

PROTEIN DISULFIDE ISOMERASE INHIBITION: A NOVEL THERAPEUTIC STRATEGY AGAINST PANCREATIC CANCER

by

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Abstract

As a lethal condition with poor prognosis, pancreatic cancer treatment needs to move away from conventional cytotoxic therapies and towards the development of novel therapies through the discovery of precise molecular targets. A potential target is protein disulfide isomerase (PDI). PDI acts to refold misfolded proteins and alleviate endoplasmic reticulum (ER) stress, a characteristic commonly recognised in cancer, as part of the unfolded protein response (UPR). This demonstrates the protective roles of PDI and highlights PDI as a potential therapeutic target for cancer treatment. The study aimed to develop novel therapeutic strategies through the investigation of the roles of PDI and the UPR pathway in pancreatic cancer progression.

Of the 21 proteins in the PDI gene family, PDIA1 and AGR2 were identified as potential influential members in pancreatic cancer progression. PDIA1 and AGR2 were significantly knocked down via small interfering RNA (siRNA) transfection. Knockdown was confirmed by Western blot, real-time quantitative polymerase chain reaction (RT-qPCR) and a PDI enzyme activity assay in pancreatic cancer cell lines: Panc-1, Mia-PaCa-2, and AsPC-1. PDI activity was also significantly inhibited after treatment with PDI inhibitors: propionic acid carbamoyl methyl amide (PACMA)-31 and CCF642. PDI inhibitor treatment and PDIA1 and AGR2 siRNA knockdown demonstrated significant inhibition of cell proliferation, cell viability and cell migration across the pancreatic cancer cell lines.

Investigations into ER stress marker mRNA and protein expression provided an insight into the effects of PDI inhibition on UPR pathway activations, particularly through the dysregulation of spliced X-box binding protein 1 (XBP-1s). PDIA1

knockdown significantly decreased XBP-1s mRNA and protein expression in AsPC-1. AGR2 knockdown significantly increased caspase-3 activity in Panc-1. These data suggest that AGR2 inhibition could trigger a pro-apoptotic response through chronic UPR signalling and caspase-3 activation and that PDIA1 inhibition may act to repress pro-survival gene XBP-1s.

Investigations of PDI inhibitor and gemcitabine combination therapy demonstrated a potential synergistic effect in driving significant inhibition of cell proliferation with 1 μ M PACMA-31 and 0.05 μ M gemcitabine combination treatment in Mia-PaCa-2. XBP-1s mRNA expression was also significantly increased with PACMA-31 and gemcitabine combination treatment, suggesting a potential role of XBP-1s in gemcitabine resistance and highlighting it as further therapeutic target.

Collectively, the data in the current study suggest that inhibition of PDI disrupts pancreatic cancer cell proliferation and migration and further contributes to UPR pathway dysregulation, driving ER stress-induced apoptosis.

Acknowledgements

I would like to acknowledge the incorporation of material from my published literature review:

Powell, L. E., & Foster, P. A. (2021). Protein disulphide isomerase inhibition as a potential cancer therapeutic strategy. *Cancer medicine*, 10(8), 2812–2825.

The material within the published review was written by myself in its entirety, with no written co-author contributions, and has been incorporated into part of this thesis to aid the discussion of current and relevant literature.

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List of Abbreviations

AJCC	American Joint Committee on Cancer
Anti-PD-1	Anti-programmed cell death 1 antibodies
AREG	Amphiregulin
ASK1	Apoptosis signal-regulating kinase
ATF4	Activating transcription factor 6
ATF6	Activating transcription factor 6
Anti-PD-1	Anti-programmed cell death 1 antibodies
BFG	Breakage-fusion-bridge
BIM	Bcl2-interacting mediator of cell death
BRCA	Breast cancer gene
bZIP	Basic-region leucine zipper
CA19-9	Carbohydrate antigen
CfDNA	Cell-free DNA
COX-2	Cyclooxygenase-2
CREB	cAMP-response element-binding
CSC	Cancer stem cell
CT	Computed tomography
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTSB	Cathepsin
DDA	Disulfide bond Disrupting Agent
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle's Medium
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERO1α	Endoplasmic reticulum oxidoreductin 1
EU	Europe
EUS	Endoscopic ultrasound
GDP	Guanosine diphosphate
GTP	Guanosine triphosphatase
GTPase	Guanosine triphosphatase
FBS	Foetal bovine serum
H₂O₂	Hydrogen peroxide
ICI	Immune checkpoint inhibitor
IGF	Insulin-like growth factor
IL-6	Interleukin-6
IPMNs	Intraductal papillary mucinous neoplasms
iRAP	Integrated RNA-Seq analysis pipeline
IRE1	Inositol-requiring enzyme 1
KRAS	Kristen rat sarcoma viral oncogene homolog
LIMMA	Linear models for microarray data
MAPK	Ras/Raf/mitogen-activated protein kinase
MCN	Mucinous cystic neoplasms
MM	Multiple myeloma
MMP	Matrix metalloproteinase

mTOR	Phosphoinositide-3 kinase/Akt/mammalian target of rapamycin
MRCP	Magnetic resonance cholangiopancreatography
MRI	Magnetic resonant imaging
mTOR	Phosphoinositide-3 kinase/Akt/mammalian target of rapamycin
MTT	Tetrazolium salt
NaHCO₃	Sodium bicarbonate
NFκB	Nuclear factor-κB
OS	Overall survival
PACMA	Propionic acid carbamoyl methyl amide
PALB2	Partner And Locator of BRCA2
PanINs	Pancreatic intraepithelial neoplasias
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-Buffered Saline
PERK	Pancreatic ER kinase (PKR)-like ER kinase
PDAC	Pancreatic ductal adenocarcinoma
PDI	Protein disulfide isomerase
PFS	Progression-free survival
PPI	Protein-protein interaction
PSC	Pancreatic stellate cell
PUMA	P53 upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
RB	Retinoblastoma
ROS	Reactive oxygen species
SDS	Sodium-dodecyl-sulphate
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
SMAD4	SMAD family member 4
SHH	Sonic hedgehog
siRNA	Small interfering RNA
SDS	Sodium dodecyl sulfate
SSB	Single-strand breaks
TBST	Tris-buffered saline with Tween 20
TCF/LCF	T-cell factor/lymphoid enhancer-binding factor
TGF-β1	Transforming growth factor beta1
TNBC	Triple negative breast cancer
TNF- α	Tumour necrosis factor-α
TNM	Tumour-node-metastasis
TP53	Tumour protein 53
TPM	Transcript per million
TRX	Thioredoxin
TSGs	Tumour suppressor genes
UICC	Union for International Cancer Control
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor
XBP-1	X-box binding protein 1
XBP-1s	Spliced X-box binding protein 1

CHAPTER 1 – GENERAL INTRODUCTION

1.1 The organ: The Pancreas anatomy and function

1.1.1 Anatomy of the Pancreas

The pancreas is a composite organ, located in the upper abdomen, behind the posterior and upper abdominal wall. The normal adult human pancreas weighs approximately 100g in healthy individuals (Longnecker et al., 2018). Weight however varies significantly, with continued growth in adults until approximately age 30 (Saisho et al., 2007). Anatomically, it is divided into five distinct parts: the head, neck, body, tail and uncinat process (Figure 1.1) (Atkinson et al., 2020). The head of the pancreas lies surrounded by the C-shaped curve of the duodenum, while the body and tail regions extend across the mid-point of the body to a point in close proximity to the splenic hilum (Talathi et al., 2023). Pancreatic secretion is emptied through the pancreatic duct which merges with the common bile duct to form the hepatopancreatic ampulla. This in turn opens up into the descending duodenum (Talathi et al., 2023). The histological structure of the pancreas is distinguished into two basic cell types: pancreatic islets (islets of Langerhans) and pancreatic acini. These cell types help to regulate the two main functions of the pancreas, those being endocrine and exocrine respectively (Karpińska and Czauderna, 2022).

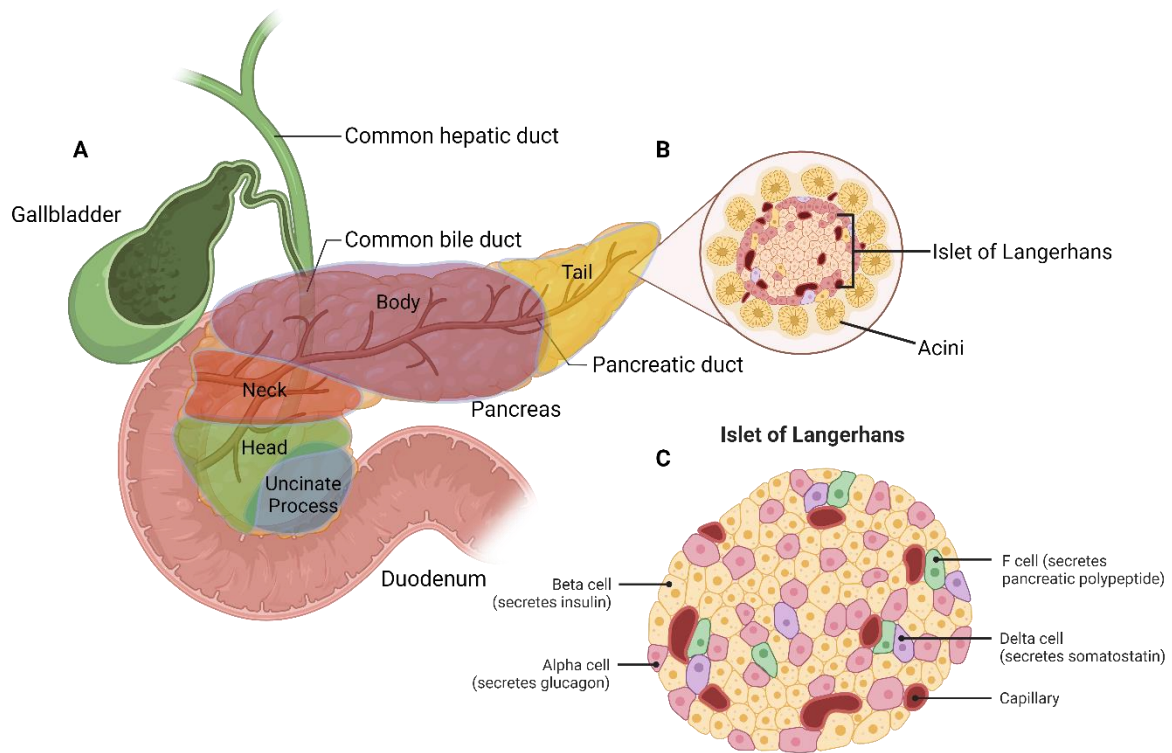


Figure 1.1 Schematic diagram showing human pancreas anatomy and cellular structure. (Adapted from Atkinson et al., 2020).

(A) Diagram detailing the distinct parts of the pancreas and its surrounding organs. (B) The pancreas at a cellular level showing the organisation of the endocrine Islet of Langerhans and exocrine Acini. (C) The Islet of Langerhans and its four endocrine cell types and respective cell functions.

1.1.2 Exocrine Function

Approximately 80% of all pancreatic tissue is devoted to exocrine functions (Talathi et al., 2023). The exocrine pancreas secretes digestive enzymes into the duodenum, playing an essential role in the digestion and processing of foodstuffs through the gastrointestinal epithelium. These digestive enzymes are essential for the absorption of nutrients in the digestive process; hence a loss of exocrine pancreatic function quickly leads to malnutrition (Padol, 2010). Acinar cells, arranged in clusters (acini), are responsible for the production of these essential proteolytic, lipolytic and

glycolytic enzymes. These include the secretion of trypsin, lipase, and amylase which respectively digest protein, fat, and carbohydrate (Atkinson et al., 2020). The exocrine pancreas also acts to support the transport of enzymes from acinar cells into the intestine through secretions of water and ions. The iso-osmotic pancreatic secretion carrying these digestive enzymes is also highly alkaline due to high concentrations of sodium bicarbonate (NaHCO_3) (Padol, 2010). The alkaline pancreatic juice, led through the pancreatic ducts to the duodenum, is essential in neutralising the acidic gastric chyme to ensure a neutral pH in the intestinal lumen and consequent optimal enzymatic function for digestion (Padol, 2010).

1.1.3 Endocrine Function

Arranged between acini, are endocrine pancreatic cells known as the islets of Langerhans. Pancreatic islets consist of multiple cell types, each responsible for the secretion of specific hormones. Approximately 40-60% of endocrine cells are beta-cells, responsible for producing insulin (Atkinson et al., 2020). Other cell types, shown in Figure 1.1, include glucagon secreting alpha-cells, somatostatin secreting delta-cells and pancreatic polypeptide secreting F cells (Mense and Rosol, 2018). Hormones enter the blood stream from the islets through a vast capillary network which merge into small veins outside the islet (Talathi et al., 2023). Crucially, the endocrine pancreas acts through the production of these hormones to regulate blood carbohydrate levels, whereby insulin acts to lower blood carbohydrate and glucagon acts to elevate it. A disruption to endocrine pancreatic function would therefore disrupt normal blood glucose regulation, leading to the onset of diabetes as the regulation of energy metabolism becomes disordered (Atkinson et al., 2020).

Endocrine pancreatic function is therefore essential for healthy carbohydrate metabolism and digestion.

1.2 The Disease: Pancreatic Cancer

1.2.1 Epidemiology of Pancreatic Cancer

Pancreatic cancer is an extremely lethal condition, recognised as the worst cancer site in terms of prognostic outcomes in the treatment of patients (McGuigan et al., 2018). Its tendency to often present at an advanced stage contributes to poor survival rates, with one-year survival rate at just 20% and with five-year survival rate being as little as 5-7% (Vincent et al., 2011). Pancreatic ductal adenocarcinoma (PDAC) and its variants account for 90% of all pancreatic malignancies (Tempero et al., 2010). Such low survival rates account for the fact that PDAC, despite being the tenth most common cancer globally, is the seventh leading cause of cancer-related death (Menini et al., 2021). In the UK, pancreatic cancer incidence rose by 9% from 2008 to 2018 and is predicted to continue becoming increasingly prevalent (Cancer Research UK) (Figure 1.2). While it is uncertain why pancreatic cancer incidence is increasing, there could be potential associations with the increasing incidence of obesity in the UK, which rose by 13% from 1993 to 2019 (Bracci, 2012; Opazo Breton and Gray, 2023). Incidence in the UK is projected to rise by 5% from 2023 to 2040. This is coupled with high mortality rates, which increased by 3% from 2008-2018, however rates are projected to fall by 5% by 2040 (Cancer Research UK). Despite this projected fall in UK mortality rate, the increasing incidence and the lack of current effective treatment strategies mean that death rates are constantly on the rise globally. It is predicted that the recorded number of pancreatic cancer related

deaths in Europe (EU) in 2010 will be increased by approximately 50% by 2025 (Ferlay et al., 2016). This dramatic rise in death brings forth further projection that pancreatic cancer will become the third leading cause of cancer-related death in the EU, only falling short of lung and colorectal cancer (Hu et al., 2021). Though pancreatic cancer is more prevalent in men than women, the trend towards increased incidence and death is observed regardless of sex (Rawla et al., 2019). In order to combat these rising trends, reflective of such poor prognosis, risk factors for pancreatic cancer onset and development must be fully understood. Outlining key risk factors allows for guidance in both prevention and earlier screening of pancreatic cancer, resulting in better survival outcomes for patients.

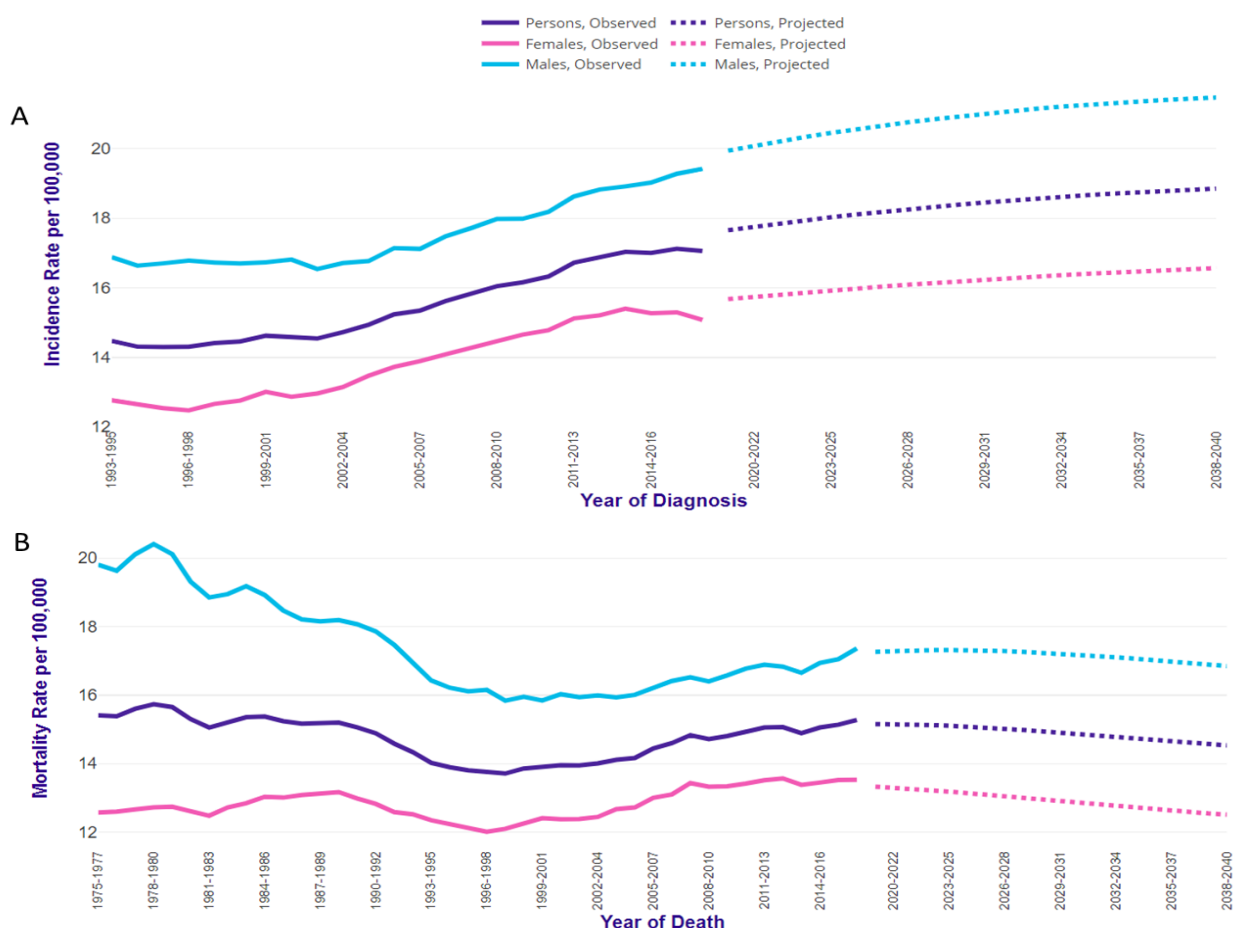


Figure 1.2 Incidence and mortality rate of pancreatic cancer per 100,000 people in the UK, 1975-2018 and the projected rates from 2020-2040. (Data obtained from Cancer Research UK).

1.2.2 Risk factors associated with Pancreatic Cancer

1.2.2.1 Age, Sex and Ethnicity

Pancreatic cancer is predominantly a disease among the elderly, with approximately 90% of new pancreatic cancer cases occurring in patients over the age of 55 (McGuigan et al., 2018). As a disease primarily found in the elderly, proper screening for early detection could be vital in improving early detection rates, especially since it is extremely rare to see the disease develop in those below the age of 30 (Hu et al., 2021). Pancreatic cancer has a male predominance, with a global incidence rate of 5.5 per 100,000 in men and 4.0 per 100,000 in women (Bray et al., 2018). With no discernible genetic link currently found, this higher incidence in men may be due to increased environmental or lifestyle risk factors such as increased alcohol consumption or smoking (Rawla et al., 2019). Recent studies have shown that even low alcohol consumption did contribute to increased risk of pancreatic cancer when in combination with smoking, therefore highlighting the combination as an important lifestyle risk factor (Rahman et al., 2015). Significant differences in pancreatic cancer incidence can be seen between ethnic groups. In general, pancreatic cancer risk is considerably higher in black people than in any other race (Yadav & Lowenfels, 2013). This difference is thought to be due to potential underlying gene interactions but also due to lifestyle factors, again including smoking and alcohol consumption, as well as diet and vitamin D deficiency (Rawla et al., 2019).

1.2.2.2 Genetic Factors

As a hereditary disease, pancreatic cancer is considered familial if the number of diagnosed first degree relatives is two or greater. Familial pancreatic cancer

accounts for approximately 10% of cases (Hruban et al., 2010). The lifetime risk of developing pancreatic cancer is 1.3% within the general population, whereas for those with two affected first degree relatives it is 8-12% (Grover & Syngal, 2010). This then rises significantly to 40% in those with three or more affected first degree relatives (Grover & Syngal, 2010). Among these patients with a family history of pancreatic cancer, a genetic predisposition can be seen in the presence of several germline mutations. Breast cancer gene 2 (BRCA2) gene mutations have been most commonly observed in familial pancreatic cancer patients, coupled with partner and locator of BRCA2 (PALB2), which has now been labelled as a pancreatic cancer susceptibility gene (Jones et al., 2009). PALB2 and BRCA2 act as tumour suppressor genes, whereby PALB2 binds to and localises with BRCA2 during double-strand DNA repair (Hofstatter et al., 2011). Therefore, mutations in PALB2 and BRCA2 lead to defective DNA damage repair and increased pancreatic cancer risk (Villarroel et al., 2011). Germline PALB2 mutations have been identified in approximately 3-4% of familial pancreatic cancer cases (Hofstatter et al., 2011). BRCA2 germline mutations have been found in 3.7-19% of patients with strong familial histories of pancreatic cancer (Rosen et al., 2021). Germline mutations associated with specific syndromes have also been shown to increase pancreatic cancer risk (Table 1.1). With so many hereditary genetic risk factors outlined, screening of this high-risk cohort could prove vital in improving rates of early detection and diagnosis.

Gene	Syndrome	Pancreatic Cancer Risk over Lifetime (%)
<i>BRCA2 & BRCA1</i>	Hereditary Breast and Ovarian Cancer	5
<i>PRSS1</i>	Hereditary Pancreatitis	40
<i>STK11</i>	Peutz-Jeghers Syndrome	35
<i>CDKN2A</i>	Familial atypical multiple mole melanoma	19
<i>MMR</i>	Hereditary nonpolyposis colorectal cancer	90%

Table 1.1 Hereditary syndromes associated with familial pancreatic cancer risk. (McGuigan et al., 2018; Karakas et al., 2018).

1.2.2.3 Pancreatitis

As shown in Table 1.1, individuals affected with hereditary pancreatitis have an increased lifetime risk of pancreatic cancer of 40%. Pancreatitis is a condition in which the pancreas becomes inflamed, inducing a destructive process whereby digestive enzymes are pre-emptively activated before their release into the small intestine (Rawla et al., 2019). Hence these activated enzymes attack and ultimately damage the pancreas, leading to malfunction. While pancreatitis can be either acute or chronic, a recurrence of acute pancreatitis leads to pancreatic tissue damage build up and the development of chronic pancreatitis (Etemad and Whitcomb, 2001). Chronic pancreatitis is a severe, painful condition, often leading to the progression of diabetes and contributing to a malabsorption of vital nutrients (Etemad and Whitcomb, 2001).

Chronic pancreatitis has been strongly linked with pancreatic cancer as a risk factor. While only 5% of patients with chronic pancreatitis will develop pancreatic cancer, in cases of hereditary pancreatitis specifically, the risk is considerably increased (Raimondi et al., 2010). Patients with hereditary pancreatitis have been recorded to have rates of pancreatic cancer that are 50-fold greater than the general population (Raimondi et al., 2010). Up to 80% of hereditary pancreatitis patients have been found to have a mutation in the PRSS1 gene (Raphael & Willingham, 2016). PRSS1 encodes cationic trypsinogen, the precursor for the most abundant digestive enzyme secreted by the human pancreas, trypsin (Németh & Sahin-Tóth, 2014). PRSS1 mutations can increase the autocatalytic conversion of trypsinogen to active trypsin, resulting in premature, intrapancreatic trypsinogen activation, or lead to ineffective deactivation of trypsin by eliminating a trypsin autodegradation site (Rosendahl et al., 2007; Grover & Syngal, 2010). These PRSS1 mutations can result in pancreatic parenchymal tissue breakdown, and increased risk of pancreatitis (Rosendahl et al., 2007; Grover & Syngal, 2010). More recent studies have confirmed a link between PRSS1 mutation and pancreatic cancer tumorigenesis. However, pathway delineation remains unclear as to whether this is due to increased trypsin activation or the direct activation of tumour pathways (Liu et al., 2019). Though this highlights trypsin as a potential target in inhibiting pancreatic cancer, more pressingly, it reinforces that screening for hereditary pancreatitis could improve rates of early detection.

It should also be noted that only 1.8% of patients with chronic pancreatitis develop pancreatic cancer within the first 10 years of diagnosis (Lowenfels et al., 1993). It is not until 30-40 years post diagnosis that this rate significantly increases (Rawla et

al., 2019). This suggests that the early onset nature of hereditary chronic pancreatitis, and consequent prolonged period of pancreatic inflammation over a patient's lifetime, may contribute to increased pancreatic cancer risk. It is well recorded that prolonged inflammation drives the survival of a tumour-promoting environment. Pancreatic inflammation has been attributed to driving pancreatic cancer growth through signalling pathways involving cyclooxygenase-2 (COX-2) and Hedgehog and Notch (Weiss, 2014). COX-2 has been an effective anti-cancer target in pancreatic cancer cells, with its inhibition suppressing cell proliferation in vitro and in vivo (Xu et al., 2008). This further solidifies that the persistent inflammatory nature of hereditary chronic pancreatitis perpetuates pancreatic carcinogenesis and promotes the investigation of new diagnostic approaches in these high risk patients. These potential pathways of chronic pancreatitis are further potentiated by smoking, a known risk factor for both chronic pancreatitis and pancreatic cancer. Exposure to cigarette smoke has been shown to also induce COX-2 activity, exerting tumorigenic, pro-inflammatory effects (Huang & Chen, 2011). It is therefore likely that a combination of both genetic and lifestyle risk factors acts in conjunction to elicit the same or associated oncogenic pathways. Determining the number of these key risk factors present in an individual would give clearer insight into their susceptibility for pancreatic cancer development.

1.2.2.4 Diabetes Mellitus and Obesity

As previously stated, pancreatitis is often a prerequisite for the development of diabetes. Both type I and type II diabetes have been associated with increased risk of pancreatic cancer (Batabyal et al., 2014). Diabetes mellitus is defined by chronic

hyperglycaemia, due to defective function in the metabolism of carbohydrates, proteins and fats (American Diabetes Association, 2004). Patients recently diagnosed with diabetes are approximately seven times more likely to develop pancreatic cancer (Huang et al., 2020). In pancreatic cancer patients, 50–80% also suffer with either diabetes or impaired glucose tolerance (Li, 2012). While diabetes is recognised as a risk factor in the predisposition of pancreatic cancer, the relationship is complex, and it can also be recognised as an early manifestation of pancreatic cancer (Fisher, 2001). Patients can be considered to have what is termed secondary diabetes, in cases where diabetes was diagnosed within 2 years prior to pancreatic cancer diagnosis (Li, 2012). Studies have shown that this patient cohort account for approximately 75% of diabetes cases in pancreatic cancer patients, suggesting that diabetes onset may be predominantly cancer-caused (Pannala et al., 2008). However, with still many diabetes cases going undiagnosed before pancreatic cancer onset, the relationship between the two diseases remains poorly defined.

Smoking and obesity are two major risk factors for both type II diabetes and pancreatic cancer. However, biomarker studies have shown that it is still likely that type II diabetes is an independent risk factor for pancreatic cancer. Type II diabetes mellitus is caused by insulin resistance and is considerably more prevalent than type I. Meta-analysis studies have shown pancreatic cancer risk to be associated with high levels of circulating C-peptide/insulin and further markers of glycaemia (Pisani, 2008). While the underlying mechanisms of diabetes associated pancreatic tumorigenesis remain ambiguous, it is commonly hypothesised that hyperinsulinemia is the defining cause of increased pancreatic cancer risk (Andersen et al., 2017). An oversecretion of insulin is often a pancreatic beta-cell response mechanism to insulin

resistance. Insulin has been shown to contribute to increased cell proliferation and glucose utilisation, both of which can contribute to tumour progression (Ding et al., 2000). Insulin also indirectly drives pancreatic cancer progression through the upregulation of insulin-like growth factors (IGFs) (Andersen et al., 2017). IGF-1 is highly expressed in pancreatic cancer cells and has been shown to exhibit more potent tumorigenic effects than insulin itself (Zeng et al., 2003). IGF-1 has been attributed to activating pathways involved in proliferation, invasion, and vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) expression – key contributors to tumour angiogenesis (Zeng et al., 2003). Hyperinsulinemia also induces expression of IGF-1 receptors (IGF-R), which activate downstream oncogenic pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase/Akt/mammalian target of rapamycin (mTOR) pathways, therefore driving tumour growth and survival (Pollak, 2008). Hyperglycaemia also has been shown to accelerate pancreatic tumour development through the activation of transforming growth factor beta1 (TGF- β 1), which promotes pancreatic epithelial cells to acquire mesenchymal and cancer stem cell properties (Rahn et al., 2018).

Obesity is recognised as a stand-alone risk factor for pancreatic cancer but more markedly known for its synergistic relationship with type II diabetes in pancreatic cancer development (Andersen et al., 2017). Obesity triggers higher levels of insulin resistance therefore promoting the onset of type II diabetes and acting concurrently in the contribution to a hormonal imbalance (Andersen et al., 2017). As previously discussed, inflammation is a key driving factor in pancreatic cancer development. Both obesity and diabetes are pro-inflammatory conditions, responsible for activating

inflammatory pathways involved in pancreatic cancer pathogenesis (George et al., 2022). It is thought that both fat and glucose intake trigger inflammation through inducing the production of reactive oxygen species (ROS), and activation of transcription factors such as nuclear factor- κ B (NF κ B) (George et al., 2022). The NF κ B pathway contributes to a tumour microenvironment by inhibiting apoptosis (Duan et al., 2021). In addition, obesity-associated hypertrophied adipocytes release increased pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) (Duan et al., 2021). Studies in the KrasG12D mouse model of pancreatic cancer have demonstrated that a high fat, high calorie diet leads to visceral obesity, pancreatic inflammation, and accelerated pancreatic neoplasia (Hertzer et al., 2016). These studies have further demonstrated that KrasG12D mice on a high fat, high calorie diet have significantly increased inflammation in the visceral adipose tissue, particularly in per-pancreatic fat (PPF), suggesting that obesity-associated inflammation in the PPF may accelerate pancreatic cancer progression (Hertzer et al., 2016). Research has shown significant links between obesity and type II diabetes in their contribution to pancreatic cancer. While the relationship between diabetes and pancreatic cancer is still not fully understood, potential mechanisms discussed are outlined in Figure 1.3.

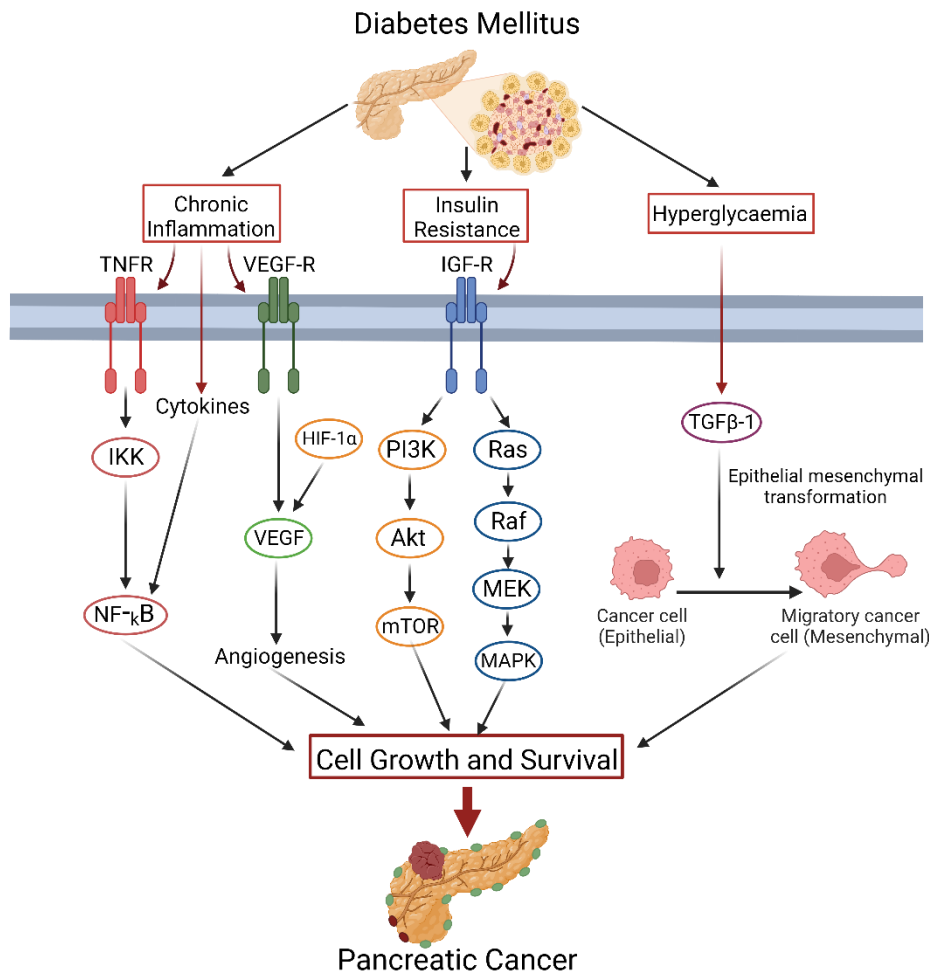


Figure 1.3 Potential Mechanisms associated with diabetes mellitus and pancreatic cancer progression. (Original figure created from references in text). Diabetes mellitus triggers oncogenic pathways through chronic inflammation, insulin resistance and hyperglycaemia to promote pancreatic cancer growth and survival. Chronic inflammation activates inflammatory cytokines and TNFR which induces NFκB. It also activates VEGF-R, resulting in angiogenesis. Insulin resistance activates IGF-R, which triggers MAPK and PI3K pathways. Hyperglycaemia triggers TGFβ-1 which leads to epithelial mesenchymal transformation.

