression in the AsPC-1 cells transiently silencing GPR35 (both sequences) and PANC-1 cell lines (sequence.3) compared to the cells non-targeting GPR35 siRNA (siControl), which shows that GPR35 has a role in PDAC apoptosis.

We have performed the cleaved caspase 3 protein detection assay by WB analysis using AsPC-1 and MIA PaCa-2 cells stably expressing less GPR35 by CRISPR/Cas9. Figure 4.11 presents the upregulation of cleaved caspase 3 in MIA PaCA-2 and AsPC-1 cell lines stably expressing

a lower GPR35 compared to controls which clearly shows that the inhibition of GPR35 induces apoptosis in PDAC.

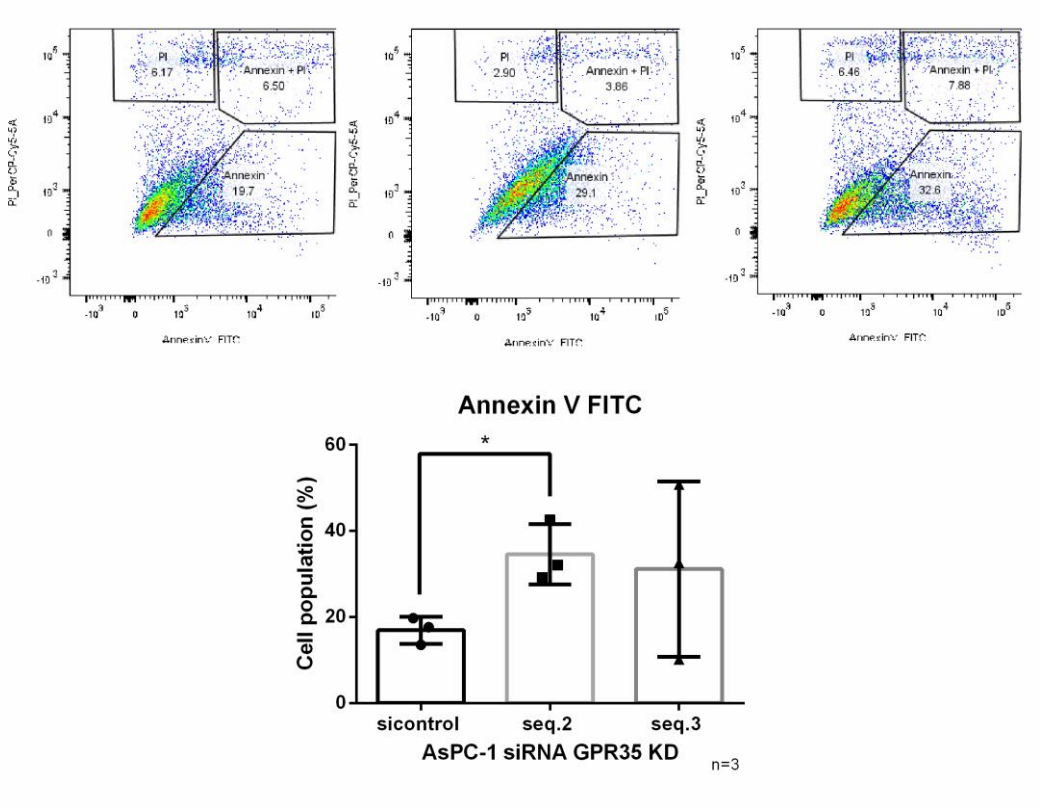


Figure 4.9. Annexin-V/-FITC/-PI FCM analysis shows that the inhibition of GPR35 induces apoptosis in AsPC-1. *AsPC-1 expressing less GPR35 by siRNA transfection (sequence.2) was showing a higher signal of Annexin-V/-FITC in FCM analysis compared to control. Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. *p-value is < 0.05(t-test).*

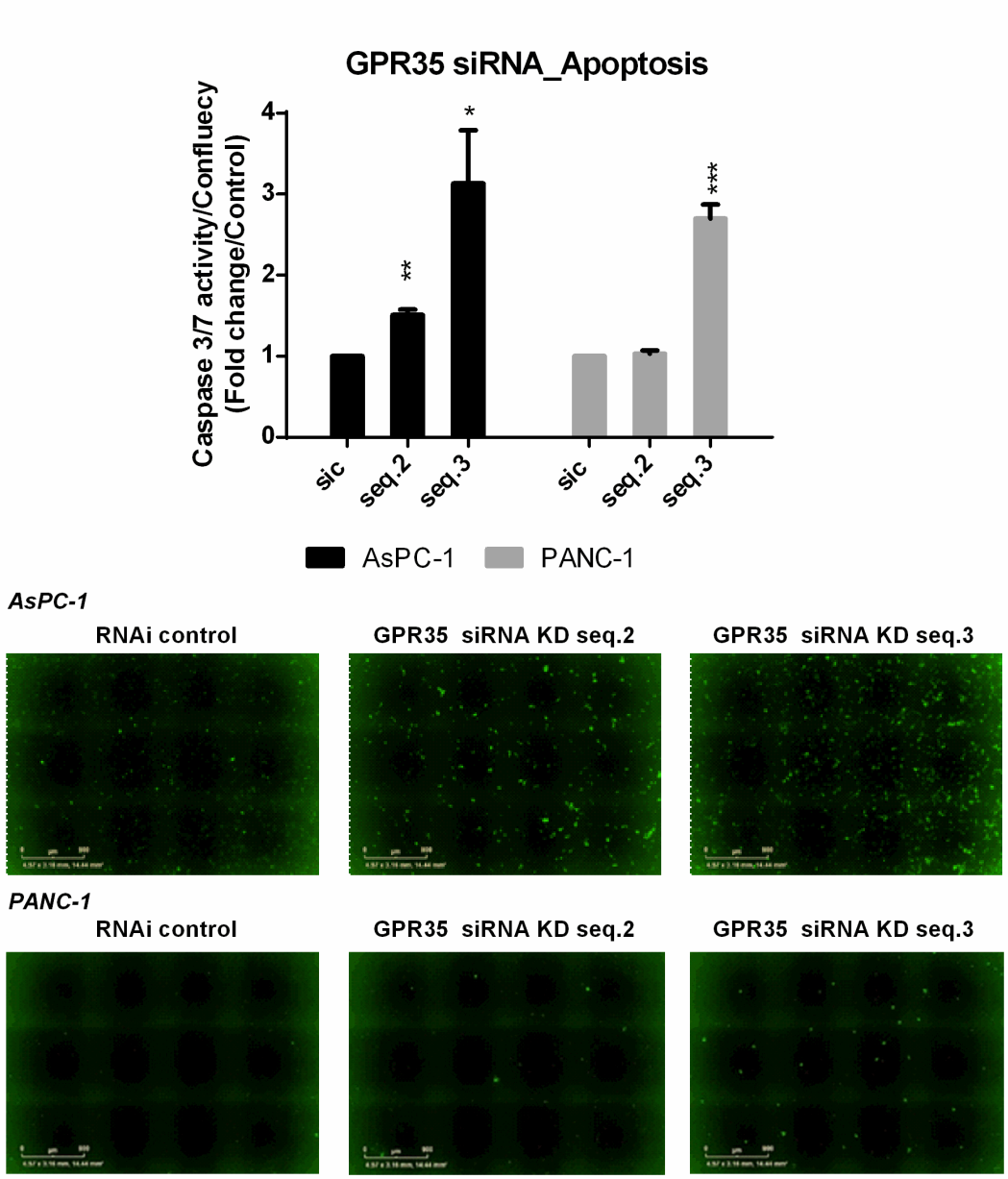


Figure 4.10. Caspase3/7 probe activity shows apoptosis induced by the inhibition of GPR35 in PDAC cell lines. *Caspase3/7 probe activity was observed after siRNA transfection of GPR35 in AsPC-1 and PANC-1. The photos after 72 hours of siRNA transfection were taken in real-time by IncuCyte ZOOM System-Essen BioScience. AsPC-1 cell lines expressing less GPR35 show significantly increased caspase 3/7 activity in both sequences and PANC-1 cells silencing GPR35 siRNA show almost three times higher caspase 3/7 signal compared to the cells non-targeting GPR35. Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. *p-value is < 0.05, **p-value is <0.01 and ***p-value is <0.001(t-test).*

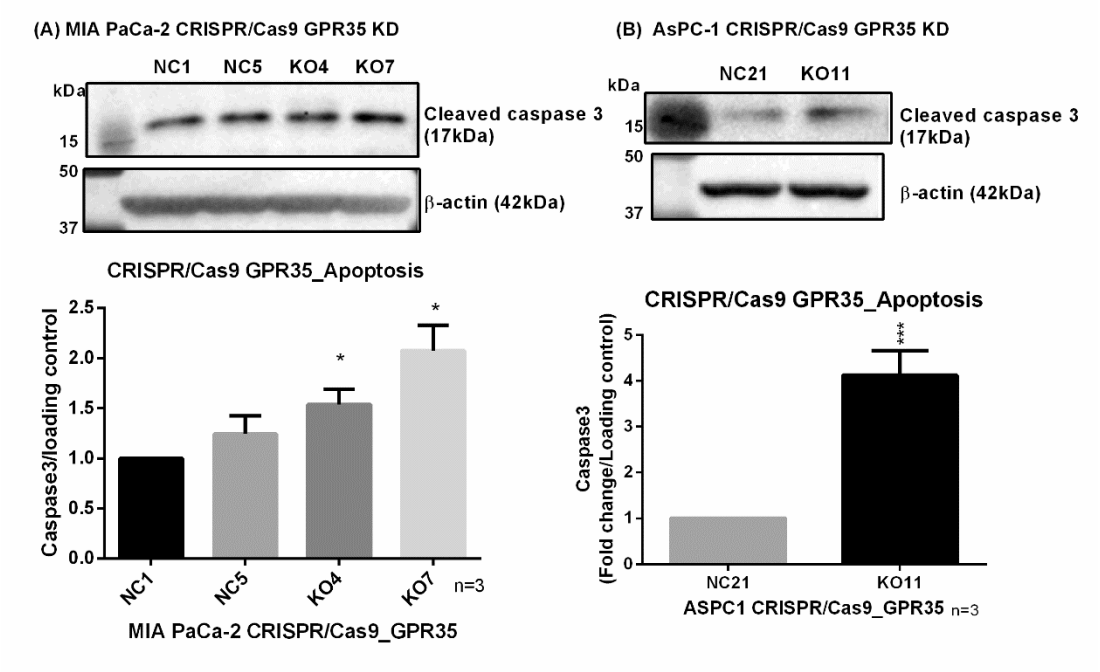


Figure 4.11. Western blot analysis shows that the inhibition of GPR35 induces apoptosis in MIA PaCa-2 (A) and AsPC-1 (B) expressing a lower GPR35 by CRISPR/Cas9 system. *Significantly higher cleaved caspase 3 molecule was detected by Western blot analysis in MIA PaCa-2 and AsPC-1 expressing less GPR35 by CRISPR/Cas9 system compared to the cells carrying an empty vector (Control). Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. *p-value is < 0.05, **p-value is <0.01, ***p-value is <0.001 and ****p-value is <0.0001(t-test).*

4.8 The role of GPR35 in autophagy

Autophagy can contribute to cell survival or programmed cell death. Once cells are damaged, the dysfunctional cells start to remove and degrade their components by autophagy. The degraded cellular components are recycled to promote cell survival. Autophagy is an evolutionarily conserved cellular strategy for normal cells to survive under metabolic and therapeutic stresses like nutrient deprivation, hypoxia and drug stimuli. Moreover, continually activated autophagy by persistent stresses, which consume important proteins and organelles, can lead to programmed cell death, but in a caspase-independent way.

Paradoxically, cancer cells also utilize the mechanism of autophagy for survival and programmed cell death by changing its machinery. The alteration of autophagy in cancer can contribute to metastasis, cancer cell proliferation as well as cancer cell survival. Due to these diverse roles of autophagy in cancer, the effects of autophagy have been conflicted: anti- vs pro-tumorigenic and anti- vs pro-metastatic. Currently, investigators presume that autophagy

has divergent roles in tumorigenesis and metastasis. Autophagy can inhibit tumour growth in the early stage by decreasing chronic inflammation and tissue damage caused by oncogenic signalling. However, once the tumour is developed, autophagy can contribute to tumorigenesis by increasing cell proliferation and metabolic changes against stresses encountered by the solid tumour, which can be poor vascularization and a hypoxic environment.

Necrosis occurs inside solid tumours due to poor vascularization and hypoxia condition. This damage by necrosis enables macrophages to infiltrate tumour sites, providing cancer cells with a favourable microenvironment disconnecting cell-cell and cell-ECM interactions, and eventually initiating metastasis. Autophagy enables tumour cells to survive under nutrition deficiency and hypoxia, thereby autophagy can have an anti-metastatic role by preventing invasion, the first step of metastasis, characterized by increased cell motility. However, autophagy also can have a pro-metastatic role by helping matrix-detached pre-metastatic tumour cells to avoid anoikis. Commonly detached cells from ECM are exposed to a specific type of apoptosis, called anoikis. In addition, autophagy provides malignant cells with the energy to survive until the cells are re-attached and colonized in the destination site.

PDAC is easy to be exposed to nutritional stress and hypoxic condition because of the surrounding stroma. Many studies highlight that autophagy is required to form pancreatic tumours and progress metastasis of PDAC. However, the mechanism of autophagy in pancreatic cancer is still unclear. Thus, it is crucial to identify whether cell death or less proliferation by the inhibition of GPR35 is caused by autophagy.

We performed an autophagy assay by detecting light chain 3 isoform B (LC3B) and P62/SQSTM-1 (sequestosome-1) expression by Western blot. LC3 is a key marker of autophagy. Once autophagy occurs LC3, located in the cytoplasm, diffusely undergoes post-translational modifications. Cleaved LC3-I converts to LC3-II by conjugating with phosphatidylethanolamine (PE) on the surface of autophagosomes. P62 is a protein binding to ubiquitin and autophagosomal membrane protein LC3/Atg8. P62 containing protein aggregates to their autophagosomes facilitates lysosomal degradation of autophagosomes, which leads to a decrease in P62 levels during autophagy. Thus, an increased expression of LC3-II, together with a decreased expression of P62, suggests induced autophagy.

Figure 4.12 shows that the selected clones from genetically modified MIA PaCa-2 cell lines expressing less GPR35 by CRISPR/Cas9, which are KO4 and KO7, have lower expression of P62 and higher expression of LC3BII compared to control NC1. This result demonstrates that GPR35 regulates autophagy in pancreatic cancer.

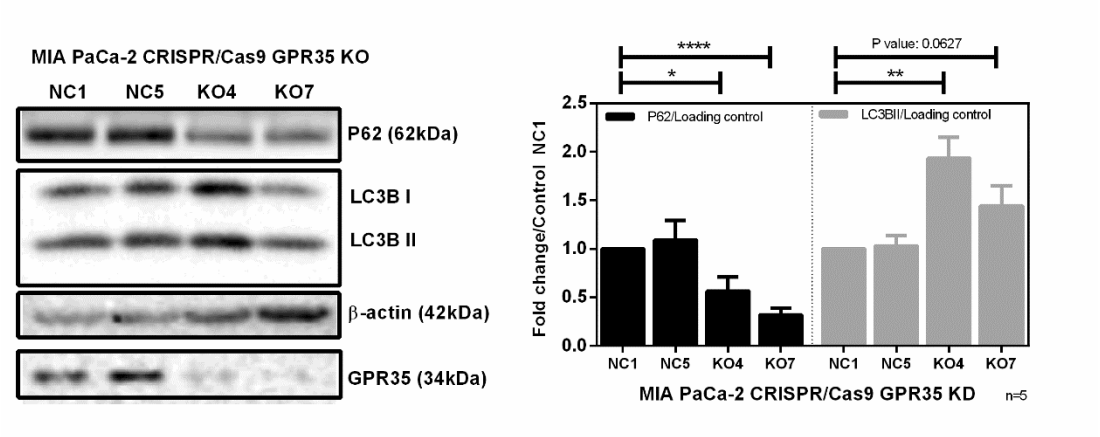


Figure 4.12. The inhibition of GPR35 induces autophagy in MIA PaCa-2. *A significantly lower expression of p62 was detected on both KO4 and KO7 cell lines. High expression of LC3BII was recorded in KO4 compared to control NC1. Five independent repeats of all the experiments were performed. Results are represented as mean ± SEM. *p-value is < 0.05, **p-value is <0.01 and ****p-value is <0.0001(t-test).*

4.9 Chapter summary and discussion

This chapter demonstrated the following:

- ML145 reduces cell proliferation in most PDAC cell lines. Serum-free condition boosts ML145 to dramatically decrease cell number in PDAC cell lines, except PANC-1.
- ML145 and CID2745687 do not affect cell proliferation in hTERT-HPNE.
- ML145 reduces 3D colony formation in AsPC-1 and MIA PaCa-2.
- The inhibition of GPR35 by siRNA silencing transfection reduces cell growth in PDAC cell lines.
- The inhibition of GPR35 by CRISPR/Cas9 system reduces colony formation in AsPC-1 and MIA PaCa-2.
- Compound 10 helps AsPC-1 and HPAF-II cell growth and/or survival in serum starvation conditions.
- The inhibition of GPR35 changes the morphology of PDAC cells and increases apoptosis and autophagy.

- G0/G1 cell cycle arrest occurs in GPR35 downregulated AsPC-1 cells by siRNA transfection.
- Apoptosis induced by the inhibition of GPR35 has been verified by annexin-V/-FITC/-PI assay, Caspase 3/7 probe assay and cleaved caspase 3 detection assay.
- Autophagy induced by the inhibition of GPR35 has been verified by P62 and LC3BII expression assay.

We have verified that both pharmacologically and genetically downregulated GPR35 expression induces PDAC cell death in both 2D and 3D conditions, suggesting the proliferation role of GPR35 in PDAC. To date, several GPR35 studies have shown its proliferation role. A proliferative response in human vascular cells, including human endothelial cells and smooth muscle cells, induced by stimulation of GPR35 (with a treatment of zaprinast or pamoic acid) has been reported (McCallum et al., 2015). Moreover, the reduced cell proliferation in xenograft mice transplanted with A549R cells having depletion of GPR35 compared to control was observed by the proliferation marker Ki-67 staining study (W. Wang et al., 2018). GPR35 modulates diverse proliferation signalling pathways, such as EGFR/Src-Ras-ERK and PI3K/AKT, to maintain intestinal epithelial cell (IEC) turnover (Schneditz et al., 2019). In this study, a reduced number of IECs in GPR35 KO mice (above 50% reduction) compared to wild-type mice was observed by G5-bromo-2'-deoxyuridine (BrdU) assay and the Ki-67 staining analysis. These previous studies strongly support our findings that GPR35 has an important role in cell proliferation.

Selectively, only PDAC cell lines, and not normal pancreas cell lines, responded effectively to ML145. ML145 has shown more than 1000-fold selectivity for GPR35 compared to GPR55 which has the highest homology with GPR35, indicating that ML145 has high affinity and selectivity to human GPR35 (Heynen-Genel et al., 2010). Together our findings suggest that ML145 is a potential new drug for PDAC, while further studies are required to confirm the characteristics and safety of this antagonist.

The inhibition of GPR35 by both pharmacological and genetical modulation induced morphological changes in this study. In fact, several studies have demonstrated the morphological alterations induced by GPR35 activation. Ruffle-ended type morphology changes showing reorganization of actin filaments were observed in neonatal mouse cardiomyocytes induced by overexpression of GPR35 (Ronkainen et al., 2014). Moreover, the cytoskeleton arrangement induced by GPR35 activation with the treatment of pamoic acid in human vascular cells was reported (McCallum et al., 2015). Chemical genomic analysis of GPR35 suggested that GPR35 may have a pivotal role in regulating the morphogenetic process

(H. H. Hu et al., 2017). Unlikely other previous studies, our findings are distinguished in terms of showing morphological changes induced by the inhibition of GPR35, and not by its activation.

We have successfully downregulated GPR35 expression in human PDAC cell lines using siRNA or the CRISPR/Cas9 system. However, the variability amongst GPR35 KD clones modified by CRISPR/Cas9 system was shown. Two main reasons for this variability can be hypothesized. First, three gRNAs used in this study may not show their activity. Three active gRNAs should result in more than 99% of cells having KO in theory. However, the clones selected by puromycin treatment in this study showed a decreased expression of GPR35 in WB; the reason for this could be that either one or two gRNAs may not be active. Second, the selected KO clones transfected by CRISPR/Cas9 may not stably suppress the expression of GPR35. We tried to select a single cell from an individual colony after puromycin selection during the establishment of this stable GPR35 KO cell line but mixed cells might be present in one clone. This leads to variability amongst clones. Nevertheless, GPR35 KD clones modified using the CRISPR/Cas9 system show successfully downregulated GPR35 expression. The PDAC cell lines with genetically modified GPR35 expression provide broad views on cell proliferative roles of this receptor, such as the regulation of cell cycle arrest, apoptosis and autophagy.

We demonstrated that the anti-proliferation effect obtained by depleting GPR35 in AsPC-1 cells is induced by cell cycle arrest. This is the first study to show the cell cycle arrest in a GPR35-dependent manner.