1.2.3 Clinical Presentation

Pancreatic cancer is predominantly clinically silent in its early stages, hence presenting major diagnostic challenges. It is often detected at a late stage, post metastasis and surrounding tissue tumour invasion, meaning that most symptoms are imputable to advanced disease (Vincent et al., 2011). This attributes to the fact

that at the time of presentation 80–85% of tumours are not able to be resected and so remain incurable (Vincent et al., 2011). In late-stage diagnosis, presenting symptoms include pain in the back and abdomen, weight loss and sleep problems (DiMagno, 1999). Back pain in particular has been shown to predict unresectable cases and shortened survival even post-surgery (DiMagno, 1999). In cases of pancreatic-duct obstruction, obstructive jaundice and pancreatitis attacks are also present manifestations (Vincent et al., 2011). Despite these symptoms being suggestive of pancreatic cancer, they are often also attributable to other diseases or conditions. One study showed that 57% of patients presenting these key symptoms had other related health problems, including various other cancer types such as ovarian, bowel and uterine (DiMagno et al., 1977). As previously discussed, a large proportion of pancreatic cancer patients have been previously diagnosed with diabetes. With few clinical, presentable early signs of pancreatic cancer, screening in diabetic patients could improve rates of diagnosis, especially in older patients where the disease is more prevalent.

1.2.4 Tumour Staging

Pancreatic cancer tumour stages are characterised using the tumour-node-metastasis (TNM) classification. In this system, tumour advancement is based on primary tumour dimensions and extensions (T), the presence of metastatic lymph nodes (N), and the presence of distant metastases (M) (Morana et al., 2010). These have been determined as key characteristics that correlate with prognostic outcomes of the disease. In terms of the T parameter, only T4 is considered unresectable due to the infiltration of the tumour into the superior mesenteric artery or the celiac axis

(Wolff et al., 2000). The N parameter highlights the detrimental effect lymph node involvement has on patient survival. Crucially, it is nodal metastases that prove most challenging in treatment as metastases to the paraaortic node, common hepatic artery, or celiac node, prevent the possibility of surgery (Maithel et al., 2007). Similarly, the presence of distant metastases, outlined by the M parameter, also prevent surgical options. The most common sites of distant metastases include the liver and peritoneum (Morana et al., 2010). With peritoneal metastases present in 50% of pancreatic cancer patients at the time of death, distant metastases are of significant detriment to chances of survival (Avula et al., 2020). The American Joint Committee on Cancer and Union for International Cancer Control (AJCC/UICC) staging system incorporates TNM classification and is the worldwide clinical tool used for staging assessment of pancreatic cancer (Cong et al., 2018). AJCC/UICC staging and associated 5-year survival rates are outlined in Table 1.2.

Stages		TNM classification	Clinical classification	Resectability	5-year survival rate (%)
0		Tis, N0, M0	Carcinoma in situ	Resectable	n/a
IA		T1, N0, M0	Localised in pancreas	Resectable	31-39
IB		T2, N0, M0	Localised in pancreas	Resectable	22-27
IIA		T3, N0, M0	Locally invasive	Potentially resectable	16-25
IIB		T1 – T3, N1, M0	Locally invasive	Potentially resectable	8-10
III		T4, Any N, M0	Locally advanced	Potentially resectable	0-7
IV		Any T, Any N, M1	Distant Metastases	Unresectable	0-4

Table 1.2 AJCC/UICC staging and survival in pancreatic cancer. (Adapted from, Hartwig and Büchler, 2013).

1.2.5 Diagnosis

As discussed, pancreatic cancer often only presents at an advanced stage, with frequent metastases, meaning surgical resection is not a treatment option at the point of diagnosis. With a low lifetime risk, screening for the general population is not viable. However, individuals with a familial pancreatic cancer risk have been defined as a higher risk population for screening, beginning at the age of 50 (Unger et al., 2018). Though screening for high risk populations has been identified, challenges remain in developing effective imaging and tumour marker detection techniques for pancreatic cancer in its early stages (Wu et al., 2022). Current diagnostic methods are outlined below.

1.2.5.1 Imaging

A range of imaging modalities are currently used for diagnosis. These include secretin enhanced magnetic resonant imaging (MRI), computed tomography (CT), endoscopic ultrasound (EUS) and magnetic resonance cholangiopancreatography (MRCP) (McGuigan et al., 2018). EUS is more sensitive than MRI and CT in identifying smaller solid tumours of less than 2cm in diameter (Yu et al., 2017). However, EUS is an invasive, wound causing examination, meaning enhanced CT is the current first choice for capturing early stage lesions. However, sensitivity remains relatively low in tumours less than 2cm diameter at 63-77% (Francis, 2007). EUS does prove advantageous in very early stage diagnosis, with its sensitivity at stage 0 being 45.5%, this is significantly higher than that of CT or MRI which are both just 9.7% (Kurihara et al., 2020). Screening methods now primarily focus on the screening of preinvasive lesions rather than early stage pancreatic cancer, to resect these lesions before the cancer becomes invasive (Vincent et al., 2011). The ability of EUS to detect these preinvasive lesions means that it is becoming more widely used as a screening test, however the development of less invasive screening techniques would be preferential (Vincet et al., 2011). Research has recently been focused on the discovery of novel biomarkers in early stage pancreatic cancer to provide an alternative, accurate and relatively non-invasive diagnostic approach.

1.2.5.2 Biomarkers

As the understanding of pancreatic cancer molecular pathology continues to grow, research efforts have been focused on the detection of potential predictive biomarkers of asymptomatic, early stage disease. At present, carbohydrate antigen

(CA19-9) is the most routinely used tumour biomarker in pancreatic cancer diagnosis, often combined with imaging methods, it proves valuable as a marker of disease (Kim et al., 2004). Despite its usefulness, a high CA19-9 level indicates advanced pancreatic cancer, and does not address the issue of a lack of early stage detection methods (Dariya et al., 2019). CA19-9 is more appropriately used in a post-diagnostic setting to measure recurrent disease or the efficacy of treatments (Fahrman et al., 2019). Recently, research has been aimed at identifying plasma-based metabolic biomarker signatures that could aid early diagnosis. A recent study identified a biomarker signature consisting of nine metabolites as well as CA19-9 (Mayerle et al., 2018). This novel biomarker signature was able to distinguish pancreatic cancer and chronic pancreatitis, with a higher diagnostic accuracy than that seen with just CA19-9 (Mayerle et al., 2018).

Genetic mutations are also potential biomarkers detected in early stage pancreatic cancer. This includes mutant Kirsten rat sarcoma viral oncogene homolog (KRAS), identified as a useful prognostic marker (Garcea et al., 2005). However, detection of mutant KRAS does have limitations in that there is a low sensitivity for its detection in circulating cell-free DNA (CfDNA), despite high sensitivity rates in tumour tissue (Riva et al., 2016). CfDNA is easily accessible in peripheral blood and can be used as biomarkers for cancer prognosis, with significant correlations observed between CfDNA concentration, tumour metastases and patient survival (Yan et al., 2021). Further research needs to be carried out to enhance sensitivity for early detection of mutant KRAS in CfDNA.

A vast range of other molecular biomarkers are currently under investigation. These include proteins from blood and pancreatic juice, DNA methylation and noncoding RNAs, all of which have exhibited potential usefulness in pancreatic cancer detection (Wu et al., 2022). With currently no potent, specific biomarker for early disease detection, advancements and novel approaches need to be made to improve pancreatic cancer diagnosis.

1.2.6 Molecular Pathogenesis

Pancreatic cancer most commonly arises in exocrine cells, with endocrine tumours accounting for less than 5% of pancreatic cancer cases (Grant et al., 2016). As previously mentioned, PDAC are the most prevalent pancreatic cancer subtype, accounting for the majority of malignancies. PDAC derives from pancreatic duct epithelial cells which undergo a series of genetic mutations, leading to the development of precursor lesions; pancreatic intraepithelial neoplasias (PanINs), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCN) (Grant et al., 2016). PanINs are microscopic lesions that occur in small pancreatic ducts (Esposito et al., 2014). Classified through grades 1-3, they are representative of the neoplastic process that occurs in the consequent development of invasive pancreatic cancer (Hruban et al., 2001). PanINs have been further classified into low grade (PanIN 1 and PanIN 2) and high grade (PanIN 3) (Basturk et al., 2015). The histological pathogenesis of PanIn to PDAC development is shown in Figure 1.4.

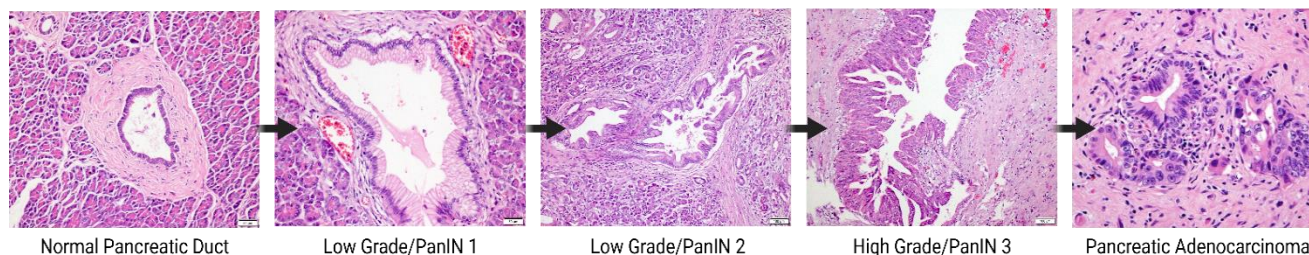


Figure 1.4 PanIN morphological changes in pancreatic adenocarcinoma progression. (Histological images taken from Pathology Outlines Database, 2023).

IPMNs and MCNs are less common and less well studied precursor lesions. Mucinous tumours represent just 10% of cases and are a less invasive subtype (Fesinmeyer et al., 2005). Research has suggested that while IPMNs and MCNs may show some variation in the molecular events observed in PanIN malignant transformation, several key up-regulated genes are shared between the three lesion types (Hezel et al., 2006). Further investigation into the genetic alterations observed in PDAC pathogenesis within each precursor lesion subtype is needed to exploit these molecular pathways for potential treatments. Commonly known pathways of molecular pathogenesis are described below.

1.2.6.1 Activation of Oncogenic KRAS Mutations

Oncogenic KRAS mutations are a hugely prevalent feature of PDAC, found in 92% of cases (Bailey et al., 2016). KRAS is a signal transducer protein from the guanosine triphosphatase (GTPase) superfamily, also recognised as a member of the family of RAS proto-oncogenes (Pantsar, 2019 & Dariya et al., 2019). In normal conditions, KRAS fluctuates between its active guanosine triphosphate (GTP)-bound state and its inactive guanosine diphosphate (GDP)-bound state (Waters and Der,

2018). Ras in a normal state is GDP-bound and inactive (Waters and Der, 2018). KRAS mutations impair the GTPase activities of KRAS, this consequently locks Ras in its GTP bound, active state, resulting in prolonged stimulation of tumorigenic-associated pathways such as MAPK and mTOR (Luo, 2021). Activating KRAS mutations are one of the earliest observed molecular alterations observed, with prevalence as high as 90% in low grade PanINs (Kanda et al., 2012). Research suggests that KRAS mutations are responsible for the initiation of pancreatic cancer through PanIN development but not independently responsible for the consequent PDAC onset (Grant et al., 2016). Research has shown that PDAC development requires a subsequent inactivation of key tumour suppressor genes (Grant et al., 2016). It is therefore proposed that the molecular pathogenesis of pancreatic cancer is a multi-step neoplastic process.

1.2.6.2 Inactivation of Tumour Suppressor Genes

As mentioned, the inactivation of tumour suppressor genes (TSGs) is a crucial oncogenic event in pancreatic cancer transformation, appearing in both PanIN and PDAC itself. TSGs act to regulate cell proliferation and prevent exaggerated cell division exhibited by cancer cells (Muller and Vousden, 2013). This inactivation therefore prevents cell growth regulation, hence resulting in pancreatic cancer development. Following activating KRAS mutations, inactivation of several TSGs can occur, the most common being tumour protein 53 (TP53), SMAD family member 4 (SMAD4) and CDK2NA (Hezel et al., 2006). TP53 encodes for transcription factor p53, a key component in the promotion of cell cycle arrest and apoptosis during episodes of cell stress, or following DNA damage (Khoo et al., 2014). Inactivation of

TP53, and hence p53, therefore promotes uncontrolled cell growth and cancer cell survival. This is also in conjunction with a decreased sensitivity to some chemotherapeutic treatments (Sarkar et al., 2007). The inactivation of TP53 has also be linked to increased invasiveness, with downstream cell migration and epithelial-mesenchymal transition (EMT) counteracting pathways no longer activated (Powell et al., 2014). With gene alterations in p53 and KRAS mutations exhibiting a somewhat collaborative relationship in the molecular pathogenesis of pancreatic cancer, both can be observed as a potential useful indicator for disease stage and potential treatment response.

Inactivation of p53 is however primarily exhibited post CDKN2A inactivation, in higher grade PanINs (Maitra et al., 2003). This implies that TP53 inactivation occurs later in the suggested multi-step tumorigenic process and that CDKN2A inactivation is an earlier indicator of disease progression. CDKN2A encodes p16, which is responsible for the inhibition of cyclin D and CDK4/6 (Sarkar et al., 2007). By inhibiting cyclin D and CDK4/6, phosphorylation of retinoblastoma (RB) is also inhibited, consequently disrupting entry into the S-phase of the cell cycle (Sarkar et al., 2007). An inactivation of CDKN2A therefore enables cells to bypass the p16-induced senescence response that occurs post activating KRAS mutations, further driving tumour progression (Maitra et al., 2003). Inactivation of CDKN2A is both the most prevalent and earliest TSG inactivation observed in PDAC, with CDKN2A loss present in 95% of tumours (Schutte et al., 1997).

SMAD4 inactivation is interestingly more specific to pancreatic cancer than other types, where it exhibits a low incidence (Sarkar et al., 2007). SMAD4 inactivation has been observed to primarily occur following CDKN2A inactivation, signifying its role in advanced PanINs and final stage PDAC development (Wilentz et al., 2000). SMAD4 is an important component in the transforming growth factor type β (TGF- β) signalling pathway, where it has an antiproliferative effect, hence regulating cell growth (Massagué et al., 2000). SMAD4 expression has been shown to be of potential clinical relevance, with one study reporting that patients with SMAD4-expressing pancreatic adenocarcinomas had a significantly longer survival rate compared with those lacking SMAD-4 expression (Tascilar et al., 2001). The TGF- β pathway is also known for its role in EMT promotion, hence increasing metastatic potential (Racu et al., 2022). TGF- β pathway signalling leads to the activation of Smad2/3 which binds to Smad4, which is thought to promote EMT through its transcriptional programs (Grant et al., 2016). This highlights how the relationship between SMAD4 and PDAC progression is complex and somewhat controversial, with some contrasting studies indicating that EMT is induced in SMAD4 inactivated cells also (Yamada, et al., 2015). Whilst molecular pathways of SMAD4 in TGF- β induced EMT are yet to be elucidated, SMAD4 can be described as a key player in the molecular pathogenesis of PDAC.

1.2.6.3 Telomere Abnormalities

Telomere shortening is the most common, early genetic event exhibited in the multi-step molecular model of PDAC progression (van Heek et al., 2002). Telomeres are specialised structures located at the end of chromosomes, to protect them from

chromosomal breakage in cell division, maintaining genome integrity (van Heek et al., 2002). Telomeres shorten over time with cell division, eventually resulting in breakage-fusion-bridge (BFG) cycles which promote chromosomal instability, associated with early stage neoplasia (Hezel et al., 2006). Telomere dysfunction has been found in approximately 90% of low grade PanINs and has shown association with p53 loss, also recognised as an early stage event (van Heek et al., 2002). It is predicted that telomere shortening precedes p53 loss, contributing to TP53 inactivation by inducing selective pressure (Greenberg et al., 1999). This highlights how telomere shortening may be a crucial predisposing factor in initiating the development of precursor lesions and consequent PDAC progression. The discussed molecular multi-step model of PDAC pathogenesis is shown in Figure 1.5.

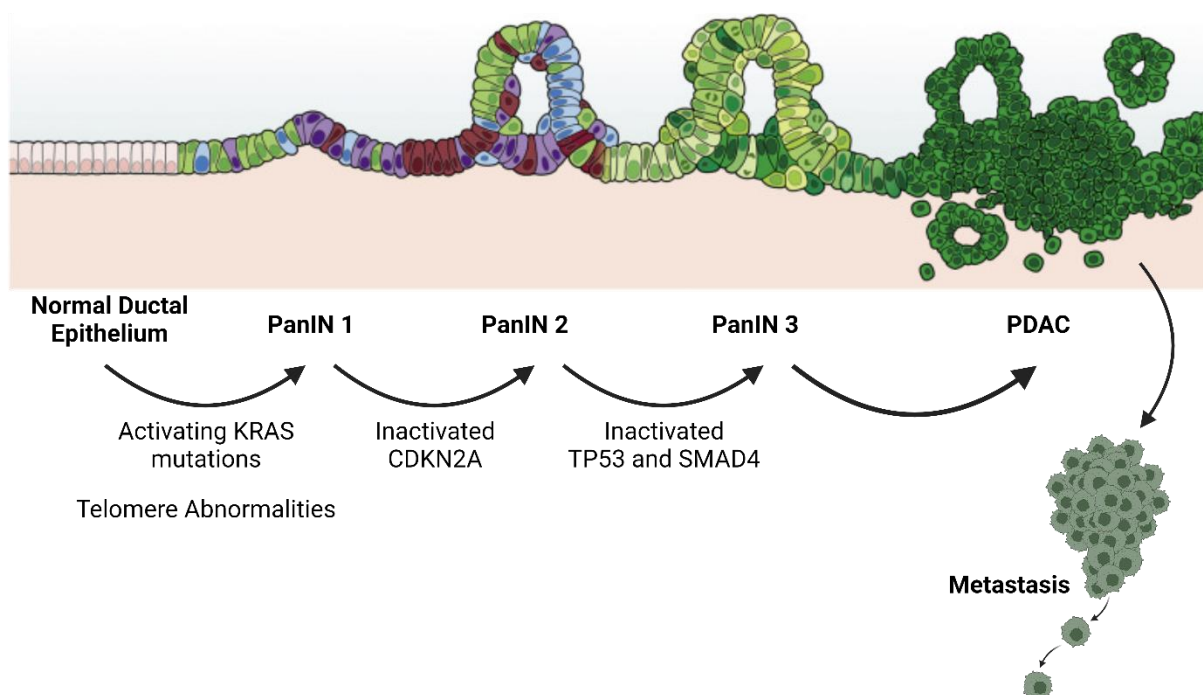


Figure 1.5. Schematic of gene alterations involved in molecular pathogenesis of pancreatic cancer. (Original Diagram, adapted from Grant et al., 2016). Activating Kras mutations and telomere abnormalities aid early-onset PanIN 1 development. Inactivation of CDKN2A aids PanIN 2 progression. The inactivation of

TP53 and SMAD4 aids progression to advanced PanIN 3 lesions and consequent invasive pancreatic adenocarcinoma.

1.2.7 Treatment

As previously mentioned, only surgical resection has the potential to cure pancreatic cancer. In recent times, the addition of neoadjuvant chemotherapy and radiotherapy has also improved survival rates (Zhao and Liu, 2020). Newer therapies such as immunotherapy and targeted therapy have also been of recent focus as potential further treatment methods (Zhao and Liu, 2020). The current landscape of pancreatic cancer treatment methods is presented in Figure 1.6.

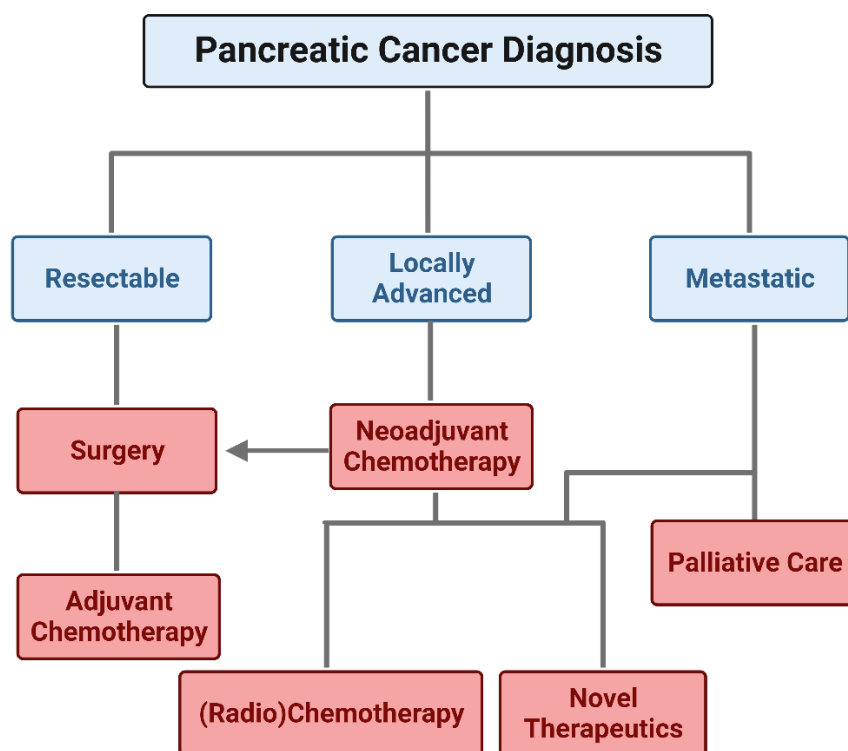


Figure 1.6. Overview of current treatment strategies for pancreatic cancer at resectable, locally advanced, and metastatic stages. (Original Diagram, adapted from Neesse et al., 2013).

1.2.7.1 Surgery and Neoadjuvant Treatment

With earlier stage detection, patients can undergo surgical resection with the aim of resecting the entire tumour mass to achieve a microscopically negative tumour margin (R0) (Demir et al., 2018). The various potential surgical techniques include total pancreatectomy, distal pancreatectomy and pancreaticoduodenectomy (Whipple's procedure) (Zhao and Liu, 2020). The choice of surgical option is dependent on the tumour's anatomical location. As previously described in Table 1.2, patients can undergo surgery up to stage IV, in which distant metastases prevent the possibility of resection. However, patients with distant metastases that are more isolated, can be reviewed for potential surgery after long-term chemotherapy and appropriate tumour shrinkage (Strobel et al., 2019). The addition of preoperative chemotherapy or radiotherapy has also proven to improve patient survival, and the resectability of tumours. One study showed that in patients with unresectable pancreatic cancer, 61% were able to have surgical treatment following neoadjuvant chemotherapy (Hackert et al., 2016). Despite a growing number of patients receiving tumour resection, survival rates remain poor. This is due to high rates of tumour recurrence post resection, with statistics showing recurrence rates of 69-75% at 2 years and 80-90% at 5 years (Lambert et al., 2019). This highlights a necessity for adjuvant treatment in addition to resection.

1.2.7.2 Adjuvant Chemotherapy

Adjuvant chemotherapy is an essential post-surgery treatment follow-up to increase patient survival rates. The CONKO-001 trial demonstrated an increased 5-year survival rate of 20.7% with adjuvant gemcitabine treatment, compared to 10.4% with

complete surgical resection alone (Oettle et al., 2013). Similarly, the ESPAC-1 trial demonstrated that adjuvant fluorouracil and folinic acid treatment contributed to a 21% 5-year survival rate, as opposed to 8% in non-chemotherapy treated patients (Lambert et al., 2019). However, in the follow-up ESPAC-3 trial, patients who received fluorouracil and folinic acid treatment exhibited significantly more adverse effects (14%) compared to gemcitabine (7.5%) (Lambert et al., 2019). Gemcitabine is therefore the preferential adjuvant chemotherapy treatment.

More recent trials have investigated survival effects of combined chemotherapy treatments. The clinical trial PRODIGE24/CCTG compared the use of stand-alone gemcitabine treatment with a combination treatment of oxaliplatin, irinotecan and leucovorin (mFOLFIRONOX) (Conroy et al., 2018). mFOLFIRONOX treatment demonstrated a significantly improved survival rate of 63.4% at 3 years versus 48.6% with gemcitabine treatment alone (Conroy et al., 2018). Despite this, mFOLFIRONOX treatment should be highly considered before use, due to a high incidence of toxic adverse effects, reflected in 75.9% of treated patients (Conroy et al., 2018). However, its increased survival benefits mean that mFOLFIRONOX treatment is recommended in patients with metastatic disease (McGuigan et al., 2018).

The addition of adjuvant radiotherapy combined with chemotherapy have also been investigated, with clinical trials gathering some conflicting results. An analysis-based study of the National Cancer Database suggests that radiotherapy addition to adjuvant chemotherapy is correlated with increased overall survival (Rutter et al.,

2015). Contrasting clinical trial results do imply however, that the impact of radiotherapy treatment on survival is dependent on the timing of postoperative treatment. It is believed that radiotherapy treatment provides the greatest survival benefit post 1-3 months of adjuvant chemotherapy treatment (Lambert et al., 2019). Further research is needed to assess the most effective strategies for adjuvant chemoradiotherapy.

1.2.7.3 Immunotherapy

The use of immunotherapy has been of recent investigation in pancreatic cancer treatment. Immunotherapy has the potential to combat the immunosuppressive nature of the tumour microenvironment, by mobilising the immune system to fight against cancer cell growth. Despite the success of immune checkpoint inhibitors (ICIs) among other various malignancies, only anti-programmed cell death 1 antibodies (anti-PD-1) have been approved for pancreatic cancer treatment use and only in a small minority of patients who also are mismatch repair-deficient (Le et al., 2017). Mismatch repair pathways play an important role in identifying and repairing mismatched bases during DNA replication and genetic recombination in cancer cells (Zhao et al., 2019). Mismatch repair-deficiency leads to the accumulation of mutations in microsatellites of cancer cells, a condition known as microsatellite instability (Zhao et al., 2019). This subsequently leads to the generation of neoantigens which can stimulate an anti-tumour immune response (Kelderman et al., 2015). The activity of anti-PD-1 therapy is therefore heightened in mismatch-repair deficient tumours as there is an increased probability of a strong neo-antigen-driven T cell response (Kelderman et al., 2015). Despite the approval of anti-PD-1 therapy,

this treatment approach does not show an effective response rate (18.2%) among advanced pancreatic cancer cases (Marabelle et al., 2020). Several factors can contribute to immunotherapy resistance such as varying expression levels of PD-1, regulatory T-cell infiltration, and T-cell exhaustion due to prolonged antigen exposure (Banerjee et al., 2018). Studies have also been carried out to assess the potential immunotherapy combination therapies with chemotherapy, targeted therapies and other immunotherapeutics. The immune therapeutic ipilimumab, which acts as an antagonist antibody to cytotoxic T-lymphocyte antigen 4 (CTLA-4) on T cells, has recently been trialled in combination with gemcitabine but did not prove to be more effective than gemcitabine alone in advanced pancreatic cancer (Yoon et al., 2021; Kamath et al., 2020). However, a phase II trial did demonstrate a slightly improved response rate with combination treatment of pembrolizumab, an antagonist antibody to PD-1 on T-cells, gemcitabine, and nab-paclitaxel in metastatic pancreatic cancer, compared with chemotherapy treatment alone (Weiss et al., 2018). While some studies have indicated potentially promising results for immunotherapy combination treatments, more research is needed to assess the potential in pancreatic cancer (Yoon et al., 2021).

1.2.7.4 Targeted Therapy

With current frontline therapy options driven by cytotoxic therapies, the discovery and development of molecular targeted therapies poses potential for a better standard of care for pancreatic cancer patients. As previously outlined, KRAS, TP53, CDKN2A and SMAD4 are hallmark gene mutations that drive pancreatic cancer progression. These genes and their products however have proven difficult to target

(Zarkavelis et al., 2019). Many targeted therapies, often combined with gemcitabine treatment, have failed to continue development past phase II and phase III clinical trials (Shaya, et al., 2023). With a lack of efficacy in these trialled targeting agents, research has recently focused on the potential of biomarker-driven molecularly targeted therapies. In patients harbouring BRCA1/2 germline mutations, the Poly (ADP-ribose) polymerase (PARP) inhibitor Olaparib has been approved for treatment of advanced, metastatic pancreatic cancer patients (Manrai et al., 2021). PARP inhibitors disrupt the repair of single-strand breaks (SSB), therefore preventing homologous recombination repair, creating a cytotoxic environment (Manrai et al., 2021). PARP inhibitors are often especially lethal in cancer cells exhibiting BRCA1/2 mutations, due to their hindered ability for DNA damage response (DDR) (Lord and Ashworth, 2016). Several recent studies suggest that by matching specific pancreatic cancer associated biomarkers with targeted therapy, this could prove a more effective therapeutic approach (Shaya, et al., 2023). With multiple molecular factors contributing to pancreatic cancer progression, combinations of matched targeted therapies may prove a novel approach. Continued research into understanding the complexities of the molecular mechanisms driving pancreatic cancer provides opportunities for the development of more precise, targeted treatments.

1.2.8 Signal Transduction Pathways of Therapeutic Potential

1.2.8.1 EGFR Pathway

The epidermal growth factor receptor (EGFR) is frequently overexpressed in pancreatic cancer (Bloomston et al., 2006). The phosphorylation of EGFR triggers

the activation of several pro-survival pathways including, MAPK, STAT and PI3K (Wong and Lemoine, 2009). As previously discussed, activating KRAS mutations are an essential driver in pancreatic cancer progression. EGFR activates Ras-mediated tumorigenic signalling pathways and so inhibition of its receptor kinase activity has been explored. Erlotinib is a developed tyrosine kinase inhibitor that acts to suppress EGFR and showed promising results in improving survival of advanced pancreatic cancer patients in combination with gemcitabine (Moore et al., 2007). One-year survival rates of pancreatic cancer patients was shown to be increased with erlotinib plus gemcitabine combination treatment (23%), compared with gemcitabine treatment alone (17%) (Moore et al., 2007). Erlotinib was the first targeted therapy approved for pancreatic cancer treatment (Wong and Lemoine, 2009). However, despite erlotinib treatment demonstrating a statistically significant improved survival, this difference compared with gemcitabine alone is small and hence its cost-effectiveness has been questioned (Grubbs et al., 2006).

Cetuximab was thought to be another promising targeted therapy. As a monoclonal antibody that inhibits EGFR, it gained attention through investigation in colorectal cancer with the mutated-KRAS biomarker (Karapetis et al., 2008). Despite this, phase III trials proved cetuximab and gemcitabine combination to be ineffective in advanced pancreatic cancer patients (Philip et al., 2010). With no EGFR inhibitor proving greatly effective it is clear that more research is needed into the inhibition of EGFR signalling, perhaps combined with the inhibition of its downstream mechanisms.

1.2.8.2 Ras Pathway

As discussed, Ras proteins are activated downstream of EGFR phosphorylation, inducing the oncogenic Ras/Raf/MAPK pathway, driving cancer cell growth and survival. Research has been carried out to target each of these key pathway components. Tipifarnib was developed for pancreatic cancer treatment to target Ras activation by inhibiting its farnesylation and hence membrane localisation (Tanaka, 2016). However, no significant survival improvement was seen in phase III trials (Van Cutsem et al., 2004).

Downstream of Ras, Raf is sequentially activated. Sorafenib also reached phase III clinical trials as an inhibitor of Raf kinase, but in fact induced a decreased survival response in patients, highlighting the complexities of targeting Ras pathway components (Gonçalves et al., 2012). Further downstream, inhibitors trametinib and selumetinib were developed to target MEK kinases in an effort to disrupt cancer cell growth and apoptotic resistance, however again no clinical survival benefit was shown (Tanaka, 2016). Despite disappointing clinical trial results, research has indicated that EGFR does play a crucial role in KRAS-driven pancreatic cancer progression (Tanaka, 2016). Further EGFR downstream signalling pathways are therefore also of therapeutic relevance and require further investigation.