Accumulated evidence described GPR35 as a key modulator of apoptosis. The deficiency of GPR35 in A549R cells increased the cellular apoptosis rate (verified by annexin V assay and caspase-3, bcl2, p21, bcl-XL expression analysis), indicating that GPR35 regulates cellular apoptosis generated by drug toxicity (W. Wang et al., 2018). The neonatal murine ventricular myocytes apoptosis induced by anoxia was inhibited with the treatment of CID2745687 (annexin V assay and caspase-3/9 expression analysis), suggesting that GPR35 has a protective role in cell apoptosis caused by hypoxia (K. Chen et al., 2020). Our apoptosis studies showed the GPR35 is regulating PDAC cell apoptosis. Therefore, therapeutically targeting GPR35 seems very promising for PDAC patients.

Chapter 5

Chapter 5: The mechanism of cell proliferation, cell survival, apoptosis and autophagy correlating the expression of GPR35

5.1 Rationale

To better understand the role of GPR35 in PDAC it is crucial to investigate the anti-proliferation, survival, apoptosis and autophagy mechanisms regulated by GPR35. We started by examining three downstream selected molecules having a well-known role in cell proliferation, survival, apoptosis and autophagy in PDAC, which are AKT, ERK and HIF1-α.

The AKT serine/threonine kinase (protein kinase B), which is the central protein in the PI3K/AKT signalling pathway, plays an important role in regulations of cancer cell survival, proliferation, apoptosis, autophagy, glucose metabolism and metastasis. AKT is activated by phosphorylation on Thr308 or Ser473. The phosphorylated AKT (pAKT) mediates a variety of downstream protein substrates regulating diverse cellular mechanisms as mentioned above. Constitutive activation of AKT has been reported as a therapeutic target for various cancers including breast cancer metastasis, prostate cancer, colon cancer and pancreatic cancer. In pancreatic cancer, overexpression of the *Akt2* gene is founded in around 20% of PDAC patients. The activation of AKT in pancreatic cancer has been reported as a mediator having an anti-apoptotic effect and tumorigenic effect. Moreover, activated AKT in PDAC is involved in the regulation of autophagy inducing cell death independently on apoptosis.

The ERK, also known as the 42-/44-kDa MAPK, is a central protein mediating the MAPK/ERK pathway regulating mainly the cell cycle. Once this pathway is dysregulated, uncontrolled regulation of the cell cycle leads to uncontrolled cell proliferation, differentiation, survival and motility, which eventually can lead to cancer. Thereby, the ERK pathway has been widely investigated in cancer research. Many pancreatic cancer studies have reported that the ERK signalling pathway regulates cell proliferation, survival, apoptosis and metastasis.

HIF-1 is a regulator of hypoxia in cells. Pancreatic cancer is normally exposed to hypoxic environments due to a desmoplastic reaction and hypoperfusion leading to the formation of a dense microenvironment resulting in oxygen shortage. Overexpression of HIF-1 has been reported in 88% of pancreatic cancer tissues (Shibaji et al., 2003). High expression of HIF-1 influences metastasis, proliferation, reprogrammed metabolism and poor prognosis in pancreatic cancer. However, the mechanism remains unclear.

Therefore, we tested the involvement of, AKT, ERK and HIF-1 as downstream effectors of GPR35. This chapter hypothesises that the mechanism of anti-cell proliferation, cell survival,

apoptosis and autophagy is correlated with GPR35 expression in PDAC that in turn activates AKT, ERK and HIF-1α.

5.2 The pharmacological inhibition of GPR35 reduces AKT activity and HIF-1α stabilization.

We next examined the levels of phospho-specific AKT (Ser-473) and HIF-1α in four cell lines, AsPC-1, HPAF-II, MIA PaCa-2 and PANC-1. We have treated cells with ML145 for 1 hour and verified the expression level of these two molecules by Western blot analysis. Figure 5.1 shows that ML145 treatment downregulated the level of pAKT S473 and HIF-1α in all four cell lines tested, especially at 10μM concentration, compared to non-treated cells. Not only ML145, but also CID2745687 was tested in AsPC-1 cell line. Figure 5.2 shows that a lower level of pAKT S473 was verified in cells treated with 10μM of CID2745687, but we could not find any significant difference in the expression of HIF-1α by Western blot analysis. We examined the expression of pERK on AsPC-1 and Capan-2 cell lines (Figure5.3). Both cell lines were incubated with ML145 (10μM) for 1 hour and the lysates were analysed by Western blot. There is no significant difference in the activation of ERK between ML145 treated cell and non-treated cell.

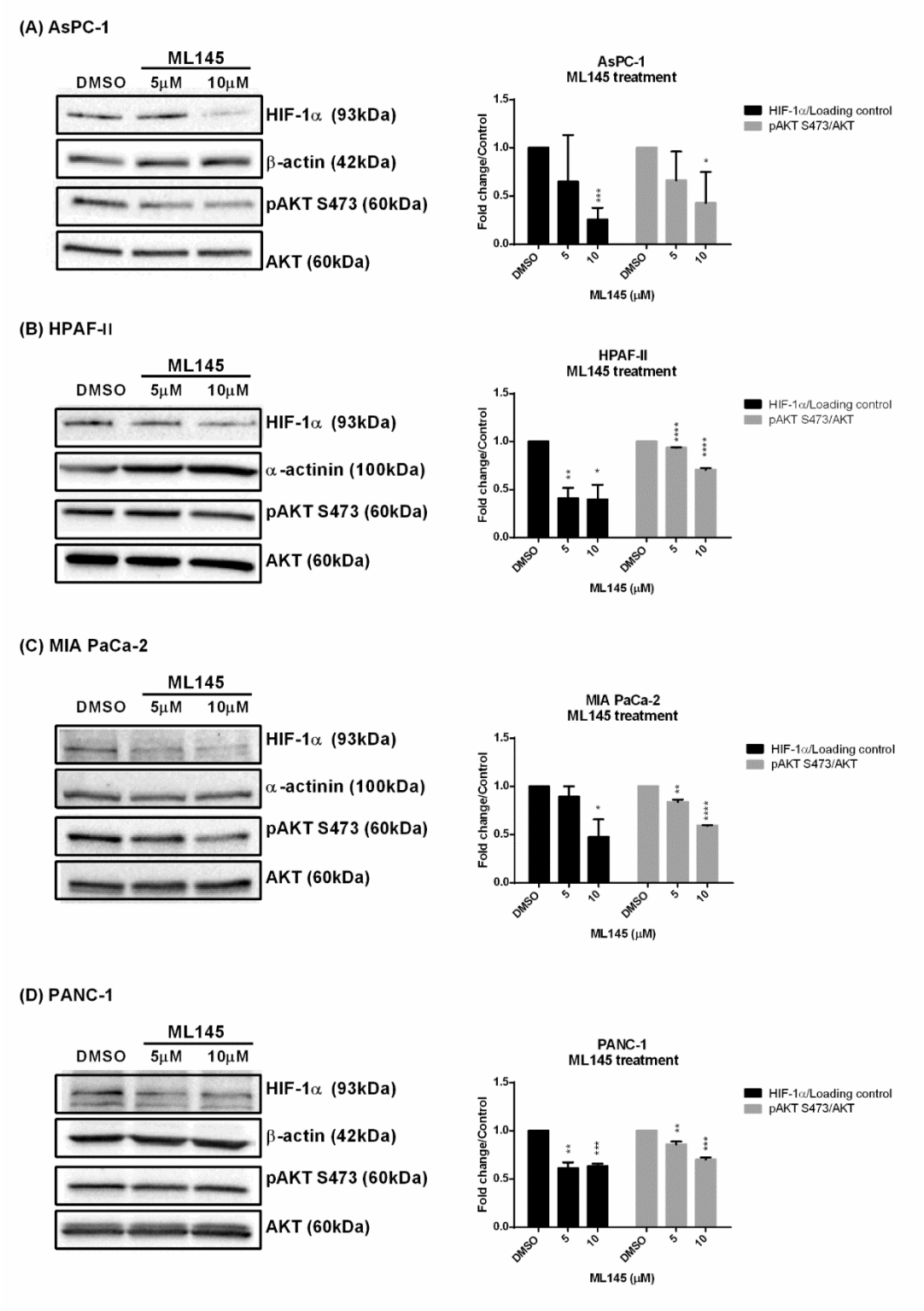


Figure 5.1. ML145 downregulates pAKT S473 and HIF-1α in PDAC. *(A) AsPC-1, (B) HPAF-II, (C) MIA PaCa-2 and (D) PANC-1 were treated with ML145 (total concentrations are 5 or 10 μM) for 24 hours. The cells were harvested and screened with pAKT S473 (CST) and HIF-1α (Novus) antibodies. The western blot shows the downregulated expression of pAKT S473 and HIF-1α in ML145 treated (A) AsPC-1, (B) HPAF-II, (C) MIA PaCa-2 and (D) PANC-1 compared to non-treated cells. Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. *p-value is < 0.05, **p-value is <0.01, ***p-value is <0.001 and ****p-value is <0.0001(t-test).*

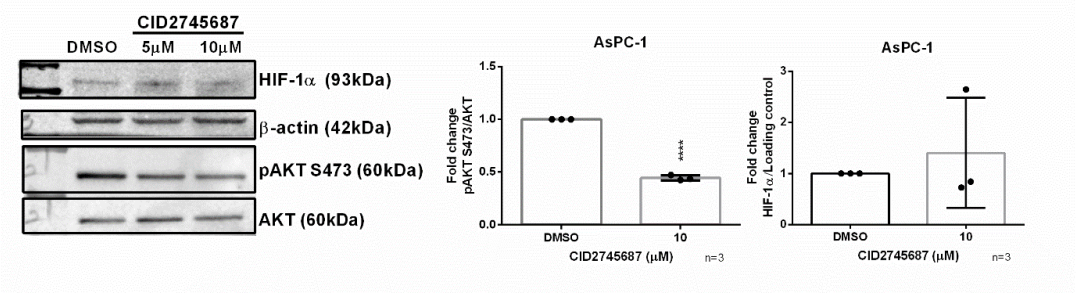


Figure 5.2. CID2735687 downregulates pAKT S473 in AsPC-1. *The cells were treated with two different concentrations of CID2745687, 5 and 10 μM. The cells were harvested after 1 hour of incubation and screened with HIF-1α (Novus) and pAKT S473 (CST) abs. Western blot analysis shows significantly less expression of pAKT S473 compared to control when the cells were treated in 10μM of concentration of CID2745687. However, the expression of HIF-1α did not show a significant difference. Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. ****p-value is <0.0001(t-test).*

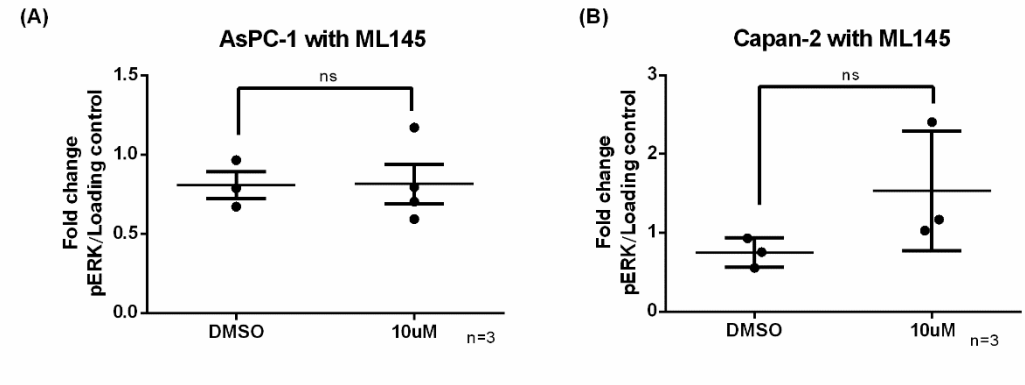


Figure 5.3. The inhibition of GPR35 is not related to ERK activity. *AsPC-1 and Capan-2 cell lines were treated with ML145 (10μM) for 1 hour in a complete medium. The lysates were tested with pERK antibody (CST) and tubulin antibody for loading control. Western blot analysis shows there is no significant difference in pERK expression between treated cells and non-treated cells. Three independent repeats of all the experiments were performed. Results are represented as mean ± SEM. ns means statistically non-significant (t-test).*

5.3 Synthetic agonists of GPR35 activate ERK and AKT.

Synthetic agonists of GPR35 which are compound 10, pamoic acid and zaprinast were tested in AsPC-1 and HPAF-II cell lines. Figure 5.4. A and B show that compound 10 induces the activity of AKT and ERK mostly between 5 to 15 minutes in AsPC-1 and HPAF-II cell lines. Pamoic acid increases the expression of pAKT S473 at 1 min and phospho-ERK1/2 (pERK) between 1 to 30 minutes in AsPC-1 (Figure 5.4.C). Zaprinast also shows a significantly increased pERK expression in AsPC-1 between 1 to 30 minutes (Figure 5.4.D).

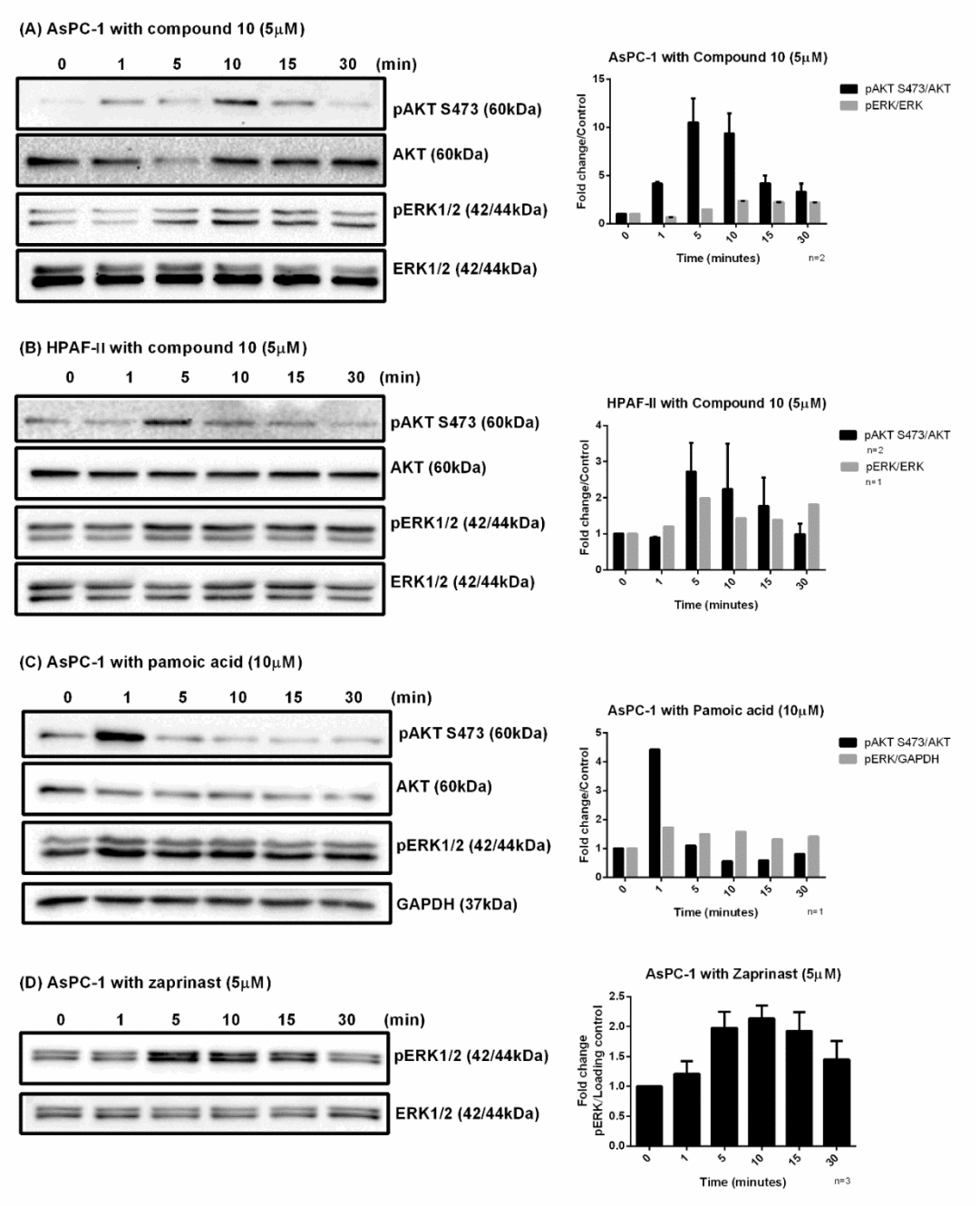


Figure 5.4. Synthetic agonists of GPR35 active ERK and AKT in AsPC-1 and HPAF-II cell lines. *Compound 10 induces the expression of pERK and pAKT S473 in AsPC-1 (A) and HPAF-II (B). (C) Pamoic acid increases the activity of ERK and AKT in AsPC-1. (D) Zaprinast shows significantly the increased expression of pERK in AsPC-1 between 1 to 30 minutes. All experiments have been repeated between 1 to 3 times. Some of the results are represented as mean ± SEM.*

5.4 GPR35 downregulation by siRNA inhibits AKT and HIF-1 activity, but not ERK activity

Inhibition of pAKT S473 and HIF-1 as verified in four cell lines, AsPC-1, HPAF-II, MIA PaCa-2 and PANC-1, expressing less GPR35 by siRNA transfection compared to the cells transfected with non-targeting GPR35 siRNA (Figure 5.5). Surprisingly, PANC-1 cell line shows upregulated HIF-1α unlike other cell lines (Figure 5.5.C).

No changes in pERK levels could be detected by GPR35 downregulation. Therefore, the downregulation of GPR35 indicates that this receptor does not regulate the expression of phospho-ERK1/2 (pERK). AsPC-1 and CFPAC-1 cell lines were transiently silenced withGPR35 siRNA transfection and examined by Western blot. After 72 hours of transfection, pERK was analysed and results are shown in Figure 5.6. Both cell lines present no difference in the levels of pERK in both sequences compared to siControl.

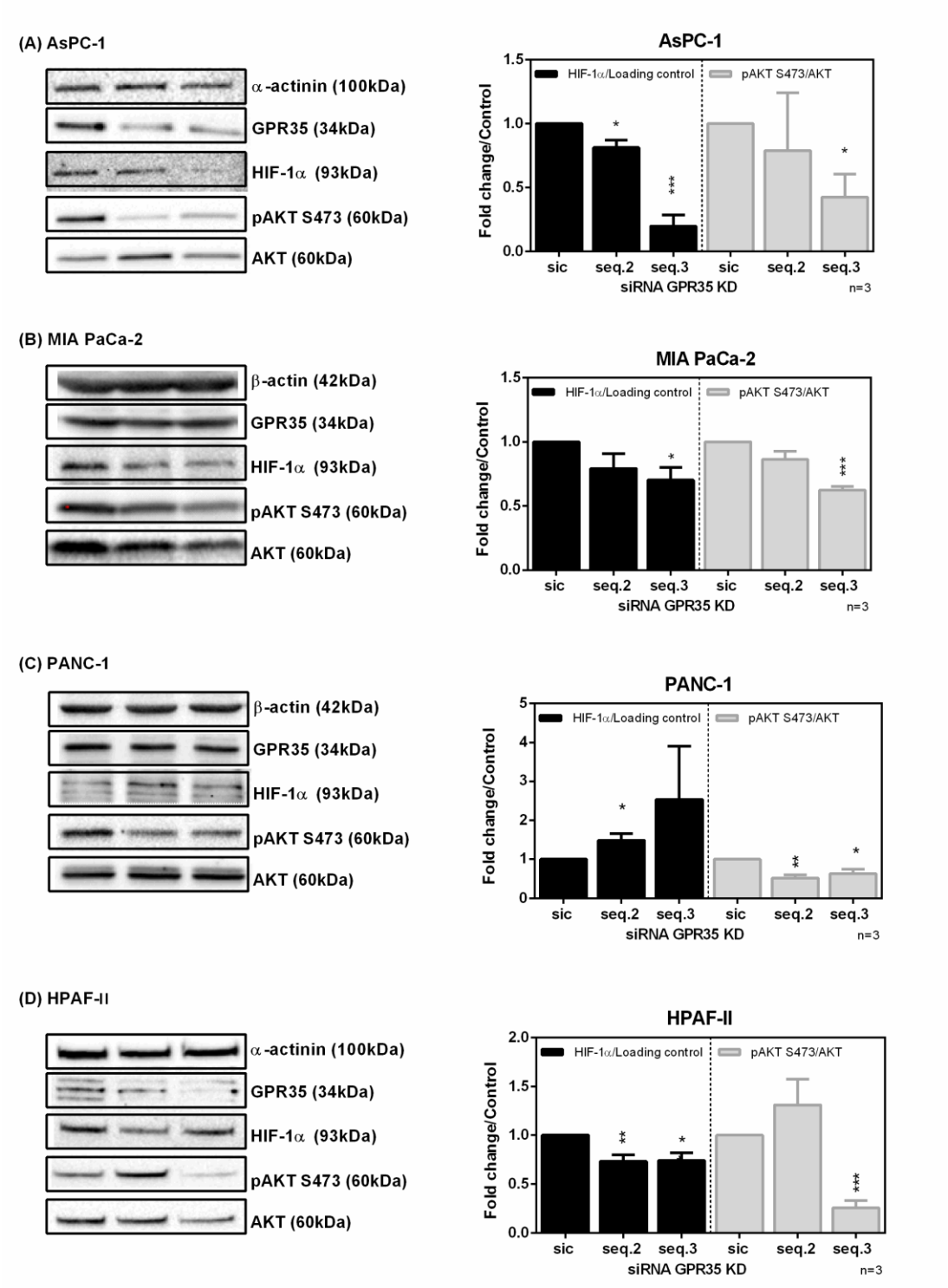


Figure 5.5. Downregulation of pAKT S473 and HIF-1α was detected in AsPC-1, HPAF-II and MIA PaCa-2 expressing a lower GPR35 by siRNA transfection. *GPR35 was silenced in AsPC-1, HPAF-II, MIA PaCa-2 and PANC-1 by siRNA transfection. The cells were harvested after 72 hours of the transfection and screened with pAKT S473 (CST) and HIF-1α (Novus) antibodies by Western blot analysis. The results show the downregulated expression of pAKT S473 and HIF-1α in most of the four cell lines (but not in the PANC-1 cell line for the downregulated expression of HIF-1α) expressing a lower GPR35 compared to the cells non-targeting GPR35 siRNA. Three independent repeats of the experiments were performed. The*