1.2.8.3 PI3K Pathway

PI3K is activated downstream of EGFR, PI3K activates Akt, which in turn activates pro-survival targets; mTOR and NF κ B (Wong and Lemoine, 2009). Akt was shown to

be activated in approximately 59% of pancreatic cancers and recognised as a key prognostic factor (Schlieman et al., 2003). Other PI3K-Akt pathway driven cancers have seen some targeted therapies approved. These include mTOR inhibitor temsirolimus and NF κ B inhibitor bortezomib, which have been approved for treatment of renal-cell carcinoma and multiple myeloma respectively (Ito et al., 2006, Alberts et al., 2005). Unfortunately, both of these inhibitors have failed to prove beneficial in pancreatic cancer treatment (Ito et al., 2006, Alberts et al., 2005). This again highlights the complexities of these signal transduction pathways in pancreatic cancer. Research is needed to better understand these pathways and explore other potential therapeutic avenues in the discovery of novel molecular targets.

1.2.8.4 Hedgehog and Wnt Pathways

Hedgehog and Wnt are key embryonic pathways in pancreatic cancer. These pathways act as regulators for EMT, enabling wound healing and tissue repair. EMT is strongly associated with increased invasiveness of cancer cells, and therefore considered a crucial step in the progression of metastatic pancreatic cancer (Grant et al., 2018). As well as promoting mesenchymal-like properties, EMT does also promote cancer stem cell (CSC)-like properties through the induction of cell surface markers CD44^{high} and CD24^{low} (Shah et al., 2007). CSCs are unique in that they possess a self-renewal ability, characteristically similar to that of normal stem cells (Ayob and Ramasamy, 2018). Not only do these characteristics promote tumour regeneration, CSCs are also extremely resistant to cytotoxic therapies, including gemcitabine (Zhou et al., 2021).

The hedgehog pathway ligand sonic hedgehog (SHH) is expressed in 70% of pancreatic cancers, and it is thought that its overexpression may contribute to tumorigenesis (Thayer et al., 2003). Hedgehog signalling is also upregulated in some subsets of pancreatic CSCs (Li et al., 2007). It has been demonstrated that use of the SHH specific inhibitor cyclopamine reduces pancreatic cancer cell growth and survival (Berman et al., 2003). Cyclopamine has also proven effective in combination with EGFR inhibitor Iressa, enhancing its antiproliferative effect (Hu et al., 2007). In conjunction with this, studies have shown that NF κ B contributes to SHH induction and hedgehog signalling pathway activation (Nakashima et al., 2006). These studies imply a crosstalk relationship between the EGFR and hedgehog pathways, suggesting that a series of signal transductions occur across several oncogenic pathways, in a complex and cooperative manner to drive pancreatic cancer progression. While cyclopamine has not been approved for clinical use due to chemical instability and poor bioavailability, further hedgehog pathway inhibitors are currently in clinical trials, shown in Figure 1.7. (Rosow et al., 2012).

The Wnt pathway is also highly implicated in pancreatic tumorigenesis, with its inhibition leading to both decreased proliferation and induced apoptosis (Pasca di Magliano et al., 2007). Wnt proteins bind with Frizzled receptors to trigger downstream activations that inhibit β -catenin phosphorylation. This enables β -catenin to enter the nucleus to bind to T-cell factor/lymphoid enhancer-binding factor TCF/LEF transcription factors, activating target genes (Pasca di Magliano et al., 2007). Several phase I clinical trials are currently investigating the use therapies targeting the Wnt protein, Frizzled receptor, and β -catenin function in pancreatic cancer (Figure 1.7) (Tanaka, 2016). Crosstalk has also been established between

the Wnt pathway and NF κ B, with this thought to significantly influence the promotion of inflammation and hence cancer progression (Ma and Hottiger, 2016). This again highlights the complex network of pancreatic cancer associated signal transduction pathways and promotes investigation into combined, targeted therapies. An outline of the described signal transduction pathways and clinically trialled targeted agents are outlined in Figure 1.7. This figure highlights molecular avenues of future therapeutic potential with continued research.

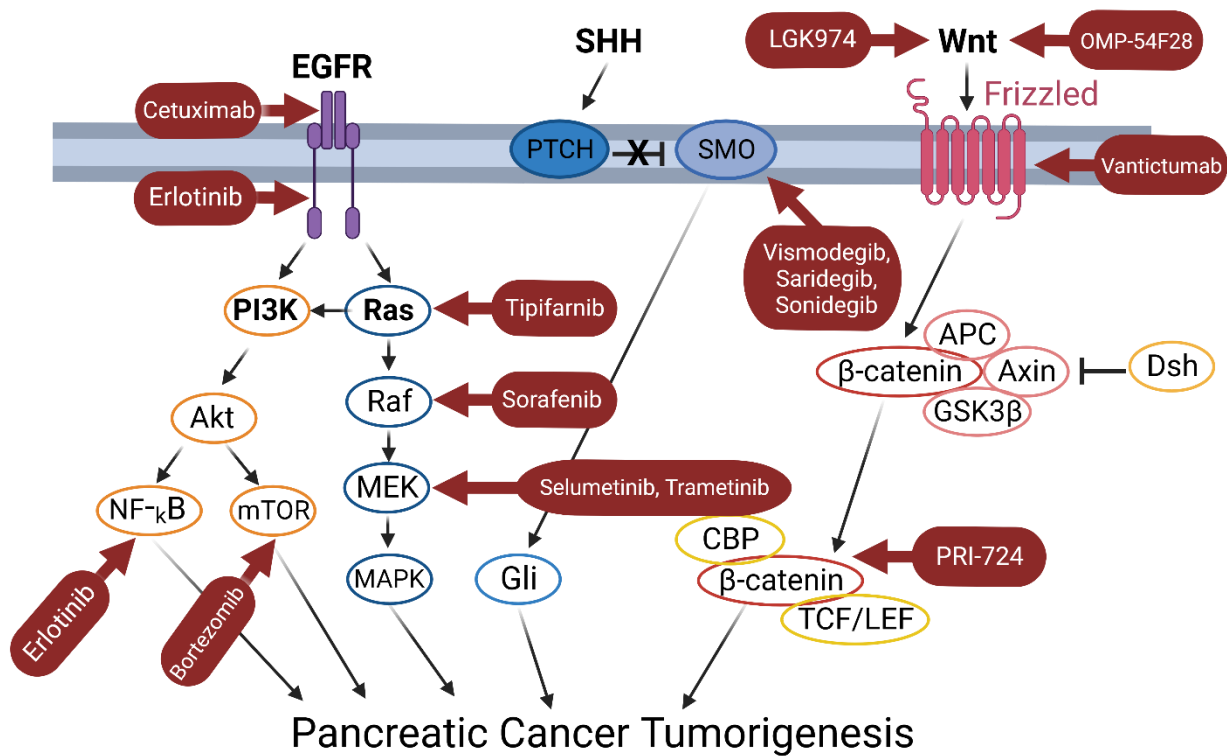


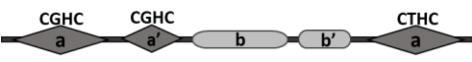
Figure 1.7. Clinically trialed targeted agents in oncogenic signal transduction pathways of pancreatic cancer. (Original Diagram, adapted from Tanaka, 2016). Cetuximab and Erlotinib target EGFR to prevent downstream activation of MAPK and PI3K pathways. Erlotinib also targets NF κ B of the PI3K pathway, Bortezomib targets mTOR. The MAPK pathway is targeted with Tipifarnib which inhibits Ras, Sorafenib which inhibits Raf and Selumetinib and Trametinib that target MEK. Vismodegib, Saridegib and Sonidegib all target SMO which is required for SHH driven responses. LGK974 and OMP-54F28 disrupt the Wnt protein, while Vantictumab prevents the binding of Wnt to the Frizzled receptor. PRI-724 prevents the binding of β -catenin and CBP. All of these targeted agents attempt to inhibit these influential oncogenic pathways in pancreatic cancer.

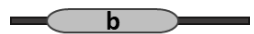
1.3 The Target: Protein Disulphide Isomerase

1.3.1 PDI Gene Family Structure

The protein disulfide isomerase (PDI) family is a group of multifunctional endoplasmic reticulum (ER) enzymes, recognised to comprise of a total of 21 members (HUGO gene nomenclature committee, 2019). The number of known human PDI members has increased rapidly over recent years as cDNA sequence data available in the public domain has continued to expand (Appenzeller-Herzog et al. 2008). Primarily PDIs are recognised to function as a catalyst in the formation, breakage and rearrangement of protein disulfide bonds (S-S bonds), however despite the implied isomerase function, not all members have been experimentally proven to demonstrate this activity (Lee et al. 2017). PDI family members are related in that they all possess at least one thioredoxin-like (TRX-like) domain (Appenzeller-Herzog et al. 2008). The TRX-like domains are categorised into two types; the a-type (a or a') domain is recognised as catalytically active, whilst the b-type (b or b') domain is recognised as catalytically inactive (Kozlov et al. 2010). Members possessing a-type domains typically possess a CXXC (Cys-X-X-Cys) motif in an active site, the cysteines are thiol-reactive and therefore allow for catalytic activity (Kozlov et al. 2010). CXXC is recognised as the consensus sequence and the most highly conserved catalytic motif is CGHC (Cys-Gly-His-Cys), typically termed the "classical" motif (Alanen et al. 2003). Whilst many members contain a combination of both a-type and b-type domains in various arrangements, there are some atypical members that only possess one domain type. Members that possess only b-type domains do not contain cysteines, and hence do not have a catalytic site sequence; they are therefore incapable of mediating S-S bond formation. These members have been shown to demonstrate a separate function believed to be involved in protein

recruitment, with roles as a molecular chaperone (Galligan et al. 2012). This demonstrates how PDI members are therefore solely unified on sequence similarity, particularly via the presence of a TRX-like domain, as opposed to their enzymatic activities. Table 1.3 demonstrates the diverse nature of the human PDI family, displaying that beyond the presence of a TRX-like domain, members differ considerably in length, substrate specificity and domain arrangement (Powell and Foster, 2021).

Gene Name	Other Aliases	Known Function	Domain Organisation	ER Retention Sequence
PDIA1	P4HB, PDI, PO4DB, ERBA2L	Forms/rearranges disulfide bonds of nascent proteins. Chaperone to inhibit aggregation of misfolded proteins.		KDEL
PDIA2	PDIp, PDA2	Intracellular estrogen-binding protein. Chaperone to inhibit aggregation of misfolded proteins.		KEEL
PDIA3	ERP57, ERP60, GRP57	Promotes formation of disulfide bonds in glycoprotein substrates. Chaperone to inhibit aggregation of misfolded proteins.		QEDL
PDIA4	ERP70, ERP72	Catalyses protein folding and thiol-disulfide interchange reactions. Enhances rate of IgG disulfide bonding and antibody assembly when bound to cyclophilin B.		KEEL

PDIA5	PDIR	Catalyses protein folding and thiol-disulfide interchange reactions. Binding site for ER chaperone calreticulin.		KEEL
PDIA6	P5, ERP5, TXNDC7	Regulates the UPR through binding to and inactivating ERN1 signalling. Chaperone to inhibit aggregation of misfolded proteins.		KDEL
PDILT	PDIA7	Chaperone involved in spermatogenesis.		KEEL
ERP27	PDIA8	Specifically binds unfolded proteins and may recruit PDIA3 to unfolded substrates.		KVEL
ERP29	PDIA9, ERP28	Processes secretory proteins in ER, possibly by folding ER proteins.		KEEL
ERP44	PDIA10, TXNDC4	Inhibits calcium channel activity of ITPR1. Retains ERO1A and ERO1B in ER and may play role in oxidative folding in ER.		RDEL
TMX1	PDIA11, TXNDC1	Catalyses protein folding and thiol-disulfide interchange reactions. Cell redox homeostasis.		-
TMX2	PDIA12, TXNDC14	Catalyses protein folding and thiol-disulfide interchange reactions. Cell redox homeostasis.		KKDK
TMX3	PDIA13, TXNDC10	Catalyses protein folding and thiol-disulfide interchange reactions.		KKKD
TMX4	PDIA14, TXNDC13	Catalyses protein folding and thiol-		RQR

		disulfide interchange reactions. Cell redox homeostasis.		
TXNDC 5	PDIA15, ERP46, Endo-PDI	Catalyses protein folding and thiol-disulfide interchange reactions. May protect hypoxic cells from apoptosis.		KDEL
TXNDC 12	PDIA16, TLP19, AGR1, ERP18, ERP19	Catalyses protein folding and thiol-disulfide interchange reactions.		EDEL
AGR2	PDIA17, XAG-2, HAG-2	Catalyses protein folding and thiol-disulfide interchange reactions. Roles in cell migration, cellular transformation and cell adhesion and is as a p53 inhibitor.		KTEL
AGR3	PDIA18, HAG-3, BCMP11	Catalyses protein folding and thiol-disulfide interchange reactions. Regulates ciliary beat frequency in multiciliated cells.		QSEL
DNAJC1 0	PDIA19, ERDJ5	Co-chaperone in ERAD. Reduces incorrect disulfide bonds in misfolded proteins, recognised by EDEM1.		KDEL
CASQ1	PDIB1	Calcium-binding protein in sarcoplasmic reticulum that acts as an internal calcium store in muscle.		-
CASQ2	PDIB2	Calcium-binding protein in sarcoplasmic reticulum that acts as an internal calcium store in muscle.		-

Table 1.3. PDI Gene Family Members. (Original Table, Powell and Foster, 2021).

As discussed, the presence of a TRX-like domain is one of the few unifying structural features of PDI family members, suggesting the occurrence of evolutionary divergence amongst human PDI proteins. Phylogenetic analysis of PDI, shown in Figure 1.8, demonstrates the existence of PDI subfamilies (Powell and Foster, 2021). Sequence analysis distinguished both the AGR subfamily and the CASQ subfamily as subsets of genes that appear most evolutionarily related. With the genetic distances represented as estimates of the number of mutations that have accumulated along each branch as they split from the common ancestor, the neighbourhood joining method of phylogenetic analysis can only be taken as an approximation. However, the data is supported by the fact that these subfamilies have also been recognised in literature for their structural differences (Powell and Foster, 2021). The AGR subfamily (AGR2, ARG3 and TXNDC12) is well-recognised as its members only carry a-type domains, without the presence of a b-type domain (Kozlov et al. 2010). The AGR subfamily members are therefore distinctly recognised for their catalytic role in disulfide bond formation. AGR2 and AGR3 are unique in that while they act to facilitate disulfide bond formation, they lack a C-terminal cysteine in their catalytic motif and so are recognised to possess non-canonical CXXS (Cys -X-X-Ser) motifs (Park et al. 2009). Recent research has demonstrated each cysteine in the consensus CXXC motif to play a unique role; the N-terminal cysteine acts to form disulfide bonds with the protein substrate; whereas the C-terminal cysteine plays a role in the release of the substrate (Xu et al. 2014). This suggests that the AGR subfamily may differ from other PDI members in substrate specificity. In contrast, studies have suggested that other PDI members such as PDIA1 and PDIA3 overlap in substrate specificity, with minimal differences (Rutkevich et al. 2010).

In contrast, CASQ1 and CASQ2 only possess b-type domains, and are recognised as their own unique subfamily. The CASQ subfamily act as the main calcium-binding proteins of the sarcoplasmic reticulum, with their primary role being the regulation of calcium release in muscle: Their role in non-muscle tissues is still unrecognised (Novák et al. 2011). They are also the only PDI members that do not possess an ER retention sequence (Shin et al. 2000). The CASQ subfamily are therefore seen as functionally distinct from other PDI members and could even be recognised to be irrelevant compared to the known functions of other PDI family members. Similarly, ERP27 and ERP29 also only possess b-type domains however, they are presumed to play important roles as a molecular chaperone to assist the conformational folding of proteins to assist proteostasis (Alanen et al. 2006). ERP29 in particular is recognised as an ER-stress inducible protein that co-localises with other ER stress associated chaperones such as BiP (Mkrtchian et al. 1998). The existence of these PDI subfamilies therefore reiterates the diverse nature of the PDI gene family and highlights the fact that sequence similarity is the primary determinate factor in the grouping of PDI members, as opposed to their enzymatic properties.

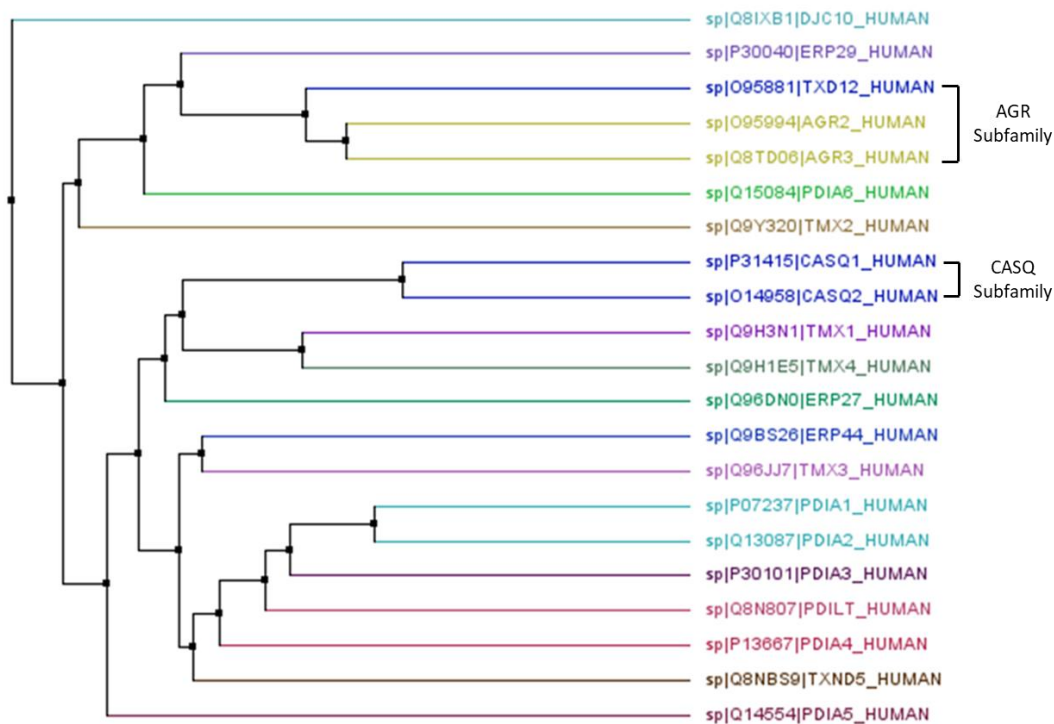


Figure 1.8. Phylogenetic tree of human PDI gene family. (Original Figure, Powell and Foster, 2021).

Alignment of protein sequences of human PDI genes was performed using ClustalW software and the phylogenetic tree was constructed by the neighbourhood joining method using Jalview software.

The evolutionary divergence of the PDI gene family is further demonstrated in DNAJC10 which appears dissociated from other PDI members in Figure 1.8. Whilst it possesses numerous TRX-like domains, DNAJC10 has been shown to exhibit no oxidase or isomerase activity and also only possesses approximately one third of the activity that PDIA1 possesses (Ushioda et al. 2008). Evolutionary divergence is also shown in PDILT, it is recognised as a completely unique member of the PDI family due to the fact that it is specifically expressed in the testis and contains unusual catalytic motifs (SKQS, SKKC) that are not present in any other PDI members (van Lith et al. 2005). PDILT has been demonstrated to attribute to S-S bond formation of the membrane-bound metalloprotease ADAM3 which acts to aid sperm migration

(Tokuhiro et al. 2012). Phylogenetic analysis of the human PDI gene family demonstrates that while members are phylogenetically related, a vast amount of evolutionary divergence is apparent. This is supported by the fact that considering the broad nature of both the domain composition and enzymatic functions, PDI members exhibit quite minimal overall sequence homology (Galligan et al. 2012).

1.3.2 PDI Function

The PDI gene family are highly abundant ER proteins, recognised as folding catalysts (Ferrari et al. 1999). With the primary function of disulfide bond formation, PDI plays a key role in the correct folding of polypeptide chains in the ER (Galligan et al. 2012). The formation of disulfide bonds occurs during oxidative folding in the ER, in which they are formed by the oxidation of thiol groups of cysteines and then isomerised to achieve the correct conformation (Depuydt et al. 2011). The post-translational modification that occurs through disulfide bond formation is essential for the stabilisation and maturation of most secretory and membrane proteins in the ER (Riemer et al. 2009). PDI therefore plays a critical role in ER proteostasis which aids the maintenance of several cellular functions such as, gluconeogenesis, calcium storage, organelle biogenesis and lipogenesis (Hetz, 2012). Disruption to ER proteostasis often therefore leads to the progression of multiple disease states through its various influences on cellular stress (Wang et al. 2019).

In addition to the role of PDI as a catalyst in disulfide bond formation and rearrangement, PDI proteins also function as molecular chaperones. In this role PDI acts to assist protein folding or refolding via the inhibition of non-productive folding or by the aggregation of damaged polypeptides or partially folded intermediates (Wang

et al. 2015). PDI is believed to inhibit the aggregation of these substrates through interaction with the portions of each substrate that have a tendency to self-associate; this is achieved by their ability to identify proteins in a non-naïve conformation (Hatahet et al. 2007). This role of PDI has been suggested to act as an entirely independent function from its role as a catalyst (Winter et al. 2002). PDI still acts to refold misfolded proteins that do not possess disulfide bonds, therefore suggesting the function of PDI as a molecular chaperone and its catalytic activity can be dissociated (Winter et al. 2002).

However, PDI can also demonstrate opposing behaviour with aggregation-prone substrates present at high concentrations, by inducing aggregation of misfolded substrates to in turn precipitate misfolded proteins (Sideraki et al. 2000). The ability of PDI to facilitate this aggregation as opposed to inhibit it is recognised as anti-chaperone activity (Puig et al. 1994). ER retention of misfolded proteins has been demonstrated in ER-chaperones that possess the KDEL retention sequence which remain in the ER with their unfolded protein cargo (Pelham, 1988). Misfolded proteins can also become trapped in the ER as large, chaperone-associated aggregates (Wilkinson et al. 2004). Whilst appropriate folding of these proteins will enable their secretion from the ER, this suggests that the anti-chaperone activity of PDI contributes to misfolded aggregate formation and accumulation in ER, resulting in significant ER stress (Lodish et al. 1993).

These observed characteristics of the PDI gene family primarily describe the archetypal PDI (PDIA1), however less is known about other PDI members. As

discussed, although structurally similar, each PDI member does possess a distinct substrate specificity. This has been shown through analysis of disulfide bond formation amongst major PDI members including PDIA1, PDIA3 and PDIA4, which revealed that each member was specialised for its own unique set of substrates (Jessop et al. 2009). For example, PDIA2 which was first identified as a pancreas specific PDI protein, although structurally very similar to PDIA1, is less effective in oxidation, differs in substrate specificity and acts to chaperone denatured substrates (Fu et al. 2010). This demonstrates how PDI members most likely differ in their functions and have divergent function within different tissues.

PDI activity is regulated by redox reactions, with the actions of PDI relying on cycles of reduction and oxidation (Schwaller et al. 2003). Whilst in a reduced state PDI acts to break non-native disulfide bonds by process of isomerisation, during an oxidised state PDI acts to correctly introduce and pair cysteines to form native disulphide bonds, and hence both reduced and oxidised pathways lead to the formation of a native protein (Schwaller et al. 2003). Redox reactions allow PDI to act in such ways through inducing changes in its conformation (Wang et al. 2015). Oxidation acts to alter PDI from a closed conformation to an open conformation, the substrate binding surface therefore becoming more greatly exposed, allowing for a higher activity level and binding affinity (Wang et al. 2012). Reduced PDI remains in a closed, compact conformation which is optimal for binding the substrates that require isomerisation (Wang et al. 2015). Disulfide formation is often error prone during early protein folding resulting in the incorrect pairing of cysteines impeding further folding (Gilbert, 1994). Thus, isomerisation is often required to amend these incorrect cysteines to

reform the cysteines to their native arrangement. This described mechanism is portrayed in Figure 1.9.

Disulfide Bond Formation

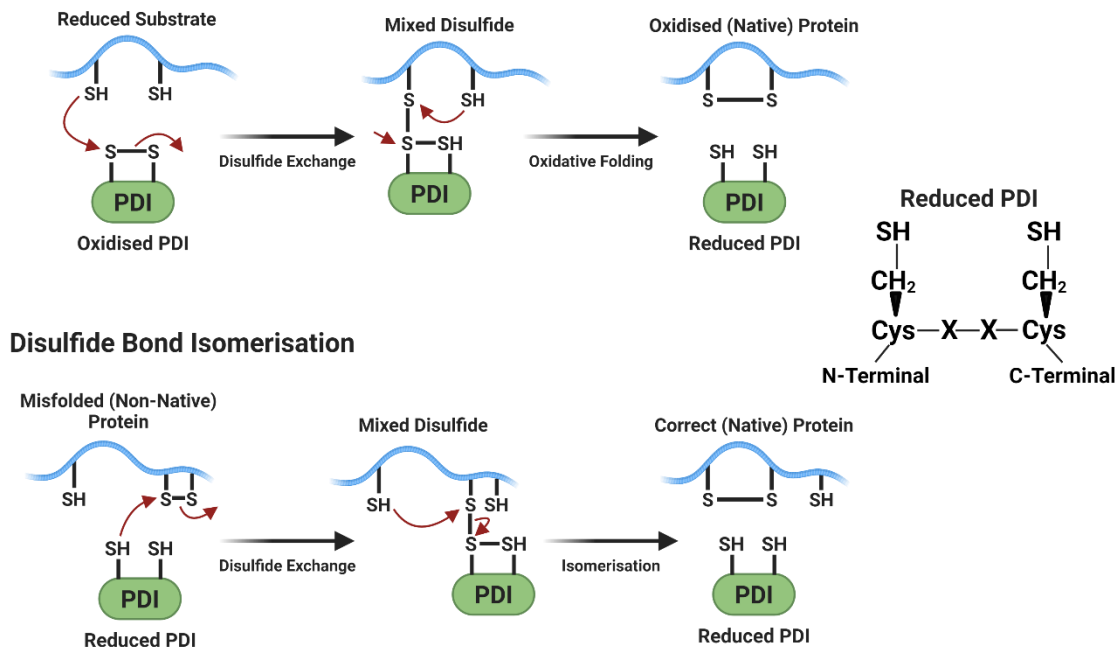


Figure 1.9. Redox reactions involved with PDI induced disulfide bond formation and isomerisation. (Original diagram, Powell and Foster, 2021). The mechanisms of disulphide exchange are shown in bond formation and isomerisation through redox reactions.

1.3.3 PDI in ER Stress

As previously discussed, PDI proteins play a key role in the regulation of proteostasis in the ER, a disruption in PDI activity would therefore result in a disturbance of proteostasis which would in turn trigger an ER stress response. With the accumulation of unfolded and misfolded proteins, ER stress then activates the unfolded protein response (UPR) (Lee et al. 2017). Initially, UPR acts to alleviate ER stress via the upregulation of chaperones for protein folding to combat the accumulation of unfolded proteins. The UPR further acts to induce endoplasmic

reticulum associated degradation (ERAD) combined with autophagy to remove misfolded proteins (Perri et al. 2016). The UPR therefore does play an important role in combatting ER stress and maintaining cell survival and proteostasis. However, this response is short-term, and a prolonged period of ER stress triggers a pro-apoptotic UPR (Perri et al. 2016). PDI is often found to be upregulated alongside other UPR proteins such as BiP and Grp94, and hence highlights the importance of both UPR and PDI, as a component of UPR, in the regulation of cell survival (Bartkowiak et al. 2010). This suggests that prolonged inhibition of PDI would potentially induce apoptosis and thus further highlights PDI as a potential therapeutic target in cancer.

Upon accumulation of unfolded/misfolded proteins, the UPR triggers the activation of three primary ER stress receptors; inositol-requiring enzyme 1 (IRE1 α), activating transcription factor 6 (ATF6) and pancreatic ER kinase (PKR)-like ER kinase (PERK), shown in Figure 1.10 (Li et al. 2011). These receptors remain in an inactive state through binding to BiP, an ER chaperone, however once UPR is triggered by ER stress BiP dissociates from the receptors, resulting in their activation (Rutkowski et al. 2006).

The activation of these receptors triggers several pro-protective pathways to aid cell survival. Activation of PERK induces the transcription of ATF4. ATF4 promotes the expression of ER chaperones such as PDI that refold misfolded proteins (Halperin et al. 2014). ATF4 also has further pro-protective effects through the activation of genes involved in autophagy and an antioxidant response (Cao and Kaufman, 2012). Similarly, activation of the IRE1 α pathway triggers the splicing of X-box binding

protein 1 (XBP-1), which belongs to the basic leucine zipper (bZIP) protein family, is a key transcription factor of UPR target genes (Park et al., 2021). XBP-1 acts to induce the accumulation of ER chaperones and ERAD-associated proteins to reduce ER stress (Park et al., 2021). IRE1 α also regulates phospholipid synthesis which triggers ER membrane expansion to help combat stress (Lee et al. 2003). Upon ATF6 activation, the cleaved ATF6 fragment also induces the expression of XBP-1, ERAD-associated proteins and ER chaperones (Schröder and Kaufman, 2005).

As discussed, in conditions of prolonged and chronic ER stress, the role of UPR can alter to pro-apoptotic rather than pro-survival. Also shown in Figure 1.10 are the pathways induced following chronic UPR. Prolonged activation of PERK elicits other responses as a consequence of ATF4 activation leading to CHOP upregulation. CHOP plays a key role in ER stress induced apoptosis through its ability to bind to several key UPR proteins, including ATF6 and ATF4 (Cao and Kaufman, 2012). CHOP also induces ERO1 α activation which acts to transfer electrons from PDI to O₂ to produce hydrogen peroxide, which causes increased oxidative stress (Cao and Kaufman, 2012). CHOP can also induce other pro-apoptotic proteins including p53 upregulated modulator of apoptosis (PUMA) and Bcl2-interacting mediator of cell death (BIM). Furthermore, CHOP inhibits survival proteins such as BCL-2 (Soo et al. 2009). Interactions with these proteins result in the activation of BAX and BAK associated apoptosis; CHOP is therefore a key player in mediating several apoptotic pathways (Hetz and Mollereau, 2014). The IRE1 α pathway also plays pro-apoptotic roles under chronic ER stress through the activation of the JNK pathway, which in turn interacts with BAX and BAK; it also induces pro-inflammatory associated apoptotic pathways (Hetz et al. 2006).

Whilst PDI plays the role of a pro-protective downstream ER chaperone, Figure 1.10 also shows how PDI members also govern UPR activation. The UPR stress receptors; IRE1 α , ATF6 and PERK are activated upon directly sensing unfolded protein accumulation. Whilst it is clear that PDI dysfunction leads to protein misfolding, recent studies have also described how PDI acts to mediate redox regulation of the luminal domains of ER stress receptors (Eletto et al. 2014). This redox regulation can trigger the activation of the receptors, which triggers the activation of downstream UPR pathways. Research suggests that PDIA5 acts to cleave disulfide bonds in ATF6 leading to its reduction (Higa et al. 2014). The reduction of ATF6, when coupled with BiP dissociation, leads to the ER to Golgi transport of ATF6, triggering the induction of downstream ATF6 target genes. (Higa et al. 2014, Nakanaka et al. 2007). Similarly, PDIA6 acts to exhibit thiol-disulphide exchange reactions with IRE1 α , leading to its reduction (Eletto et al. 2016, 2014). This leads to the direct binding of both PDIA6 and BiP to IRE1 α which inactivates the IRE1 α pathway, therefore acting to prevent exaggerated and chronic UPR signalling (Eletto et al. 2016, 2014). PDIA6 has also been described to interact with PERK, but its interaction is still yet to be fully understood (Eletto et al. 2016, 2014). However, there has also been evidence that PDIA3 interacts with PERK through redox regulation, via interaction with PDIA1 (Kranz et al. 2017). PDIA3 has been described to form a complex with PDIA1 in which it helps to maintain PDIA1 in its reduced state and prevent PERK activation (Appenzeller-Herzog et al. 2008). The absence of PDIA3 leads to an accumulation of oxidised PDIA1 which triggers PERK activation, suggesting that PDIA3 plays an important role in preventing chronic UPR activation. (Kranz et al. 2017). Further to this, PDIA1 has been shown to also interact with the

IRE1 α pathway through alteration in its activity via phosphorylation (Yu et al. 2020). The phosphorylation of Ser357 structurally alters PDIA1 to exhibit an open conformation which helps to prevent protein misfolding and also allows PDIA1 to bind to IRE1 α , where it acts to attenuate excessive and chronic UPR signalling (Yu et al. 2020). These recent studies demonstrate various ways in which PDI members are also acting as upstream regulators of the UPR pathway, further demonstrating their protective properties.

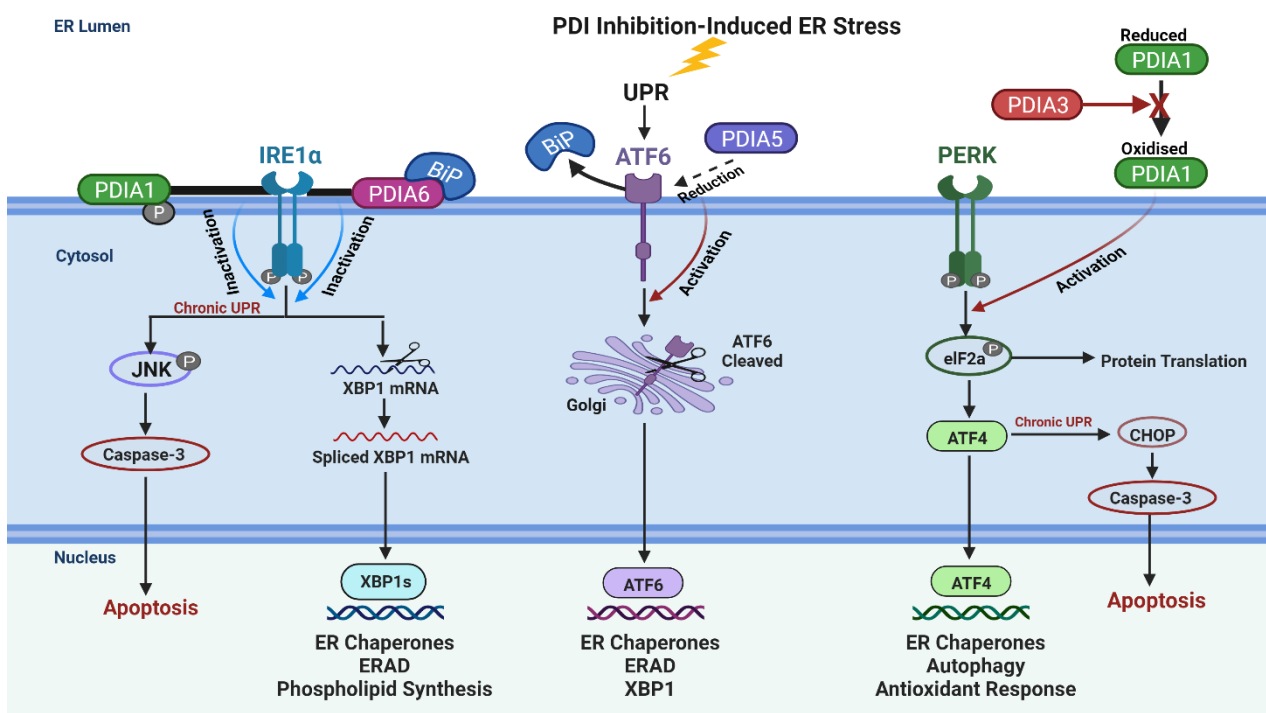


Figure 1.10. The roles of PDI members in the unfolded protein response pathway. (Original diagram, Powell and Foster, 2021).

PDI inhibition triggers a chronic UPR, triggering JNK and CHOP apoptotic pathways. IRE1 α , ATF6 and PERK UPR signalling arms under normal ER stress conditions trigger pro-protective mechanisms. BiP is shown in regulating IRE1 α and ATF6 pathway activations. PDIA1 and PDIA6 are shown to regulate IRE1 α , PDIA5 is shown in the activation of ATF6, and PDIA3 and PDIA1 are shown in the activation of PERK.

1.3.4 The role of UPR in Pancreatic Cancer

Recent research has demonstrated an association between UPR signalling and tumorigenesis. The contribution of ER stress and consequent UPR pathway activation has been well established in many cancer types including breast, colon, and prostate cancer (Madden et al., 2019). With the tumour microenvironment often under conditions of intrinsic and extrinsic stress, the UPR is frequently activated (Malhotra and Kaufman, 2007). Recent research has further suggested that extrinsic stress factors, hypoxia and nutrient deficiency are potential drivers of UPR activation and consequent tumour progression in pancreatic cancer (Long et al., 2021). By maintaining cellular homeostasis, the UPR has been implicated in each crucial stage of tumour progression, from initial transformation to uncontrolled cell growth, angiogenesis, and metastasis (Madden et al., 2019). It has also been suggested that the UPR may regulate differentiation in CSCs. (Liang et al., 2021).

Pancreatic cancer is known for its highly immunosuppressive tumour microenvironment which allows for survival under conditions of stress (Zhou et al., 2021). This implies that the UPR may play a crucial role in the ability of pancreatic cancer to regulate and tolerate high stress conditions. The ability of pancreatic cancer to tolerate intense conditions of stress can be observed through long-term gemcitabine treatment, whereby adaptation of the tumour microenvironment results in chemoresistance and decreased efficacy of treatment (Long et al., 2021).

Pathways of stress regulation such as UPR could also therefore contribute to chemoresistance, potentially through the upregulation of CSCs. While the specific mechanisms by which the UPR drives pancreatic cancer progression remain not fully

elucidated, research has shown that each of the three main UPR signalling branches are implicated in the hallmarks of cancer, shown in Figure 1.11.

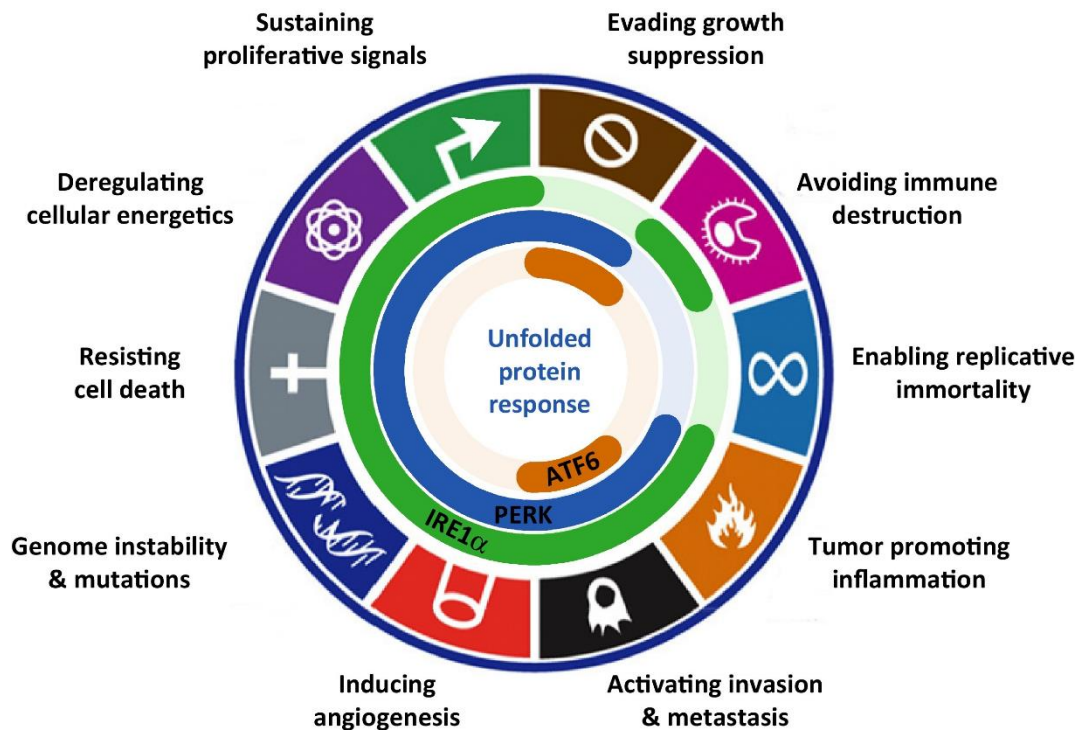


Figure 1.11. The role of UPR signalling branches, IRE1 α , PERK and ATF6 in the hallmarks of cancer. (Urrea et al., 2016).

The therapeutic potential of UPR inhibition is beginning to be explored. BiP, a key activator of UPR signalling pathways, is highly expressed in pancreatic cancer, coinciding with predictions of poor prognosis (Shen et al., 2017). Research has shown that haploinsufficiency of BiP disrupts KRAS-driven pancreatic cancer progression in Kras^{G12D/+} mice, without harmful effects on the pancreas (Shen et al., 2017). Though the importance of BiP in cell survival through the UPR has been highlighted, BiP has also been associated with previously described pancreatic oncogenic pathways; EGFR and PI3K. BiP has been reported to form a direct complex with EGFR under conditions of stress (Cai et al., 1998). Also, in

glioblastoma cells, small interfering RNA (siRNA) knockdown of BiP attenuated ERK activation, further suggesting a potential role in EGFR signalling (Zhang et al., 2011). Thus, BiP may be affecting downstream signalling of the Ras pathway and consequent KRAS-driven tumorigenesis. BiP inhibition has also been shown to suppress Akt signalling in tumours, suggesting influence in the PI3K signalling pathway, also important for pancreatic cancer development (Liu et al., 2013). Further investigations need to be made to assess the potential crosstalk and shared mechanisms between the UPR and other oncogenic signalling pathways in pancreatic cancer. This could lead to the development of novel therapeutic strategies, aimed at disrupting multiple oncogenic pathways.

One study demonstrated that treatment of PERK inhibitor, GSK2656157, induced an antitumour effect, reducing pancreatic tumour size in vivo (Atkins et al., 2013). However, with PERK inhibition also severely disrupting normal pancreatic function, more research is needed for such targets to be clinically trialled (Atkins et al., 2013). IRE1 α inhibitors have also proven to be beneficial in disrupting pancreatic cancer cell growth through both cell cycle arrest and induction of apoptosis (Thakur et al., 2018).

STF-083010 is another inhibitor that affects IRE1 α signalling through the disruption of XBP-1 splicing (Papandreou et al., 2011). As mentioned, spliced XBP-1 (XBP-1s), as an active transcription factor, induces the accumulation of ER chaperones and ERAD, reducing ER stress levels. STF-083010 treatment has been shown to decrease the viability of pancreatic cancer cells, also highlighting XBP-1s as a downstream potential therapeutic target (Chien et al., 2014).

Interestingly, the multiple kinase inhibitor sunitinib, which is clinically approved for treatment of various solid tumours, is also thought to disrupt IRE1 α autophosphorylation, although the exact mechanisms are not fully understood (Thakur et al., 2018). Sunitinib has recently been shown to increase survival in in vivo pancreatic cancer mouse models in combination with gemcitabine, with limited toxic effects (Thakur et al., 2018). These results therefore suggest that IRE1 α signalling inhibition could also enhance the efficacy of chemotherapy treatment. With a range of UPR-associated targeted therapies currently under investigation, but no direct UPR-targeted therapies approved for pancreatic cancer treatment, continued investigations into novel UPR targets are needed.

1.3.5 PDI in Cancer

Although PDI proteins are recognised as some of the most abundant cellular proteins, they are frequently upregulated in a variety of cancer types. Microarray data analysis has demonstrated the upregulation of several typical PDI members including PDIA1, PDIA3, PDIA4 and PDIA6 in multiple cancers such as breast, colorectal, liver, brain and prostate (Chawsheen et al. 2018). PDIA1, PDIA3, PDIA4 and PDIA6 demonstrate a higher level of expression compared with normal tissue across various cancer types (Powell & Foster, 2021). The overexpression of PDI is consistent throughout the majority of cancer types and PDI members. This implies that PDI could play an important role in promoting cancer cell survival.

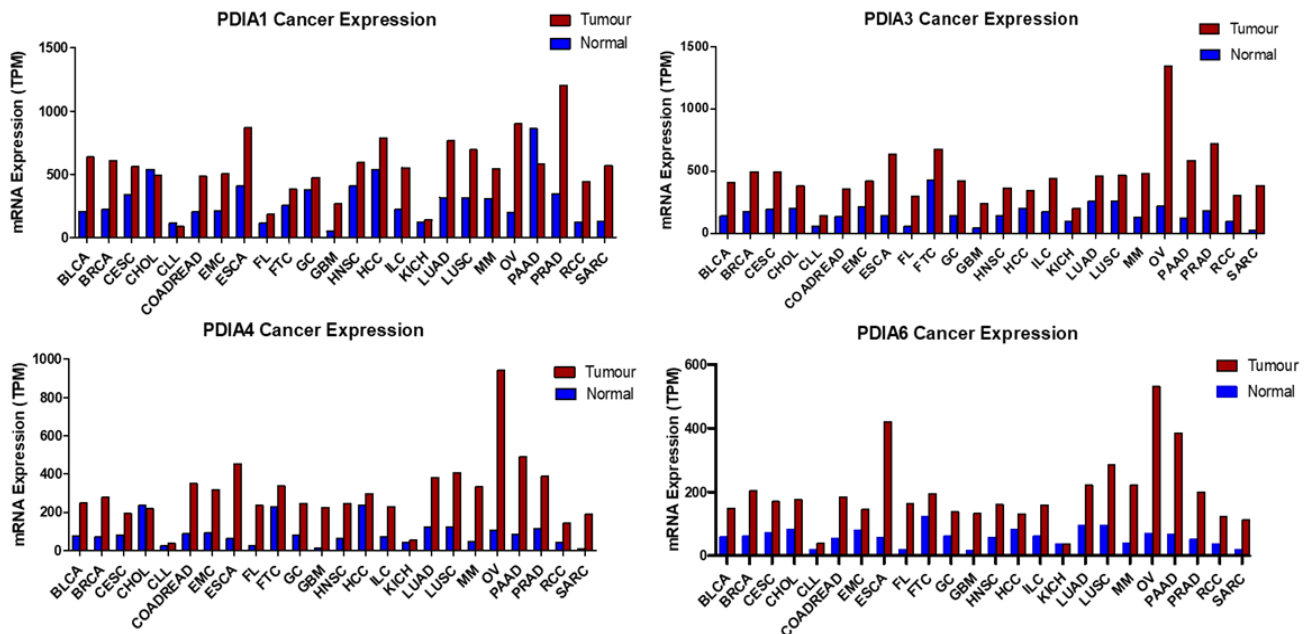


Figure 1.12. mRNA Expression of PDIA1, PDIA3, PDIA4 and PDIA6 in Tumour vs Normal Tissue across 24 cancer types; see TCGA abbreviations (Powell and Foster, 2021).

The pro-survival role of PDI has been demonstrated in recent research in lung cancer cells, in which both PDIA4 and PDIA6 were overexpressed and shown to mediate resistance to cisplatin-induced apoptosis (Tufo et al. 2014). Further research has shown that the knockdown of PDIA1 in breast cancer and neuroblastoma cell lines induced cytotoxicity via caspase activation (Hashida et al. 2011). This supports the hypothesis that the oncogenic effects of PDI are mediated by their role in the UPR signalling pathway and regulation of apoptosis. An association between PDI and the redox regulation of the UPR sensors has been recognised in recent research and therefore demonstrates the ability of PDI to influence the UPR (Kranz et al. 2017). For example, PDIA5 can act to cleave the disulfide bridges of the UPR sensor ATF6 to aid its transport to the nucleus (Higa et al. 2014). ATF6 has been associated with tumour cell resistance to chemotherapy, in

particular PDIA5/AFT6 signalling is associated with resistance to imatinib treatment in leukaemia cells, therefore demonstrating a pro-survival role of PDI (Higa et al. 2014). Additionally, PDIA6 was demonstrated to directly bind to the luminal domain of the UPR sensor IRE1 α through disulfide bond formation, in which it acted to control its activation and aid its return to its inactive state (Eletto et al. 2014). PDIA6 therefore acts to attenuate IRE1 α and thus alleviates excessive UPR activity, again demonstrating the pro-survival role of PDI.

Whilst PDI is recognised for its roles in UPR signalling and hence cancer cell survival, PDI also aids the activation of metalloproteases at the cell surface which act to catalyse the shedding of membrane-associated proteins (Willems et al. 2010). Surface PDI proteins act to catalyse the formation of disulfide bonds through their interaction with client proteins, including integrins, selectins and metalloproteins (Benham, 2012). Interestingly, research has shown PDI expression to be associated with the promotion of metastasis and invasiveness. For example, PDIA1 protein expression is significantly higher in the metastatic auxiliary lymph node breast tumour than in primary breast tumours (Thongwatchara et al. 2011). The catalysation of disulfide bonds in these client proteins by PDI members could be a factor contributing towards the positive correlation between PDI expression and cancer cell metastasis. Furthermore, PDI members may activate membrane proteins such as integrins or proteolytic enzymes such as matrix metalloproteinases (MMPs), both of which mediate cell migration and adhesion and ultimately contribute to metastatic spread (Xu et al. 2014).

PDI proteins have also been shown to contribute towards cancer progression through their involvement in other cancer-associated signalling pathways, aside from the UPR pathway (Lee et al. 2017). With PDI protein interaction being so vast, each PDI member can be seen as mechanistically distinct. For example, TXNDC5 is recognised to play a role in angiogenesis and is involved in the activation of the MAPK pathway. As previously mentioned, the MAPK pathway is well recognised for its association with various pro-oncogenic effects; with its activation playing key roles in cell proliferation, apoptosis, differentiation and cell migration (Santarpia et al. 2012). This highlights the fact that further research needs to be carried out into the pro-oncogenic roles of each PDI member, focusing on key associated downstream oncogenic pathways such as UPR, to determine their potential as a therapeutic target for cancer treatment.

1.3.6 The role of AGR2 in pancreatic cancer pathogenesis

Whilst the roles of PDI members in pancreatic cancer progression are still not fully understood, AGR2 could be considered the most studied in terms of its oncogenic nature. AGR2 is found to be overexpressed in many cancer types, including pancreatic cancer (Chevet et al., 2003). In terms of its role in the UPR, AGR2 has been shown to be upregulated through activation of both IRE1 α and ATF6 signalling arms (Higa et al., 2011). AGR2 plays an important protective role in maintaining ER homeostasis, with AGR-silenced cells showing an increased sensitivity to ER stress (Higa et al., 2011). Studies have found AGR2 expressed across all stages of pancreatic cancer progression, including the earliest PanIN lesions to metastatic lesions (Dumartin et al., 2011). Research has further suggested that ER-stress

induced AGR2 could be overexpressed prior to activating-KRAS mutations, highlighting it as a potential biomarker for early stage pancreatic cancer development (Dumartin et al., 2017).

AGR2 is also considered to potentially play an important role in later stage pancreatic cancer development, where it is found overexpressed in PanIN 3 lesions, post loss of SMAD4 (Norris et al., 2013). It has been proposed that loss of SMAD4 may lead to the upregulation of AGR2 and consequent increased mucin 1 (MUC1) expression, which drives metastasis (Norris et al., 2013). AGR2 has been further found overexpressed in pancreatic CSCs, suggesting its influence in promoting a chemoresistant tumour environment through the maintenance of cell stemness (Dumartin et al., 2017). With AGR2 likely to play several roles in pancreatic cancer pathogenesis, it can be considered a promising therapeutic target. The potential key influences of AGR2 overexpression in pancreatic cancer pathogenesis are shown in Figure 1.13.

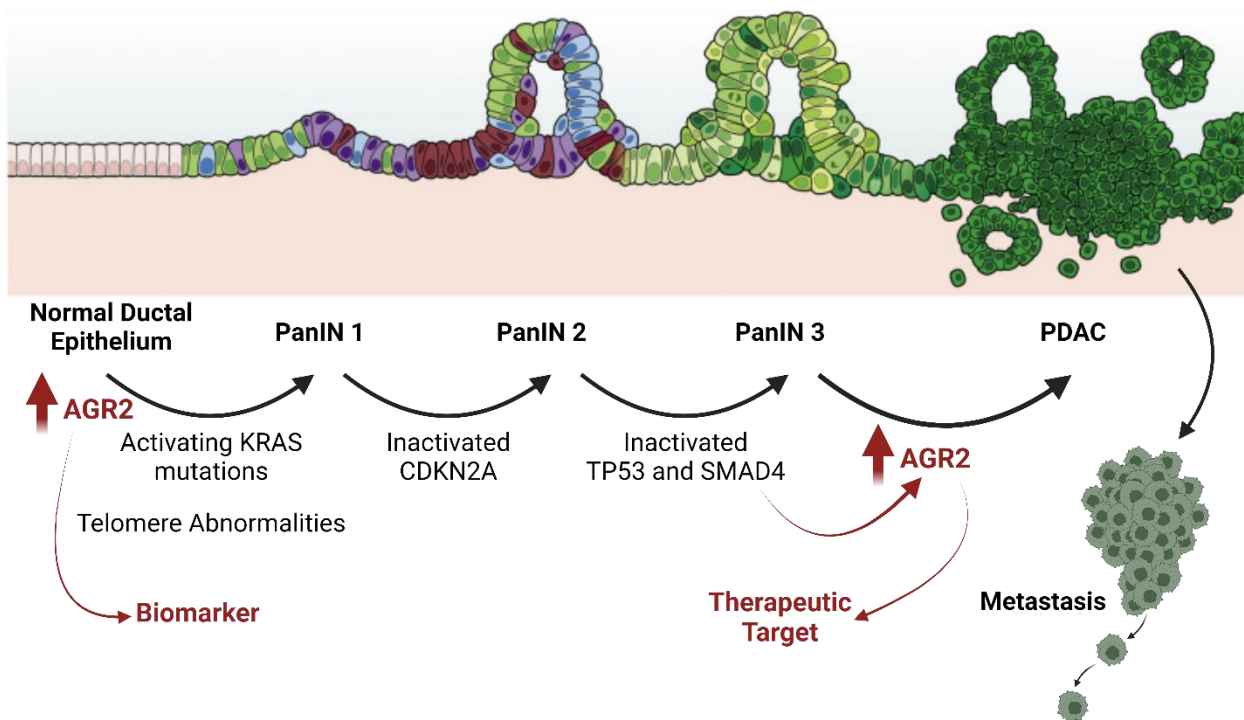


Figure 1.13. Role of AGR2 in pancreatic cancer pathogenesis. (Original diagram adapted from Grant et al., 2016 and references in text). AGR2 is upregulated prior to activating KRAS mutations, highlighting its biomarker potential. Inactivation of SMAD4 triggers AGR2 overexpression, highlighting it as a therapeutic target in pancreatic adenocarcinoma progression.

1.3.7 The role of AGR2 in oncogenic signalling pathways

The underlying mechanisms by which AGR2 may promote tumour progression remains poorly understood. While AGR2 has been outlined as a protective agent in the regulation of ER stress and hence cancer cell survival, AGR2 has shown some evidence of its implication in other oncogenic signalling pathways. One study has shown AGR2 to induce the expression of growth-promoting EGFR ligand, amphiregulin (AREG) (Dong et al., 2011). This therefore implies a role of UPR in EGFR signalling, an important oncogenic pathway of pancreatic cancer, as previously described. It has also been shown that AGR2 can act as an inhibitor of

p53, silencing its tumour suppressor abilities and promoting cancer cell survival (Pohler et al., 2004). With the inactivation of TP53 highlighted as a key event in pancreatic cancer pathogenesis, this implies further oncogenic influence of AGR2. Another study recently demonstrated the ability of AGR2 to activate cathepsins B and D (CTSB/D), which are known for their role in promoting tumour dissemination and consequent invasion (Dumartin et al., 2011). Both CTSB and CTSD have been shown to be upregulated in pancreatic cancer (Tumminello et al., 1996). This then highlights a potential pathway of AGR2-induced metastasis through CTSB/D activations. Studies in breast cancer have also outlined that AGR2 activates the tumour driver, cyclin D1 (Vanderlaag et al., 2010). The overexpression of cyclin D1 has been attributed to poor prognosis in pancreatic cancer patients, thought to promote both tumour growth and resistance to drug-induced apoptosis (Biliran et al., 2005). These studies further emphasise the tumorigenic role that AGR2 is likely to play in pancreatic cancer. The various oncogenic-associated pathways of AGR2 are outlined in Figure 1.14.

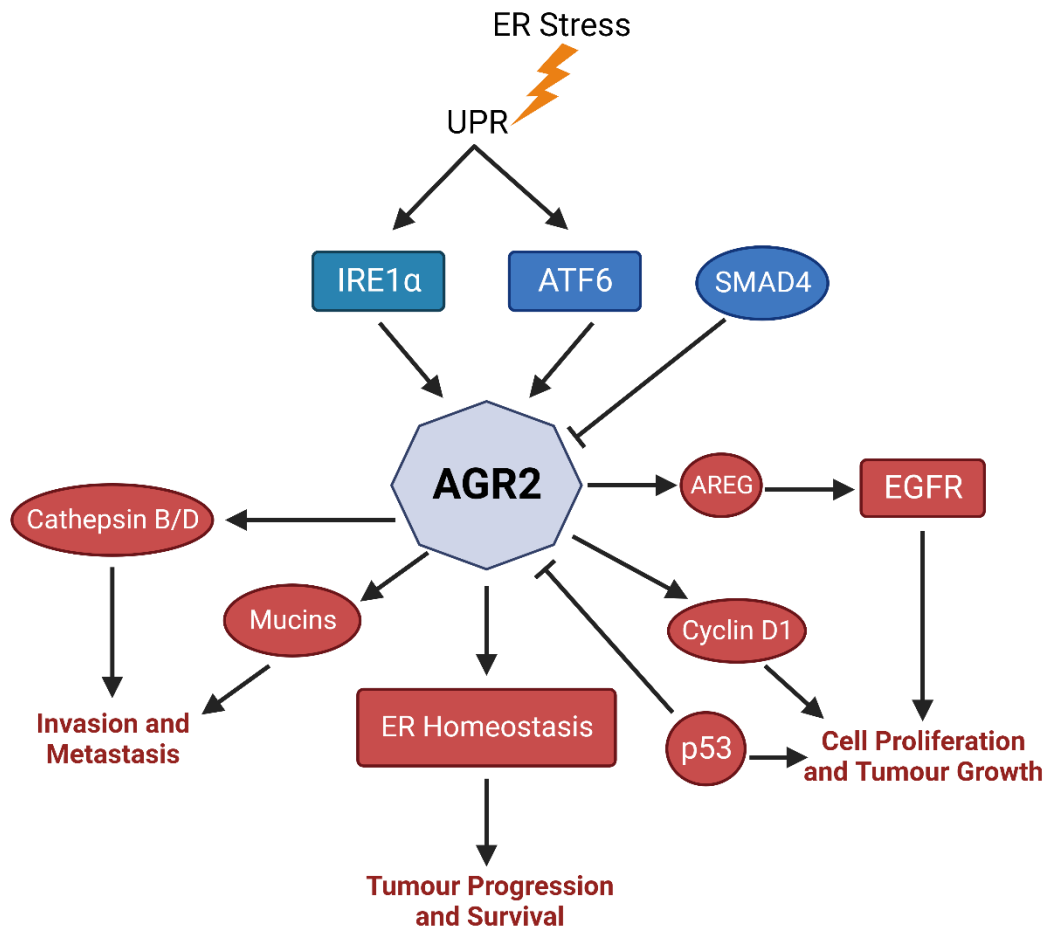


Figure 1.14. AGR2 oncogenic signalling pathways. (Original diagram, developed from references in text).

AGR2 is induced by IRE1 α and ATF6 signalling arms of the UPR. It is also induced by a loss of SMAD4. AGR2 acts to maintain ER homeostasis, enabling tumour progression and survival. It also induces EGFR and cyclin D1 pathway signalling and inhibits p53 to aid tumour growth. It also triggers pathways of metastasis through cathepsin B/D and mucin activations.

1.3.7 PDI Inhibitors

There is a clear potential therapeutic role for the use of PDI inhibitors in cancer treatment. Various groups worldwide have identified PDI inhibitors of numerous chemical varieties including both antibiotics and oestrogen polyphenols (Badolato et al. 2017). The use of the antibiotic bacitracin as a PDI inhibitor induces apoptosis

through the accumulation of ER stress in melanoma cells (Lovat et al. 2008). Apoptosis has also been observed in human breast cancer MCF-7 cells and human neuroblastoma SH-SY5Y cells as a result of PDI knockdown (Hashida et al. 2011). Contradictory to this, PDI knockdown in human cervical cancer HeLa cells did not demonstrate any effects on cell viability (Hashida et al. 2011). This suggests that the effects of PDI inhibition on apoptosis may be cell-type or cancer-type specific, therefore the development of PDI inhibitors may also be specified to certain cancer types. However, it is unclear from the research which PDI family member is the target of these knockdowns, this reiterates the need for further research into the roles of each specific PDI member to determine their potential as individual therapeutic targets. Bacitracin was additionally shown to inhibit cell migration and invasion in glioblastoma cells, therefore demonstrating the potential of PDI inhibition in combatting metastasis, as well as cancer cell growth and survival (Goplen et al. 2006). Despite these promising effects of bacitracin, it is not PDI specific as it has been shown to inhibit other proteins in the absence of PDI activity (Xu et al. 2014). With bacitracin exhibiting many off-target effects and although it has been a focus of research, it has not entered clinical trials for PDI-associated diseases due to its toxicity and poor cell permeability (Godin et al. 2004). Consequently, there is an unmet medical need for the development of function-specific, small molecule PDI inhibitors for a greater efficacy and more potent treatment.

Propionic acid carbamoyl methyl amides (PACMAs) have been recently recognised as small molecule irreversible PDI inhibitors that have demonstrated cytotoxic effects in a broad range of human cancer cell lines (Yamada et al. 2011). Most significantly, a series of PACMA derivatives were demonstrated to result in substantial cytotoxicity

in human ovarian cancer cells (Xu et al. 2012). The IC₅₀ values for these PACMA derivatives ranged from 0.2µM to >10µM, demonstrating a lack of potency across many compounds, this highlights the need for the development of compounds with greater efficacy. PACMAs act to irreversibly inhibit PDI by disrupting the PDI CXXC motif through the formation of a covalent bond (C-S) with the cysteines in the active site (Badolato et al. 2017). The disruption of the CXXC motif in PDI appears to be a very proficient method of PDI inhibition. This was demonstrated in PDIA1, which contains two independent active sites, in which it has been shown that the disruption of cysteines in either active site leads to a 50% loss of PDIA1 activity, whilst disruption at both active sites completely abolishes all PDIA1 activity (Vuori et al. 1992). Among these PACMA molecules, PACMA-31 was identified to be an orally active irreversible PDI inhibitor that has demonstrated both oral bioavailability and in vivo activity within a mouse xenograft model of human ovarian cancer (Xu et al. 2012). PACMA-31 has shown anticancer activity both in vitro and in vivo in human ovarian cancer, with no substantial toxicity to normal tissue; furthermore, it has also shown effectiveness against chemoresistant cell lines (Xu et al. 2012). This highlights PDI as a druggable target and further promotes research into the use of small molecule PDI inhibitors in cancer treatment.

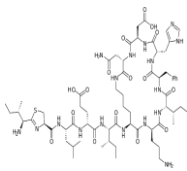
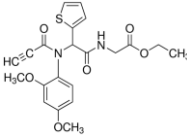
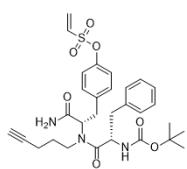
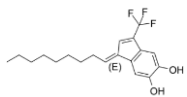
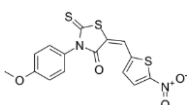
Table 1.4 shows the characteristics of several PDI inhibitors of which have previously shown therapeutic potential in cancer treatment. Like PACMA-31, P1 is a similarly acting, irreversible PDI inhibitor. However, it exhibits a greater potency than PACMA-31 with an IC₅₀ of 1.7µM, measured via an in vitro insulin aggregation assay (Ge et al. 2013). P1 also demonstrates inhibition of cell growth across numerous cancer cell lines (Ge et al. 2013). It is again notable however, that current research

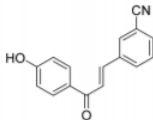
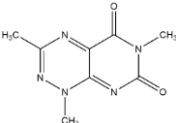
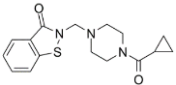
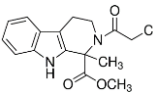
has only shown P1 to have the ability to inhibit PDIA1, but this is not selective (Ge et al. 2013). PACMA-31 on the other hand has shown the ability to bind to and inhibit further PDI members. With a similar mechanism of action, it can be suggested that P1 would also target these members. This reiterates the need to conduct further research to target specific PDI members in order to establish which members are crucial for their role in tumorigenesis.

Further observations of the role of PDIA1 in cancer can be made with the use of selective PDIA1 inhibitors; KSC-34 and RB-11-ca. Both of these compounds act to inhibit PDIA1 by inhibiting a and a' domain sites (Cole et al. 2018). Comparison of these selective PDIA1 inhibitors with E64FC26; a potent pan-style inhibitor which inhibits; PDIA1, PDIA3, PDIA4, PDIA6 and TXNDC5, would give an indication as to whether inhibition of PDIA1 alone is sufficient for suppressing cancer cell growth (Robinson et al. 2019). This then promotes further studies into the selective inhibition of other PDI members.

Table 1.4 demonstrates how different PDI inhibitors vary in their mode of action. Whilst KSC-34 and RB-11-ca specifically inhibit a-type domains of PDIA1, BAP2 specifically inhibits b-type domains of PDIA1 (Xu et al. 2019). BAP2 has demonstrated apoptotic effects in glioblastoma cells, therefore comparison with KSC-34 and RB-11-ca could give insight into the roles of catalytic a-type sites and non-catalytic b-type sites in driving cancer cell growth. Research has also shown 35G8 to have anti-cancer effects in glioblastoma through the induction of cell death via autophagy and ferroptosis rather than apoptosis (Kyani et al. 2018). Although the

mechanism of action of 35G8 is unknown, it appears to lack drug-like properties as rather than specifically targeting PDI, it acts to repress PDI target genes such as TXNIP and EGR1 (Kyani et al. 2018). 35G8 is also recognised as a redox cycling molecule (Xu et al. 2019). This suggests a potential use of 35G8 as an adjuvant in combination with other PDI inhibitors such as BAP2 as a potential therapeutic strategy. The vast range of PDI inhibitors promotes further research into the discovery of the most effective mode of action of PDI inhibition and the most important PDI members to target in order to develop novel drugs and improved therapeutic strategies.