*results are represented as mean ± SEM. *p-value is < 0.05, **p-value is <0.01 and ***p-value is <0.001(t-test).*

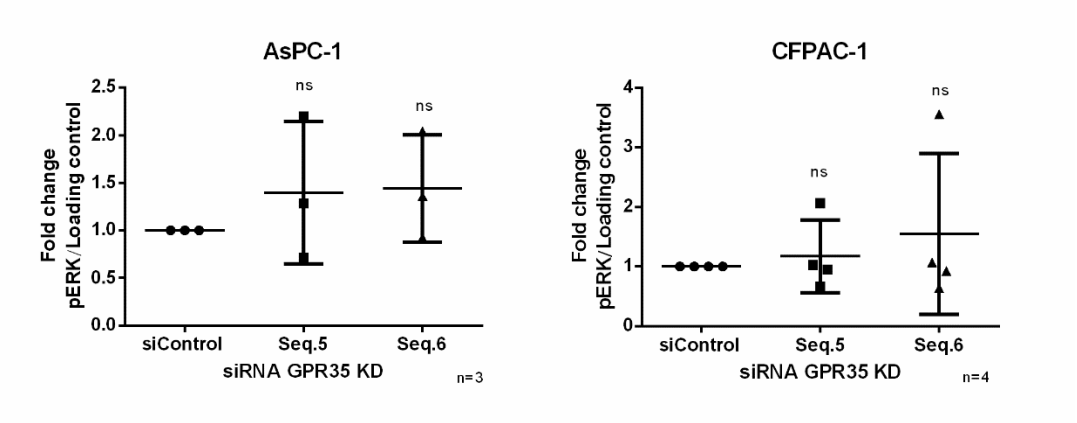


Figure 5.6. The inhibition of GPR35 by gene silencing is not related to ERK activation*. AsPC-1 and CFPAC-1 cell lines were transfected to silence GPR35 transiently by siRNA. After 72 hours, the cells were harvested and the expression level of pERK (CST) was screened by Western blot analysis. Both cell lines show there is not a significant difference in the expression level of pERK between control and sequence 5/6. All experiments were repeated 3 or 4 times. The results are represented as mean ± SEM. Ns means statistically non-significant (t-test).*

5.5 GPR35 is controlled by hypoxia

HIF-1, one of the transcription factors induced by hypoxia, forms a heterodimer that consists of an alpha subunit (HIF-1α) and a beta subunit (HIF-1β). Under normoxic conditions, HIF-1α undergoes post-translational modification by VHL, also known as a tumour suppressor protein, which results in their degradation by the ubiquitin protease pathway. Once cells are exposed to oxygen deprivation stress, O_2-depedent HIF-1α is unable to be degraded, which leads to the HIF-1α stabilization. The HIF-1 heterodimers can induce transcription of genes related to diverse cellular processes including angiogenesis, erythropoiesis and metabolism, eventually helping to deliver oxygen to less oxygenated areas. However, HIF-1α can be stabilized continuously even in the presence of oxygen by increased oncogenic signalling such as PI3K and MAPK pathways in cancer cells (reviewed by (Semenza, 2003). Overexpression of HIF-1α has been reported as an important indicator of cancer progression, especially pro-metastatic effects in PDAC (Hoffmann et al., 2008; T. Zhao et al., 2012).

Based on our observed HIF-1α downregulation following GPR35 inhibition, we could hypothesize that the expression of GPR35 might be strictly correlated to hypoxia in PDAC. We performed additional hypoxic experiments, such as HIF-1α knocking down transfection and hypoxia incubation to further identifying the GPR35 - HIF-1 mutual connection.

To evaluate the GPR35 regulation by the oxygen-independent HIF-1 activity, HIF-1α was transiently silenced by siRNA transfection in MIA PaCa-2 and AsPC-1 cell lines. The expression of GPR35 was screened by Western blot analysis. As shown in Figure 5.7. A and B both siRNA sequences knocking down HIF-1αdownregulated GPR35 compared to control siRNA.

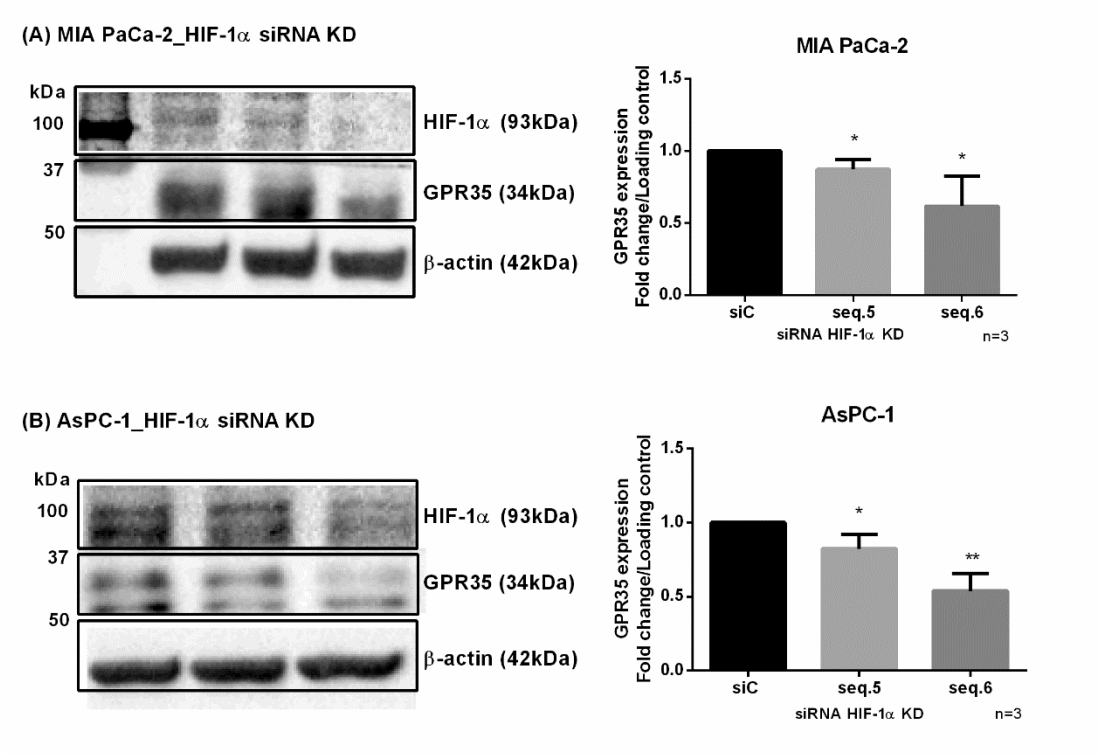


Figure 5.7. GPR35 is related to the expression of HIF-1. *(A)(B) Silencing HIF-1α induces downregulation of GPR35 in AsPC-1 and MIA PaCa-2. All experiments have been performed 3 times. The results are represented as mean ± SEM. *p-value is < 0.05 and **p-value is <0.01.*

Moreover, we incubated MIA PaCa-2 and AsPC-1 cell lines expressing less GPR35 by CRISPR/Cas9 in hypoxic conditions to understand the role of GPR35 in cell proliferation in oxygen-deprived conditions. Cells were incubated in a hypoxia chamber (1% oxygen) for 72 hours and the number of cells was counted manually. Interestingly, Figure 5.8.A presents a significantly higher number of cells in MIA PaCa-2_KO7 cell lines compared to control NC5. AsPC-1_KO11 also shows a higher number of counted cells compared to control (NC21) after hypoxic incubation (Figure 5.8.B).

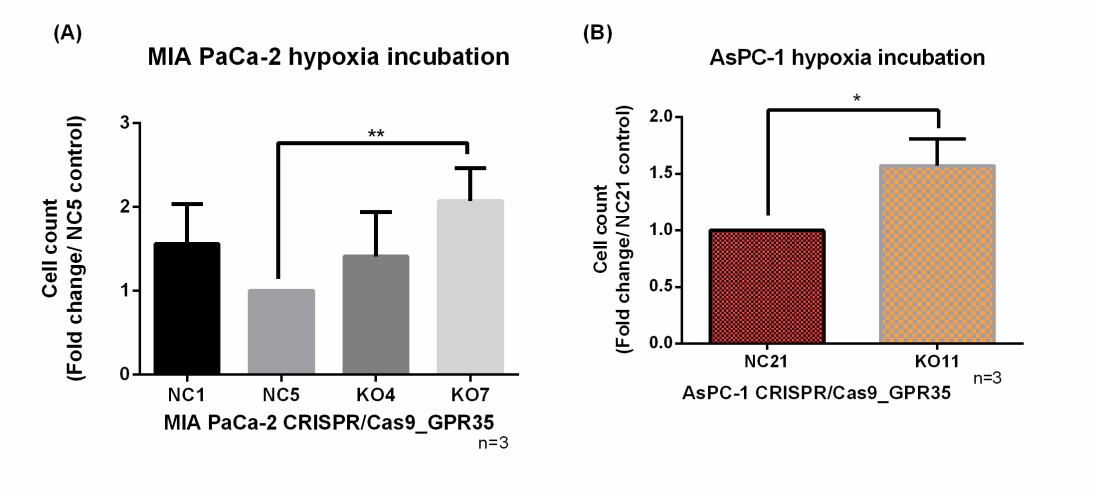


Figure 5.8. *A higher number of MIA PaCa-2 and AsPC-1 cells expressing a lower GPR35 was counted after hypoxic incubation (1% oxygen) for 72 hours compared to control. All experiments repeated 3 times. The results are represented as mean ± SEM. *p-value is < 0.05 and **p-value is <0.01 (t-test).*

5.6 Mutated p53 via miR-34b induces overexpression of GPR35

The tumour suppressor protein p53 plays a crucial role in the regulation of the cell cycle and apoptosis. The *TP53* gene is mutated in 50 to 70% of human pancreatic cancers, following an initiating alteration in the *KRAS* gene (Scarpa et al., 1993). Experimental evidence in the last decades reported that mutant p53 correlates with cancer progression, metastasis and autophagy in pancreatic cancer. Missense mutations occurring predominantly in PDAC result in mutant p53 protein accumulation, leading to tumour growth (J. M. Bailey et al., 2016). Mutation of *TP53* overcomes the growth arrest/senescence of premalignant pancreatic cells driven by the expression of $Kras^{G12D}$ and accelerates PDAC progression in the mouse pancreas (Morton et al., 2010). Another study has verified that the accumulation of mutant p53 promotes pancreatic cancer metastasis in a murine model (Morton et al., 2010; Weissmueller et al., 2014). Moreover, a correlation between autophagy and p53 in pancreatic cancer has been demonstrated, indicating that the inhibition of autophagy, due to the loss of p53, drives a more rapid PDAC formation (Rosenfeldt et al., 2013). Our previous data have shown that GPR35 is related to cell proliferation controlled by apoptosis, cell cycle arrest, and autophagy. Moreover, the expression of GPR35 was lower in Capan-2, which contains wild type p53, compared to the other PDAC cell lines investigated that possess mutant p53. Thus, we next assessed the correlation between the p53 status and the expression of GPR35. A panel of murine pancreatic cell lines bearing different status of p53, wild type p53 (PZR1), mutant p53 (PZPR1) and

deleted p53 (PZPflR), were analysed by WB. Figure 5.9 shows that the expression of GPR35 was lower in the PZR1 cell line (WT p53) while the highest level of GPR35 expression among the three cell lines was detected in the PZPR1 cell line (mutant p53). In addition, higher levels of P62 were observed in PZPR1 and PZPflR cell lines, indicating that p53 mutation or deletion regulates autophagy. Similarly, higher levels of HIF-1α were shown in cell lines having mutated and deleted p53. Taken together, these data suggest that the presence of a fully functional p53 protein can negatively regulate the expression of GPR35 in normal pancreatic cells. Consequently, GPR35 is overexpressed in pancreatic cancer cells possessing mutated p53, present in most PDAC cases, possibly leading to increased pancreatic cancer proliferation and metastasis.

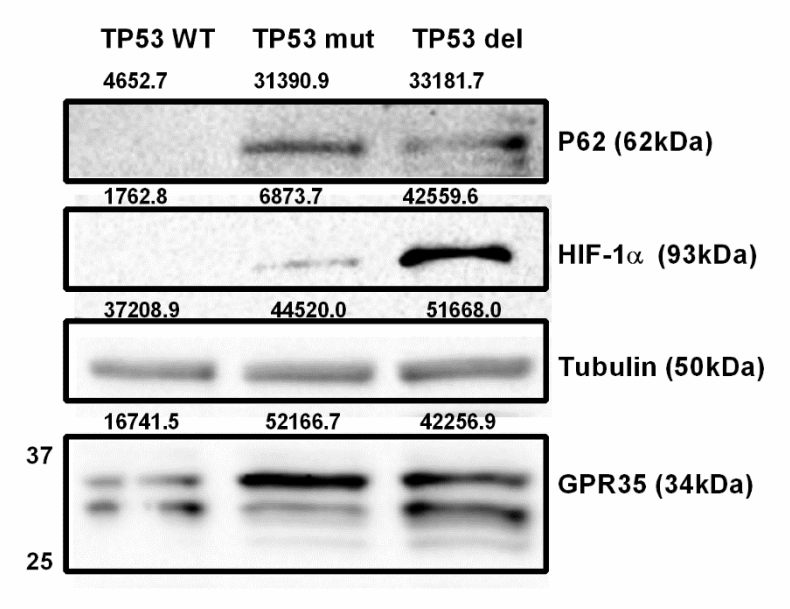


Figure 5.9 GPR35 expression is dependent on TP53 mutations. *The murine pancreatic cell lines containing different p53 status TP53 wild type (WT), mutated $TP53^{R172H/+}$ (mut) and deleted $TP53^{fl/+}$ (del), were analysed by WB. The expression of p62, HIF-1α and GPR35 was tested. The number above the blots indicates the intensity of the expressed bands measured by ImageJ.*

MicroRNAs (miRNAs or miRs) are high-conserved short (about 22 nucleotides in length) single-stranded noncoding RNAs that can bind to specific sites within three prime untranslated regions (3'-UTR) in messenger RNA (mRNA) for negatively regulating mRNA translation. A single miRNA can bind with hundreds of different mRNAs and simultaneously, because one mRNA bears multiple binding sites in 3'-UTR for different miRNAs. The miR-34 family consists of three processed miRs: miR-34a, miR-34b and miR-34c. The latter is directly

regulated by p53 to inhibit tumorigenesis. Inactivation of miR-34 has been observed in PDAC patients and most of PDAC cell lines as mutant p53 dysregulates miR-34 expression. We next investigated the potential involvement of the miR-34in p53-dependent downregulation of GPR35. To this end, we reintroduce miR-34b by transfection in AsPC-1 cell line. Interestingly, the expression of GPR35 is significantly decreased compared to the control and the vehicle in miR-34b transfected cells (Figure 5.10).

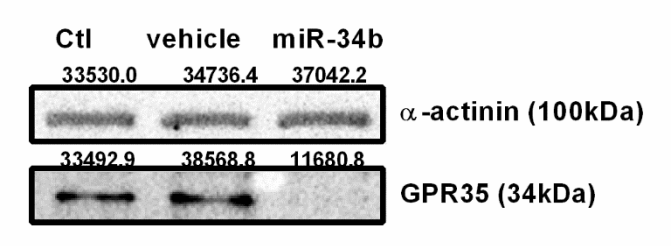


Figure 5.10. The reintroduction of miRNA-34b in AsPC-1 negatively regulates the expression of GPR35. *AsPC-1 cells were transfected with miR-34b-3p (Ambion) and the lysates were collected 72 hours post-transfection. The expression of GPR35 (Cayman) is significantly decreased in the transfected cells. The number above the blots indicates the intensity of the expressed bands measured by ImageJ.*

5.7 GPR35 reprograms PDAC cell metabolism

Cancer cells rewire their metabolic programs to drive tumour growth and survival. Otto Warburg was the first to find in the 1920s, that cancer cells are likely to metabolize glucose to lactate by glycolysis even under aerobic conditions, while normal cells do so only in the absence of oxygen. This phenomenon, called the Warburg effect or aerobic glycolysis, has been observed in diverse cancer types even though this ATP production by glycolysis is far less efficient compared to oxidative phosphorylation because an increase in glucose uptake is required. Based on the concomitant increase in glucose uptake, fluorodeoxyglucose positron emission tomography (FDG-PET) is clinically exploited for detecting tumours. Even though aerobic glycolysis is currently accepted as a hallmark of cancer metabolism, the correlation of aerobic glycolysis with cancer progression is still under investigation to better explain the Warburg effect.

AKT kinase has a pivotal role in the regulation of metabolism for cancer cell growth. AKT stimulates glucose uptake through glucose transporter translocation and utilizes it for cancer growth. AKT inhibits the activation of thioredoxin-interacting protein (TXNIP), which generally promotes endocytosis of GLUT1 to inhibit glucose uptake, resulting in a rich GLUT1 and GLUT4 environment at the plasma membrane (Parikh et al., 2007). The enhanced

glucose uptake by regulation of TXNIP by AKT activation has been reported in non-small-cell lung cancer cell lines and in vivo (Ancey, Contat, & Meylan, 2018; Hong, Yu, Luo, & Hagen, 2016). Moreover, AKT directly controls glycolytic enzymes such as hexokinase 2 (HK2) to regulate glycolysis. The overexpression of HK2 in many human cancers including pancreatic, colorectal and liver cancer, has been found (Y. Liu et al., 2016). Not only tumour growth but also metastasis is promoted by the upregulated HK2 in pancreatic cancer (Anderson, Marayati, Moffitt, & Yeh, 2017).

Activation of HIF1 in presence of oxygen can be mediated by the PI3K/AKT signalling pathway and other oncogene mutations. The activated HIF1 increases the expression of glucose transporters and glycolytic enzymes to shift cell metabolism toward glycolysis. HIF1 reduces the glucose-derived pyruvate by activating pyruvate dehydrogenase kinases (PDKs), resulting in less utilization of tricarboxylic acid (TCA) cycle and eventually decreased oxidative phosphorylation and oxygen consumption. The expression of hypoxia-related glucose transporters has been verified in malignant lesions in the pancreas (J. Chen et al., 2003; Ren et al., 2013), brain (Labak et al., 2016) and thyroid (Jozwiak, Krzeslak, Pomorski, & Lipinska, 2012).

Tumour suppressor protein p53 also regulates cell metabolism. Loss or mutation of protein p53 downregulates SCO2 which mediates the synthesis of cytochrome c oxidase protein (SCO2) and TP53-induced glycolysis and apoptosis regulator (TIGAR), leading to prevent partially mitochondrial respiration (Matoba et al., 2006) and glycolysis (Bensaad et al., 2006).

Therefore, in order to fully understand the role of GPR35 in pancreatic cancer, we investigated the effects of GPR35 regulation on cancer cell metabolism. Based on our previous data, GPR35 is directly involved in the activation of AKT, HIF1 and p53, which simultaneously are related to cancer cell metabolism. Thus, we performed a metabolism assay using the XF^{e}96 Agilent Seahorse Extracellular Flux Analyzer, for analysing glycolysis and oxidative phosphorylation.

Oxygen consumption rate (OCR) with the addition of diverse mitochondrial stressors, such as oligomycin, carbonyl cyanide-4 (trifluorometoxy) phenylhydrazone (FCCP) and antimycin A/rotenone, were measured in this study to understand oxidative phosphorylation. Oligomycin is an ATP synthesis inhibitor that decreases electron flow through the electron transport chain (ETC), eventually negatively impacting mitochondrial respiration and oxygen consumption. This decrease of OCR reflects ATP-linked respiration and/or cellular ATP production (Figure 5.11.A). FCCP uncouples the oxygen consumption of ATP synthase by disturbing the proton gradient at the mitochondrial membrane, resulting in a maximal value of OCR (maximal respiration). Calculation of the difference between maximal respiration and basal respiration

tells us the spare respiratory capacity, which indicates an amount of extra ATP generated in response to a sudden increase in energy demand (Figure 5.11.A). The combination of antimycin A and rotenone inhibits ETC and reduces OCR to a minimal value, presenting non-mitochondrial respiration (Figure 5.11.A).

Measurements of extracellular acidification rate (ECAR) upon treatment with glucose and oligomycin were analysed in this study to understand glycolysis (Figure 5.11.B). Supplied glucose for running glycolysis enables to measure the glycolytic rate by calculating the difference between ECAR before and after treatment of glucose. As explained above, oligomycin inhibits ATP synthesis in ETC, leading to glycolysis. The difference between ECAR before and after treatment of oligomycin provides the glycolytic reserve capacity (Figure 5.11.B). The basal line of ECAR was calculated by averaging the first three measurements before glucose addition instead of using 2-deoxyglucose in this study (Figure 5.11.B).

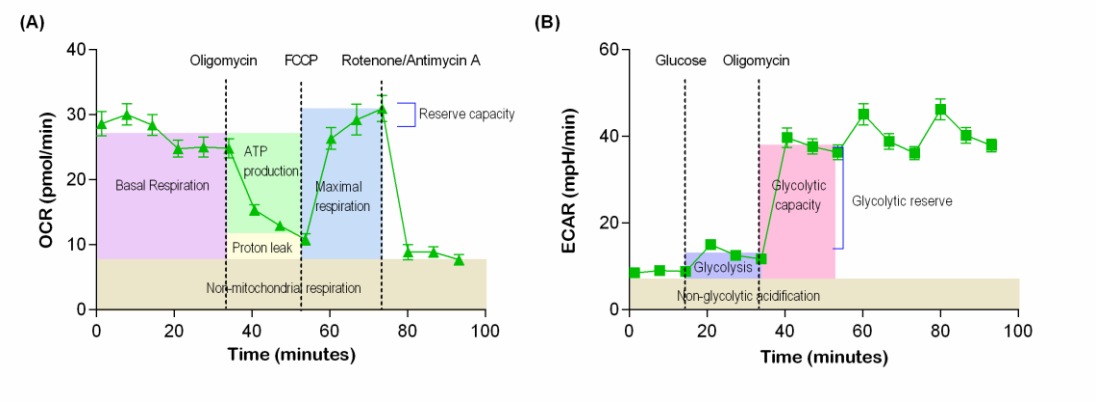


Figure 5.11. (A) *Measurements of OCR provide information on mitochondrial respiration including basal respiration, ATP production, maximal respiration, reserve capacity and non-mitochondrial respiration.* ***(B)*** *ECAR measurements indicate glycolysis, glycolytic capacity, glycolytic reserve and non-glycolytic acidification.*

MIA PaCa-2 and AsPC-1 cell lines with GPR35 downregulated by CRISPR/Cas9 system were tested by a metabolic assay. In a mitochondrial respiration test, the basal OCR in cells expressing high GPR35 (NC cell types) protein levels are greater than that in KO cell types in both MIA PaCa-2 (Figure 5.12.A) and AsPC-1 cell lines (Figure 5.13.A). This shows that the inhibition of GPR35 significantly decreases basal respiration in PDAC (Figure 5.12&13.B). Reduction of OCR after the addition of oligomycin occurred in all cell types of both cell lines (Figure 5.12&13.A). However, only KO4 showed higher ATP production compared to NC5 in MIA PaCa-2 (Figure 5.12.B) while all NC cell types showed significantly higher ATP

production compared to KO cell types in AsPC-1 (Figure 5.13.B). In response to FCCP, increased OCR from decreased OCR by oligomycin was detected in all cell types of both cell lines (Figure 5.12&13.A). Based on the increased OCR by FCCP, maximal respiration and spare capacity were analysed. Both NC cell types show much higher maximal respiration compared to KO cell types in both MIA PaCa-2 and AsPC-1 cell lines (Figure 5.12&13.B). However, most of the KO cell types, except KO6 AsPC-1, had little response to FCCP and the OCR values were less than the basal line (Figure 5.12&13.B). For testing glycolysis, measurements after and before the addition of glucose or oligomycin were analysed (Figure 5.12&13.C&D). All NC cell types have greater glycolysis, glycolic capacity and glycolic reserve compared to KO cell types in both MIA PaCa-2 (Figure 5.12.D) and AsPC-1 cell lines (Figure 5.13.D). Figure 5.14 shows the basal line of OCR against basal line ECAR in MIA PaCa-2 (A) and AsPC-1 (B) cell lines. The inhibitory effects on mitochondrial energy metabolism and glycolysis can be explained by the fact that GPR35 inhibition leads to a quiescent state. This data demonstrates that cell proliferation in PDAC is mediated by GPR35 that induces metabolism reprogramming.

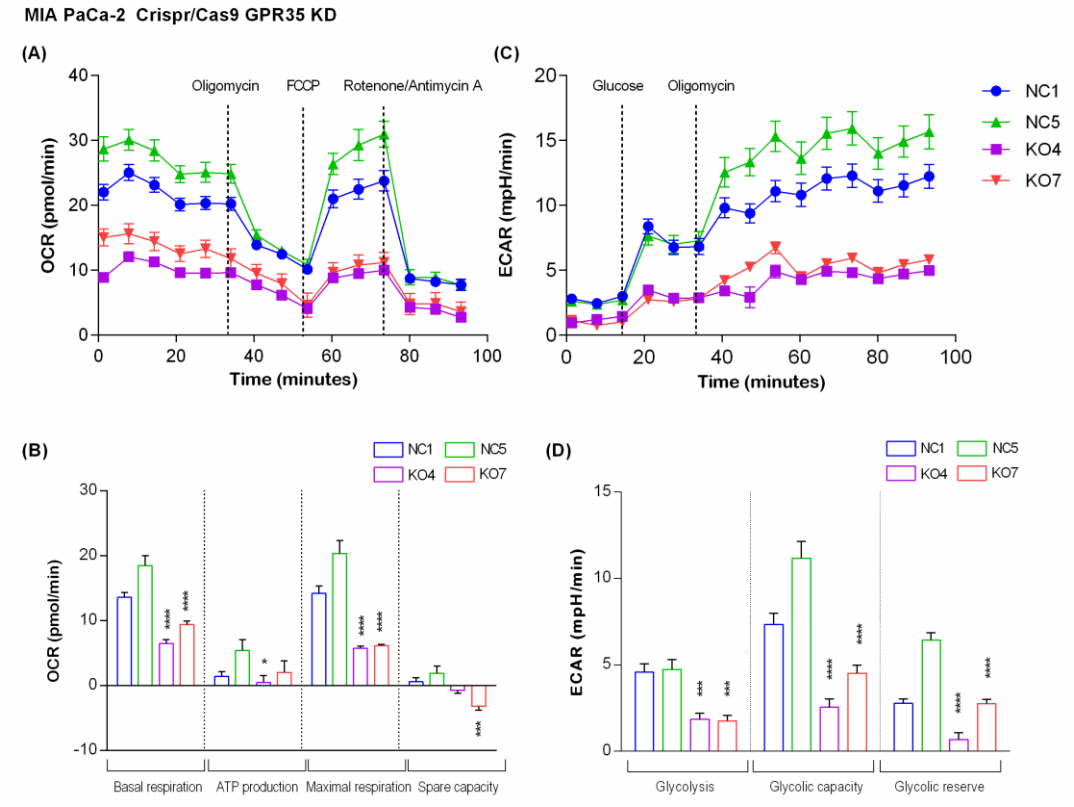


Figure 5.12. Influence of the GPR35 inhibition on cellular metabolism of MIA PaCa-2. *(A) OCR under mitochondrial stresses was recorded. (B) Basal respiration, ATP production, maximal respiration and spare capacity were calculated based on OCR measurement. (C) ECAR before and after the addition of glucose and oligomycin was measured. (D) Glycolysis, glycolic capacity and glycolic reserve were calculated based on ECAR measurement. Data from one replicative experiment is shown (n=minimum 11). All experiments were repeated 3*

*times. The results are represented as mean ± SEM. P-value compared to NC5 is shown. *p-value is < 0.05, p*** is <0.001 and ****p-value is <0.0001(t-test).*

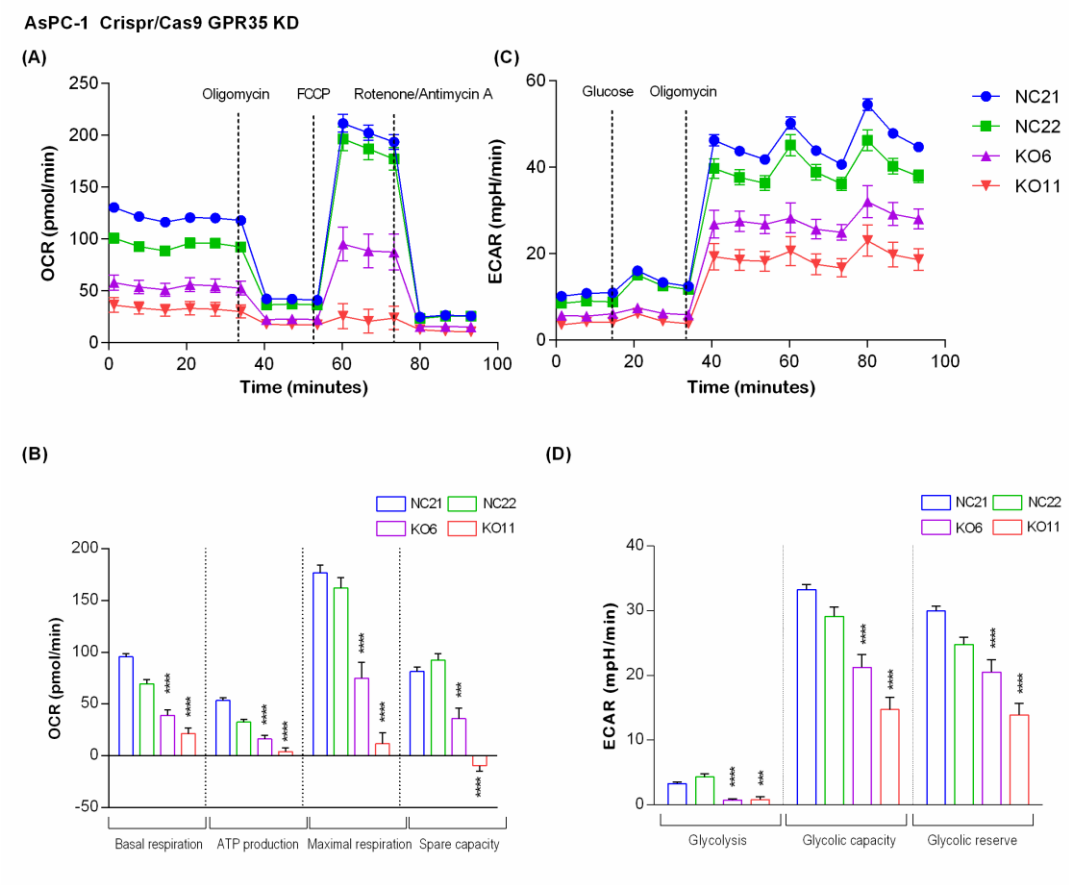


Figure 5.13. Influence of the GPR35 inhibition on cellular metabolism of AsPC-1. *(A) OCR under mitochondrial stresses was recorded. (B) Basal respiration, ATP production, maximal respiration and spare capacity were calculated based on OCR measurement. (C) ECAR before and after the addition of glucose and oligomycin was measured. (D) Glycolysis, glycolic capacity and glycolic reserve were calculated based on ECAR measurement. Data from one replicative experiment is shown (n= minimum 4). All experiments were repeated 3 times. The results are represented as mean ± SEM. P-value compared to NC21 is shown. p*** is <0.001 and ****p-value is <0.0001(t-test).*

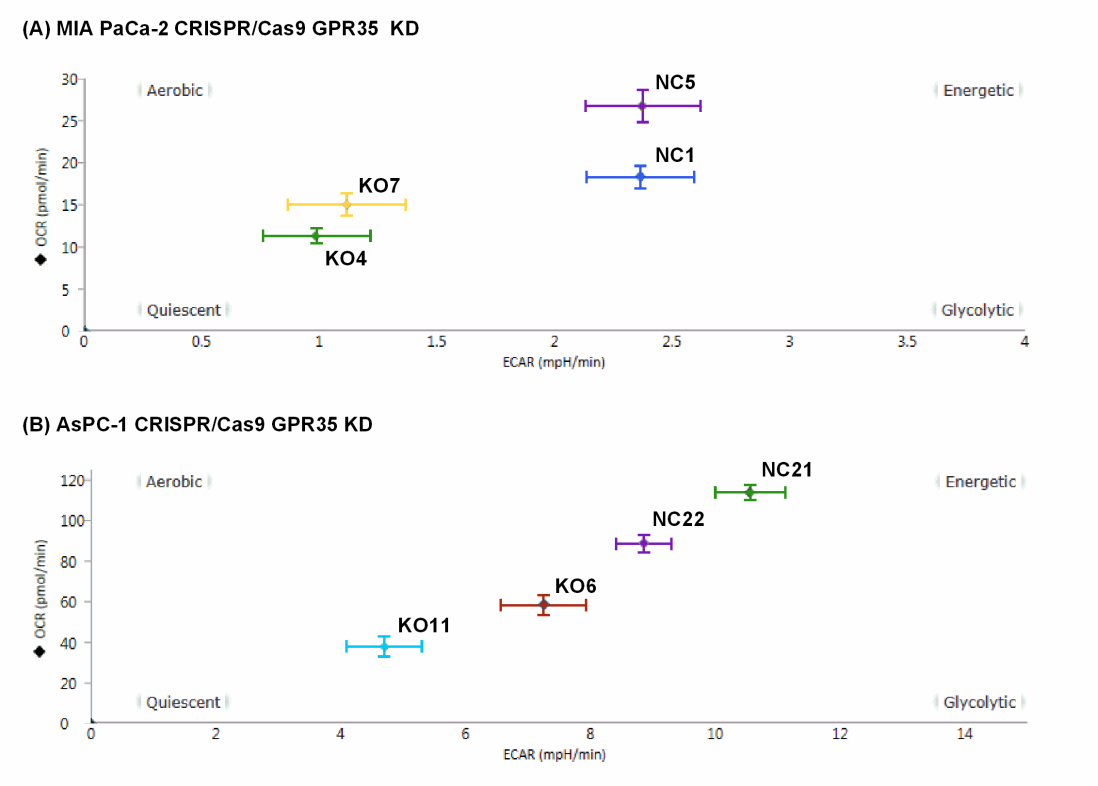


Figure 5.14. The inhibition of GPR35 induces PDAC cell to a quiescent state. *Cell respiration (OCR) against glycolysis (ECAR) in MIA PaCa-2 (A) and AsPC-1 (B). Data from one replicative experiment is shown (n= minimum 7). All experiments were repeated 3 times. The results are represented as mean ± SEM*

5.8 Chapter summary and discussion

This chapter demonstrated the following:

- Pharmacological inhibition of GPR35 decreases pAKT S473 and HIF-1α levels but not pERK.
- Activation of GPR35 by synthetic agonists up-regulates pAKT S473 and pERK.
- Downregulation of GPR35 by siRNA transfection decreases pAKT S473 and HIF-1α levels but not pERK.
- The expression of GPR35 is regulated by oxygen-independent HIF-1.
- A high number of MIA PaCa-2 and AsPC-1 cells expressing low GPR35 was counted under hypoxia.
- Mutated tumour suppressor p53 via miR-34b induces overexpression of GPR35.
- The inhibitory effect on OXPHOS and glycolysis is confirmed in MIA PaCa-2 and AsPC-1 cells expressing low GPR35 by CRISPR/Cas9 system.

We found that the expression of GPR35 is highly correlated with cell proliferation and survival in PDAC cells and that diverse cell functions including apoptosis, cell cycle arrest and autophagy, mediate the growth arrest in PDAC cells upon genetical and pharmacological GPR35 inhibition`. In this chapter, we made efforts to explore the key downstream factors regulating the proliferation induced by GPR35 in PDAC, which are demonstrated to be dependent on AKT activation and stabilization of HIF-1α.

Phosphorylation of AKT induced by GPR35 in diverse cells has been reported. The reduced activation of AKT (S473) was detected in A549R cells (human lung cancer cell line, A549 doxorubicin-resistant cells) with deficient GPR35 expression induced by siRNA silencing transfection (W. Wang et al., 2018). In the following year, another study also revealed that the deletion of GPR35 by siRNA silencing transfection reduced the phosphorylation of AKT at both threonine 308 and serine 473 in Caco2 cells (human epithelial colorectal adenocarcinoma cells) and murine bone marrow-derived macrophages (BMDM) (Schneditz et al., 2019). The authors suggested that GPR35 itself regulates not only AKT but also Src, MAPK and EGFR signal transduction in a Na/K-ATPase-dependent manner. However, the reduced expression of pERK1/2 (MAPK) only in BMDM, but not in Caco2 cells was presented. We also could not observe a reduced pERK expression in PDAC cell lines expressing low GPR35 by siRNA silencing transfection, even though the enhanced pERK upon the treatment of compound 10 (a potential agonist of GPR35) in a time-dependent manner in PDAC cell lines was detected. Therefore, we can estimate that the activation of GPR35 by agonists increased pERK activation, but GPR35 depletion does not reduce the basal expression of pERK. Indeed, KYNA treatment enhanced activation of ERK in adipocytes at 5 min, suggesting MAPK/ERK signalling is induced by GPR35 activation (Agudelo et al., 2018). However, the deficiency of GPR35 expression in BMDMs ($Gpr35^{-/-}$ BMDMs) has not affected on phosphorylation of ERK compared to Gpr35 wild type BMDMs (Kaya et al., 2020), which shows similar results to our findings.

Therefore, we can conclude that: 1) GPR35 regulates AKT signalling in both ligand-independent and dependent manner in PDAC. 2) MAPK/ERK signalling pathway is regulated by the activation of GPR35 upon agonists' activation, but not by a ligand-independent GPR35 in PDAC.

In addition, we verified the reciprocal regulation between GPR35 and HIF-1. Previous studies have shown the GPR35 is characterized as a hypoxia-sensitive gene in mouse cardiomyocytes (Ronkainen et al., 2014) and in colon cancer cell lines (H. H. Hu et al., 2017). Ronkainen et al. observed the overexpression of GPR35 mRNA and the translated GPR35 in membrane induced by both oxygen-dependent and independent (using a chemical activator of HIF, a

prolyl hydroxylase inhibitor, DFO) activation of HIF-1. Another individual study showed that the activation of GPR35 by synthetic agonists of GPR35, pamoic acid and zaprinast, upregulated HIF-1α protein expression under normoxic conditions in HT-29 and HCC-2998 cells (H. H. Hu et al., 2017). This regulation of HIF-1α expression in HT-29 cells was inhibited by ML145, the selective MEK inhibitor (U0126), protein synthesis inhibitor (cycloheximide) or transcription inhibitor (actinomycin D), suggesting that the increased HIF-1α is specific to GPR35 activation (H. H. Hu et al., 2017). However, so far, no publications are showing that GPR35 inhibition downregulates HIF-1α protein expression. Therefore, our findings will provide a different view to understand the regulation of the already stabilized HIF-1α, often observed in cancer cells, by GPR35 inhibition. Furthermore, reciprocal regulation of HIF-1 was indicated in our studies by showing a decreased expression of GPR35 under HIF-1α inhibition in PDAC cell lines. Interestingly, PDAC cells exposed to hypoxic conditions (1% oxygen) showed a different role in cell proliferation. Indeed, the number MIA PaCa-2 cells and AsPC-1 expressing low GPR35 by CRISPR/Cas9 system was increased under hypoxic conditions. This result indicates that the expression of GPR35 is upregulated by oxygen-independent HIF-1 in PDAC, whereas in oxygen-dependent HIF-1 condition GPR35 plays an anti-cell proliferation role in PDAC.

The expression of GPR35 is regulated not only by HIF-1, but also by the mutation status of the tumour suppressor gene *TP53* via miR-34b. These findings will be the first report about the association between GPR35 and p53. Our previous studies (Figure 3.3.A), showing lower expression levels of GPR35 in Capan-2 cells (expressing the functional p53) and no or moderate expression of GPR35 in normal pancreatic cells HPDE and hTERT-HPNE cells, enable us to hypothesize that GPR35 expression might be associated with p53 mutation in PDAC. Consistently, our WB analysis (Figure 5.9) revealed that a higher expression of GPR35 is induced by mutated p53 cells compared to cells having p53 wildtype.

Moreover, we demonstrated that GPR35 has a metabolic reprogramming role in PDAC, leading to the utilization of both OXPHOS and glycolysis to survive or grow. In fact, the deletion of GPR35 by siRNA transfection in Caco2 cells changed their cell metabolism (Schneditz et al., 2019). The decreased cellular respiration was observed in Caco2 cells with deficiency of GPR35 expression by siRNA silencing transfection compared to its control. The study by Schneditz et al. showed results consistent with our findings in MIA PaCa-2 cells expressing low GPR35 by CRISPR/Cas9 transfection. Another study also demonstrated the metabolic reprogramming role of GPR35 in adipocytes (Agudelo et al., 2018). An increased maximal respiratory capacity was observed in primary adipocytes (derived from C57BL/6J mice) upon KYNA treatment. Moreover, additional treatment of isoproterenol, a β-AR

agonist, showed enhanced oxygen consumption while there were no OCR changes in GPR35 silencing adipocytes with the same treatment, together suggesting that the activation of GPR35 by KYNA affects β-AR signalling regulating adipocytes' metabolism.