PDI Inhibitor	Chemical Structure	Mode of Action	IC50	Known PDI Members Inhibited	Cell Based and Pre-clinical Studies	Ref.
Bacitracin		- Competitive inhibitor - Binds to free thiols in substrate binding domain - Cell impermeable - Reversible	150-200µM	PDIA1	Enhances apoptosis in melanoma cells and inhibits migration and invasion of glioblastoma cells.	Dickerhof et al., 2011; Kaplan et al., 2015.
PACMA-31		- Binds to cysteine residues in active site - Cell permeable - Irreversible	10µM	PDIA1, PDIA3, PDIA4, PDIA6, TXNDC5.	Inhibits human ovarian cancer cell growth. Inhibits proliferation of OVCAR-8 in culture and in a tumour xenograft model.	Robinson et al., 2019; Xu et al., 2012.
P1		- Binds to cysteine residues in active site. - Cell permeable - Irreversible	1.7µM	PDIA1	Inhibits proliferation of cancer cell lines; MCF-7, Hep-G2, MDA-MB-231, UACC-257, T47D.	Ge et al., 2013.
E64FC26		- Pan-style inhibitor - Mechanism unknown - Cell Permeable	1.9µM	PDIA1, PDIA3, PDIA4, PDIA6, TXNDC5	Induces apoptosis and cytotoxic effects in multiple myeloma cells.	Robinson et al., 2019.
CCF642		- Allosteric inhibitor - Binds to conserved lysine directly adjacent to the active site - Cell permeable	2.9µM	PDIA1, PDIA3, PDIA4	Induces apoptosis and cytotoxic effects in multiple myeloma cells. Prolongs the lifespan in multiple	van Lith et al., 2005.

		- Irreversible			myeloma mouse model.	
BAP2		- Allosteric inhibitor - Binds to b' domain - Cell permeable	0.9µM	PDIA1, PDIA2	Inhibits tumour growth both in vitro and in vivo in glioblastoma.	Xu et al., 2019; Yang et al., 2019.
35G8		- Mechanism Unknown	0.17µM	Represses PDI target genes such as TXNIP and EGR1.	Induces cell death via autophagy and ferroptosis in glioblastoma cells.	Kyani et al., 2018.
LOC-14		- Allosteric inhibitor - Binds adjacent to active site, forces protein to maintain oxidized conformation - Cell permeable - Reversible	5µM	PDIA3	Antiapoptotic, neuroprotective function on nerve cells in a model of Huntington disease.	Chamberlain et al., 2019; Kaplan et al., 2015.
16F16		- Binds to cysteine residues in active site - Cell permeable - Irreversible	~70 µM	PDIA1, PDIA3	Prevent apoptosis induced by mutant huntingtin protein and neuroprotective in rat neurons.	Ge et al., 2013; Hoffstrom et al., 2010.

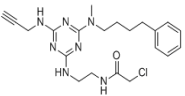
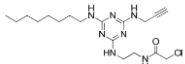
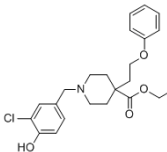
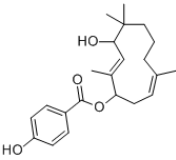
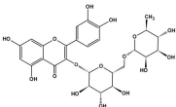
KSC-34		- Selective PDIA1 inhibitor - Inhibits C53 in a domain active site. - Cell permeable	N/A	PDIA1	30-fold selectivity for the a-site over the a' site and shows time-dependent inhibition of PDIA1 reductase activity in vitro.	Cole et al., 2018.
RB-11-ca		- Selective PDIA1 inhibitor - Inhibits C53 in a domain active site. - Cell permeable - Irreversible	30-50μM	PDIA1	Inhibits proliferation of HeLa cells.	Cole et al., 2018.
ML359		- Likely binds to b' domain - Cell permeable - Reversible	0.25μM	PDIA1	Inhibits platelet aggregation. Not cytotoxic in human cell lines.	Bendapudi et al., 2014; Khodier et al., 2010.
Juniferdin		- Mechanism unknown. - Cell permeable - Reversible	0.16μM	PDIA1	Inhibits reduction of HIV-1 gp120 and reduces influenza virus replication. Cytotoxic in several cell lines.	Khodier et al., 2010.
Quercetin-3-rutinoside		- Binds to b' domain - Cell impermeable - Reversible	6μM	PDIA1	Inhibits platelet aggregation and blocks thrombus formation in vivo. Not cytotoxic.	Jasuja et al., 2012.

Table 1.4. PDI Inhibitor Characteristics. (Original Table, Powell and Foster, 2021; References in Table).

1.4 Hypothesis and Aims

There is compelling evidence that the UPR is frequently activated in pancreatic cancer to regulate ER homeostasis under conditions of ER stress. The UPR is thought to therefore contribute to the chemoresistant nature of the pancreatic tumour microenvironment, which is synonymous with the lack of effective treatments in advanced and metastatic disease. PDI acts as a vital component of the UPR, relieving ER stress through its protein folding and chaperone abilities, promoting cell survival. This highlights PDI as a potential therapeutic target. However, the specific impact of each PDI family member on pancreatic cancer progression is not yet fully understood. Nor is it understood how these PDI members interact with specific UPR pathway genes to promote pancreatic cancer cell survival. It is hypothesised that the inhibition of PDI promotes prolonged UPR pathway activation, resulting in a cellular signalling switch from pro-survival mechanisms to ER stress-induced apoptosis. With PDI also implicated throughout several pancreatic cancer associated-oncogenic pathways, it is also hypothesised that PDI inhibition will disrupt cell proliferation and migration.

The overall aim of the study is to develop novel therapeutic strategies through the investigation of the roles of PDI and UPR pathway members in pancreatic cancer progression. This was investigated through the following objectives:

- To investigate how PDI expression is dysregulated in pancreatic cancer and identify which members are potential key therapeutic targets.
- To investigate how PDI inhibition affects pancreatic cancer cell proliferation and migration.

- To better elucidate the downstream UPR mechanisms of PDI inhibition in pancreatic cancer through the measurement of ER stress marker expression and apoptosis.
- To explore the potential of PDI inhibition and chemotherapy combination treatments.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Description of Pancreatic Cell Lines

Pancreatic cancer cell lines used in experiments were Panc-1, Mia-PaCa-2 and AsPC-1 (ATCC). Panc-1 was established from a 56-year-old male with an adenocarcinoma, isolated from the pancreatic duct. Mia-PaCa-2 and AsPC-1 were both derived from an adenocarcinoma within the head of the pancreas, with Mia-PaCa-2 being isolated from a 65-year-old male patient and AsPC-1 a 62-year-old female. Panc-1 and AsPC-1 both showed evidence of metastasis, whilst this was undetermined in Mia-PaCa-2.

Panc-1 and Mia-PaCa-2 were cultured under standard conditions in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Thermo Fisher, UK), supplemented with 10% foetal bovine serum (FBS) (Sigma) and 1% Penicillin (100units/ml)/Streptomycin (100µg/ml) (Gibco, Thermo Fisher, UK). AsPC-1 was grown in Roswell Park Memorial Institute (RPMI) medium (Gibco, Thermo Fisher, UK), supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, UK) and 1% Penicillin/Streptomycin (100 µg/mL) (Gibco, Thermo Fisher, UK). Cell lines were maintained at 37°C in a humidified 5% CO₂ incubator and media was refreshed twice a week. Cells were passaged routinely, according to ATCC culture methods. Cells were grown in either 75cm² or 25cm² cell culture flasks (Corning Ltd, UK) and passaged into new flasks once the cells had reached 80% confluence. Once confluent, the media was removed, and cells were washed in 5ml of 1x Phosphate-Buffered Saline (PBS) (Sigma-Aldrich, UK). Trypsin (Thermo Fisher, UK) was added at a volume of 1.5ml (1X working concentration in phosphate buffered saline with 1

mM EDTA) and the cells were incubated at 37°C for 3 minutes. Media was then added to inactivate and neutralise the trypsin (Thermo Fisher, UK) and cells were centrifuged at 1500 rpm for 4 minutes. The cell pellet was then resuspended and transferred into a new flask at a ratio of 1:3-1:6. Cells were also plated into 96-well, 24-well and 6-well plates (Corning Ltd, UK) during experimental work.

2.2 Drug Treatments

Cells were treated with the PDI inhibitors PACMA-31 (Bio techne, UK) and CCF642 (Bio-technique, UK) at concentrations shown in Table 1.5. Cells were also treated with Gemcitabine (Thermo Fisher, UK) at concentrations shown in Table 2.1. All drug compounds were of experimental grade and stored according to the manufacturer's instructions. DMSO was used as a vehicle for both PACMA-31 and CCF642.

PACMA-31 and CCF642 were dissolved in DMSO and aliquoted into 5ml Eppendorf tubes at a concentration of 1mM and stored at -20°C. All treatments were directly added to DMEM or RPMI with 10% FBS and 1% Penicillin (100units/ml)/Streptomycin (100µg/ml). A vehicle control of 0.1% DMSO was added to the culture medium of untreated cells in all drug treatment-based studies. All cells were treated for 48 hours, unless stated otherwise. Treatment concentrations were chosen based on the IC₅₀, shown in Table 2.1. PACMA-31 and CCF642 were chosen based on PDI inhibition results shown in previous studies in ovarian cancer and multiple myeloma, as stated in Table 1.4. Gemcitabine was used in combination studies, as the most commonly used chemotherapy treatment in pancreatic cancer patients, to emulate this cytotoxic effect.

Treatment		IC50 (μM)	Final Concentrations (μM)				
PACMA-31		10 (MedChemExpress, 2024)	1	5	10	50	100
CCF642		2.9 (MedChemExpress, 2024)	0.1	0.5	1	5	10
Gemcitabine	Panc-1	58.2 (Huanwen et al., 2009)	0.5		50		100
	Mia-PaCa-2	11.4 (Huanwen et al., 2009)	0.05		10		50
	AsPC-1	0.31 (Huanwen et al., 2009)	0.05		0.5		10

Table 2.1. Treatments and Concentrations Used.

2.3 Small interfering RNA (siRNA) Transfection

Cells were transfected with siRNA to suppress the PDI genes of interest, PDIA1 and AGR2. siRNA transfection was carried out using ON-TARGET plus SMART pool siRNA (Dharmacon, UK). Cells were seeded at 120,000 cells/well in 6-well plates (Corning, UK) and transfected 24 hours post culture using DharmaFECT Transfection Reagent 1 (Dharmacon, UK) in serum and antibiotic-free media, following the manufacturer's instructions. Transfections were optimised for PDIA1 and AGR2 siRNA to achieve a final concentration of 6.25nM with 48 hours treatment. A non-targeting scrambled siRNA (Dharmacon, UK) was used as negative control at 6.25nM. Table 2.2 shows the siRNAs used in the study.

First, the siRNAs were diluted in RNase-free water (Sigma Aldrich, UK) to a working concentration of 5 μ M. Using the working solution, the siRNA was again diluted to the optimal treatment concentration of 6.25nM in serum and antibiotic-free media (DMEM medium for Panc-1 and Mia-PaCa-2 cell lines, and RPMI medium for AsPC-1, with no foetal bovine serum or antibiotic added). The working solution of DharmaFECT Transfection Reagent 1 was then prepared by diluting 4 μ l in 196 μ l serum and antibiotic-free DMEM/RPMI media. Both the siRNA and transfection reagent solutions were mixed and incubated at room temperature for 5 minutes. The transfection reagent solution was then added to the siRNA solution at a 1:1 ratio and thoroughly mixed. The prepared siRNA complex was then incubated at room temperature for 20 minutes. The siRNA complex (400 μ l) was added to each well of the 6 well plate, in addition to 1.6ml of complete media (DMEM/RPMI with 10% foetal bovine serum and 1% Penicillin [100units/ml]/Streptomycin [100 μ g/ml]). The wells were mixed gently and incubated for 48 hours. Following the 48-hour transfection, cells were either reseeded for use in cell proliferation (see sections 2.10.2 and 2.10.3) and migration (see section 2.13) assays or extracted for RNA (see section 2.4) or protein (see section 2.7) collection for use in RTq-PCR (see section 2.6), Western blotting (see section 2.9) or caspase-3 activity assays (see section 2.12).

siRNA Product	ID
ON-TARGETplus Human P4HB siRNA	L-003690-00-0005
ON-TARGETplus Human AGR2 siRNA	L-003626-00-0005
ON-TARGETplus Non-targeting Control siRNAs	D-001810-01-05
DharmaFECT 1 Transfection Reagent	T-2001-01

Table 2.2 Details of siRNA products used.

2.4. RNA Extraction

RNA was harvested using the RNeasy® Mini RNA extraction kit (Qiagen, UK) following the protocol for 'Animal Cells Spin'. RNA was harvested from cells in 6-well plates (Corning, UK) following 48-hour PDI inhibitor, siRNA knockdown or combination experimental treatments. Media was aspirated from the wells and 117µl RLT buffer, containing 1:100 β-mercaptoethanol, added to each well of the 6 well plate. Cells were lysed in RLT buffer using a cell scraper from 3 wells of the 6 wells plate and combined into one Eppendorf tube to equate to 350µl total RLT Buffer per sample. This allowed for a high concentration of RNA to be collected in each sample. Ethanol (350µl) was added at 70% concentration to each sample and homogenised. The sample was then transferred to a RNeasy spin column with a 2ml collection tube and centrifuged at 10,000rpm for 15 seconds. The flow-through was discarded and 700µl of RW1 buffer was then added to the RNAeasy column for washing of the membrane-bound RNA. The RNeasy column was again centrifuged at 10,000rpm for 15 seconds, and flow-through discarded. RPE buffer (500µl) was added as a concentrated washing buffer, followed by centrifugation at 10,000rpm for 15 seconds, followed by 2 minutes with a second addition and the flow-through

discarded. To ensure the pellet was dry, the column was centrifuged for a further 1 minute at full speed. To elute the RNA, the column was placed in a new 1.5ml Eppendorf for collection and 40µl of RNase free water was added. The column was then centrifuged at 10,000rpm for 1 minute to elute the RNA. Eluted RNA was stored at -80°C.

2.5 cDNA Synthesis

Concentration and purity (according the ratio of absorbance at 260–280nm) of extracted RNA was measured using a Nanodrop spectrophotometer (Nanodrop ND-1000, Thermo Scientific). cDNA synthesis was then performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. RT Master Mix (2x) was prepared at a volume of 10µl for a 20µl reaction. The 2x RT Master Mix was comprised of; 2µl RT buffer, 0.8µl 25x dNTP Mix (100mM), 2µl 10x RT random primers, 1µl MultiScribe Reverse Transcriptase and 4.2µl Nuclease-free H₂O. 10µl of 2x RT Master Mix was pipetted into a PCR reaction tube, with the addition of 10µl of RNA (diluted in RNase free water to a final concentration of 100ng/µl), to equate to one sample. The PCR tubes were then put into a thermal cycler, programmed with the following optimised conditions for 35 cycles, shown in Table 2.3. cDNA samples were stored at -20°C.

Stage	Conditions	Function
1	25°C for 10 minutes	Annealing and pre-primer extension
2	37°C for 120 minutes	Incubation
3	85°C for 5 minutes	Inactivation
4	Cool to 4°C	

Table 2.3. Thermal Cycler Conditions for cDNA Synthesis.

2.6 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR reactions were performed on Applied Biosystems™ MicroAmp™ fast optical 96-well reaction plates. For every reaction, 1µl of cDNA was used and added to a reaction mix containing 5µl TaqMan fast advanced PCR Master Mix, 0.5µl TaqMan Gene Expression Assay and 3.5µl of RNase free water (Applied Biosystems, UK). mRNA levels were determined by real-time PCR using TaqMan gene expression assays (Thermo Fisher, UK), listed in Table 2.4. Relative mRNA levels were normalised to the mean CT value of the housekeeping gene RPLP0 Hs99999902_m1 (ThermoFisher, UK). PCR amplification was carried out in 50 cycles at the following conditions; enzyme activation for 10 minutes at 95°C, denaturation for 15 seconds at 95°C and extension for 1 minute at 60°C. Reactions for each gene were run in triplicate and included a non-template control. Results were analysed in a QuantStudio 5 Real Time PCR machine (Applied Biosystems, UK) and thereafter ΔCT and $\Delta\Delta CT$ were calculated according to the $2^{-\Delta\Delta CT}$

method. The following formulas were used to calculate the relative expression using the $2^{-\Delta\Delta CT}$ method:

$$\Delta Ct = Ct (\text{target gene}) - Ct (\text{housekeeping gene})$$

Ct (target gene) indicates the value of the threshold cycle for the gene of interest (e.g. PDIA1 or AGR2), and Ct (housekeeping gene) indicates the value of the threshold cycle for the housekeeping gene used as normaliser (RPLP0). The following formula was then used to calculate the relative expression of all samples in comparison with the Ct values obtained from the corresponding untreated cells (control):

$$\text{Relative expression} = 2^{-\Delta\Delta Ct} [\text{where } \Delta\Delta Ct = \Delta Ct (\text{sample}) - \Delta Ct (\text{control})]$$

ΔCt (sample) indicates the difference between Ct values of the target gene and the housekeeping gene calculated for the test sample, ΔCt (control) indicates the difference between the Ct values of the target gene and the Ct values of the housekeeping gene(s) obtained from the corresponding untreated cells (control).

GENE	CODE
RPLP0	Hs99999902_m1
PDIA1	Hs01050257_m1
AGR2	Hs00356521_m1
XBP-1S	Hs03929085_g1
CASPASE-3	Hs00234387_m1

Table 2.4. TaqMan gene expression assays used.

2.7 Protein Extraction

Using RIPA lysis buffer (Sigma Aldrich, UK) containing protease inhibitor cocktail (Sigma Aldrich, UK), protein was extracted from cell lines. The protease inhibitor was added to the RIPA buffer at a 1:10 dilution. Post either PDI inhibitor, siRNA knockdown or combination experimental treatment, protein samples were collected from two wells of a 6 well plate (Corning, UK) where 50µl of the buffer solution was added to each well and cells were detached by scraping. Total protein lysate at a volume of 100µl was collected for each sample and stored at -80°C.

2.8 Protein Concentration Measurement

Protein concentration was determined colorimetrically using the BCA Protein Assay Kit (Thermo Fisher, UK). The assay determines protein concentration through the protein mediated reduction of Cu^{2+} to Cu^{+} which occurs in an alkaline environment. Cu^{+} then reacts with bicinchoninic acid (BCA) which generates a colorimetrically detectable end-product which is measured by absorbance at 562nm. Protein samples were diluted 1:10 in RIPA buffer and 5µl of each diluted protein sample was loaded into one well of a flat-bottomed, transparent 96 well plate (Corning, UK). Serial stock protein solutions (5µl) were also loaded, ranging in concentration from 0-2000 µg/ml, which were used to generate a standard curve. BCA Reagent B was added to BCA Reagent A at a ratio of 1:50 to produce a working reagent which was then added to each well at a volume of 200µl. The plate was then incubated for 25 minutes at 37°C and then a multi-label plate reader (Wallac, Victor3 1420) was used to measure at 562nm to determine protein concentration.

2.9 Western Blot

Western Blotting was used to assess protein expression. The process of a western blot involves the generation of an electrical current through a gel in which denatured proteins travel through which leads to their separation according to the net charge, size, and shape of their native structure. The desired protein is then detected using protein-specific primary and secondary antibodies.

Samples at volumes which corresponded to 30µg of protein were mixed with a loading buffer at a 1:1 ratio. The loading buffer was made by mixing 950µl of 1x Laemmli buffer, containing SDS (sodium dodecyl sulfate) detergent (Bio-rad, UK) with 50µl of β-mercaptoethanol (Sigma Aldrich, UK). SDS has a strong protein-denaturing effect. In the presence of both SDS and β-mercaptoethanol (a reducing agent that cleaves disulfide bonds), proteins unfold into linear chains with a negative charge, facilitating the separation of polypeptides.

Proteins were separated by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were loaded into a 15% SDS-PAGE gel. The gel constituents (15% resolving gel and 5% stacking gel) were prepared, and the gels were handcast using Mini-PROTEAN spacer plates and apparatus (Bio-Rad, UK). An equal amount of protein was separated for each sample by SDS-PAGE. Gels were first run for 40 minutes at 70V until the sample dye had entered the resolving section of the gel. The gels were then run for further 40 minutes at 130V until the sample dye had reached the bottom of the gel. Tanks were filled with 1X running buffer. The samples were run alongside the Precision Plus Protein™ Dual Colour Standard (Bio-Rad, UK) in order to determine the sizes of the proteins detected in the samples.

The separated proteins were then transferred from the acrylamide gel onto a polyvinylidene difluoride (PVDF) membrane (Merck, UK). The membrane was firstly pre-soaked in 100% methanol for 30 seconds, washed in distilled water for 2 minutes and then soaked in transfer buffer. The gels and membranes were then placed within a transfer cassette in between pre-soaked filter paper and fibre pads, and then transfer was performed using a Mini Trans-Blot Cell (Bio-Rad, UK), running in transfer buffer at 100V for 90 minutes.

Following the protein transfer, the membranes were blocked in 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) for 1 hour at room temperature to prevent non-specific binding. Membranes were then washed in TBST in 10 minute intervals, thrice. The membranes were then incubated with primary antibodies for the protein of interest at 4°C overnight. The primary antibodies were diluted in 5% milk with TBST at the concentrations shown in Table 2.5. Incubation at 4°C overnight was found to be the most optimal incubation time across all primary antibodies used, to produce the strongest signal. Membranes were then washed again in TBST as previously described and incubated with the corresponding secondary antibody (dilution; 1:4000) for 1 hour at room temperature. The details of all primary and secondary antibodies used, and their concentrations are shown in Table 2.5. The membranes were then washed again in TBST as previously described to remove unbound antibody.

Antibody-antigen complexes were detected using enhanced chemiluminescence (ECL) detection reagent (Merck, UK), according to the manufacturer's instructions. The ECL detection reagent was added to the membrane in a drop wise manner (1ml) and incubated at room temperature for 1 minute. The membrane was then exposed to an x-ray film in a cassette for an appropriate length of time dependent on the

strength of protein expression. Exposed films were developed on a Xenograph Compact X4.

To allow for subsequent detection of beta-actin, membranes were stripped of current antibodies by incubating in a mild stripping buffer (250mM glycine, 1% w/v SDS, pH2.2) for 15 minutes at room temperature. The membranes were then washed in TBST in 10 minute intervals, twice. The membranes were then blocked again in 5% non-fat milk in TBST, and reapplied with the beta-actin primary antibody, following the methods previously described for consequent detection.

Antibody	Antibody Type	Concentration	Supplier
Beta-actin (A5441)	Mouse monoclonal	1:10,000	Sigma Aldrich
PDIA1 (11245-1-AP)	Rabbit Polyclonal	1:500	Proteintech
AGR2 (12275-1-AP)	Rabbit Polyclonal	1:1000	Proteintech
BiP (11587-1-AP)	Rabbit Polyclonal	1:1000	Proteintech
XBP-1S (24868-1-AP)	Rabbit Polyclonal	1:1000	Proteintech
Goat anti-mouse (sc-2005)	Polyclonal	1:4000	Santa Cruz
Goat anti-rabbit (7074S)	Polyclonal	1:4000	Cell Signalling Technology

Table 2.5. Details of Antibodies used in Western Blotting.

Densitometry analysis of Western blot analysis was performed using Image J software (imagej.nih.gov/ij). The intensity of the bands for the control protein, (β -actin), and proteins of interest were analysed using the box tool at a set size. The background intensity of each sample was also measured and subtracted from the band intensities. The proteins of interest were then normalized to beta-actin, which served as a loading control.

2.10 Cell Proliferation

2.10.1 Optimisation of BrdU Proliferation Assay

Cell seeding density was optimised to obtain the most linear time-proliferation logarithmic curve in untreated cells over 72 hours for Panc-1, AsPC-1, and Mia-PaCa-2. Initial seeding density ranged from 500 to 10,000 cells per well (with initial increment of 500 then 1000). The length of time of detection reagent incubation was also optimised before measuring fluorescence. The plate was read at 5-minute intervals up to 30 minutes. The optimal conditions for all cell lines were a cell seeding density of 4000 cells per well and a 25 minute detection reagent incubation.

2.10.2 BrdU Proliferation Assay

Proliferation was measured using Cell Proliferation ELISA, BrdU (Colorimetric) kit (Roche, UK) following the manufacturer's protocol. Fluorescence, indicating BrdU incorporation into cell DNA during DNA replication, and therefore evidence of cell proliferation, was measured at 370nm. For PDI inhibitor studies AsPc-1, Panc-1 and Mia-PaCa-2 were seeded into 96 well plates (Corning, UK) at 4000 cells/well and treated with two PDI inhibitors: PACMA-31 and CCF642. Details of PACMA-31 and CCF642 treatment doses are described in Table 2.1. For combination studies, cells were treated with Gemcitabine, simultaneously with PDI inhibitor treatments. Gemcitabine treatment doses for each cell line are described in Table 2.1. Cell proliferation was measured for each cell line after treatment at varying concentrations listed in table 2.1. Cells were treated after 24 hours incubation and again after 48 hours incubation. After seeding and incubation for a total of 72 hours with or without inhibitor treatment, cells were treated with the BrdU labelling solution.

For knockdown studies siRNA transfection was carried out as described above, cells were then reseeded into 96 well plates and incubated also for a total of 72 hours post media change. For combination studies, Gemcitabine treatment was applied for 48 hours following reseeding into 96 well plates and post media change. The final volume of DMEM/RPMI media, containing cell treatments, was 100µl per well of the 96 well plate.

BrdU labelling solution (10µl) was added to each well of the 96 well plate, at a final concentration of 10µM. The cells were then incubated for a further 4 hours at 37°C. After 4 hours the BrdU labelling solution was removed and plates were covered in foil and left to dry overnight at 4°C. FixDenat solution (Roche, UK) was then added to each well at a volume of 200µl. Plates were fixed for 30 minutes at room temperature. The FixDenat solution was removed, and cells were then treated with 100µl of anti-BrdU-peroxidase working antibody solution per well. Plates were then incubated for a further 90 minutes at room temperature. The antibody solution was then washed off with a washing dilution containing a 1:10 dilution of the washing buffer (Roche, UK) in 1x PBS (Roche, UK). 100µl of the tetramethyl-benzidine (TMB) peroxidase substrate solution was added to each well. Fluorescence, proportional to the amount of BrdU incorporation in the proliferating cells, was then recorded after 25 minutes of substrate incubation, using an ELISA multi-label reader (Wallac, Victor3 1420) at wavelength of 370nm.

2.10.3 MTT Proliferation Assay

Cell viability was measured using the MTT cell viability assay kit (Biotum, UK). The MTT is a versatile assay, still commonly used to determine the chemosensitivity of cancer cells following cytotoxic agent addition and drug action, it proves a low-cost and efficient method. The MTT assay is based on the ability of metabolically active cells to cleave the yellow tetrazolium salt (MTT) to produce a purple formazan crystal. The concentration of solubilised formazan is then determined by the measurement of optical density at 570nm. The colorimetric signal, measured on a spectrometer, is then proportional to the cell number, consequently determining cell viability.

For PDI inhibitor studies AsPc-1, Panc-1 and Mia-PaCa-2 were seeded into 96 well plates (Corning, UK) at 4000 cells/well and treated with PDI inhibitors, siRNA knockdown and Gemcitabine combinations as previously described in BrdU proliferation assays. Cells were treated to the final concentrations shown in Table 2.1 and to a final volume of 100µl per well. After cell treatments, 10µl of the MTT solution was then added to the 100µl of cell culture medium per well. The solution was mixed by gentle shaking of the plates. The plates were then incubated for 4 hours at 37°C. DMSO (200µl) was then added into each well and gently pipetted several times to ensure dissolvment of the formazan crystal. Absorbance was then measured on an multi-label plate reader (Wallac, Victor3 1420) at 570nm. Background absorbance was also measured at 630nm and subtracted to normalise signal absorbance values.

2.11 PDI Enzyme Activity

PDI activity was measured using the PDI activity assay kit (fluorometric) (Abcam, UK), according to the manufacturer's instructions, which was adapted to be used with Pacma-31 and CCF642 and siRNA knockdown treatment. The reductase activity of PDI is utilised to determine its activity, by employing a synthetic quenched fluorescent substrate which is cleaved in the presence of PDI, producing a fluorometric signal. Cells were grown in 25cm² cell culture flasks (T25) (Corning Ltd, UK). PDI inhibitor and siRNA knockdown treatments were applied to the final concentrations listed in Table 2.1. Following respective 48-hour treatments, cells were homogenised with 200µl of ice-cold PDI assay buffer to produce a cell lysate. Protein concentration of cells in T25 flasks was also measured using the BCA assay, as previously described (see section 2.8), to determine a consistent level of protein between cell types and treatments. Cell lysates were then incubated on ice for 10 minutes. Each cell lysate sample was then centrifuged at 10,000 x g for 5 minutes at 4°C to remove any cell debris. The supernatant (100µl) was transferred to a new Eppendorf tube. Saturated ammonium sulphate (4.1M) at a volume of 200µl was added to the fresh lysate at a final concentration of 2.05M and incubated on ice for 20 minutes, to remove small molecules that may interfere with the assay. The cell lysate was then centrifuged again at 10,000 x g at 4°C for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 200µl of ice-cold PDI assay buffer. Each sample (5µl) was plated into two parallel wells (sample and background control) of a black 96 well plate (Grenier, UK), with 3 replicates. The fluorescence standard (25µM) was diluted in PDI assay buffer to create a series of 6 fluorescent standards, shown in Table 2.6, to produce a standard curve. A representative standard curve is shown in Figure 2.1.

Standard	Volume of 25 μ M Fluorescence standard (μ l)	Volume of PDI Assay Buffer (μ l)	Concentration of Fluorescence standard (pmol/well)
1	0	100	0
2	2	98	50
3	4	96	100
4	6	94	150
5	8	92	200
6	10	90	250

Table 2.6. Fluorescent standards used to generate PDI activity assay standard curve.

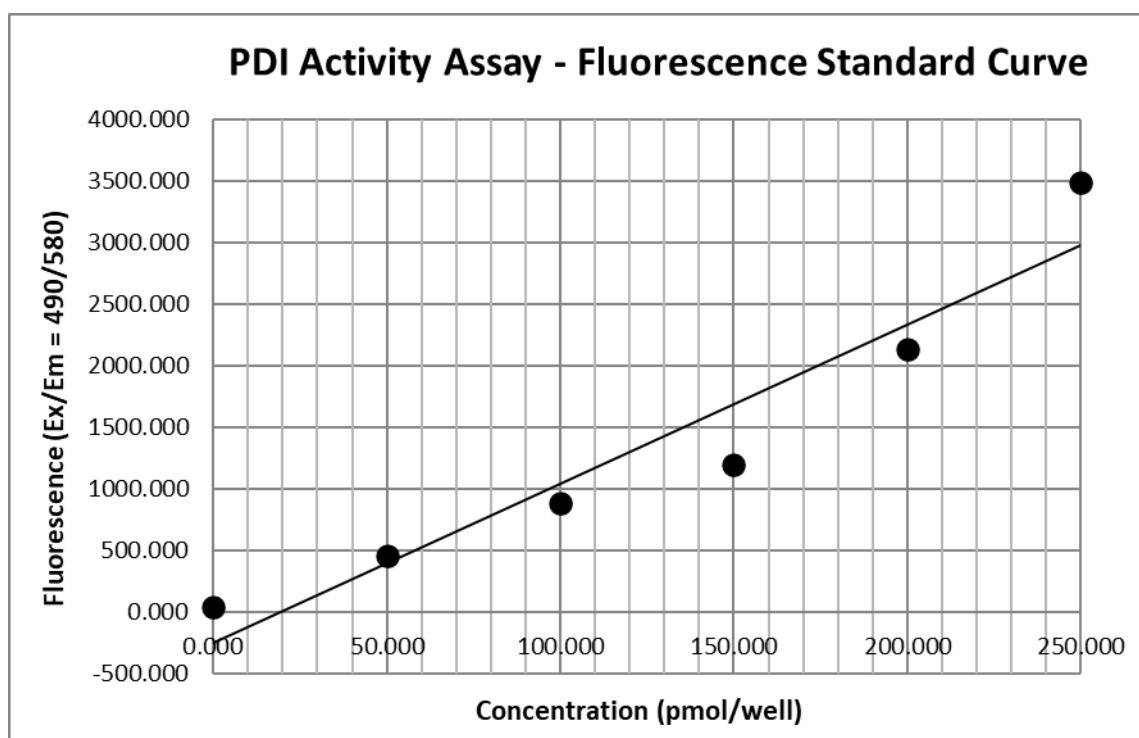


Figure 2.1. Typical standard curve of 6 fluorescent standard used in PDI activity assay.