Analysing our observations altogether, it has become clear that: 1) mutant p53 protein increases the expression of GPR35 and 2) the inhibition of GPR35 causes cellular metabolic reprogramming, and eventually leads to anti-cell proliferation, anti-survival, apoptosis and autophagy through activation of AKT and oxygen-independent HIF-1.

Chapter 6

Chapter 6: The metastatic role of GPR35 in PDAC

6.1 Rationale

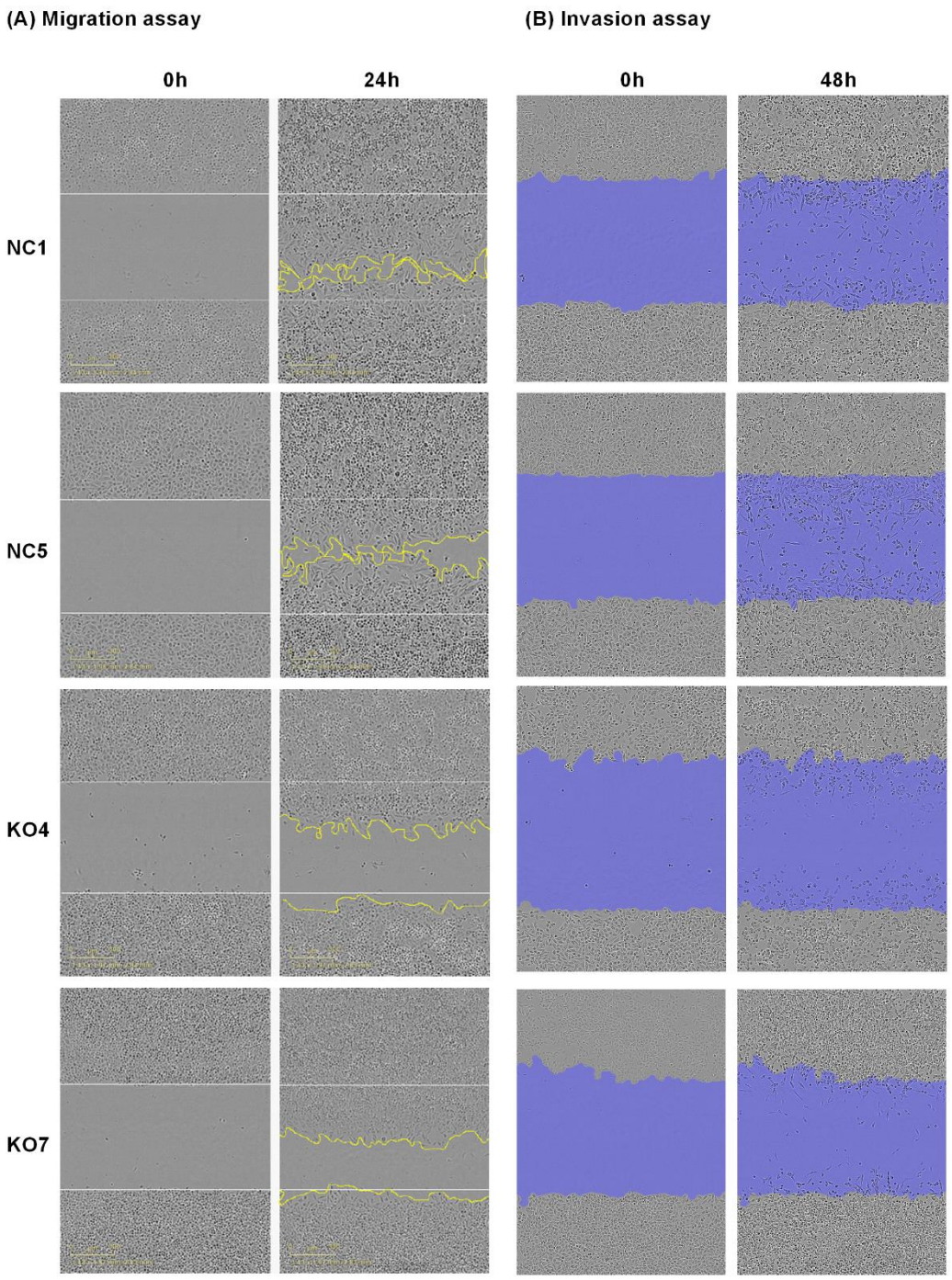
Metastasis is a long and difficult process for cells originated from the primary tumour, requiring several steps: 1) activation of epithelial-mesenchymal transition (EMT), 2) intravasation, 3) extravasation and 4) colonization of metastases. Changes in cell properties by metastasis-involved molecules are essential in each of these steps. The identification of these molecules playing critical roles in each of these metastasis steps for PDAC still remains unclear.

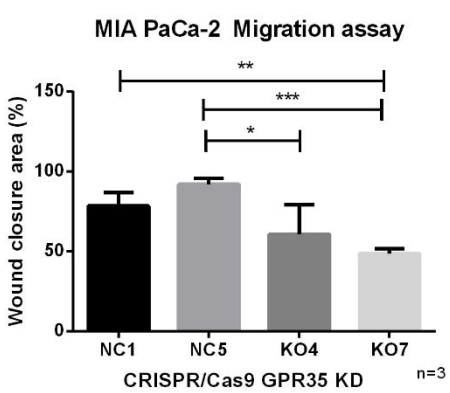
PDAC is one of the most aggressive cancers characterized by high metastatic potential. In addition, it is well known that HIF-1α has an important role in cancer cell survival and metastasis in PDAC. Our previous data shows that GPR35 is highly related to the expression of HIF-1α and the inhibition of GPR35 reduces cell survival and anti-apoptosis abilities. Moreover, the high expression of GPR35 in PDX cell lines characterizing mesenchymal morphology was verified in this study. The survival plot also supports this potential metastatic role of GPR35 in PDAC by showing the low survival rate of patients having high expression of this receptor. Overexpression of GPR35 detected in mutant TP53 cell line also supports the theory of a metastatic role of this receptor because it is known that mutation on TP53 gene more likely occurs in invasive carcinomas. Thus, we will continue researching the metastatic role of GPR35 in PDAC by multiple metastatic assays showing cellular properties on migration, invasion, cancer stem cells and EMT.

6.2 The inhibition of GPR35 decreases cell migration and invasion in PDAC

The most common assay to investigate the metastatic capacity of cancer cells *in vitro* is the scratch wound assay. It is a straightforward and simple method for the evaluation of cell migration. An artificial gap is created on a confluent cell monolayer, called a "scratch wound" and the cells will move toward the gap to close the wound and fill up the gap. The images are taken regularly with certain time intervals from the beginning point. The comparisons among the cells under different conditions are analysed. This assay can be further developed to study cell invasion by filling the gap with Matrigel mimicking extracellular matrix (ECM) in real environments.

The selected clones from MIA PaCa-2 cell lines expressing a lower GPR35 by CRISPR/Cas9 system were seeded in a 96 well plate and grown until fully confluent. Once the high cell density was established, we scratched a wound (IncuCyte® WoundMaker) and incubated the cells for 24 hours. Figure 6.1.A shows the representative photos and the graph including the results from three independent experiments. Both clones, KO4 and KO7, show significantly less migration compared to control NC5. The clone KO7 has less than 50% wound closure area compared to both controls, NC1 and NC5. We next measured the effect of GPR35 downregulation on cell invasion, the capacity of cancer cells to migrate and penetrate in the surrounding tissues. For the invasion assay, we covered the wound area with 5mg/ml of Matrigel and the number of invaded cells in the initial wound area was counted after 48 hours of incubation. Figure 6.1.B shows that both clones KO4 and KO7 have a smaller number of invading cells compared to controls NC1 and NC5.


(A) Migration assay
(B) Invasion assay
0h
24h
0h
48h
NC1
NC5
KO4
KO7


MIA PaCa-2 Migration assay
Wound closure area (%)
150
100
50
0
**
***
*
NC1
NC5
KO4
KO7
CRISPR/Cas9 GPR35 KD
n=3

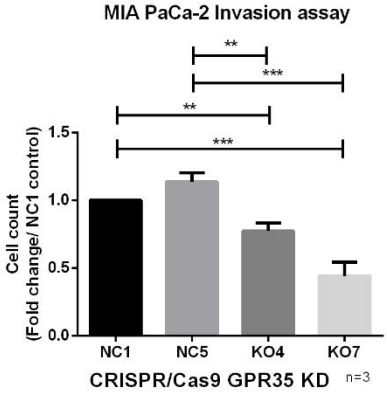

MIA PaCa-2 Invasion assay
Cell count
(Fold change/ NC1 control)
1.5
1.0
0.5
0.0
**
***
**
***
NC1
NC5
KO4
KO7
CRISPR/Cas9 GPR35 KD
n=3

Figure 6.1. The inhibition of GPR35 in MIA PaCA-2 by CRISPR/Cas9 system decreases the cells' abilities of migration and invasion. *(A) The selected clones of MIA PaCa-2 cells expressing lower GPR35 by CRISPR/Cas9 system, KO4 and KO7, show a significantly smaller wound closure area compared to control NC5. Especially, KO7 recorded around 50% less wound closure area compared to NC1 and NC5. (B) The invading cells in the initial wound area were counted manually by using Image J. A smaller number of KO4 and KO7 cells were shown compared to controls. All experiments were performed 3 times. The results are represented as mean ± SEM. *p-value is < 0.05, **p-value is <0.01 and ***p-value is <0.001.*

6.3 The inhibition of GPR35 reduces the growth of CSCs in PDAC

To better understand the role of GPR35 in PDAC progression, we have tested pancreatic cancer stem-like cells (CSCs), defined as subpopulations of cancer cells characterized by slow cell cycle and self-renewal capacity. For this study, we established tumorspheres, which represent CSCs by a distinct morphology such as spheroid-like structures and aggregated form, as described in Material & Methods. To test the effects of the inhibition of GPR35 on the tumorspheres, the same number of tumorspheres generated from the selected clones of MIA PaCa-2 expressing lower GPR35 by CRISPR/Cas9 and controls was incubated in the same conditions. The number of cells was counted after 72 hours of incubation and Figure 6.2.A shows that KO4 has a significant decrease in cell number compared to control NC1. For testing the effect of GPR35 inhibition on tumorspheres of AsPC-1using another method, we incubated them with CID2745687 for 72 hours and the number of cells was counted. Figure 6.2.B indicates that the number of cells treated with CID2745687, (5, 10 or 20 μM of concentration), was significantly less than non-treated cells. However, the combination (G1+C5) of CID2745687 (concentration 5 μM) and gemcitabine (concentration 1 μM) shows similar effects to gemcitabine itself (concentration 1 μM).

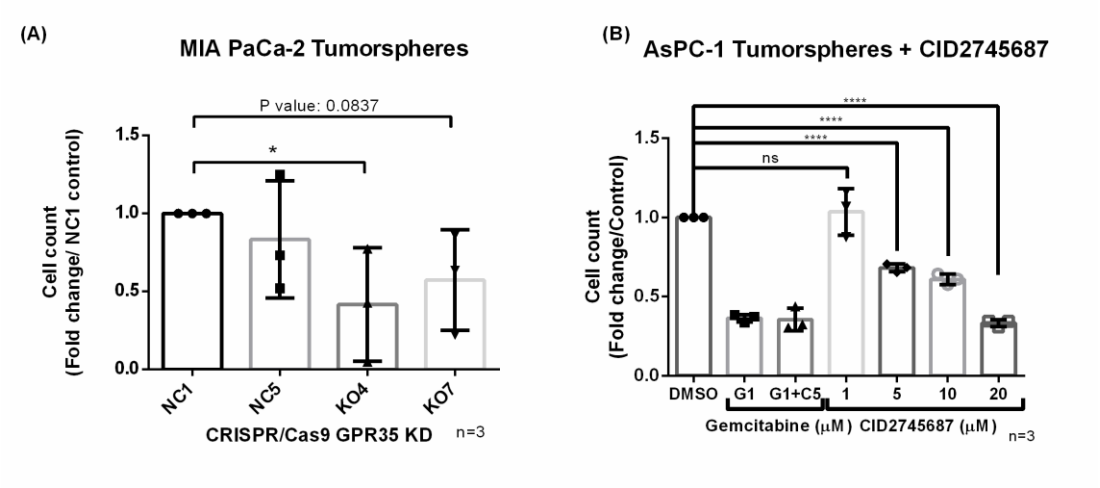


Figure 6.2. The inhibition of GPR35 reduces the number of PDAC tumorspheres cells. *(A) The number of cells from tumorspheres established from the selected clones of MIA PaCa-2 were counted manually after 72 hours of incubation. The tumorspheres expressing lower GPR35 by CRISPR/Cas9 system were less compared to controls. (B) Tumorspheres generated from AsPC-1 cell lines were incubated with CID2745687 (concentrations 1, 5, 10 and 20 μM) and/or gemcitabine (concentration 1 μM) for 72 hours and the number of cells was counted. The number of cells from tumorspheres with CID2745687 was significantly less compared to non-treated cells. All experiments performed 3 times. The results are represented as mean ± SEM. *p-value is < 0.05 and ****p-value is <0.0001.*

6.4 The inhibition of GPR35 plays a role in EMT of PDAC CSCs

CSCs highly contribute to cancer metastasis. To migrate to a distant site from primary tumours and to invade a new site in other organs, the epithelial to mesenchymal transition (EMT) is the essential step for metastatic CSCs. Tumorspheres tend to express more mesenchymal markers representing the characteristics of strong invasion, less proliferation, and increased cell survival. The expression of vimentin, one representative mesenchymal marker, was tested on tumorspheres or their parental adherent cells from the selected clones of MIA PaCa-2. Interestingly, a significantly lower expression of vimentin was detected in both parental adherent cells and tumorspheres from clones expressing lower GPR35 KO4 (tumorspheres only but the p-value was closed to 0.05 on parental adherent cells) and KO7 compared to controls (Figure 6.3.C). However, the expression of E-cadherin, well known as an epithelial marker, with the same MIA PaCa-2 samples gave inconsistent results (data not shown). The expression of E-cadherin in these cell lines has been controversial and much experimental evidence show its deficient expression (Caca et al., 1999; Frixen et al., 1991; Winter et al., 2008). CXCR4 was used as a pancreatic CSC marker in this study based on several publications (Hermann et al., 2007; Kure et al., 2012). Therefore, the data clearly demonstrates

that GPR35 induces the mesenchymal transition of PDAC in order to eventually help cell migration. These results consistently show that GPR35 may have a pivotal metastatic role in PDAC.

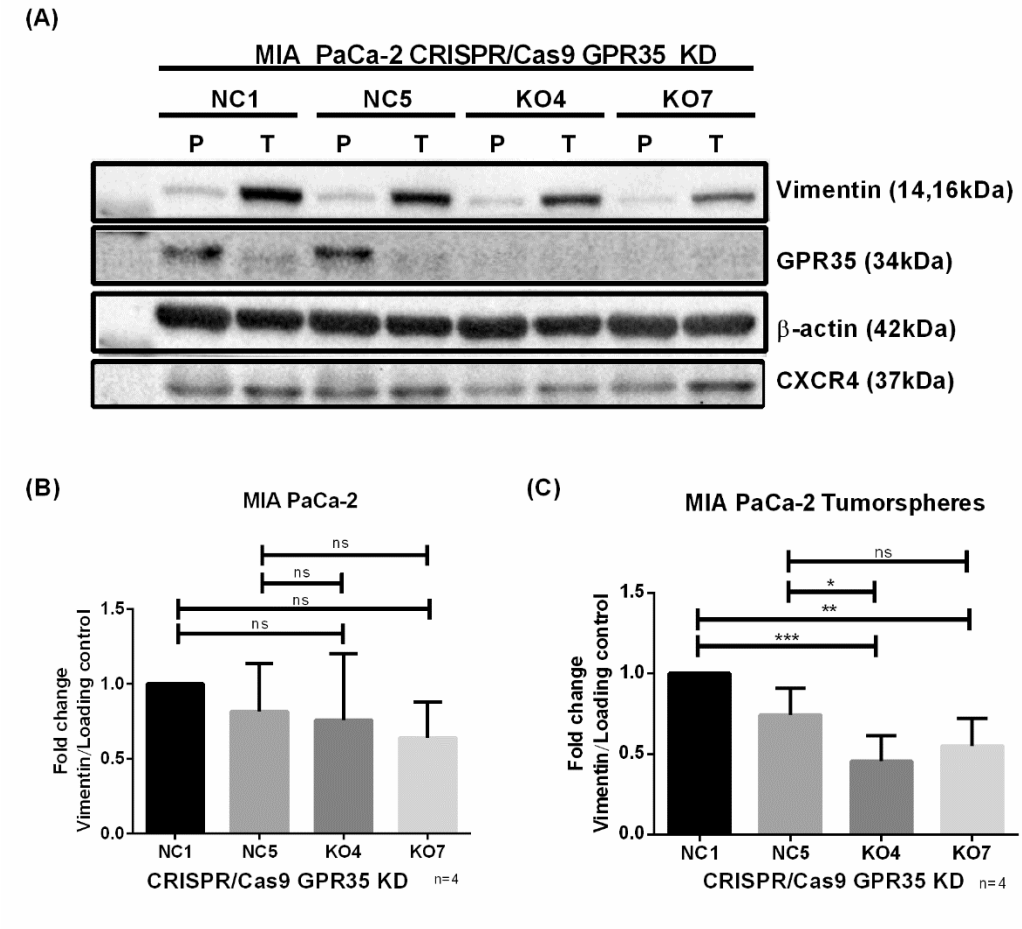


Figure 6.3. The inhibition of GPR35 reduces the mesenchymal transition of both parental adherent cells (P) and tumorspheres (T) in PDAC. *Lysates were analysed for the expression of vimentin by Western blot. Lower expression of vimentin was detected on parental adherent cells (B) and tumorspheres (C) from MIA PaCa-2 expressing lower GPR35. Higher expression of CXCR4 was found in tumorspheres. Antibody β-actin was used as a loading control. (A) A representative blot showing relative expression of vimentin, β-actin, CXCR4 and GPR35. All experiments were performed 3 times. The results are represented as mean ± SEM. *p-value is < 0.05 and **p-value is <0.01.*

6.5 Chapter summary and discussion

This chapter demonstrated the following:

- It is verified that GPR35 downregulation by CRISPR/Cas9 reduces cell migration and invasion in MIA PaCa-2 cell lines using a scratch wound assay.
- MIA PaCa-2 tumorspheres expressing lower GPR35 grew relatively less than control tumorspheres.
- The number of cells from AsPC-1 tumorspheres with ML145 incubation was less than control tumorspheres.
- Downregulation of vimentin expression was detected on both parental adherent cells and tumorspheres of MIA PaCa-2 expressing lower GPR35 by CRISPR/Cas9 system.

In this chapter, we demonstrated that GPR35 has a migration role in PDAC. A similar study verifying the role of GPR35 was performed in human saphenous vein smooth muscle cells (HSV SMC) by a scratch wound assay (McCallum et al., 2015). The migration was induced by the activation of GPR35 under pamoic acid and zaprinast treatment. Moreover, the migration in response to GPR35 agonists was inhibited by CID2745687 or ML145 exposure. However, the indicated mRNA expression of GPR35 in human vascular cells was relatively low compared to HT-29 cells, denoting that using these vascular cells expressing a low amount of endogenous GPR35 may not be suitable to test migration effects. Also, the selectivity for GPR35 synthetic agonists used in this study, especially pamoic acid, accepted as a partial agonist of this receptor, still remains unclear. Another study demonstrated that lodoxamide, a potential synthetic agonist of GPR35, reduces cell migration in a dose-dependent manner in human THP-1 cells (Park et al., 2018). Interestingly, an opposite migration role was presented in THP-1 cells compared to our study which showed a decreased migration rate by GPR35 inhibition. Consistently with Park et al.'s study, an increased baseline of migration in *Gpr35*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and the GPR35 knockdown in human THP-1 cells by siRNA silencing transfection was observed (Schneditz et al., 2019). Taken together, this data clearly shows the important role of GPR35 in cellular migration, even though it might act differently depending on cell types.

Our findings provide a promising result on the invasion role of GPR35 in PDAC using the stably downregulated GPR35 expressing cells. The metastatic roles of GPR35 in cancer have never been directly determined in other publications. These significant findings will enable us to understand the important role of GPR35 in metastatic pancreatic cancer, even though further study using other diverse PDAC cell lines is required. We have used MIA PaCa-2 cell line originated from undifferentiated adenocarcinomas and it is known that this cell line, unlikely

the AsPC-1 cell line, has a characteristic of a very invasive growing pattern. Due to this heterogeneity of PDAC cell lines, additional studies related to cell differentiation is essential for future research.

Nevertheless, these findings, especially the tumorspheres experiments, suggest a strong relationship of GPR35 with pancreatic cancer stem cells, which has been reported as a major cause of PDAC metastasis (Hermann et al., 2007) and PDAC recurrence. Therefore, our results show the potential of GPR35 as a new therapeutic strategy to overcome the limitation of current therapies for PDAC.