A reaction mix and background control mix were each made to a total volume of 25 μ l per well. The reaction mix was made by the addition of 1 μ l of 1mM PDI activator to 24 μ l of PDI assay buffer. The PDI activator contains Tris(2-carboxyethyl)phosphine

hydrochloride (TCEP), a strong reducing agent. The background control mix was made by the addition of 1µl of 1mM PDI activator to 22µl of PDI assay buffer and 2µl of the PDI inhibitor per well. In the presence of the specific inhibitor, the quenched fluorescent substrate cannot be cleaved by PDI, hence not producing an enhanced fluorometric signal. The reaction mix (25µl) and 25µl of the background control mix were both added to each corresponding well. The wells were mixed well, and the plates were incubated for 15 minutes at room temperature. A detection mix was then made by the addition of 2µl of the PDI substrate to 23µl of PDI assay buffer per well. The detection mix (25µl) was added to all wells and mixed. Fluorescence was then measured immediately on a plate reader in kinetic mode for 30 minutes at Ex/Em = 490/580. Background control readings were subtracted and the change in RFU over 30 minutes was calculated to determine relative PDI activity.

2.12 Caspase-3 Activity Assay

Caspase-3 activity was measured using the caspase-3 assay kit (Colorimetric) (Abcam), according to the manufacturer's instructions. Caspase 3 activity was determined by the detection of chromophore p-nitroaniline (p-NA) absorbance after cleavage from the labelled substrate DEVD-p-NA. Cells were grown in 6 well plates (Corning, UK) with PDI inhibitor, siRNA knockdown and gemcitabine combination 48 hour treatments as listed in Table 2.1. Following treatment and at approximately 80% confluency, cells were homogenised to produce a cell lysate. Protein concentration was measured using a BCA assay, as previously described (see section 2.8), and the concentration was adjusted to 200µg of protein in 50µL cell lysis buffer per well of a 96 well plate (Corning, UK). Reaction Buffer (2x), containing 10mM DTT, was

added to each sample at a volume of 50µl. DEVD-p-NA substrate (4mM) was also added to each sample at a volume of 5µl to a final concentration of 200µM. The plate was then incubated for 2 hours at 37°C and absorbance was measured at 405nm on a microplate reader.

Due to discontinuation of the caspase-3 assay kit (Colorimetric) (Abcam), the Amplite® Colorimetric caspase 3/7 assay kit (AAT Bioquest, UK) was also used in caspase activity studies. In this live-cell assay, (Z-DEVD)2R110 is used as a chromogenic indicator for caspase 3/7 activity. Caspase cleaves R110 peptides. R110, induced by caspases, as a yellow colour dye, absorbance of R110 can be measured at 490nm, to proportionally indicate the level of caspase 3/7 activity. Cells were plated in 96 well plates at 4000 cells per well. Treatments were carried out as previously described for 48 hours. Following treatment to induce apoptosis, 100µl of the caspase 3/7 working solution was added to 100µl of cell culture medium in each well. The plates were then incubated for 1 hour at room temperature and protected by the light by covering with foil. Plates were then centrifuged at 800rpm for 2 minutes. Absorbance was then measured at 490nm on a microplate reader.

2.13 Cell Migration Assay

Panc-1, Mia-PaCa-2 and AsPC-1 cell lines were plated into 24 well plates (Corning, UK) and incubated for 24 hours to allow cell growth to approximately 80% confluency. The surface of each well was then scratched twice with a sterile 20µl Gilson pipette tip to produce a cross shape. The cells were then incubated for a further 30 minutes to minimise cell stress and loss of adhesion. Media from each well

(500µl) was aspirated and replaced with 500µl of fresh media (control) or the drug dilutions of Pacma-31 and CCF642. For siRNA knockdown studies cells were transfected as previously described with volumes altered due to cells being plated in a 24 well plate. All wells were photographed using a EVOS XL core system imaging microscope (ThermoFisher Scientific, UK) at 5X magnification. Images were taken at the time points of 0, 24 and 48 hours post scratch. The area of the scratch was measured using Image J software to assess cell migration.

2.14 Data Mining

RNA-sequencing data for mRNA expression of PDI genes was obtained from The Cancer Genome Atlas (TCGA) (TCGA, 2024) and Genotype-Tissue Expression (GTEx Portal, 2024) datasets. Due to the TCGA pancreatic adenocarcinoma (PAAD) dataset comprising of 179 tumour samples and only 4 normal samples (TCGA, 2024), RNA-sequencing data of 171 normal pancreas specimens from GTEx dataset was also integrated (GTEx Portal, 2024). The gene expression data were converted to Transcripts per million reads (TPM) format. The online tool GEPIA (Gene Expression Profiling Interactive Analysis) (Tang et al., 2017) was used to compare differential expression of PDI family members between pancreatic carcinoma and normal samples, using the TCGA and GTEx combined dataset. GEPIA was further used to compare PDI expression to AJCC/UICC tumour stage (Tang et al., 2017). These datasets were also used to produce Kaplan-Meier Survival Curves. Pancreatic cell line specific RNA-sequencing data was downloaded from the European Bioinformatics Institute (EBI) database (The UniProt Consortium, 2023). Immunohistochemistry data demonstrating PDI protein expression was obtained

from the Human Protein Atlas database (Uhlén et al., 2015). The protein-protein interaction network was constructed using the Pancreatic Expression Database (PED) (Marzec et al., 2018). The Cancer Cell Line Encyclopaedia database was used to obtain copy number alteration data for PDI genes (Ghandi et al., 2019).

2.15 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software 5 (GraphPad Software; California, USA). The presence of a normal or abnormal Gaussian distribution was determined using column statistics analysis. A one-way ANOVA was used to determine significance and compare between two or more independent treatment groups, with post-hoc Tukey's multiple comparison tests or Dunnett's multiple comparison tests to determine which treatment groups were significantly different if one-way ANOVA test showed significance. Tukey's multiple comparison test was used in cases where every mean was compared to every other mean, whereas Dunnett's multiple comparison was used for the comparison of every mean to a control mean. In experiments where the comparison to the control mean was the only important result, Dunnett's multiple comparison was used in order to perform fewer comparisons and maintain a better power. Significance is represented by; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

CHAPTER 3 – IDENTIFICATION OF KEY PDI MEMBERS AS POTENTIAL THERAPEUTIC TARGETS IN PANCREATIC CANCER THROUGH DATA MINING

Chapter 3 - Identification of key PDI members as potential therapeutic targets in Pancreatic Cancer through data mining

3.1 Introduction

3.1.1 The Role of the PDI Gene Family in Cancer

The PDI family is a group of multifunctional ER enzymes that comprise of 21 total members, shown in Table 1.3. PDI gene family members exhibit a diverse nature, with members differing considerably in length, substrate specificity and domain arrangement. This vastitude of differences between PDI members brings forth a need to evaluate the potential roles of each PDI member to assess their importance in pancreatic cancer development. PDI members are consistently overexpressed in several cancer types, emphasising their potential influence in cancer growth and survival. While the potential oncogenic influence of PDI has been investigated in multiple cancer types including breast, colorectal, liver, brain and prostate, fewer insights have been made into its role in pancreatic cancer (Chawsheen et al. 2018). Recent research has only clearly identified one PDI family member to be of potential oncogenic influence in pancreatic cancer – AGR2. However, the mechanisms by which AGR2 may drive pancreatic cancer progression are not well defined. AGR2 has been found to be overexpressed in both early PanIn lesions and later-stage lesions (Dumartin et al., 2011). These studies, combined with evidence of interactions between AGR2 and several known oncogenic pathways (Figure 1.14), demonstrate the potential of AGR2 as an oncogenic driver for pancreatic cancer progression. However, further investigations are needed to assess its therapeutic potential.

PDI aids cancer cell survival through its enzymatic functions as a catalyst for disulphide bond rearrangement, acting to facilitate protein folding and in turn, maintaining ER homeostasis. PDI has been attributed to protective roles through activation of the unfolded protein response (UPR) pathway, which in periods of ER stress, acts to alleviate stress and hence maintain cell survival. PDI members have been shown to act as both downstream chaperones of the UPR and upstream governors of UPR activation. While some observations of how specific PDI members could contribute in the UPR have been made (Figure 1.10), the roles of each PDI member are not well defined, especially in a pancreatic carcinogenic environment. A more definitive understanding of how each PDI member contributes to cell survival through activation of the UPR pathway would enable the development of more precise therapeutic targets.

With the maintenance of ER homeostasis being a primary function of PDI through protein folding, an inhibition of PDI leads to an accumulation of misfolded proteins and consequent ER stress. As a result, the UPR is activated to combat this. It is hypothesised that continued inhibition of PDI would lead to persistent UPR activation, triggering downstream 'chronic' UPR pathways, which drive apoptosis. PDI inhibition could therefore be a potential therapeutic strategy, leading to apoptotic ramifications in cancer cells. Recent research has shown PDIA1 inhibition in breast cancer and neuroblastoma cell lines to induce cytotoxicity via caspase activation (Hashida et al. 2011). This supports the hypothesised therapeutic potential of PDI, through its involvement in the UPR pathway and regulation of apoptosis. Investigation into the potential of PDI members as therapeutic targets through their role in the UPR pathway is the primary aim of this study.

3.1.2 The Role of the UPR in Cancer

Though the focus of the study is centred on the therapeutic potential of PDI, its proposed oncogenic effects are intertwined with its interactions within the UPR pathway. UPR pathway signalling alone has been linked to tumorigenesis. The UPR is frequently upregulated in cancer due to extrinsic stress factors in the tumour microenvironment such as hypoxia and nutrient deficiency, which have been observed in pancreatic tumours (Long et al., 2021). With the UPR acting to maintain ER homeostasis, it has been implicated to play roles in tumour growth and survival and is believed to also contribute to chemoresistance (Long et al., 2021). The specific mechanisms by which UPR activation drives pancreatic cancer progression are not well understood. Investigations into the expression and potential roles of downstream UPR genes may give insight into the tumorigenic functions of the UPR, particularly as many also exhibit interactions with PDI members.

The UPR is comprised of three main signalling arms; each activate a predominant downstream gene that exhibits various protective effects. These include XBP-1, ATF4 and ATF6. XBP-1 induces the accumulation of ERAD-associated proteins and ER chaperones, such as PDI, to refold misfolded proteins and alleviate ER stress (Lee et al. 2003). ATF4 further promotes ER chaperone expression and triggers an antioxidant response (Cao and Kaufman, 2012). ATF6 further drives XBP-1, ERAD-associated proteins and ER chaperone expression (Schröder and Kaufman, 2005). These pro-survival mechanisms, highlight XBP-1, ATF4 and ATF6 as therapeutic targets of the UPR pathway and promotes investigations into how PDI inhibition may affect their activations. Apoptotic-associated UPR genes including CHOP and caspase-3 can also be studied to assess the prevalence of chronic UPR activations and provide evidence of prolonged ER stress. As PDI inhibition is hypothesised to

induce these apoptotic pathways, CHOP and caspase-3 are potential useful indicators for the study of UPR-associated apoptosis.

3.1.3 Hypothesis and Aims

It is hypothesised that the upregulation of PDI in pancreatic cancer will be varied across differing PDI members, highlighting which members are most likely to be potential key therapeutic targets. It is further hypothesised that certain ER stress markers of the UPR pathway may be dysregulated in pancreatic cancer, highlighting further potential downstream targets.

To investigate the potential roles of PDI family members and UPR pathway members in pancreatic cancer progression, the aims of the current study were as follows:

- To identify upregulated PDI family members in pancreatic cancer via analysis of RNA-sequencing data
- To determine which pancreatic cancer cell lines would be most appropriate for experimental use by analysis of PDI member mRNA expression
- To compare the mRNA and protein expression of upregulated PDI family members in pancreatic cancer with normal pancreas tissue expression
- To assess the effect of high PDI member expression on patient survival through generation of Kaplan-Meier curves
- To determine the key PDI members which are potential therapeutic targets in pancreatic cancer and therefore a focus of further experimental work, through data mining
- To investigate the expression of UPR pathway members in pancreatic cancer and their potential correlations with PDI expression to highlight potential UPR pathway activations for further experimental investigation

3.2 Methods

3.2.1 Databases Used for Analysis

3.2.1.1 The Cancer Genome Atlas (TCGA)

The TCGA database was used for analysis of mRNA expression, prognostic, and clinical data (TCGA, 2024). The dataset 'PAAD mRNA seq normalised' was downloaded and used for analysis of PDI member expression (TCGA, 2024). Gene expression was represented as Transcripts per million reads (TPM). Raw clinical data was used to produce Kaplan-Meier survival curves in comparison to PDI member mRNA expression. Raw clinical data was further analysed in the comparison of PDI member mRNA expression with patient therapy outcomes. mRNA expression for PDIA1, PDIA3, PDIA4, PDIA6 and AGR2 were used for comparisons with clinical data. The clinical characteristics of TCGA pancreatic cancer patients are shown in Table 3.1.

Clinical Characteristic		No. of patients (n, %) n=179
Sex	Female	80 (44.7)
	Male	99 (55.3)
Age	≥65	97 (54.2)
	<65	82 (45.8)
Status	Alive	85 (45)
	Dead	92 (52)
Stage	I	21 (11.7)
	II	147 (82.1)

	III	4 (2.2)
	IV	5 (2.8)
	Unknown	2 (1.1)
Histological type	Adenocarcinoma	147 (82.1)
	Colloid (mucinous non-cystic) carcinoma	4 (2.2)
	Neuroendocrine carcinoma	8 (4.5)
	Other subtypes	19 (10.6)
	Unknown	1 (0.6)

Table 3.1. TCGA pancreatic cancer patient clinical characteristics (TCGA, 2024).

3.2.1.2 European Bioinformatics Institute (EBI)

The EBI database (The UniProt Consortium, 2023) was used for analysis of RNA-sequencing of pancreatic cell lines Panc-1, Mia-PaCa-2, AsPC-1, Capan-2 and HPAF-II from the Cancer Cell Line Encyclopaedia (Ghandi et al., 2019). Data is expressed as normalised counts per gene. TPMs are calculated from the raw counts by an integrated RNA-Seq analysis pipeline (iRAP) that allows the user to select and apply their preferred combination of existing tools for mapping reads, quantifying expression, testing for differential expression. The TPMs were averaged for each set of technical replicates. Values were then quantile normalized within each set of biological replicates using linear models for microarray data (LIMMA), an R/Bioconductor software package that provides an integrated solution for analysing data from gene expression experiments.

3.2.1.3 Genotype-Tissue Expression (GTEx)

RNA sequencing data from the GTEx dataset were combined with the TCGA dataset (GTEx Portal, 2024; TCGA, 2024). Since the TCGA dataset only comprised of 4 normal samples, the GTEx dataset was used to supplement this, comprising of 171 normal pancreatic samples (GTEx Portal, 2024; TCGA, 2024). Both datasets were integrated to compare pancreatic tumour and normal tissue mRNA expression of PDI members: PDIA1, PDIA3, PDIA4, PDIA6 and AGR2, as well as UPR pathway genes: BiP, ATF4, ATF6, XBP-1, CHOP and Caspase-3. Gene expression data were represented as TPMs and log2 transformed.

3.2.1.3 Human Protein Atlas (HPA)

The HPA database was used to obtain immunohistochemistry data from pancreatic cancer and normal tissue samples (Uhlén et al., 2015). Protein expression was compared between PDI members: PDIA1, PDIA3, PDIA4, PDIA6 and AGR2. Level of protein expression is represented by the strength of staining in normal pancreas or tumour cells. Staining is represented as medium, high, or not detected. All samples were selected from male patients aged 63-69 for the best possible consistency in comparisons (Uhlén et al., 2015).

3.2.1.4 Gene Expression Profiling Interactive Analysis (GEPIA)

The online tool GEPIA was used to generate graphs of analysis for mRNA expression data comprising of combined TCGA and GTEx datasets (Tang et al., 2017). GEPIA was used to generate expression boxplots to compare differential expression of PDI members between pancreatic carcinoma and normal samples. It

was also used to generate violin expression plots of PDI members in comparison to AJCC tumour stages I, II, III and IV. mRNA expression is shown as transcripts per million reads and Log2 transformed.

3.2.1.5 Pancreatic Expression Database (PED)

The PED was used as a tool to construct a protein-protein interaction (PPI) network to exhibit interactions of PDI proteins: PDIA1, PDIA3, PDIA4 and AGR2 (Marzec et al., 2018). The PED uses a combination of TCGA and the International Cancer Genome Consortium (ICGC) datasets to form a pancreatic cancer database. The network was constructed to show protein interactions with a minimum interaction score of 0.4. Upregulated (red) and downregulated (blue) genes are shown through comparison of pancreatic cancer and normal sample datasets. The size of circles is equal to the log fold change (FC) value of the Z score of gene expression. The PED was also used to generate a heat map that visualised gene correlations of PDI members and UPR pathway genes. The PDI members previously studied were analysed with UPR genes: ATF4, ATF6, Caspase-3 (CASP3), CHOP (DDIT3), BiP (HSPA5) and XBP-1. Correlations were calculated using the Pearson Product Moment Correlation Coefficient (PMCC) value. Correlations were analysed in both pancreatic tumour and normal tissue, for comparison. Red represents a positive correlation (1); blue represents a negative correlation (-1) and white represents no correlation (0).

3.3 Results

3.3.1 PDI Gene Family Expression in Pancreatic Cancer

mRNA gene expression levels were assessed across all 21 PDI members in pancreatic cancer using the TCGA database (TCGA, 2024) (Figure 3.1A). The data demonstrates a relatively low level of expression among several PDI members, with a select few members showing a higher level of expression. The members demonstrating a higher level of expression were identified as potential key PDI genes related to pancreatic cancer progression. PDIA1 showed the highest level of expression, followed by AGR2. PDIA3, PDIA4 and PDIA6 also showed a higher level of expression, compared to other PDI members, and so were made the focus of subsequent research.

PDI gene expression was compared across pancreatic cancer cell lines; Panc-1, Mia-PaCa-2, AsPC-1, Capan-2 and Hapan-II (Figure 3.1B). The aim of comparison of PDI gene family expression across these cells lines was to determine the suitability of each cell line for use in further PDI gene expression experiments, as well as their relative representation of PDI expression in pancreatic cancer through comparison to expression seen in tumour samples (Figure 3.1A). Data was obtained from the European Bioinformatics Institute (EBI) database (The UniProt Consortium, 2023). The levels of gene expression strongly correlate to those seen in Figure 3.1A, with Panc-1 showing the highest levels of expression of PDIA1 and high expression levels in PDIA3, PDIA4 and PDIA6 also. Though Capan-2 shows the highest levels of expression in PDIA4, with Panc-1 showing high expression levels across PDI members including the most highly expressed member PDIA1, Panc-1 was chosen as a more suitable cell line for this study. AGR2 is shown to be highly expressed in

AsPC-1 and interestingly, not naturally expressed in Mia-PaCa-2. With both AsPC-1 and Mia-PaCa-2 also showing correlating expressions to the tumour expression data in Figure 3.1A, these cell lines were also chosen to aid a comparative study on the influence of natural AGR2 expression.

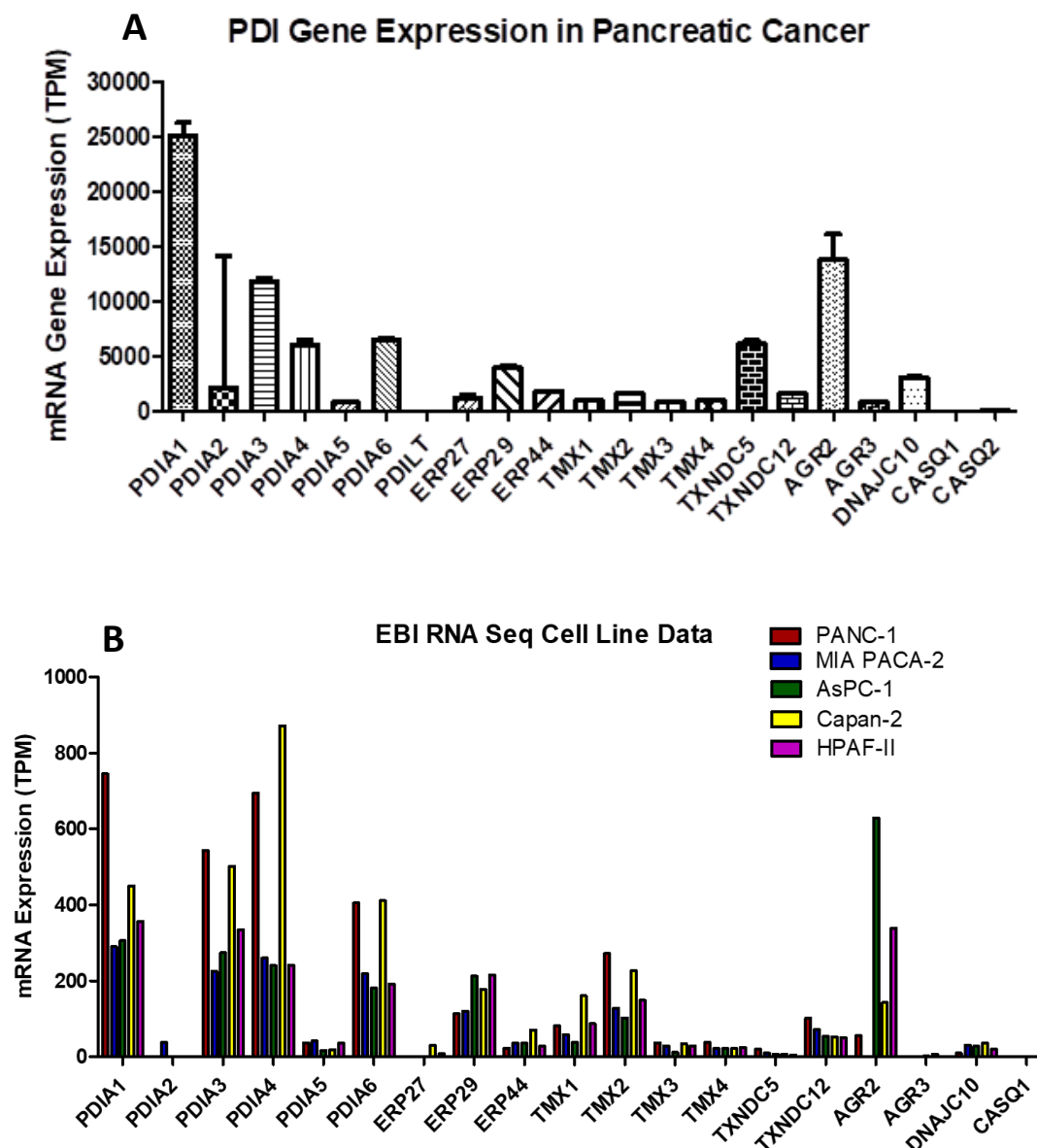


Figure 3.1. PDI Gene Family Expression in Pancreatic Cancer and Pancreatic Cancer Cell Lines.

(A) PDI gene expression values were obtained from normalised RNA-sequencing data using The Cancer Genome Atlas (TCGA) database (TCGA, 2024). The dataset 'PAAD mRNA seq normalised' was used for analysis. The mean expression is shown, and error bars denote standard deviation, $n=179$. (B) PDI gene expression in cell lines; Panc-1, Mia-PaCa-2, AsPC-1, Capan-2 and HPAF-II were obtained from RNA-sequencing data from the Cancer Cell Line Encyclopaedia, using the European

Bioinformatics Institute (EBI) database (The UniProt Consortium, 2023). mRNA expression is shown as transcripts per million reads (TPM).

3.3.2 Differential PDI Gene Expression Between Pancreatic Tumour and Normal Tissue

3.3.2.1 Comparison of PDI mRNA Expression in Tumour Vs Normal Samples

A combination of the TCGA database (TCG, 2024) and the GTEx database (GTEx Portal, 2024) was used to compare the mRNA gene expression levels of potential key PDI members; PDIA1, PDIA3, PDIA4, PDIA6 and AGR2, identified in Figure 3.1, in samples from pancreatic cancer tumour tissue and normal pancreas tissue (Figure 3.2). PDIA3, PDIA4 and AGR2 all showed a significant overexpression ($p < 0.05$) in tumour samples compared to normal, with AGR2 showing the greatest comparative difference. This overexpression highlights the potential role that the expression of these PDI members may play in pancreatic cancer progression, in particular AGR2. PDIA1 and PDIA6 showed no significant difference in expression between tumour and normal tissue, suggesting its high expression seen in pancreatic cancer could account for a potential role in normal pancreas functions.

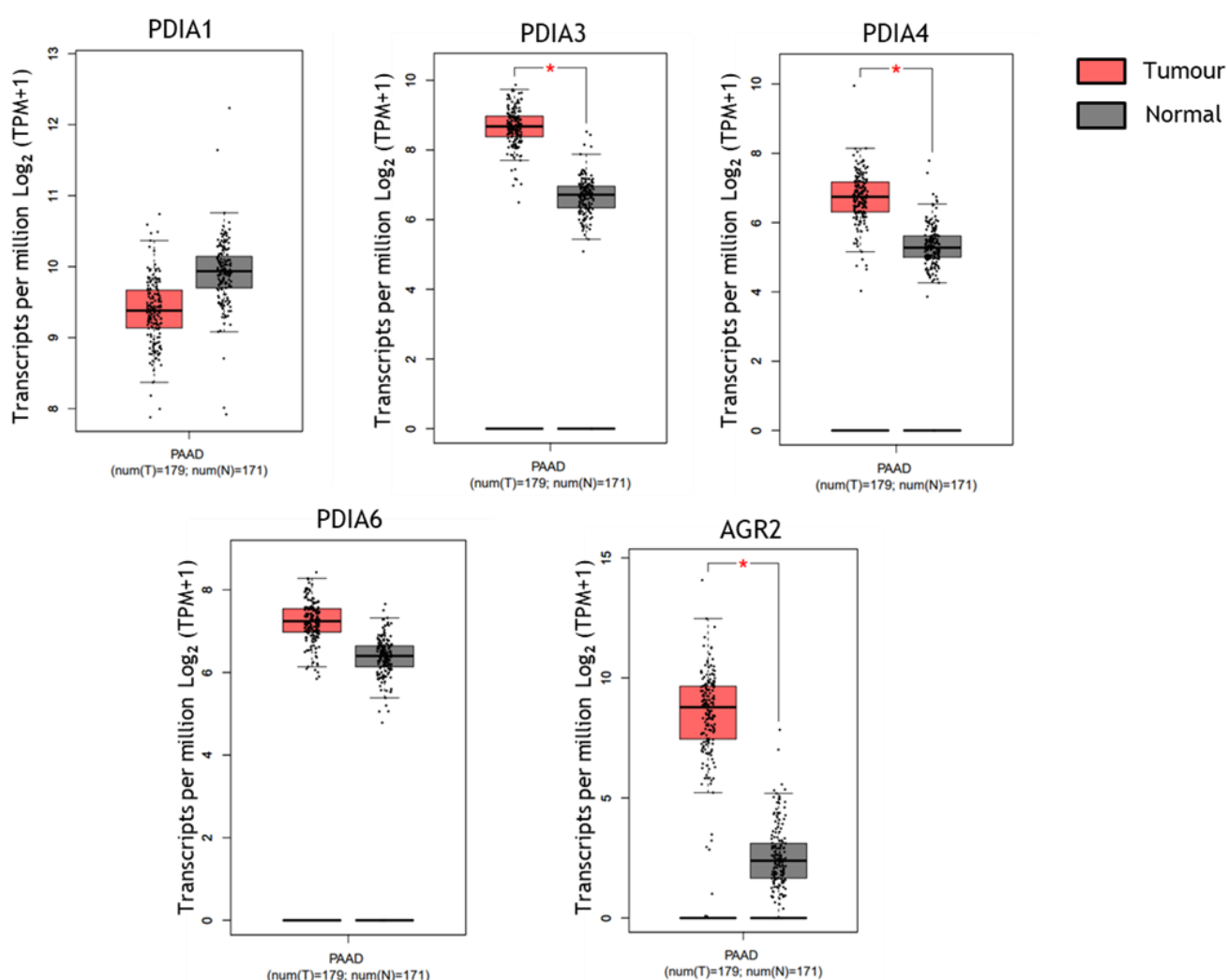


Figure 3.2. Comparison of Pancreatic Tumour Vs Normal PDI mRNA Expression.

PDIA1, PDIA3, PDIA4, PDIA6 and AGR2 mRNA gene expression levels were obtained from pancreatic cancer tumour samples (T) (n=179) and normal pancreas tissue samples (N) (n=171) using a combination of TCGA (TCGA, 2024) and GTEx databases (GTEx Portal, 2024). PDIA3, PDIA4 and AGR2 showed significantly higher expression in tumour samples than in normal samples (*P<0.05). mRNA expression is shown as transcripts per million reads and Log₂ transformed ((log₂(TPM + 1))).

3.3.2.2 Comparison of PDI Protein Expression in Tumour Vs Normal Samples

PDI protein expression was observed in tumour vs normal samples using immunohistochemistry data from the Human Protein Atlas database (Uhlén et al., 2015) (Figure 3.3) . All samples are images selected from individual male patients (n=1) aged 63-69 (Uhlén et al., 2015). The data shows correlation with gene expression data observed in Figure 3.2, with AGR2 showing the greatest difference in expression when comparing tumour and normal samples. AGR2 protein expression shows high expression in tumour samples, whilst it was not detected in normal samples. Interestingly no difference in protein expression in PDIA4 or PDIA3 is shown between tumour and normal samples, despite the significant difference shown in mRNA expression in Figure 3.2. All PDI members also show at least a medium level of protein expression in normal pancreas tissue, with the exception of AGR2. While this further highlights AGR2 as a potential key oncogene in pancreatic cancer, these data are not statistically significant due to the small sample size of only n=1, and results should therefore be interpreted with caution.

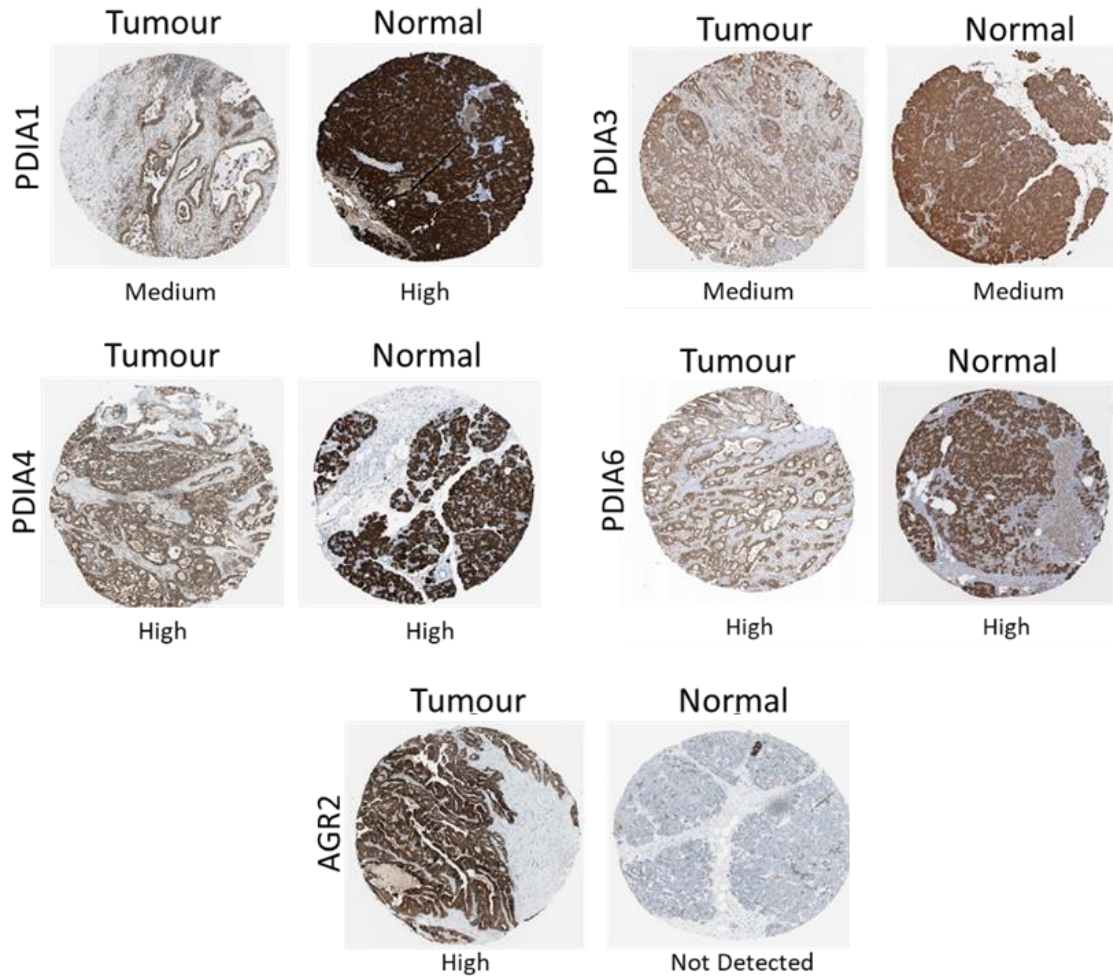


Figure 3.3. Comparison of Pancreatic Tumour Vs Normal PDI Protein Expression.

PDI protein expression was observed from immunohistochemistry data obtained from the Human Protein Atlas Database (Uhlén et al., 2015). Staining is represented as medium, high, or not detected to reflect protein expression levels. All shown samples were selected from individual male patients (n=1) aged 63-69. These patient samples were chosen to apply the greatest possible level of consistency in comparing protein expression in differing PDI members. AGR2 showed the greatest difference in protein expression levels, with a high expression in the tumour sample, but not detected in normal tissue. However, these findings were not statistically significant due to the small sample size of only n=1, and results should therefore be interpreted with caution.

3.3.3 Prognostic analysis of PDI Expression in Pancreatic Cancer

3.3.3.1 PDI mRNA Expression based on Pancreatic Tumour Stage

mRNA expression of PDI members was compared based on AJCC tumour stage, between stages I and IV (Figure 3.4). mRNA expression violin plots were generated using the GEPIA database (Tang et al., 2017). AGR2 showed a significant difference in expression between tumour stages ($P(>F)=0.000145$). While its expression at stage I tumours varied from no expression at all, to high expression levels. AGR2 expression at stage IV remained high with little variation. This suggests an increase in AGR2 expression in stage IV tumours, perhaps suggesting its influence in pancreatic cancer progression and migration. Whilst variance was seen in the expression levels of other PDI members in each tumour stage, expression levels remained relatively consistent with no significant over/under expression observed.

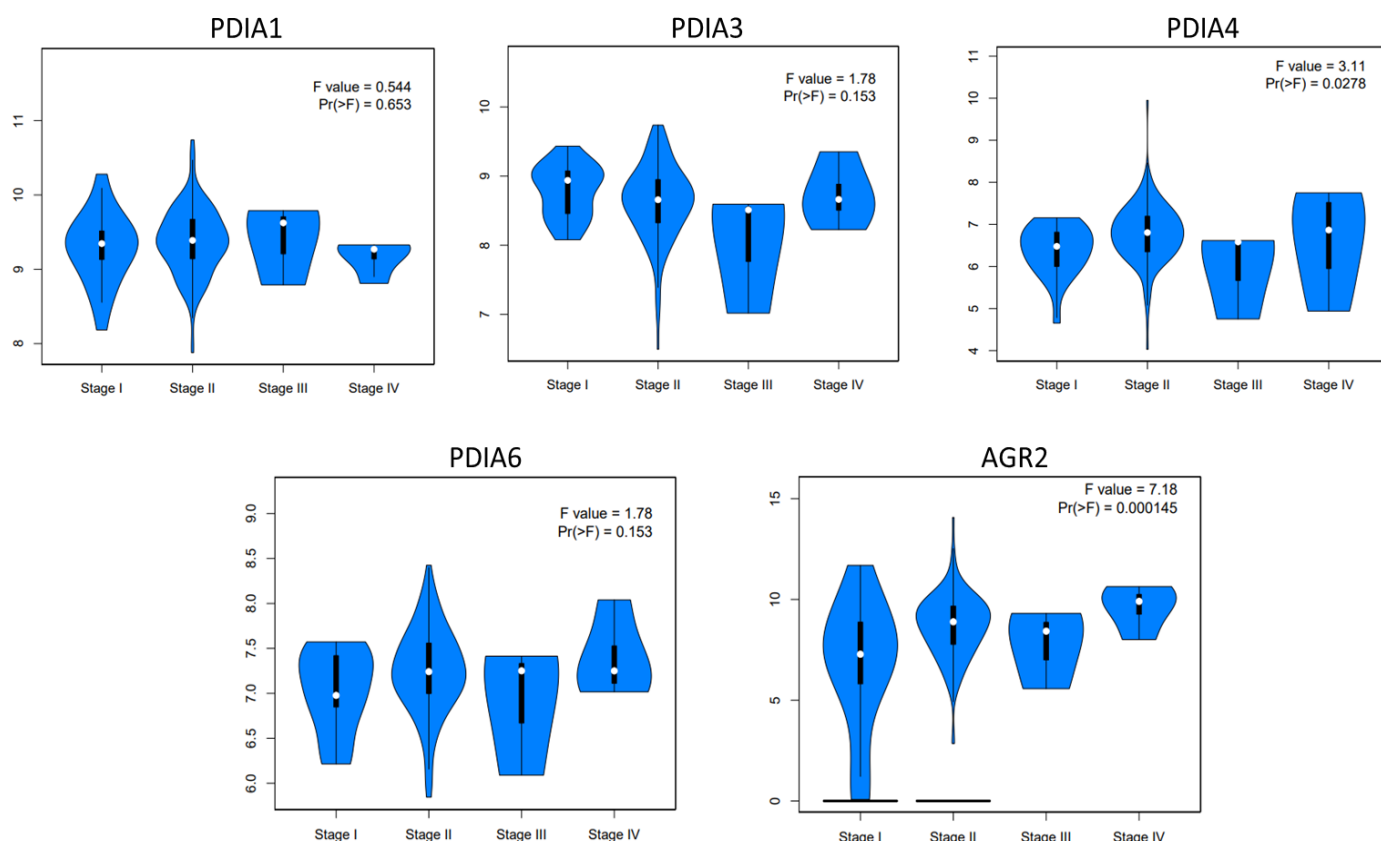


Figure 3.4. PDI mRNA expression based on TNM Tumour Stage.

mRNA expression violin plots of PDIA1, PDIA3, PDIA4, PDIA6 and AGR2 based on patient AJCC/UICC tumour stage (I-IV). Data is generated from the GEPIA dataset (Tang et al., 2017) (n=179). RNA expression is shown as transcripts per million reads and Log₂ transformed (log₂(TPM + 1)). F value denotes the ratio of between group variation and within group variation. Pr(>F) denotes significance at >0.05. AGR2 shows the most significant difference in expression between tumour stages (Pr(>F)=0.000145).

3.3.3.2 PDI mRNA Expression Based on Patient Therapy Outcome

mRNA expression of PDI members based on patient outcome post-therapy was assessed by analysis of the TCGA database (TCGA, 2024) (Figure 3.5). All patients analysed had received chemotherapy treatment, where the specific type of chemotherapy regimen received was not specified. Expression levels were compared in patients that showed progressive disease, stable disease, partial remission, or complete remission, following treatment. While no significant differences were observed, AGR2 did demonstrate the most differential expression between therapy outcomes, showing a higher expression in progressive disease than other therapy outcomes. This again demonstrates AGR2 as an outlier compared to other PDI members.

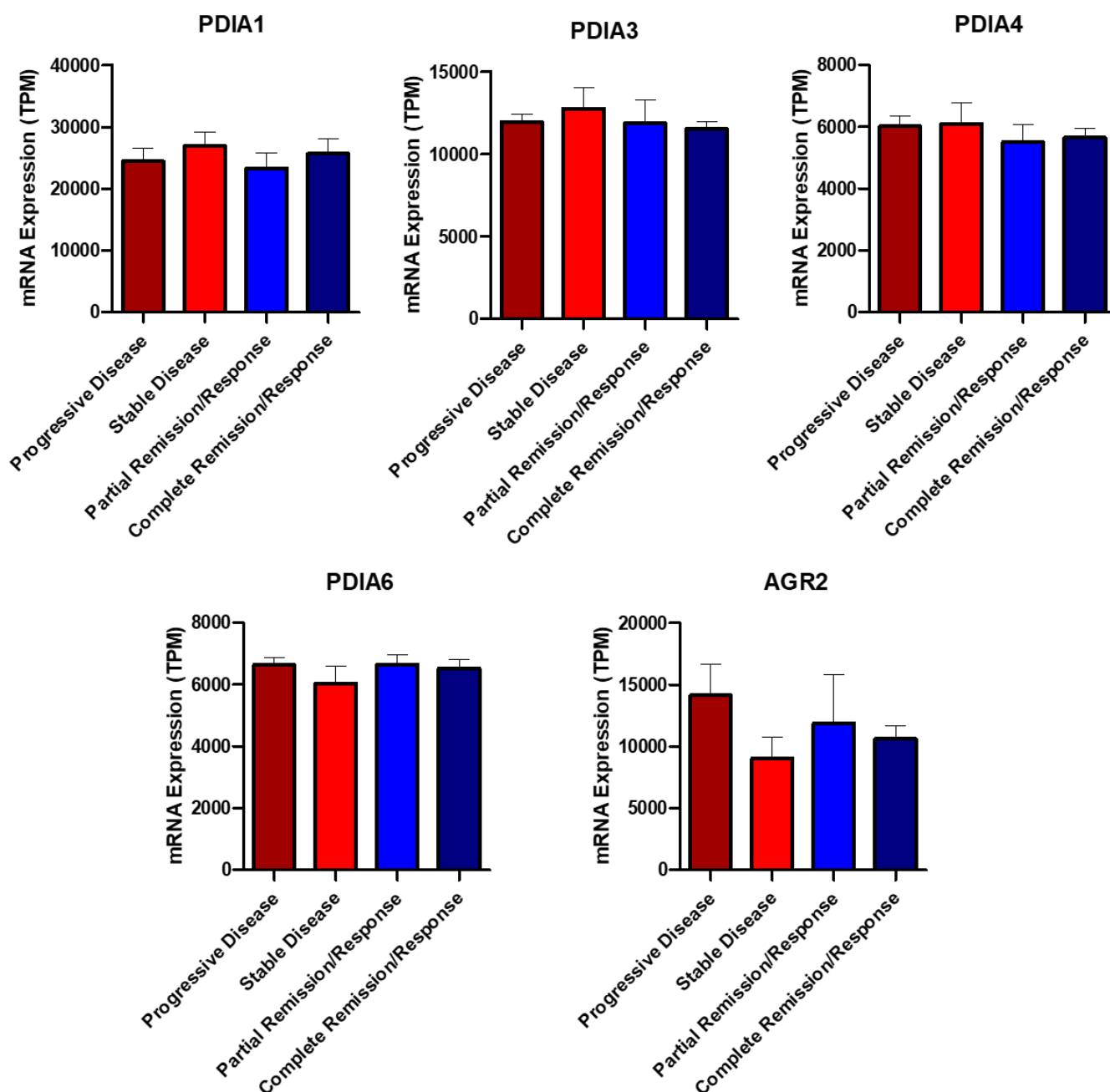


Figure 3.5. PDI mRNA expression based on Patient Therapy Outcome.

PDIA1, PDIA3, PDIA4, PDIA6 and AGR2 mRNA gene expression values based on patient therapy outcome were obtained from normalised RNA-sequencing data using the TCGA database (TCGA, 2024). The dataset 'PAAD mRNA seq normalised' was used for analysis. mRNA expression is shown as transcripts per million reads (TPM). The mean expression is shown, and error bars denote standard deviation, n=179.

3.3.3.3 PDI Expression Survival Analysis

Kaplan-Meier survival curves were created using the TCGA pancreatic cancer patient database (TCGA, 2024) to observe the correlation of survival with PDI gene expression (Figure 3.6). The most significant decrease in survival rate was shown in PDIA6 in correlation with high expression (* $p=0.0203$). A similar decrease in survival rate was seen with high PDIA3 ($p=0.0509$) and PDIA4 ($p=0.0552$) expression. This demonstrates their potential role in promoting tumorigenesis and identification as a therapeutic target. PDIA1 did not show any significant difference in survival rate between high and low expression, while AGR2 only showed a drop in survival rate with high expression post 1000 days. This could suggest that high AGR2 expression is more associated with advanced stage disease.

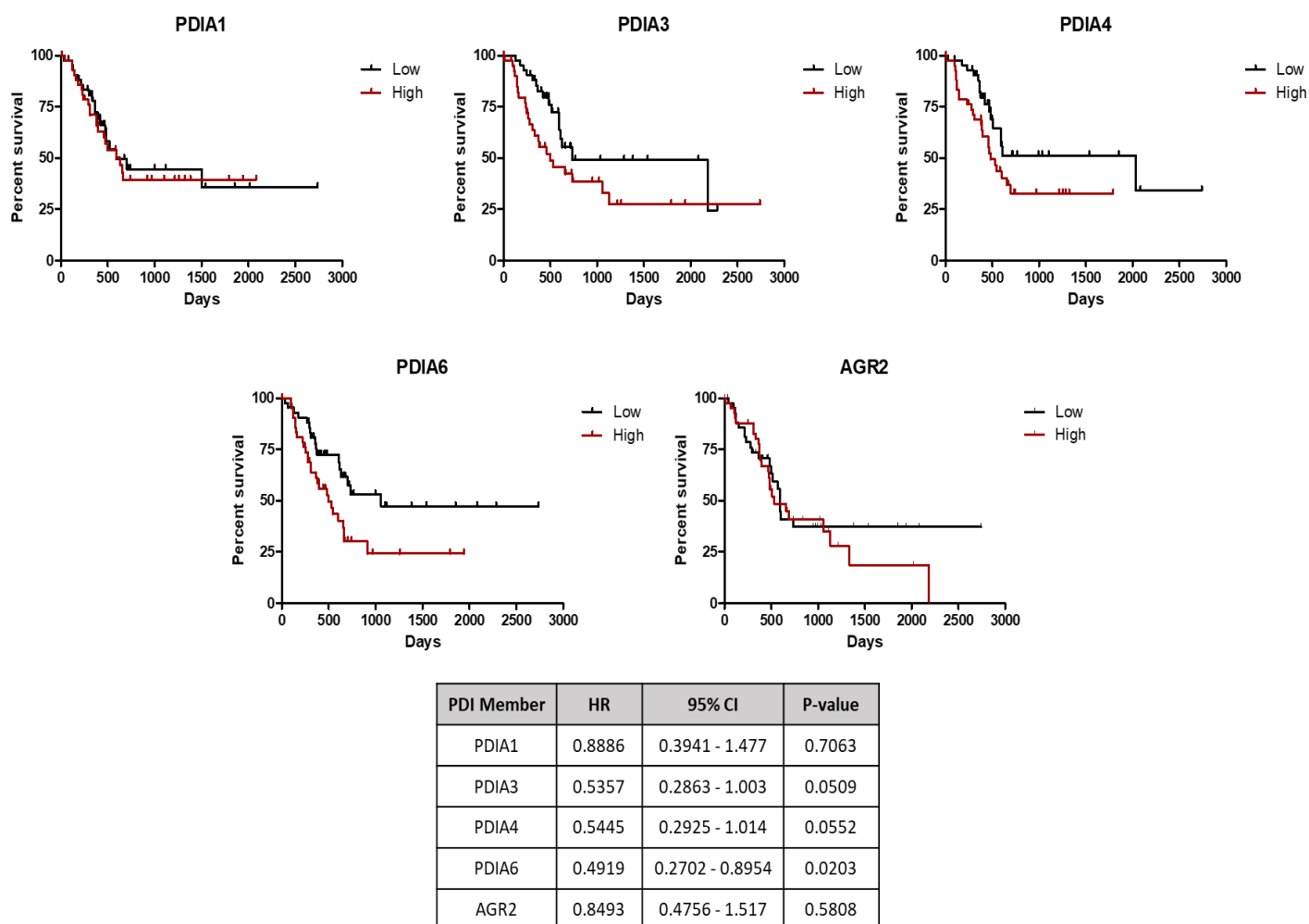


Figure 3.6. Kaplan-Meier survival analysis for high and low expression levels of PDI.

Correlation of PDIA1, PDIA3, PDIA4, PDIA6 and AGR2 gene expression with prognosis of pancreatic cancer survival. Clinical outcome data for patient survival was obtained from the TCGA database (TCGA, 2024). These data were converted into binary code for survival analysis, where 0=dead and 1=alive. Survival curves were generated by inputting this data into GraphPad Prism software 5 (GraphPad Software; California, USA) to compare patients with high (red) and low (black) gene expression. Expression was classified across all patients (n=179) as high if above the median expression (>50% expression quantile) and classified as low if below the median expression (<50% expression quantile). Significance was determined by comparison of the curves using the Log-rank (Mantel-Cox) Test. *p<0.05, HR = hazard ratio, CI = confidence interval.

3.3.4 PDI Protein-Protein Interaction Network Analysis

Protein-Protein Interactions (PPIs) were observed for PDIA1 (P4HB), PDIA3, PDIA4, PDIA6 and AGR2. The PPI network was created using the Pancreatic Expression Database (PED), which utilised the Search Tool for the Retrieval of Interacting Genes (STRING; string-db.org), which integrates both known and predicted PPIs, can be applied to predict functional interactions of proteins. Active interaction sources, including experimental repositories, computational prediction methods, and public text collections were applied as part of the analysis. A threshold confidence of interaction score of >0.4 was applied which is the default score for medium confidence in STRING. The PED used the Cytoscape plugin cytoHubba software (Cytoscape; Cytoscape.org) to create a visual representation of the PPI network, shown in Figure 3.7. The size of the nodes is related to the number of connections; the larger the number of connections is, the larger the size of the node. The colour intensity changes are related to fold change, with blue representing downregulation and red representing upregulation.

Studies have shown PPIs to be crucial events in various processes involved in cell signalling and in turn, play roles in processes such as cell proliferation, which may drive tumorigenesis. PDIA6 did not show any significant interactions, however interactions were determined with the other PDI members. The assessment of the upregulation and downregulation of these proteins highlights key proteins and downstream pathways potentially associated with PDI expression and consequential tumour progression.

PDIA4 shows interaction with upregulation of BiP (HSPA5), suggesting potential association in UPR pathway activation in pancreatic cancer. PDIA4 also shows an

interaction with upregulated peptidylprolyl cis-trans isomerase B (PPIB). PPIB is another protein upregulated by UPR activation (Zhang et al., 2022). AGR2 and PDIA1 both show interaction with upregulated ubiquilin 1 (UBQLN1), which has also been implicated to play a role in the UPR (Lu et al., 2009). These protein interactions highlight how UPR pathway activation may play a key role pancreatic tumorigenesis.

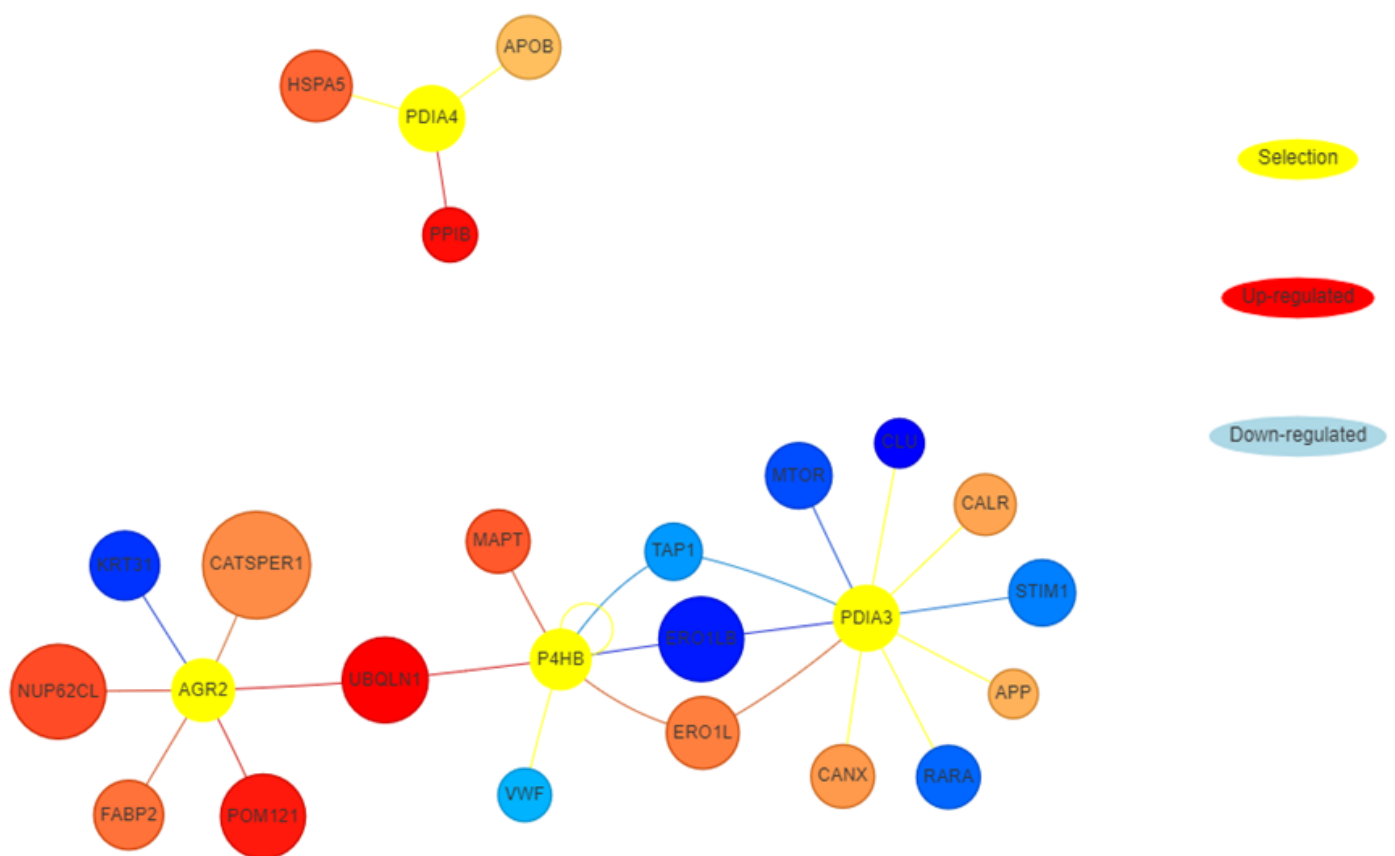


Figure 3.7. PDI Protein-Protein Interaction (PPI) Network.

The PPI network was constructed using the Pancreatic Expression Database (PED) using STRING (string-db.org) database. The PED used the Cytoscape plugin cytoHubba software (Cytoscape.org) to create a visual representation of the PPI network. The network shows protein interactions with an interaction score of >0.4 for genes; PDIA1 (P4HB), PDIA3, PDIA4 and AGR2. The size of the nodes is related to the number of connections; the larger the number of connections is, the larger the size of the node. The colour intensity changes are related to fold change, with blue representing downregulation and red representing upregulation.

3.3.5 Differential UPR Pathway Gene Expression Between Pancreatic Tumour and Normal Tissue

A combination of the TCGA database (TCGA, 2024) and the GTEx database (GTEx Portal, 2024) was used to compare the mRNA gene expression levels of potential key UPR pathway members; BiP, ATF4, ATF6, XBP-1, CHOP and Caspase-3 in samples from pancreatic cancer tumour tissue and normal pancreas tissue (Figure 3.8). BiP, CHOP and Caspase-3 all show a significantly increased ($p < 0.05$) expression in pancreatic tumours compared to normal tissue. This may indicate UPR pathway activation in pancreatic tumours, however both ATF4 and ATF6 show no significantly increased levels of expression in tumours. Furthermore, XBP-1 shows a significantly higher ($p < 0.05$) expression in normal tissue, suggesting potential suppression of the UPR receptor IRE1 in tumours. More investigation is needed to understand the roles of these key UPR pathway genes in pancreatic cancer.

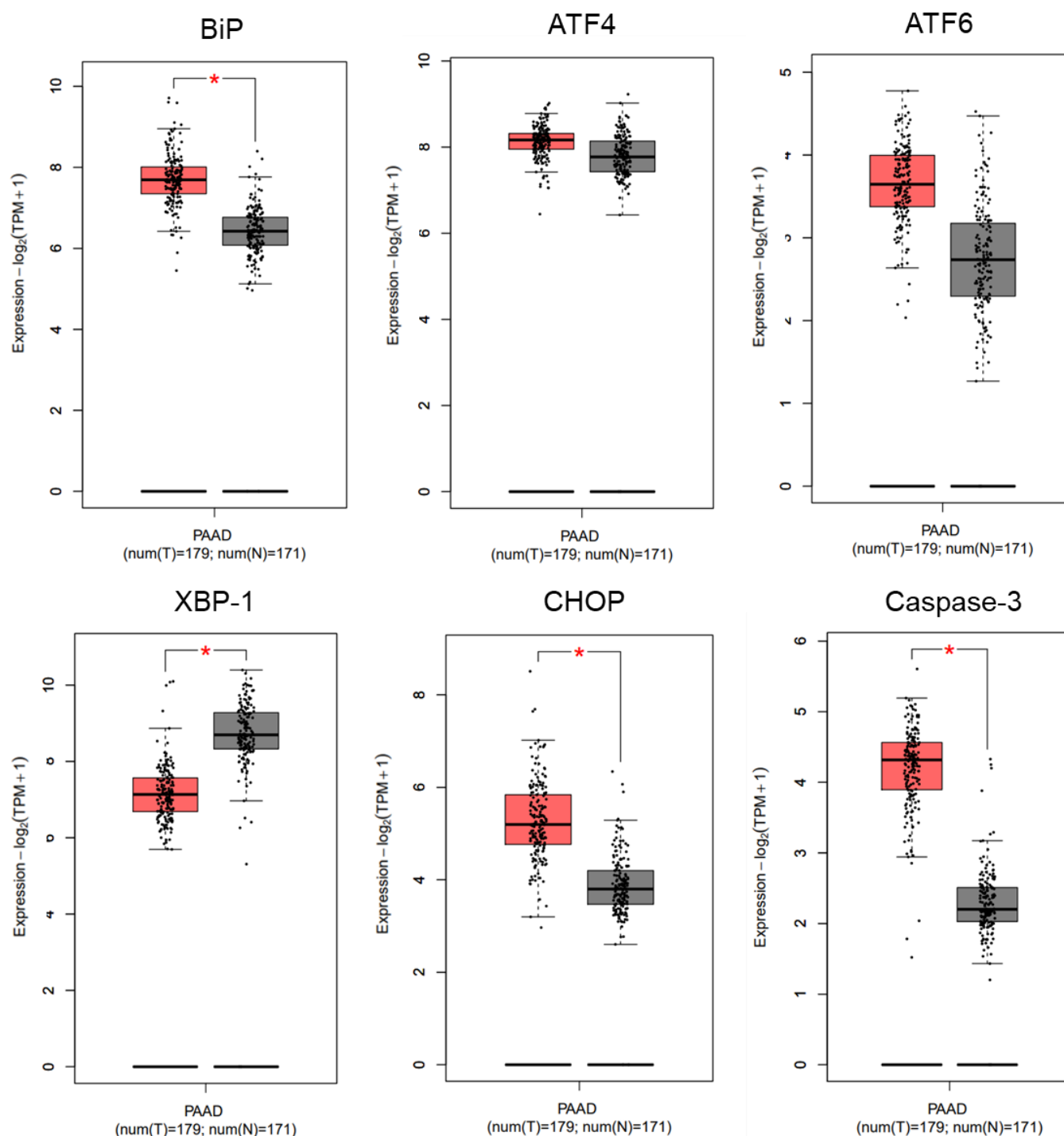


Figure 3.8. Comparison of Pancreatic Tumour Vs Normal UPR Gene mRNA Expression.

BiP, ATF4, ATF6, XBP-1, CHOP and Caspase-3 mRNA gene expression levels were obtained from pancreatic cancer tumour samples (T) (n=179) and normal pancreas tissue samples (N) (n=171) using a combination of TCGA (TCGA, 2024) and GTEx databases (GTEx Portal, 2024). BiP, XBP-1, CHOP and Caspase-3 showed significantly higher expression in tumour samples than in normal samples (*P<0.05). RNA expression is shown as transcripts per million reads and Log₂ transformed ((log₂(TPM + 1))).

3.3.6 Heat map of PDI and UPR gene correlation analysis

Correlations for PDI and UPR genes were calculated using the Pearson Product Moment Correlation Coefficient (PMCC) value. A heatmap was generated using the PED database (Marzec et al., 2018) which used mRNA gene expression data from a combination of the TCGA (TCGA, 2024) and GTEx databases (GTEx Portal, 2024) (n=179). The heatmap was representative of PDI and UPR gene correlations in pancreatic cancer and normal tissue (Figure 3.9). In normal tissue positive correlations are shown between all PDI and UPR genes with the exception of caspase-3, which shows a negative correlation among all PDI members and other UPR-associated genes. In pancreatic cancer tissue positive correlations between PDI members and UPR genes are shown to be weaker. This suggests that the UPR pathway is potentially being suppressed in a tumorigenic environment. Caspase-3 also shows some positive correlations with PDI members, particularly with AGR2, as opposed to the negative correlation seen in normal tissue. In tumours CHOP is shown to be negatively correlated with XBP-1 and ATF6, whereas these showed a positive correlation in normal tissue. ATF4 and ATF6 also switch to a negative correlation in tumours. These changes in PDI and UPR expression correlations, again highlight the need for further investigation into how UPR pathway activation is changed in pancreatic cancer.

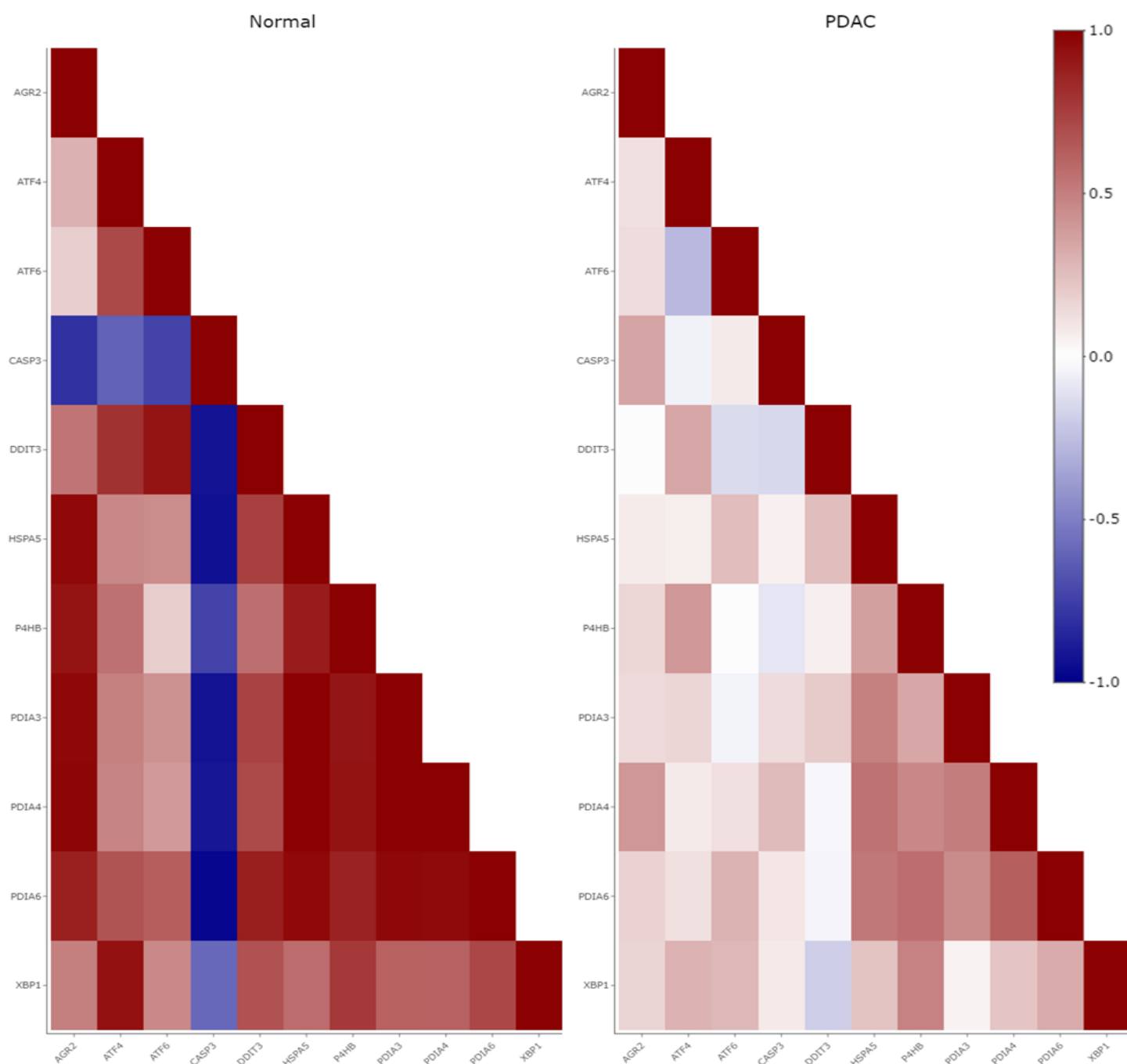


Figure 3.9. Heat map of PDI and UPR related gene correlation analysis.

Correlations for PDI members: PDIA1, PDIA3, PDIA4, PDIA6 and AGR2, and UPR pathway genes: ATF4, ATF6, Caspase-3 (CASP3), CHOP (DDIT3), BiP (HSPA5) and XBP-1, are calculated using the Pearson Product Moment Correlation Coefficient (PMCC) value. The pancreatic expression database (PED) (Marzec et al., 2018) was used to generate a heatmap, used mRNA gene expression data from a combination of the TCGA (TCGA, 2024) and GTEx databases (GTEx Portal, 2024) (n=179). Results are presented for each comparative group in the study in both pancreatic tumour (PDAC) and normal tissue. Red represents a positive correlation; blue represents a negative correlation and white represents no correlation. The colour reflects the intensity of the correlation. The closer the correlation coefficient is to 1, the more it represents the red colour.