Chapter 7

Chapter 7: Assessment of GPR35 role in PDAC by *in vivo* assay

7.1 Rationale

In the previous *in vitro* experiments was presented the potential of GPR35 as a new therapeutic target for PDAC. The pharmacological inhibition of GPR35 using its commercially available synthetic antagonists, ML145 and CID2745687, has shown selectively dramatic decreases of PDAC cells. The successfully achieved genetically modified PDAC cell lines expressing lower GPR35 by CRIPSR/Cas9 show consistent promising results. To determine the ultimate goal of this project, the assessment of GPR35 as a novel therapeutic target for PDAC, we performed *in vivo* experiments using zebrafish and mouse model.

Zebrafish, *Danio rerio*, has been used in past decades for evaluating environmental toxicity. The interest in zebrafish as a new model to replace traditional mammalian models for assessing safety in pharmacology has increased due to the limitation of large animal models. Additionally, its fully sequenced genome shows that 70% of human genes share a zebrafish homologue. This high homology allows zebrafish to be a suitable model for studying pharmaceutical toxicology. Unlikely large animal models such as mice, rats and rabbits, zebrafish have time and cost benefits as a toxicological tool. Once the mating of a single pair is obtained, hundreds of fertilized embryos are available, whereas rodents typically give birth to up to 12 pups at once. The completed development of most organs takes only 96 hpf, while rodents need almost three weeks. Its small size, 5 cm for an adult, enables to house hundreds of zebrafish in an aquarium which can have an automatic feeding machine and water purifier. These characteristics, including high homology with human genes, high fecundity, fast development and ease of maintenance, are enough to consider the zebrafish model as a pharmaceutical toxicological tool.

Xenograft mouse models play a pivotal role in human cancer research to evaluate new anticancer drugs or to study tumorigenesis. These mouse models, which are derived from human cancer cells or murine cancer cells, are classified depending on the transplant sites of the immunocompromised mouse, as ectopic and orthotopic xenograft. Generally, in the ectopic tumour xenograft model, human or murine cancer cells are injected on the flank or back of the nude or severe combined immunodeficient mouse subcutaneously. The tumour size can be measured regularly using a calliper without sacrificing the mouse. The ectopic model, accepted as a standard model, has been widely used to monitor tumorigenicity and tumour growth because of its simple and easy technique requirements compared to the orthotopic model. However, this model has a limitation in the study of cancer metastasis due to the different organ origins of the transplanted tumour cells. The orthotopic model can solve this drawback

of the ectopic model because is generated by transplantation into the same origin site of the tumour. For example, pancreatic cancer cells are directly inoculated into the murine pancreas. It is an advanced model, but surgical skills are highly required to generate orthotopic mouse models. Moreover, monitoring tumour size is difficult compared to the ectopic model due to invisible tumours, unless an optical imaging system with genetically modified cancer cells expressing bioluminescence is used.

Bioluminescence, the internally generated light by a living animal, serves as an excellent tool for studying tumorigenesis and metastasis in both *in vitro* and *in vivo* cancer research. Generally, luciferase, the oxidative enzyme-producing bioluminescence, is widely used for imaging in small living animals like mice.

The following described mouse models that use bioluminescence can be simply applied to establish metastatic mouse models, thus avoiding the use of the orthotopic model, which requires surgical experience. Metastatic mouse models can be generated by the introduction of genetically modified cells producing luciferase reporter genes either intravenously (IV) or via intraperitoneal injection (IP). The mouse model by the IV route is useful in understanding the extravasation of human metastasis. The behaviour of CTCs in the bloodstream can be studied because the cells will directly be introduced into the blood circulation. The IP injected mouse model is a good one to analyse intravasation in human metastasis. The cells, introduced directly in the peritoneum, will demonstrate cancer cells ability to penetrate the blood vessels.

In this study, we evaluate the toxicity of commercially available synthetic antagonists of GPR35, ML145 and CID2745687, using a zebrafish model. Then, we provide the details of our ongoing *in vivo* mouse experiments using genetically modified PDAC cells expressing luciferase reporter and lower GPR35.

7.2 The safety of ML145 is verified by a zebrafish-based toxicological assay.

The developmental toxicity of ML145 was tested in a zebrafish-based assay. The embryos of zebrafish were incubated with diverse dose of ML145 (0, 25, 50, 100, 150 and 200μM) or with corresponding concentration of its solvent, DMSO (0, 0.25, 0.5, 1, 1.5 and 2%). The ratio of hatching was analysed after 24 hours. For the ML145 treated group, a hatching rate similar to the DMSO group was recorded, showing that ML145 has very low toxicity (Figure 7.1.A&B). However, CID2745687 showed high toxicity in the same assay (data is not shown). The cardio toxicological assay, performed by measuring the heartbeats of zebrafish also proved the safety of ML145 (Figure 7.1.C), but not CID2745687 which kills all embryos at the lowest

concentration tested (25 μM, data is not shown). The morphological assay also shows the same pattern (Figure 7.2).

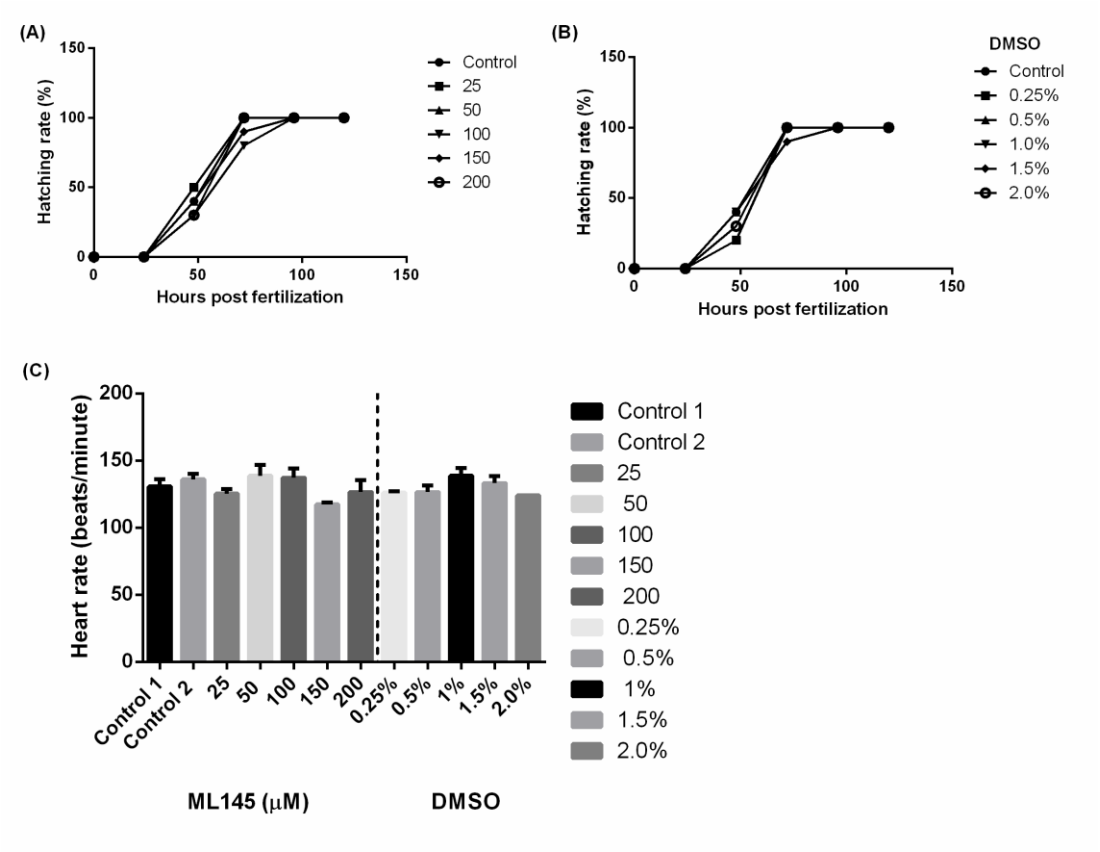


Figure 7.1 The toxicity of ML145 is analysed by developmental (A and B) and cardio-toxicological assays using a zebrafish model. *This experiment was performed by Prof. Marco Falasca and Ilaria Casari.*

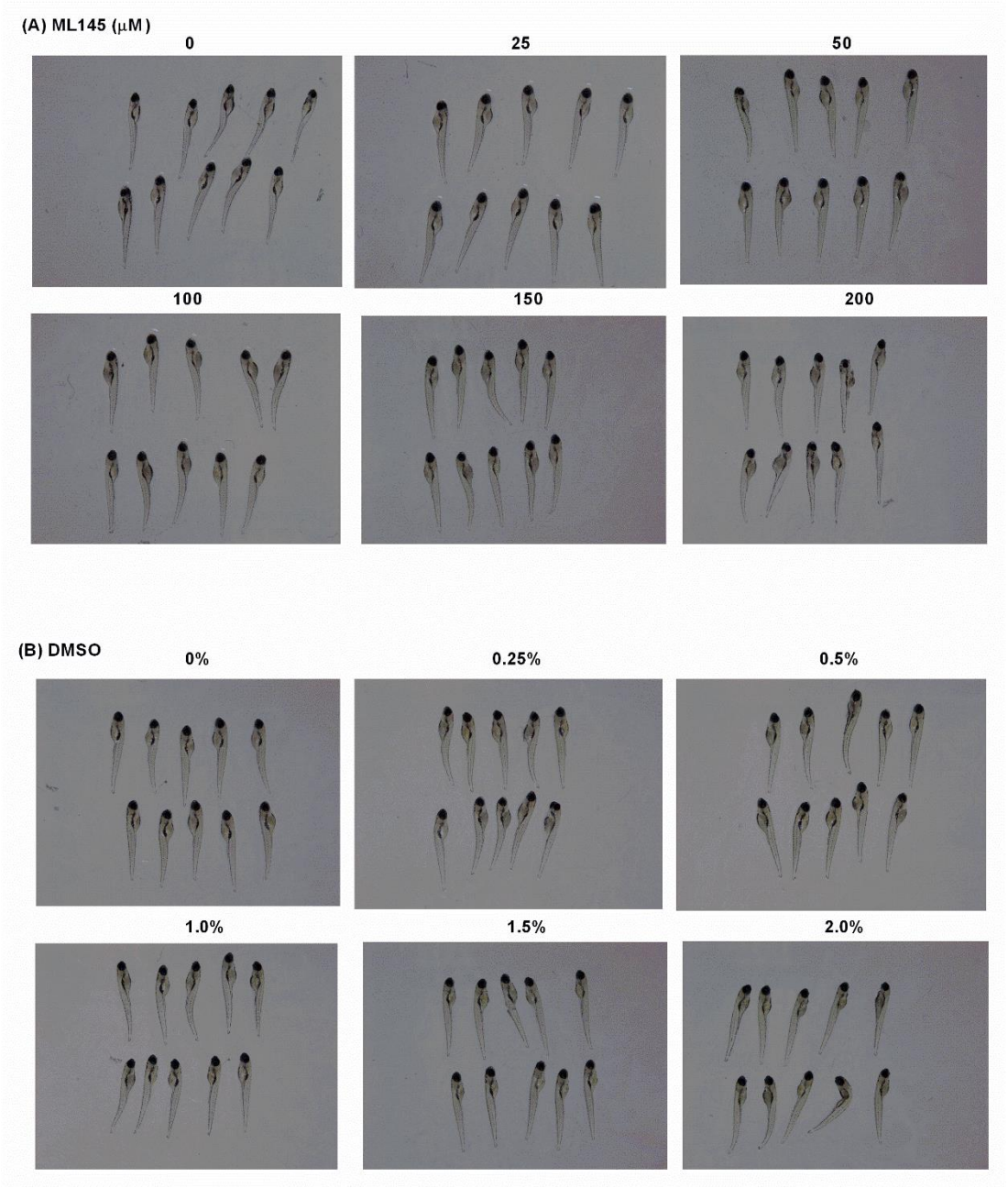


Figure 7.2 The toxicity of ML145 is analysed by a morphological assay using a zebrafish model. *This experiment was performed by Prof. Marco Falasca and Ilaria Casari.*

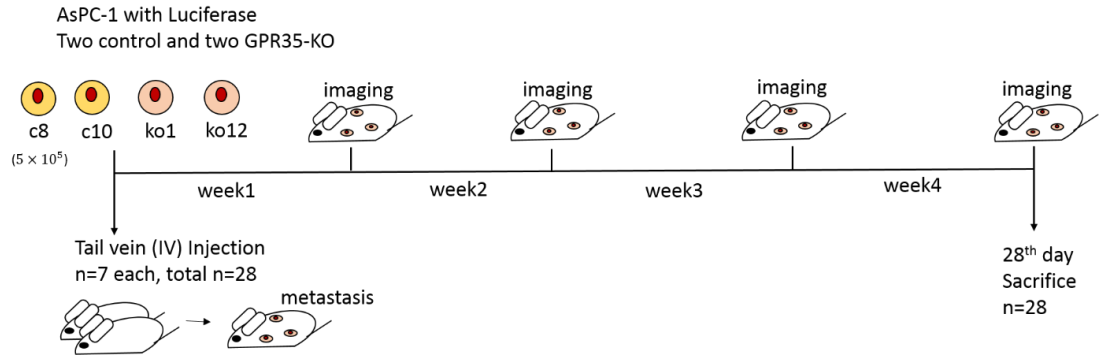
7.3 Development of AsPC-1-Luc-GPR35KD and MIA PaCa-2-Luc-GPR35KD cell lines for *in vivo* experiments

To continue studying the role of GPR35 in PDAC, we designed the following *in vivo* experiments and the animal ethic approval was successfully received (Harry Perkins Institute of medical research animal ethics committee (AEC) permit number is AE184). Because of the COVID-19 pandemic, the following *in vivo* experiments that we have designed were unfortunately delayed so I could not obtain the data from these mouse experiments. However, successful ethic approval has been already gained and these experiments are ongoing. I will provide the details of these *in vivo* experiments and discuss more about them in the future direction chapter.

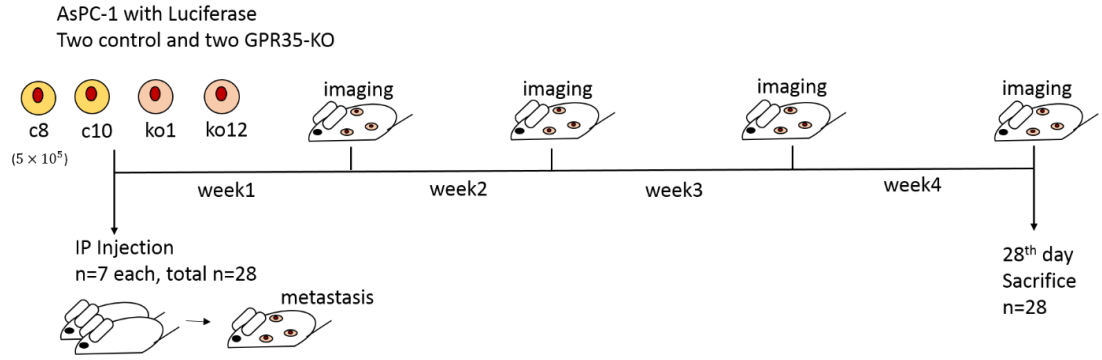
To identify whether our previous *in vitro* results showing the proliferation and metastatic role of GPR35 in PDAC are consistent in *in vivo* assay, we have designed four different mouse models (illustrated in Figure 7.3). Genetically modified cells expressing luciferase reporter gene and lower GPR35 (both AsPC-1 and MIA PaCa-2 cell lines) will be injected into immunocompromised mice via three different injection routes: IV (tail vein injection), IP and SC.

First, to mimic an extravasation process, a key step in metastasis development, the selected clones (two controls and two GPR35 KOs) will be injected via tail vein. We expect that a small number of cells will survive in circulations and grow as metastases in internal organs, especially in the lungs. D-luciferin at a dose of 150mg/kg will be injected intraperitoneally (volume will be 10μl/g of animal weight) in the mouse activating luciferase 10-15 minutes before bioluminescence imaging. Second, to mimic a second key step in metastasis, the intravasation, we will use same the clones but via IP. The rest of the steps will be identical to the previously explained IV injection model. Third, for characterising the anti-tumour effects of GPR35 genetical inhibition in PDAC, we will inject the genetically modified cells (same clones used in metastatic models) via SC and the size of tumours will be measured. Lastly, cells expressing only luciferase reporter will be injected via SC and the mice will be treated with ML145 to validate the efficacy of this drug for evaluating anti-tumour effects of GPR35 pharmacological inhibition. The total luminescence radiance of tumour bearing areas will be recorded by Calliper IVIS Lumina II at the Centre for Microscopy, Characterisation and Analysis (CMCA) in the Harry Perkins Institute. To analyse whether GPR35 inhibition can impede the dissemination of pancreatic cancer cells *in vivo*, we will use an additional model of xenotransplant in zebrafish embryos already established in our research group.

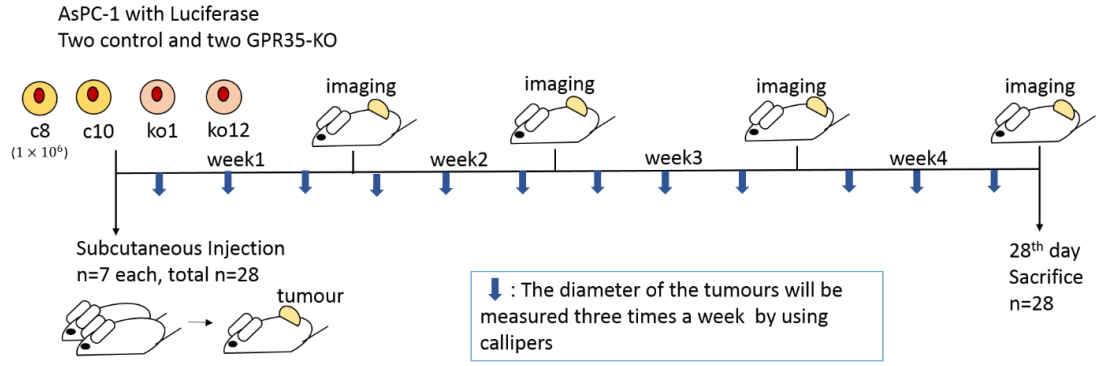
(A) Metastatic model using IV injection to mimic extravasation in human metastasis



(B) Metastatic model using IP injection to mimic intravasation in human metastasis



(C) Ectopic xenograft model to test the genetical inhibition of GPR35



(D) Ectopic xenograft model to test pharmacological inhibition of GPR35

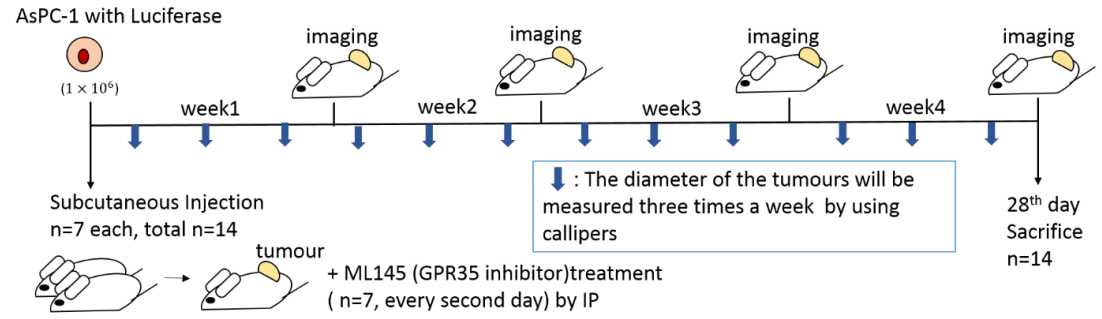


Figure 7.3 Experimental schematic diagrams for in vivo mouse models. *The schematic illustrations show four different mouse models to understand the role of GPR35 in PDAC. (A) The metastatic model using IV injection is designed to mimic the extravasation step in human metastasis. (B) The IP injection will provide information related to the intravasation step in human metastasis. (C) The ectopic xenograft mouse model will be used for evaluating tumorigenesis of GPR35 (D) The ectopic xenograft mouse model will be treated with ML145 and the efficacy of this drug will be assessed. These experiments will be repeated with MIA PaCa-2 cell line.*

To perform these *in vivo* experiments, we prepared the genetically modified cells lacking GPR35 and simultaneously expressing luminescence. For AsPC-1 cells expressing luminescence with GPR35 deficiency, I have performed a CRISPR/Cas9 transfection with the purchased AsPC-1-Luc cell line (Cellbank Australia).

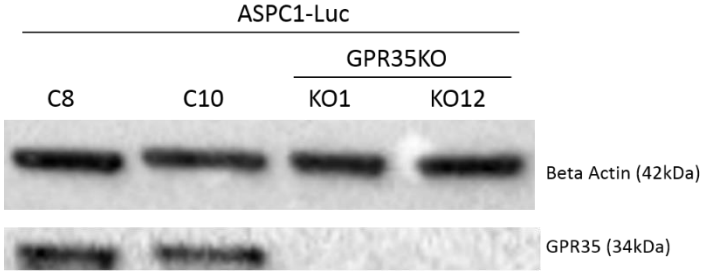


Figure 7.4 GPR35 expression in selected clones of AsPC-1-Luc-GPR35KD or control cells was analysed by WB. *The successful CRISPR/Cas9 transfection to manipulate GPR35 expression in AsPC-1-Luc cells was verified by WB. Selected KD clones KO1 and KO12 showed lower GPR35 expression compared to control clones C8 and C10.*

For the development of MIA PaCa-2-Luc-GPR35 cells, two successive transfections, using first the plasmid containing the luciferase reporter genes and then GPR35 KO plasmid or vehicle, were required because of the absence of a commercially available MIA PaCa-2-Luc cell line. This development of a new genetically modified MIA PaCa-2 cell line was challenging but it is essential as the MIA PaCa-2 cell line showed the best result from *in vitro* migration and invasion assay. The successful luciferase knock-in transfected MIA PaCa-2 cells, named MIA PaCa-2-Luc, was established. Table 11 shows the measurement of bioluminescence from MIA PaCa-2-Luc treated with D luciferin (100mg/ml). The high bioluminescent sources could be measured in MIA PaCa-2-Luc cells with treatment of D luciferin only compared to without treatment of it, while the un-transfected MIA PaCa-2 cells treated with or without D luciferin show almost the absence of bioluminescent signals.

TABLE 11 QUANTIFICATION OF LUCIFERASE BIOLUMINESCENT SOURCES

Cell lines		20,000/ a well	40,000/ a well
MIA PaCa-2_Luc	+D luciferin 100mg/ml	4785 (RLU)	8016
	-D luciferin 100mg/ml	28	26
MIA PaCa-2	+D luciferin 100mg/ml	19	17
	-D luciferin 100mg/ml	11	13

Two different cell densities of MIA PaCa-2-Luc or MIA PaCa-2 cells were treated with D luciferin at a dose of 100mg/ml (+D luciferin) or without it (-D luciferin) and the bioluminescence was measured by EnSight (PerkinElmer) with spectral response from 450nm up to 645nm. Successfully increased relative luminescence (RLU) was detected on MIA PaCa-2-Luc cells.

MIA PaCa-2-Luc cells were then transfected to manipulate GPR35 expression by CRISPR/Cas9 system. The successfully transfected cells were named MIA PaCa-2-Luc-GPR35 KD. Figure 7.5 shows the protein expression of GPR35 from lysates of the MIA PaCa-2-Luc-GPR35 KD clones and control clones. The majority of KD clones expressed less GPR35 protein compared to control clones. Based on this WB analysis, we have selected the least GPR35 expressed KD clones, which are number 2, 8, 11 and 12, and the highest GPR35 expressed control clones, which are C2, C5 and C6.

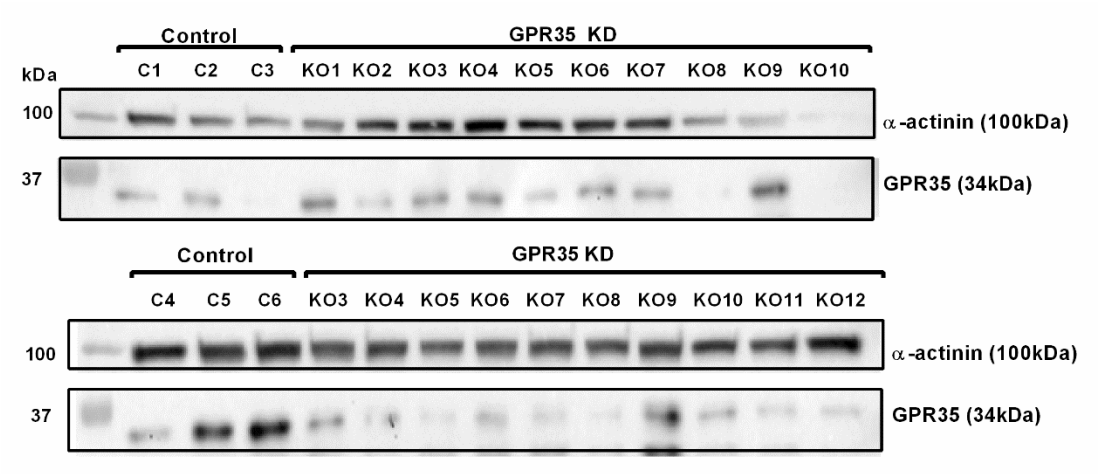


Figure 7.5 The expression of GPR35 in MIA PaCa-2-Luc-GPR35 KD cell line was screened by WB. *We selected four GPR35 KD clones, KO2, KO8, KO11 and KO12, and three control clones C2, C5 and C6, based on this WB analysis. This WB blot was performed by Xin Chien Lee.*

7.4 Chapter summary

This chapter demonstrated the following:

- The safety of ML145 was evaluated in zebrafish based toxicological assays.
- The high toxicity of CID2745687 was observed in zebrafish based toxicological assays.
- AsPC-1-Luc-GPR35KD and MIA PaCa-2-Luc-GPR35KD cell lines were successfully established for *in vivo* mouse experiments.
- Four different mouse models designed to provide a view of the role of GPR35 in PDAC are suggested.

ML145 showed high selectivity (>1,080-fold selective) toward human GPR35 compared to GPR55, suggesting to be the best antagonist of GPR35 to date (Heynen-Genel et al., 2010). For *in vivo* use, ML145 was identified as a good tool compound; its good solubility at the physiological pH (pH7.4), good membrane permeability and plasma stability were identified. Moreover, no toxicity of ML145 at a dose up to 50μM has been reported in their *in vitro* study on human hepatocytes. Therefore, our initial study using a zebrafish toxicological model showing low toxicity of ML145, together with this previous report, paved the way for the use of ML145 in *in vivo* studies.

Surprisingly, ML145, the high toxicity of CID2745687 was shown in a zebrafish-based toxicological assays. This secondary effect can arise from high affinity or selectivity toward other cell-surface receptors.

Two successfully established genetically modified PDAC cell lines, AsPC-1-Luc-GPR35KD and MIA PaCa-2-Luc-GPR35KD, will provide a broad view to understanding GPR35 by offering an excellent *in vivo* tool. Unfortunately, the COVID-19 pandemic hampered to finish our well-planned *in vivo* studies within my expected timeline; nevertheless, the details of our ongoing *in vivo* studies using these cell lines are explained in this chapter.

To date, only one xenograft model was used to show the chemoresistance and tumorigenesis role of GPR35 in lung cancer (W. Wang et al., 2018) and other studies have used a GPR35 KO mouse model to demonstrate diverse physiological roles (Agudelo et al., 2018; Divorty et al., 2018; Farooq et al., 2018; Min et al., 2010; Schneditz et al., 2019). However, no mouse experiment showing the metastatic role of GPR35 has been described in any other study. Therefore, our ongoing *in vivo* experiments will provide novel and promising results.

Chapter 8

Chapter 8: The evaluation of CXCL17 as a ligand for GPR35

8.1 Rationale

C-X-C motif chemokine 17 (CXCL17) is the latest described chemokine in CXCL family and remains as an orphan chemokine. Recently, GPR35 has been suggested as the potential endogenous receptor for CXCL17 (Maravillas-Montero et al., 2015). The first reported authors have used calcium mobilization assays to show CXCL17 response activity in the GPR35 transfected Ba/F3 and HEK293 cells. Moreover, CXCL17 has shown its strong macrophage recruitment both in their *in vitro* and *in vivo* chemotaxis assays. This paper was enough to catch our attention, prompting additional studies on GPR35 at the beginning of this project. In fact, chemokines are binding to GPCR and activate their corresponding signal cascades. Most chemokines are secreted from immobilized endothelium cells in the extracellular matrix and these secretory proteins generally recruit diverse immune cells and interact with nearby stromal cells. It is widely acknowledged that the pancreatic tumour microenvironment consists of dense stromal cells. These diverse stromal cells such as macrophages, lymphocytes and fibroblasts, interact with pancreatic cancer cells to promote cancer development and progression. Chemokines are known to act as major signals to connect these non-cancerous cells and cancerous cells for promoting carcinogenesis and metastasis, including cell migration and invasion in PDAC. For instance, CXCL12, a ligand of CXCR4, has been reported as a regulator of pancreatic tumour growth and metastasis (Roy et al., 2014). The CXCL8/CCL2 axis contributes to pancreatic cancer metastasis by promoting angiogenesis (Pausch et al., 2020). Moreover, PDAC cell migration is mediated by CCL21, CXCL10 and CX3CL1 (Hirth et al., 2020; Marchesi et al., 2008).

Therefore, we hypothesized that CXCL17 might be an endogenous ligand for GPR35 in PDAC, even though increasing evidence points to a CXCL17 receptor dissimilar to GPR35. The main purposes of this chapter are 1) to evaluate CXCL17 as an endogenous ligand for GPR35 in PDAC and 2) to identify the role of CXCL17 in PDAC.

8.2 The expression of CXCL17 in PDAC

The RNA and protein expression of CXCL17 has been shown in diverse human organs including lung, proximal digestive tract and gastrointestinal tract, according to the database "The Human Protein Atlas" (Figure 8.1.A). In particular, CXCL17 tends to be highly expressed in the lung, oesophagus and stomach, but also moderately in the pancreas. Interestingly, CXCL17 shows different expression patterns in normal tissues and cancer tissues. Higher expression of CXCL17 is observed in pancreatic cancer tissues (39.43) compared to normal pancreas tissues (18.2), while a similar expression of CXCL17 is shown in normal and cancer lung tissues. Another database shows the survival plot of pancreatic cancer patients depending on the expression of CXCL17 (Figure 8.1.B). The 77 patients possessing low expression of this molecule showed better survival rates than the 99 patients having high expression. Based on data analysis, we have performed Western blot analysis with several human PDAC cell lines and human colorectal cancer cell line (Figure 8.1.C). All cell lines that we screened showed distinct bands at a slightly higher molecular size than its estimated size. The highest expression level was detected on AsPC-1 and HPAF-II cells among six cell lines in this study.

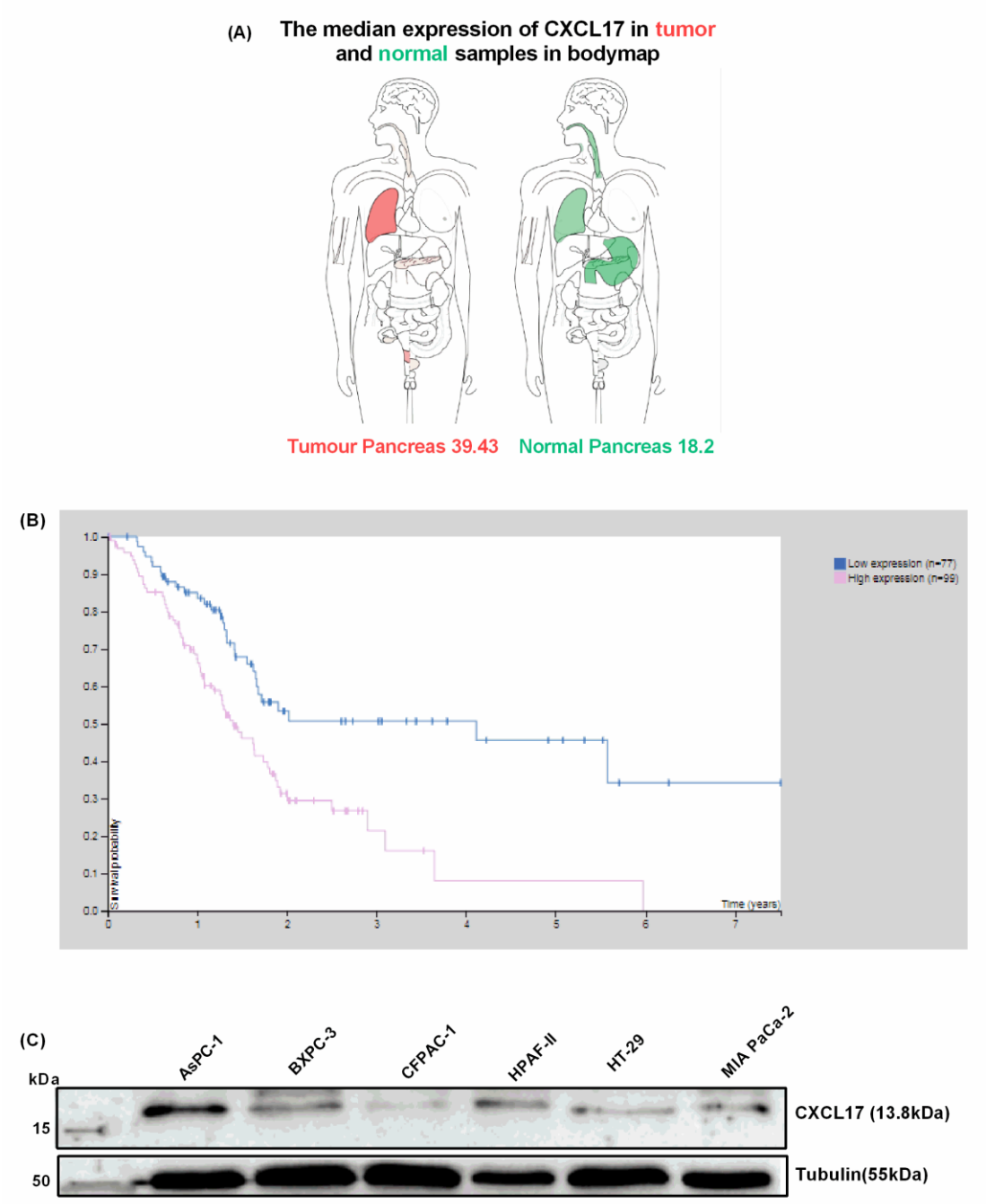


Figure 8.1 The expression of CXCL17 in human pancreas *(A) Interactive body-map of expression of CXCL17. Log_2 (TPM+1) scale. Data from GEPIA.* http://gepia.cancer-pku.cn/detail.php?gene=CXCL17 *(B) Survival plot of CXCL17 high expression cohort vs low expression cohort. Data from The Human Protein Atlas https://www.proteinatlas.org/ (C) CXCL17 protein expression on PDAC cell lines (AsPC-1, BXPC-3, CFPAC-1, HPAF-II and MIA PaCa-2) and colon cancer cell line (HT-29) was analysed by WB. Tubulin is used for loading control.*

8.3 CXCL17 induces ERK and AKT activity in PDAC

To examine the efficacy of CXCL17 on major signalling pathways in cancer, such as MAPK/ERK and PI3K-PKB/AKT pathways, we have performed time-course experiments in several PDAC cell lines using Western blot (Figure 8.2). Four PDAC cell lines, AsPC-1, HPAF-II, Capan-2 and BXPC-3 were incubated in serum free condition for 24 hours before the assay to avoid the basal activity of cells caused by different cell cycle state. CXCL17, with a dose of 50ng/ml in HBSS, was used for this experiment. Cells were treated with CXCL17 for different lengths of time (between 0 to 30 minutes), and the lysates were collected in RIPA supplemented with phosphatase inhibitors. AsPC-1 and HPAF-II were screened with p-AKT (S473), AKT, p-ERK and ERK antibody, while Capan-2 and BXPC-3 were screened with p-ERK and ERK only. In all cell lines, rapid phosphorylation of ERK was detected at 1 min (AsPC-1 and HPAF-II) or 5 min (Capan-2 and BXPC-3) sustained until 15min. The stimulation of AKT phosphorylation by CXCL17 was observed in both AsPC-1 and HPAF-II cell lines. The strongest signal for pAKT was detected at 5 min time point. These findings indicate that CXCL17 activates ERK and AKT pathways by binding the existing receptors in PDAC. Subsequently, we have further explored the role of CXCL17 in PDAC. AsPC-1 cells were incubated with CXCL17 at diverse doses (10, 20, 50, 100 and 200 ng/ml) in serum-free condition for 72 hours and the number of cells was manually counted. Interestingly, cells with CXCL17 showed better survival in comparison with untreated cells under identical conditions (Figure 8.3). This result indicates that CXCL17 has a role in cell survival in PDAC via AKT and ERK pathways.

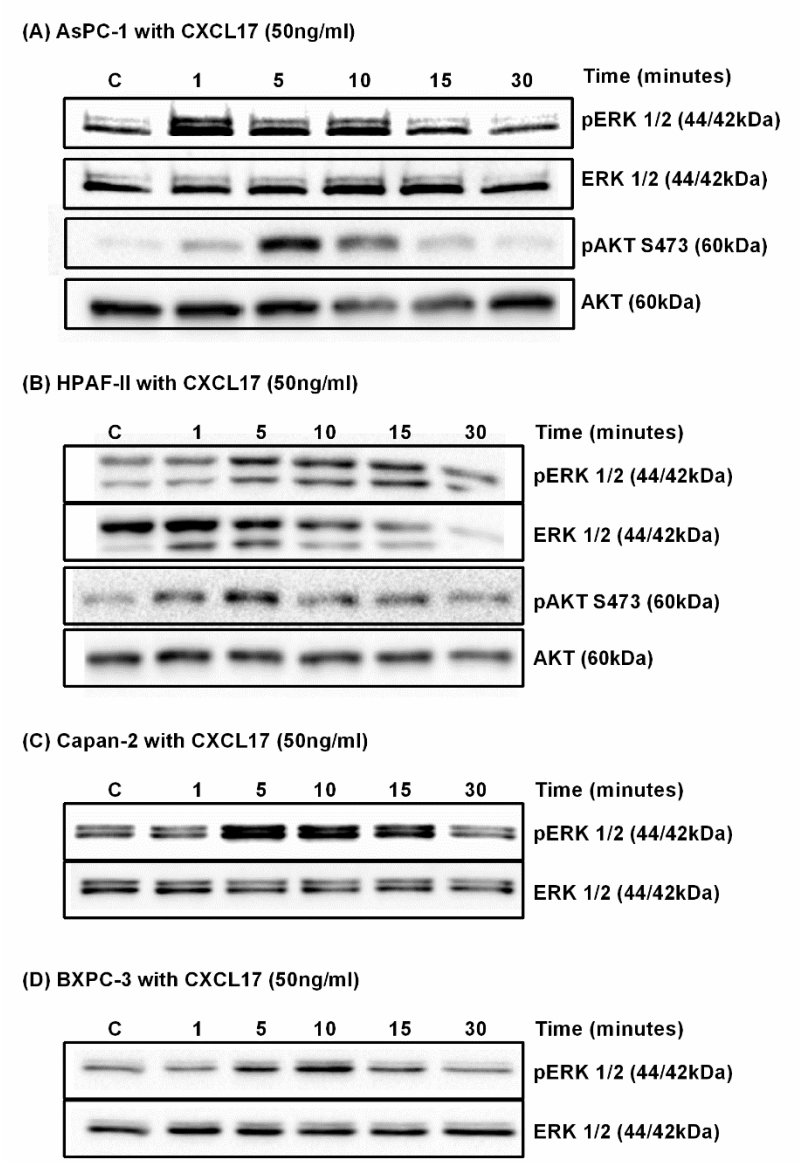


Figure 8.2 Time-course effect of CXCL17 on AKT S473 and ERK phosphorylation. *AsPC-1 cells (A), HPAF-II cells (B), Capan-2 (C) and BXPC-3 (D) were treated with CXCL17 (50ng/ml) for the times indicated. Cell lysates were harvested and P-AKT at S 473, total AKT, P-ERK and/or total ERK were analysed by Western blot. These representative blots were selected from experiments repeated at least three times.*

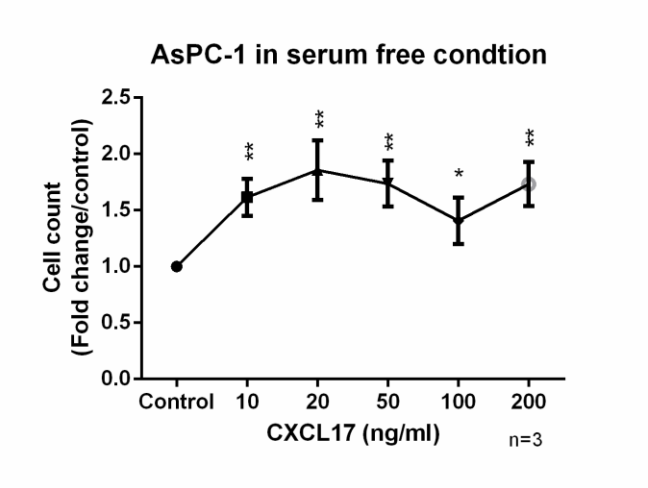


Figure 8.3 CXCL17 induces AsPC-1 cell survival in serum starvation condition. *AsPC-1 cells were incubated in serum-free condition with CXCL17 (doses indicated) for 72 hours. The number of cells was counted manually. The graph represents the relative cell numbers of CXCL17 treated cells compared to untreated cells. Four independent experiments were performed. *p-value is <0.05 and **p-value is <0.01.*

8.4 CXCL17 is not an endogenous ligand for GPR35

The receptor of CXCL17 has not been identified and it is still controversial. To verify the potential of CXCL17 as an endogenous ligand for GPR35, we designed a specific *in vitro* experiment. According to our previous *in vitro* experiments, phosphorylation of AKT at S473 is reduced by the inhibition of GPR35 and CXCL17 activates its phosphorylation. Therefore, we hypothesized that CXCL17 might not activate pAKT S473 in AsPC-1 cells expressing lower GPR35 if CXCL17 were the endogenous ligand for GPR35 in PDAC. To verify our hypothesis, AsPc-1 cells were transfected with siRNA to reduce GPR35 expression and the cells were incubated with serum free-media for 24 hours before being treated with CXCL17. The transfected cells were incubated with CXCL17 for 5 minutes and the lysates were harvested rapidly. Interestingly, the activation of AKT was observed in GPR35 knocked down AsPC-1 cells under CXCL17 treatment (Figure 8.4). This result indicates that the activation of AKT by CXCL17 is mediated by other receptors, and not by GPR35, in AsPC-1 cells.

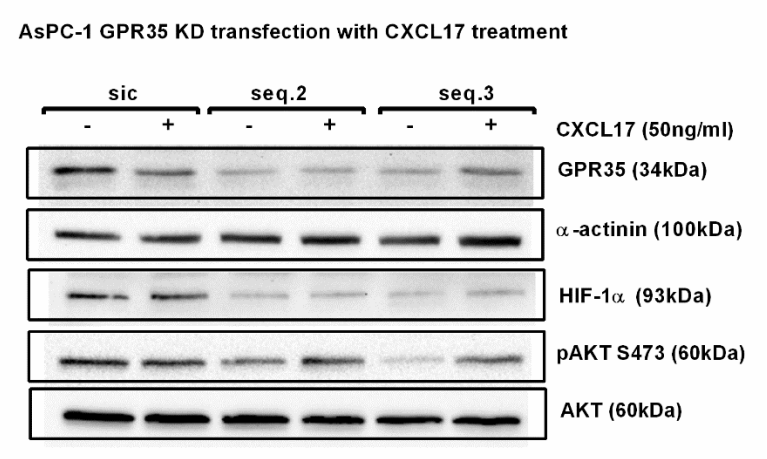


Figure 8.4 CXCL17 is not the endogenous ligand for GPR35. *AsPC-1 cells were genetically modified by siRNA transfection to reduce the expression of GPR35. CXCL17 was added at 72 hours post-transfection and lysates were harvested. Western blot analysis was performed to identify relative protein levels of GPR35, HIF-1α, pAKT S473, total AKT and α-actinin (used as loading control). CXCL17 induces phosphorylation of AKT S473 on AsPC-1 cells expressing lower GPR35 independently. This result means that CXCL17 is not related to the activation of GPR35. This representative blot has been chosen from experiments repeated at least twice.*

8.5 Chapter summary

This chapter demonstrated the following:

- The database analysis shows that CXCL17 expression has relevance to pancreatic cancer.
- The expression of CXCL17 in PDAC cell lines was verified by WB.
- Phosphorylation of AKT and ERK is induced by CXCL17 in PDAC cell lines.
- CXCL17 helps AsPC-1 cell survival in serum-free condition.
- Phosphorylation of AKT by CXCL17 is still detected in AsPC-1 cells expressing low GPR35.

Our findings provided the potential for CXCL17 as a key mediator of PDAC cell survival mechanism through the AKT and ERK pathways, despite many other approaches being required to support this study. However, our findings are still remarkable as they represent the first report about the role of CXCL17 in PDAC. In fact, only one single publication regarding CXCL17 in pancreatic cancer could be found to date to the best of my knowledge, but it is focused on immune surveillance mediated by the expression of CXCL17 in intraductal papillary mucinous adenoma (IPMA) (Hiraoka et al., 2011).

We failed to prove CXCL17 as an endogenous ligand of GPR35 in PDAC. Maravillas-Montero et al. reported for the first time that CXCL17 might be the endogenous ligand for GPR35 (Maravillas-Montero et al., 2015). They demonstrated the chemotactic responses in PGE2-pretreated THP-1 cells induced by CXCL17 in a pertussis toxin-sensitive manner. Through the calcium mobilization assay, the increased calcium flux in THP-1 cells, GPR35-transfected BA/F3 cells and GPR35-transfected HEK293 cells by CXCL17 was verified. In addition, the reduced GPR35 mRNA expression was observed in the lungs of CXCL17 KO mice. However, other following studies have shown that CXCL17 is distinct from a ligand for GPR35. Diverse assays to test signals generated by GPR35 in response to CXCL17 showed no sensitivity toward CXCL17 in the transfected cells readily expressing GPR35 (Binti Mohd Amir et al., 2018). The authors also verified no inhibitory chemotactic responses in human monocytes upon a range of CXCL17 concentration with the treatment of ML145 by chemotaxis assay. In the same year, another paper was released to examine the potential of CXCL17 as an endogenous ligand for GPR35 (Park et al., 2018). Both mouse and human CXCL17 failed to show the signals compared to lodoxamide, which was presented as a synthetic agonist of GPR35 in HEK293 cells overexpressing GPR35. The migration of THP-1 cells by CXCL17 was verified, but CID2745687 did not show the inhibitory effects on those cells. Also, transfected THP-1 cells expressing low GPR35 by siRNA silencing did not show the inhibitory chemotactic responses to CXCL17. Therefore, Park et al. suggested that the migration of THP-1 cells induced by CXCL17 in both Maravillas-Montero et al. and Park et al. studies might occur because of undefined receptors of CXCL17, not because of GPR35.

On the other hand, few studies have demonstrated the expression correlation between GPR35 and CXCL17. The role of the CXCL17-GPR35 axis in breast cancer was described (Y. J. Guo et al., 2017). They showed a high protein expression of both CXCL17 and GPR35 in breast cell lines, suggesting that both molecules endogenously exist in breast cancer cells, but comparison with normal breast cell lines was not indicated in their study. The IHC analysis using breast cancer tissues and normal adjacent tissues revealed a higher expression of both GPR35 and CXCL17 in breast cancer compared to normal tissues, but the authors failed to show the expression correlation between two molecules. Two representative data of their *in vitro* assays showing the relationship between GPR35 and CXCL17 were presented, even though there are ambiguous experimental images. First, the IF images of MCF-7 cells treated with DMSO or human recombinant CXCL17 were from one single experiment, which makes it difficult to fully show that CXCL17 causes internalization of GPR35. Second, the loading controls are missing on the WB images showing the downregulated pERK expression in GPR35 knockdown MCF-7 cells compared to control. In addition, the images showing the efficiency of shRNA transfection (successful downregulation of GPR35) were not presented.

Another study suggested the correlation between CXCL17 and GPR35 in a mouse neuropathic pain model (Rojewska et al., 2019). Strong pain-related behaviour after intrathecal injection of CXCL17 was dismissed by the administration of both KYNA and zaprinast in naïve mice. However, it remains unclear whether the pronociceptive properties of CXCL17 are blocked by the competitive ligands, KYNA and zaprinast. The authors also noted that activation of CXCL17 might occur through multiple receptors. A stronger pairing of GPR35 with CXCL17 was verified in *Gpr35*$^{+/+}$ murine bone marrow-derived macrophages (BMDMs) compared to *Gpr35*$^{-/-}$ BMDMs by intracellular calcium assay, IP_3 assay and cAMP assay (Schneditz et al., 2019). However, the pairing of CXCL17 with human GPR35 was not presented in this study.

Therefore, it seems clear that CXCL17 is not an endogenous ligand of human GPR35 in diverse organs. Our findings also clearly showed that the activation of AKT is induced by other endogenous receptors of CXCL17, and not by GPR35, in AsPC-1 cells. However, the investigation of CXCL17 in PDAC seems very interesting, based on our study showing the survival and proliferation role of CXCL17 in PDAC.

Chapter 9

Chapter 9: Discussion

9.1 GPR35 as a novel therapeutic target for pancreatic cancer

Pancreatic cancer has been a massive challenge for cancer researchers because it has characteristics of high heterogeneity and plasticity contributing to the frequent recurrences. Conventional chemotherapy has shown a limited efficacy toward pancreatic tumours and has not improved in the last decades. Therefore, for a long time, there has been a continuous demand for new therapeutic targets, although oncologists have attempted to propose numerous molecules. In this study, we identified GPR35 as a novel therapeutic target for PDAC. Previous studies have suggested therapeutic opportunities by targeting GPR35 in few different types of cancer. The cancer-relevant mechanisms regulated by GPR35 have been described, ensuring GPR35 to be a key modulator in carcinogenesis. Schneditz et al. showed that GPR35 promotes the Na/K-ATPase mediated ion transport, subsequently activating EGFR/Src/Ras/ERK and PI3K/AKT signalling pathways (Schneditz et al., 2019). Moreover, GPR35 expression regulated by HIF-1α in cardiac myocytes from cardiac failure patients was demonstrated (Ronkainen et al., 2014), suggesting that GPR35 is related to hypoxia, a major driver of carcinogenesis and metastasis. In addition, GPR35 expression has been reported to be associated with gastric, breast, colon and lung cancer, even though its role remains unclear.

We have verified that GPR35 regulates cancer cell proliferation, survival, migration and invasion in PDAC via AKT and HIF-1 signalling pathways. The PI3K/AKT/mTOR pathway is well known to play pivotal roles in pancreatic cancer. Around 1% of cancer patients (total over 1000 cases) showed non-silent somatic mutations on the PI3K/AKT/mTOR pathway, revealing that AKT is a critical signalling component for cancer initiation and progression (Y. Zhang et al., 2017). In particular, higher phospho-AKT levels in specimens from pancreatic tumour-resected patients who had shorter survival time (~4 months shorter) were observed compared to patients with low phospho-AKT expression (Massihnia et al., 2017). Furthermore, the suppression of both PI3K/AKT pathway and sonic hedgehog pathway (one representative embryonic pathway) reduced human pancreatic CSC characteristics (Sharma et al., 2015), indicating that AKT has an important role in the maintenance of CSC traits. Therefore, our findings showing the regulatory role of GPR35 in AKT signalling pathways will have significant implications in PDAC research.

We have demonstrated that HIF-1 is highly associated with GPR35 expression in PDAC. HIF-1 has been widely studied showing its important roles in PDAC tumorigenesis, cancer metabolism, and metastasis. The fact that HIF-1 expression is contributing to tumorigenesis is frequently observed in PDAC. Even in normoxic condition, high expression levels of the HIF-

1 protein were observed in 75% of pancreatic cancer cell lines (15 out of 20) (Akakura et al., 2001) and the $Kras^{G12D}$ murine model with pancreas-specific *Hif1a* gene deletion showed an accelerated PDAC initiation (K. E. Lee et al., 2016). HIF-1α is known as a key mediator for metabolic reprogramming in PDAC. The induced expression of glutaminase 2, which is an important modulator of glycolysis, was observed in PDAC cells under hypoxic condition (Guillaumond et al., 2013). Consistently, in our studies, the metabolic changes regulated by GPR35 were verified by seahorse assay. Concerning the metastatic role of HIF-1α in PDAC, several studies revealed that EMT is induced by hypoxia (S. Chen et al., 2016; Salnikov et al., 2012). Moreover, it is widely known that CSCs have a high resistance to hypoxia in PDAC and HIF-1 enhanced the metastatic ability of pancreatic CSCs (Maeda et al., 2016; H. Zhu et al., 2014).

Collectively, our extensive work showing the correlation between GPR35, AKT and HIF-1 in PDAC proliferation, survival, metabolism, migration and invasion, can be applied to develop new therapeutic strategies for PDAC.

9.2 Future directions

The results presented in this thesis have inspired new experimental aims in broad research areas including morphological alterations regulated by GPR35, the role of GPR35 in PDAC microenvironments, the association of tumour suppressor gene p53 with GPR35, the role of GPR35 in pancreatic CSCs, identification of GPR35 in drug-resistant PDAC, evaluation of potential GPR35 agonists in PDAC and *in vivo* assay.

We briefly demonstrated the morphological changes induced by the inhibition of GPR35 in PDAC cell lines. Several other individual studies also showed morphological alterations of cells activating GPR35 upon exposure to agonists, particularly KYNA and zaprinast. In addition, our findings revealed the significant impact of GPR35 on PDAC cellular morphology. Additional immunofluorescence and immunoblot studies will provide a clear view of this role of GPR35 in PDAC, which might also contribute to a better understating of migration and invasion in PDAC. Two representative essential compounds controlling cellular mobility, myosin and filamentous actin (F-actin), can be examined to show the structural roles of GPR35 in PDAC. Myosin is a superfamily of motor proteins walking along actin filaments important for cancer tumorigenesis and metastasis (Y. R. Li & Yang, 2016; Makowska, Hughes, White, Wells, & Peckham, 2015; Ouderkirk & Krendel, 2014). F-actin is a key component of stress fibres, lamellipodia and filopodia involved in cancer cell migration (Roy et al., 2015; Yamaguchi & Condeelis, 2007).

The role of GPR35 in PDAC tumour environments remains ambiguous. The expression of GPR35 in THP-1 cells was already reported in several studies, suggesting the high potential of the reciprocal role of GPR35 in PDAC microenvironments. Therefore, we can expand this study by co-culturing PDAC cell lines with tumour-associated macrophages (TAMs) to identify differential expression of GPR35. The investigation of the cross-talk between cancer cells and TAMs will provide a better understating of GPR35 in PDAC desmoplasia. Human monocyte THP-1 cells can be used as a model for TAMs, according to the described protocols (H. Ye et al., 2018; R. Zhang et al., 2019).

High expression of GPR35 was observed in mutant and deleted p53 cells; thereby, future studies will aim to verify this preliminary finding showing the association between tumour suppressor gene p53 and GPR35 in PDAC. We can simply evaluate the expression level of GPR35 in the SW1990 cell line which, unlikely other PDAC cell lines including MIA PaCa-2, PANC-1 and BXPC-3, has wild type TP53 gene. In addition, a siRNA transfection silencing TP53, or introducing mutant p53 plasmids, in SW1990 cells will also provide a clear view on the relationship between the expression of GPR35 and p53.

It has been recently proposed that(W. Wang et al., 2018) GPR35 has an important role in chemoresistance through β-arrestin/AKT signalling in lung cancer. The AKT signalling pathway was verified in our study to be regulated by GPR35 in PDAC, indicating a high possibility that it has a chemo-resistant role in PDAC as well. Moreover, higher resistance to gemcitabine in MIA PaCa-2 cells, compared to SW1990 cells that have wild type p53 gene, was reported (Nakamura et al., 2016).

Our established pancreatic CSCs model demonstrated that GPR35 mediate tumorspheres formation. Moreover, the significantly decreased expression of vimentin in MIA PaCa-2 tumorspheres expressing low GPR35 indicated that GPR35 might influence PDAC cells to gain cancer stem cell properties. Only the CXCR4 stem cell marker was indicated in this study but many other stem cell markers such as CD133, CD44, CD24, ESA, ALDH1, DCLK1 and ABCG2, can be tested on PDAC cell lines with or without depletion of GPR35 by diverse methods including immunoblots, FCM and IF.

We barely tested potential agonists of GPR35 in this study because each compound is showing a different selectivity, efficacy and potency. The evaluation of the suggested synthetic agonists and potential endogenous ligands of GPR35 is still ongoing in many studies. To date, three agonists of GPR35, KYNA, zaprinast and LPA showed reliable experimental evidence. Therefore, we can test these selected agonists of GPR35 to understand the function of GPR35 in PDAC.

Unfortunately, we could not present our *in vivo* results due to the delay caused by the COVID-19 pandemic. However, our *in vivo* experiments have been successfully approved and will identify our ultimate goals. Once we collect the tumour tissues from the xenograft mice, the Ki-67 staining assay and BrdU assay can be employed to distinguish proliferating cells. Additionally, we can provide the link between PDAC metastasis and tumour glycolysis in a GPR35 expression dependent manner with immunostaining of glut1, glut3 and HK2 in tumour tissues from a metastatic mouse model.

9.3 Final conclusions

Our investigation of GPR35 in PDAC has contributed to suggest a new therapeutic strategy to cure pancreatic cancer patients. Significantly, we have shown anti-proliferation and anti-migration of PDAC cells under the inhibition of GPR35. For the first time, we have reported that GPR35 has roles in controlling apoptosis, autophagy, cell cycle arrests, migration and invasion in PDAC cell lines. In addition, this work serves as a support to the previous study showing that GPR35 regulates the AKT signalling pathway, and could be effectively adopted to develop a new therapeutic strategy for PDAC. Furthermore, we have verified the dual role of GPR35 in HIF-1 accumulations in PDAC, which advances the current understanding of GPR35 in hypoxia. Although the *in vivo* study has yet to be conducted, we postulate that GPR35 has important roles in controlling PDAC tumorigenesis.

Chapter 10

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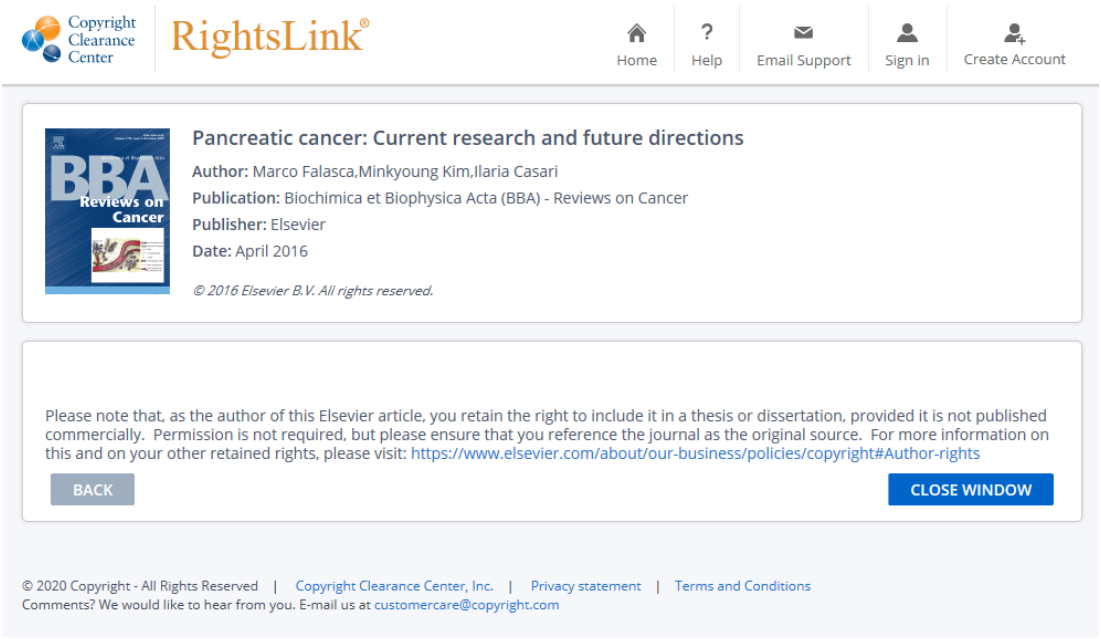

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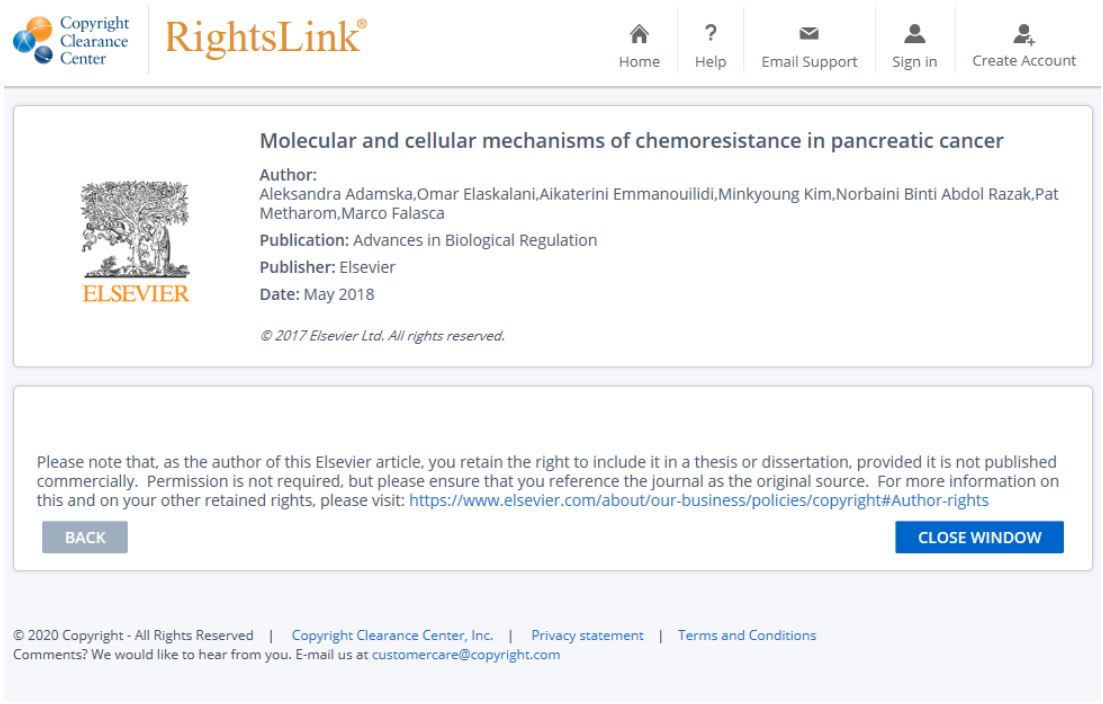

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