3.4 Discussion

3.4.1 Determination of Key PDI Members

Analysis of RNA-sequencing data for PDI gene expression in pancreatic cancer demonstrated higher levels of expression in certain PDI members (Figure 3.1A). These included; PDIA1, PDIA3, PDIA4, PDIA6 and AGR2. This was also reflected in RNA-sequencing data demonstrating PDI expression in pancreatic cancer cell lines (Figure 3.1B). These five PDI members were therefore chosen out of the 21 PDI members (Table 1.3) to be the focus of research; to analyse their potential as a target for pancreatic cancer treatment. The potential influence of the expression of these PDI members was then analysed using TCGA patient survival data. All members except PDIA1 showed a decreased survival rate with high expression, however only PDIA6 was shown to be significant (Figure 3.6). Whilst survival analysis is useful to give an indication into the potential influence of PDI in tumorigenesis, the relatively low patient sample number (n=179) suggests that the individual influence of each member cannot be completely accurately assessed and compared.

The potential role of PDI in pancreatic cancer was also demonstrated through comparison of tumour vs normal gene expression. Figure 3.2 demonstrates a significantly higher gene expression in tumour samples in PDIA3, PDIA4 and AGR2, with AGR2 demonstrating the greatest difference in expression compared with normal tissue. Figure 3.3 further compares PDI expression in tumour vs normal tissue through analysis of protein expression via immunohistochemistry. These data show only AGR2 to have a higher level of expression in tumour samples compared to normal tissue. Notably, AGR2 was the only PDI member to show a significant difference in expression between tumour stages (Figure 3.4), and the only member

to show an increased expression in progressive disease post therapy (Figure 3.5), though this was not statistically significant. However, in conjunction with the overexpression observed in tumour samples, this highlights the potential role of AGR2 in tumorigenesis and demonstrates its potential as a therapeutic target for further research.

3.4.2 AGR2 as a Potential Therapeutic Target

The comparative use of the AsPC-1 and Mia-PaCa-2 cell lines could give potential insight into the influence of AGR2 expression in tumorigenesis. RNA-sequencing data shows high expression of AGR2 in AsPC-1, and a very low expression of AGR2 in Mia-PaCa-2 (Figure 3.1B). The expression levels of the other key PDI members are shown to be very similar in both cell lines, and hence were chosen as suitable cell lines for study. As previously discussed, AGR2 has been shown to be expressed across all stages of pancreatic cancer progression. This was observed in Figure 3.4, where AGR2 was expressed throughout each tumour stage. However, significantly high expression was shown in stage IV tumours. This supports recent research demonstrating an overexpression of AGR2 in PanIN 3 lesions, consequently implying the potential role of AGR2 in the development of advanced, progressive disease (Norris et al., 2013). Whilst AGR2 can therefore be highlighted as a potential therapeutic target, the mRNA and protein overexpression of AGR2 in tumours, demonstrated in Figures 3.2 and 3.3, also implies its potential as a pancreatic cancer biomarker. However, it is also acknowledged that the findings in Figure 3.3 should be interpreted with caution due to the small sample size of only $n=1$. Research has suggested that AGR2 could be overexpressed prior to KRAS-activating mutations,

therefore serving as an early-stage biomarker, as demonstrated in Figure 1.13 (Dumartin et al., 2017). With Figures 3.3 and 3.4 showing an increased AGR2 expression in tumours compared to other PDI members, its distinction as a potential biomarker and therapeutic target are a focus of further research.

The distinction of AGR2 from other PDI family members can be also observed structurally. Figure 1.8 demonstrates AGR2 to be evolutionarily diverse from other PDI members as it is recognised as part of the AGR2 subfamily. Other structural differences are apparent in AGR2 in that it only possesses a non-catalytically active b-type domain. Non-catalytic PDI members remain poorly understood. AGR2 is evolutionarily divergent from the classic CXXC active site motif, it instead possesses a CXXS motif. The CXXS motif has been associated with more activity in disulfide bond isomerisation rather than rearrangement (Norgaard et al. 2001). A CXXS motif may also contribute to specialised ER functions such as retrograde transport of secretory proteins out of the ER and interactions with intermediates in redox reactions (Norgaard et al. 2001). ER associated functions of AGR2 have been demonstrated to contribute to ER homeostasis through UPR signalling and hence implies its associated role in cell survival and potential as a therapeutic target (Higa et al. 2011).

Figure 3.7 shows a protein-protein interaction network of AGR2 with several potential protein targets that show upregulation in pancreatic cancer. Several of these proteins have also been highlighted as potential therapeutic targets in other cancer types. UBQLN1 was shown to be upregulated in gastric cancer where it has been

associated with malignant behaviour including, metastasis, poor differentiation, and drug resistance (Bao et al. 2018). POM121 was shown to be upregulated in aggressive prostate cancers and promotes tumour growth (Bradley, 2018). Interestingly the depletion of POM121 was shown to lead to the downregulation of the oncogenic transcription factor E2F1, which plays a key role in cell survival in the UPR pathway. E2F1 knockdown sensitises cells to ER stress-dependent apoptosis, demonstrating both POM121 and E2F1 as key therapeutic targets (Pagliarini et al. 2015). In addition, UBQLN1 has also been shown to exert a pro-protective effect through UPR pathway signalling, suppressing CHOP induction and increasing cell viability (Lu et al., 2009). It has further been shown that downregulation of UBQLN1 does trigger an induction of PDI (Lu et al., 2009).

Figure 3.8 also shows a PDIA1 (P4HB) interaction with upregulated UBQLN1. This insinuates that perhaps UBQLN1 and PDI members work in conjunction to exert UPR-associated protective mechanisms. This demonstrates how PDI interactions and the UPR pathway can be linked to better understand their potential roles in pancreatic cancer progression. Figure 3.8 further shows the interaction of PDIA4 with upregulated BiP (HSPA5) and PPIB. As previously discussed, BiP is well recognised as an activator of UPR pathway receptors, implying that PDIA4 may also contribute to UPR activation through BiP interactions. PPIB has been shown to act as the UPR activates its downstream targets, acting to degrade CHOP, inhibiting UPR-associated apoptosis (Zhang et al., 2022). With several PDI members exhibiting protein interactions with varying UPR pathway-associated proteins, it is clear that further research needs to be carried out to understand the relationship

between PDI and the UPR to develop the most effective anti-cancer therapeutic strategies.

3.4.3 UPR Pathway Genes as potential therapeutic targets

Correlations in expression between PDI members and UPR genes were analysed in pancreatic tumour and normal tissue via calculation of PMCC value and visualised on a PED-generated (Marzec et al., 2018) heatmap. Normal pancreatic tissue shows positive correlations in the expression of PDI members and UPR genes, except for caspase-3 which is exclusively negatively correlated with all PDI members and other UPR genes. This suggests that the UPR pathway is being activated to maintain ER homeostasis in normal pancreatic tissue, and caspase-3 driven apoptosis is suppressed. In contrast, in pancreatic tumours positive expression correlations between PDI members and UPR genes are consistently weaker. This suggests that the UPR pathway is potentially being suppressed in a tumorigenic environment.

Caspase-3 correlations are also significantly altered in tumours, showing weaker negative correlations, and some positive correlations with PDI members, in particular AGR2. Recent research in prostate cancer have shown AGR2 and caspase-3 to exhibit a strong relationship in caspase-3 driven pathways of apoptosis (Chanda et al., 2014). More research is needed to define this relationship in pancreatic cancer. These significant alterations in expression correlation in tumours between PDI and UPR genes again emphasise the need for a clearer understanding of how UPR pathway activation is changed in pancreatic cancer.

Further investigations were made into pancreatic tumour expression levels of key UPR pathway genes: BiP, ATF4, ATF6, XBP-1, CHOP and Caspase-3 (Figure 3.8). BiP shows a significantly increased expression in tumour compared to normal tissue, as BiP is bound to UPR receptors in its inactive state, this suggests that in tumorigenic conditions the UPR is activated causing elevated levels of BiP due to dissociation from UPR activated receptors. Despite this suggested elevated UPR pathway activation, ATF4 and ATF6 do not show any significant difference in tumour expression. XBP-1 also shows a significantly higher expression in normal tissue, suggesting that downstream UPR pathway genes are not being overexpressed and UPR pathway activation may even be repressed in a tumorigenic environment. Interestingly though, both apoptosis-associated CHOP and Caspase-3 are shown to be overexpressed in tumours. Whilst this could indicate the activation of chronic UPR, upstream ATF4 does not show overexpression. CHOP and Caspase-3 could therefore be overexpressed through other unknown oncogenic pathways. A better understanding of the role of the UPR in pancreatic tumorigenesis is needed to determine genes of therapeutic potential.

3.4.4 Determination of Pancreatic Cancer Cell Lines for Experimental Use

The current study aimed to compare PDI gene expression across pancreatic cell lines; Panc-1, Mia-PaCa-2, AsPC-1, Capan-2 and Hapan-II (Figure 3.1B), to determine the suitability of each cell line for experimental use. Cell lines were determined based on their expression levels of the key PDI members identified as potential targets for further research which included, PDIA1, PDIA3, PDIA4, PDIA6

and AGR2. Analysis of mRNA expression (EBI database) showed that Panc-1 had a high level of expression across all PDI members of interest, including the most highly expressed member PDIA1, and so was chosen as a suitable cell line for study. With the identification of AGR2 as a potential primary therapeutic target through data mining analyses as previously described, AsPC-1 was chosen for its high level of AGR2 expression. In contrast, Mia-PaCa-2 was shown to not naturally express AGR2. Mia-PaCa-2 was therefore also chosen for further study as a comparative cell line to AsPC-1 and Panc-1 to assess the influence of AGR2 expression.

CHAPTER 4 – THE EFFECTS OF PDI INHIBITOR TREATMENT AND PDI KNOCKDOWN ON PDI ACTIVITY, PANCREATIC CANCER CELL PROLIFERATION AND MIGRATION

Chapter 4 - Effects of PDI inhibitor treatment and PDI knockdown on PDI activity, pancreatic cancer cell proliferation and cell migration

4.1 Introduction

4.1.1 Pacma-31 and CCF642

As a druggable target, PDI has been a recent focus of research as a potential, novel anticancer therapy. This has therefore promoted research into the use of small molecule PDI inhibitors for cancer treatment. Table 1.4 shows several PDI inhibitors that have been used in research, each possessing differences in mode of action and therapeutic potential in varying cancer types. With little known about the effects of PDI inhibitors in pancreatic cancer, PDI inhibitors Pacma-31 and CCF642 were chosen for use in this study based on their potential seen in other cancer types.

Pacma-31 was identified as an effective PDI inhibitor through studies in ovarian cancer in which a series of PACMA (propionic acid carbamoyl methyl amide) derivatives were employed to demonstrate significant cytotoxicity in ovarian cancer cell lines (Xu et al., 2012). Pacma-31 was found to exhibit anticancer effects in ovarian cancer, both in vitro, in known chemoresistant cell line, and in vivo, coupled with limited toxicity to normal tissue (Xu et al., 2012). Furthermore, the use of Pacma-31 as a potential anticancer treatment is complimented by the fact that it has demonstrated oral bioavailability (Xu et al., 2012). Pacma-31 acts as an irreversible PDI inhibitor, possessing an IC_{50} value of 10 μ M. Pacma-31 inhibits PDIA1 through disruption of the CXXC motif by binding to the Cys397 and Cys400 residues in the α' domain of the active site (Xiong et al., 2020). Disruption of cysteines at the active site has proven to be an efficient method in disrupting PDI activity. Disruption at a single active site demonstrated a 50% loss in of activity in PDIA1, while disruption of

both active sites completely abolishes PDIA1 activity (Vuori et al., 1992). Pacma-31 is therefore applicable for inhibition of PDI members possessing a canonical CXXC motif such as PDIA1. Whilst it is not clear how many PDI members Pacma-31 targets, it has been shown to irreversibly inhibit PDIA1 while also showing interactions with other members including, PDIA2, PDIA3, PDIA4, PDIA6, and TXNDC5 (Xu et al., 2019). This research emphasises the potential of Pacma-31 as an effective PDI inhibitor in pancreatic cancer.

CCF642 is also recognised as an irreversible PDI inhibitor, sharing a similar mode of action to Pacma-31, however it has been recognised to be of a higher potency. CCF642 has an IC₅₀ value of 2.9µM and differs slightly in its inhibition action in that it covalently modifies the lysine residue (Lys401 or Lys57) at the catalytic active site of PDIA1 (Hasipek et al., 2021). It therefore still acts to disrupt the CXXC motif as shown with Pacma-31 PDI inhibition. The binding sites in which CCF642 and Pacma-31 target PDIs with a CXXC motif such as PDIA1 are shown in Figure 4.1. CCF642 has been shown to disrupt PDIA1 activity in multiple myeloma (MM) cells, causing acute ER stress and consequent apoptosis (Vatolin et al., 2016). CCF642 treatment was shown to induce apoptosis through pro-apoptotic ER stress mediated CHOP induction (Vatolin et al., 2016). This suggests that CCF642 induces activation of chronic UPR signalling, and consequent UPR-associated apoptosis. The anticancer effects of CCF642 were shown to be in line with that of first-line MM therapeutic, bortezomib, with CCF642 even maintaining efficacy in bortezomib resistant cells (Hasipek et al., 2021). Research has shown that inhibition of PDIA1 specifically drives these anticancer effects in MM cells. Like Pacma-31, CCF642 is non-selective, and has additionally been shown to bind to PDIA3 and PDIA4 (Yang

et al., 2022). More recently an optimised analogue of CCF642, CCF642-34, has been developed for specific PDIA1 inhibition in the treatment of MM (Hasipek et al., 2021). Due to the higher potency of CCF642 and evidence of involvement in ER-stress induced apoptosis through CHOP induction, CCF642 provides further potential for the investigation into the effects of PDI inhibition in pancreatic cancer.

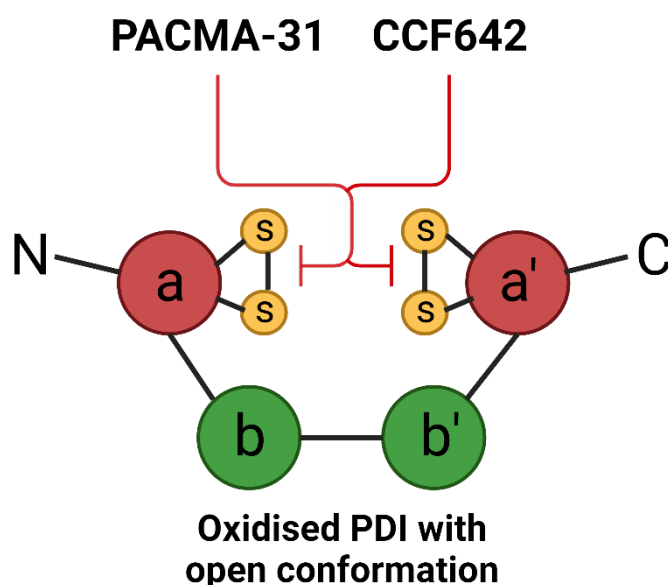


Figure 4.1. PDI binding sites of Pacma-31 and CCF642. (Original diagram created from references in text).

Since both Pacma-31 and CCF642 are strongly linked to PDIA1 inhibition, comparison of their effects with the selective siRNA knockdown of PDIA1 will give insight into the importance of PDIA1 in pancreatic cancer progression, as the most highly expressed PDI member. Results shown in PDI inhibitor studies will reflect the combined inhibition of several PDI members as well as other off-target effects. Pacma-31 and CCF642 were chosen as PDI inhibitors for this study due to their anticancer effects as described, but also due to their ability to bind to PDI members; PDIA1, PDIA3, PDIA4 and PDIA6, all of which were outlined as the most highly

expressed PDI members in pancreatic cancer (Figure 3.1). While most PDI inhibitors have been developed to target PDIA1 and other canonical CXXC motif possessing PDI members, specific inhibitors for the structurally differing AGR subfamily have only recently been under development. A recent study has discovered the ability of Disulfide bond Disrupting Agents (DDAs), previously identified as anticancer compounds driving the simultaneous downregulation of EGFR and activation of death receptors 4 and 5 (DR4/5), to target AGR2 (Law et al., 2022). This study identified DDAs as the first small molecule to act as active site inhibitors of AGR2 (Law et al., 2022). However, inhibition of AGR2 is non-specific with DDAs also shown to target PDIA1 and ERp44 (Law et al., 2022). With the emphasis of the current study highlighting AGR2 as a potential therapeutic target for pancreatic cancer, specific AGR2 inhibition has been explored through siRNA knockdown.

4.1.2 PDIA1 and AGR2 in Cancer

PDIA1 and AGR2 are the two most highly expressed PDI family members in pancreatic cancer (Figure 3.1), highlighting them as potential therapeutic targets. The potential oncogenic influence of AGR2 in pancreatic cancer pathogenesis has been previously described, whereby an overexpression of AGR2 has been observed in both early and late PanIN lesions (Figure 1.13). This suggests potential implications of AGR2 as both a biomarker and therapeutic target in pancreatic cancer. Despite these implications, the role of AGR2 in pancreatic cancer progression remains poorly understood and further investigations are needed into its influence, particularly as a target of the UPR pathway. AGR2 has been described to play a protective role in the UPR, aiding the maintenance of ER homeostasis, and

has been shown to be upregulated through activations of IRE1 α and ATF6 signalling arms (Higa et al., 2011). It was therefore an aim of the current study to inhibit AGR2 by method of siRNA knockdown to examine the impact this has on ER stress and consequent detriment to pancreatic cancer cell survival through the induction of apoptotic markers. Aside from its role in the UPR, AGR2 has also been associated with various oncogenic pathways, shown in Figure 1.14. These include pathways promoting cell proliferation such as the induction of EGFR and cyclin D1, as well as the inhibition of p53 (Dong et al., 2011, Vanderlaag et al., 2010, Pohler et al., 2004). AGR2 has also been shown to promote pathways of migration and metastasis through the activation of cathepsin B/D and mucins (Dumartin et al., 2011). The study therefore aims to assess the influence of AGR2 inhibition, on not only ER-stress associated apoptosis, but also on pancreatic cancer cell proliferation and migration.

PDIA1 has shown influence across many stages of carcinogenesis and various cancer types, thought to be attributed to its roles in protein folding and ER homeostasis (Bakker et al., 2022). This is reflected in Figure 1.12 which demonstrates an overexpression of PDIA1 across most tumour types. While research has so far demonstrated little investigation into the roles of PDIA1 in pancreatic cancer, a recent study has suggested PDIA1 as a potential biomarker for type I diabetes (Syed et al., 2023). An overexpression of PDIA1 was identified as an early marker of β cell stress, hence indicating the onset of type 1 diabetes (Syed et al., 2023). This study is of note in the context of pancreatic cancer due to the number of diabetes-associated oncogenic pathways, shown in Figure 1.3. It could be

hypothesised that PDIA1 may influence both diabetes and pancreatic cancer onset through these shared pathways.

Though this evidence of PDIA1 influence in pancreatic cancer remains unclear, PDIA1 has been studied more extensively in other cancer types. PDIA1 has shown to have particular influence in breast cancer, where PDIA1 has been shown to induce a diverse range of oncogenic pathways. PDIA1 has demonstrated regulation of NF κ B, p53 and HIF-1 α signalling, therefore influencing pathways of both cell proliferation and migration (Bakker et al., 2022). Research has shown considerable evidence of PDIA1 involvement in the promotion of metastasis across several cancer types. In axillary lymph node metastatic breast tumours, PDIA1 expression is found to be significantly higher than in primary tumours (Thongwatchara et al., 2011). In breast cancer, PDIA1 metastatic influence has been attributed to activations of HIF-1 α and metalloproteases (MMPs) (Bakker et al., 2022). PDIA1 expression has also been associated with the metastasis of malignant gliomas and increased invasiveness of liver cancer cells through EMT induction (Bakker et al., 2022). These studies highlight how PDIA1 could have influence on later-stage metastatic pancreatic tumours and encourage the investigation into the effects of PDIA1 inhibition of pancreatic cancer cell migration.

Further to this, the knockdown of PDIA1 has been shown to induce cytotoxicity in breast cancer and neuroblastoma cell lines, via caspase activation (Hashida et al. 2011). This supports the primary hypothesis of the study which states that PDIA1 inhibition induces chronic UPR signalling and consequent activation of apoptotic

pathways. Whilst the role of PDIA1 in UPR signalling is yet to be fully elucidated, it has been proposed to influence activation of both the PERK and IRE1 α signalling arms. Oxidised PDIA1 has been shown to trigger PERK activation, while reduced PDIA1 shows PERK inhibition (Kranz et al. 2017). This demonstrates how the redox regulation of PDIA1 influences UPR pathway activation. PDIA1 has also been shown to bind with IRE1 α , with which it acts to attenuate prolonged and chronic UPR signalling (Yu et al. 2020). PDIA1 therefore differs in its known UPR interactions compared to AGR2 which acts as a downstream protective UPR gene. In contrast, PDIA1 has shown evidence of influence upstream, regulating UPR pathway activation. This study therefore aims to trigger the induction of chronic UPR signalling through targeted PDIA1 inhibition.

4.1.3 Hypothesis and Aims

It is hypothesised that the inhibition of PDI by both PACMA-31 and CCF642 treatment and selective inhibition of PDIA1 and AGR2 by siRNA knockdown will disrupt cell proliferation and cell migration in Panc-1, Mia-PaCa-2 and AsPC-1 pancreatic cancer cell lines.

To investigate the effects of PDI inhibition on pancreatic cancer cell proliferation and migration, the aims of the current study were as follows:

- To assess the effect of PACMA-31 and CCF642 treatment on PDI activity in pancreatic cancer cell lines
- To assess the effect of PACMA-31 and CCF642 treatment on pancreatic cancer cell proliferation by BrdU assay and cell viability by MTT assay

- To assess the effect of PACMA-31 and CCF642 treatment on pancreatic cancer cell migration by scratch assay
- To significantly knockdown PDIA1 and AGR2 by siRNA transfection and to confirm knockdown by measurement of PDIA1 and AGR2 mRNA expression via RT-qPCR and protein expression via Western blotting
- To assess the effect of PDIA1 and AGR2 siRNA knockdown on PDI activity in pancreatic cancer cell lines
- To assess the effect of PDIA1 and AGR2 siRNA knockdown on pancreatic cancer cell proliferation by BrdU assay and cell viability by MTT assay
- To assess the effect of PDIA1 and AGR2 siRNA knockdown on pancreatic cancer cell migration by scratch assay

4.2 Materials and Methods

4.2.1 Cell Culture and Seeding

Panc-1, Mia-PaCa-2 and AsPC-1 pancreatic cancer cell lines were cultured as described in section 2.1. Cell lines were maintained at 37°C in a humidified 5% CO₂ incubator in either 75cm² flasks or 25cm² flasks for PDI activity assays. Cells were seeded into 6 well plates for protein or RNA extraction, 96 well plates for cell proliferation assays or 24 well plates for cell migration assays.

4.2.2 PDI Inhibitor Treatment

Pacma-31 and CCF642 (Bio techne, UK) PDI inhibitors were dissolved in DMSO to prepare 10mM stock solutions. The stock solutions were diluted in DMEM or RPMI

medium to concentrations for treatment, described in Table 2.1. Cells were treated 24 hours after seeding and a second time after 48 hours. The medium was changed after 72 hours. Cells were therefore treated with PDI inhibitors for a total of 48 hours prior to proliferation and migration assays or protein and RNA extractions.

4.2.3 siRNA Transfection

siRNA transfection was carried out using ON-TARGET plus SMART pool siRNA (Dharmacon, UK) according to manufacturer's instructions. AGR2 and PDIA1 were subsequently knocked down as described in section 2.3. The details of siRNA products used are shown in Table 2.5.

4.2.4 Protein Extraction and Western Blot

Protein extraction and Western blotting was carried out as described in sections 2.7, 2.8 and 2.9. Cells were transfected with siRNA prior to protein harvesting to detect AGR2 and PDIA1 knockdown. 30µg protein lysates were measured using 15% SDS-PAGE and membranes were probed with corresponding antibodies. Details of antibodies and their dilutions are described in Table 2.4.

4.2.5 RNA Extraction, cDNA synthesis and RT-qPCR

RNA extraction was performed with the RNeasy® Mini RNA extraction kit (Qiagen, UK), following the manufacturer's instructions and as described in section 2.4. cDNA synthesis was then performed using the High-Capacity cDNA Reverse Transcription

Kit (Applied Biosystems, UK) according to the manufacturer's instructions and as described in section 2.5. mRNA levels were determined by real-time PCR using TaqMan gene expression assays (ThermoFisher, UK) for PDIA1 and AGR2. Relative mRNA levels were normalised to the mean CT value of the housekeeping gene RPLP0 (ThermoFisher, UK). Details of gene expression assay are shown in Table 2.3. Reactions were analysed and ΔCT and $\Delta\Delta CT$ were calculated according to the $2^{-\Delta\Delta CT}$ method to determine relative mRNA expression.

4.2.6 PDI Activity Assay

PDI activity was measured using the PDI activity assay kit (fluorometric) (Abcam, UK), according to the manufacturer's instructions and as described in section 2.11. The PDI activity assay is applied to measure the reductase activity of PDI. PDI activity was assessed following PDI inhibitor or AGR2 and PDIA1 siRNA knockdown treatments. Fluorescent standards were used to generate a standard curve for analysis according to Table 2.5. A synthetic quenched fluorescent substrate was added, which is cleaved in the presence of PDI to produce a fluorometric signal. Change in RFU over 30 minutes was calculated to determine relative PDI activity.

4.2.7 BrdU Proliferation Assay

Proliferation was measured using Cell Proliferation ELISA, BrdU (Colorimetric) kit (Roche, UK) following the manufacturer's protocol and as described in section 2.10.2. Cell seeding density was optimised for the 72 hour assay, to seed 4000 cells per well of a 96 well plate. Cell proliferation was measured by fluorescence indicating

BrdU incorporation into cell DNA during DNA replication. Cell proliferation was measured following both Pacma-31 and CCF642 PDI inhibitor treatment as described in section 2.2 or PDIA1 and AGR2 siRNA knockdown as described in section 2.3.

4.2.8 MTT Proliferation Assay

Cell viability was measured using the MTT cell viability assay kit (Biotum, UK) according to the manufacturer's instructions and as described in section 2.10.3. Cell viability was assessed based on the ability of metabolically active cells to cleave the yellow tetrazolium salt (MTT) to produce a purple formazan crystal. Optical density of the consequent colorimetric signal, which is therefore proportional to cell number, was measured to determine cell viability following both 48-hour PDI inhibitor and PDIA1 and AGR2 siRNA knockdown treatments.

4.2.9 Scratch Assay

Scratch assays were carried out to assess cell migration as described in section 2.13. Following 48-hour PDI inhibitor or PDIA1 and AGR2 siRNA knockdown treatment, the surface of each well was then scratched twice with a sterile 20µl Gilson pipette tip to produce a cross-shaped scratch wound. Cells were incubated post scratch for 30 minutes to minimise loss of adhesion. Each well of a 24 well plate was photographed using a EVOS XL core system imaging microscope (ThermoFisher Scientific, UK) at 5X magnification at the time points of 0, 24 and 48

hours post scratch. The area of the scratch was measured using Image J software to assess cell migration.

4.2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software 5 (GraphPad Software, USA). A one-way ANOVA was used to determine significance and analyse various between independent treatment groups, with post-hoc Tukey's multiple comparison tests to determine which treatment groups were significantly different if one-way ANOVA test showed significance. Significance is represented by; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.3 Results

4.3.1 Effects of PDI Inhibitor Treatment on PDI Activity

The effect of treatment with PDI inhibitors Pacma-31 and CCF642 on PDI activity was investigated (Figure 4.2). Panc-1, Mia-PaCa-2 and AsPC-1 were all treated with Pacma-31 and CCF642 for 48 hours at the concentrations detailed in Table 2.1. PDI reductase activity was measured over 30 minutes and the change in RFU was recorded to determine relative PDI activity. PDI activity was significantly decreased in a dose dependent manner, with 100 μ M Pacma-31 and 10 μ M CCF642 treatments showing the greatest decrease in PDI activity (**** $p < 0.0001$) across all three cell lines.

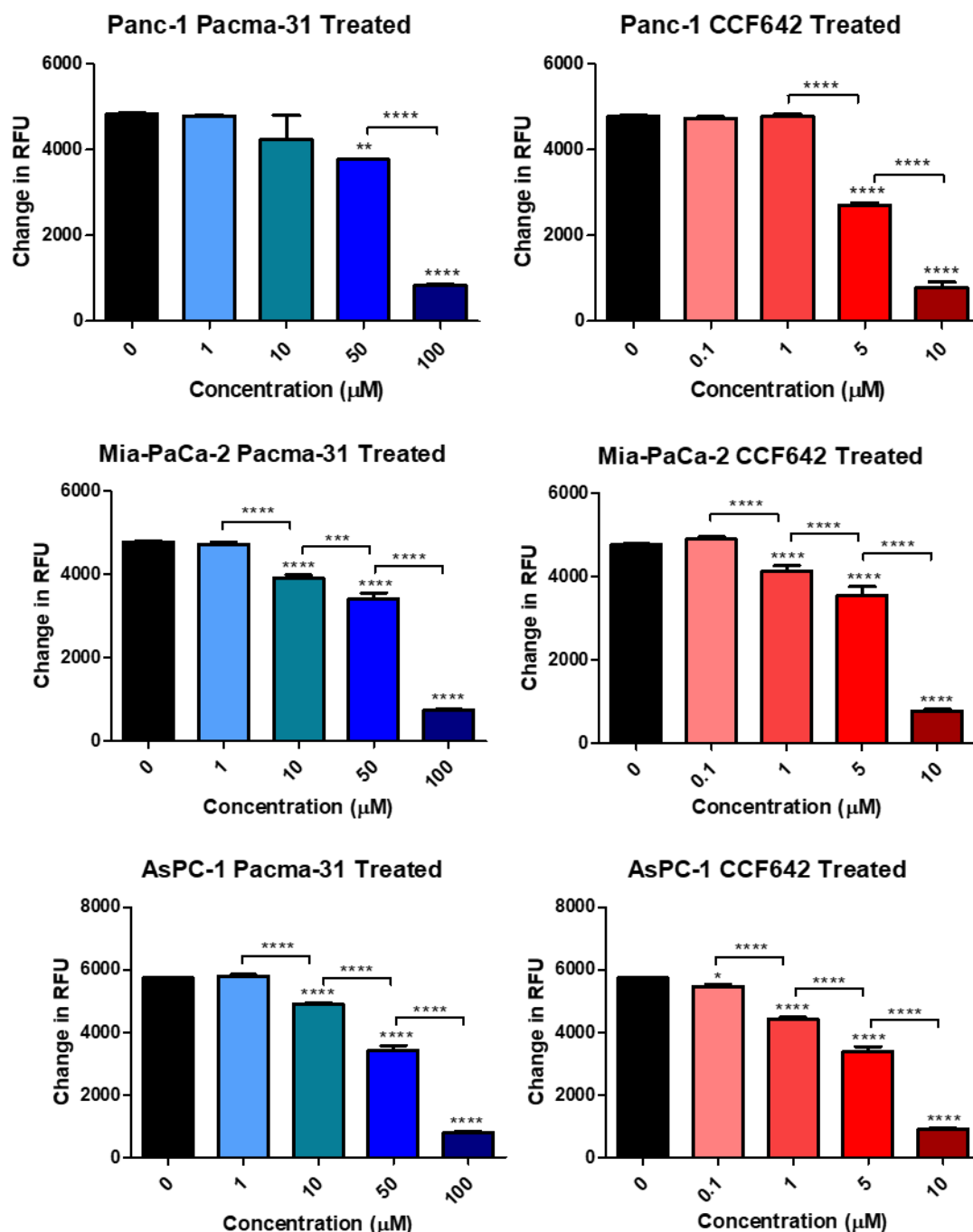


Figure 4.2. Pacma-31 and CCF642 treatment decreases PDI activity in pancreatic cancer cell lines.

PDI activity was measured following 48-hour Pacma-31 and CCF642 treatment. Following substrate addition, fluorescence was measured immediately in kinetic mode for 30 minutes at Ex/Em = 490/580. Change in RFU after 30 minutes was calculated to determine relative PDI activity. N=3 for each treatment group. Error bars denote standard deviation (SD). **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

4.3.2 Effects of PDI Inhibitor Treatment on Pancreatic Cancer Cell Proliferation

4.3.2.2 BrdU Proliferation Assay

Panc-1, Mia-PaCa-2 and AsPC-1 were treated with Pacma-31 and CCF642 for 48 hours. Absorption was measured to reflect the rate of cell proliferation, through Brdu incorporation into proliferating cells (Figure 4.3). Pacma-31 and CCF642 treatment resulted in a significant (**** $p < 0.0001$) dose-dependent inhibition of proliferation in all cell lines, with respective 100 μ M and 10 μ M treatments showing the greatest decrease in proliferation rate compared to the control. Pacma-31 and CCF642 treatment both showed the most significant inhibition of proliferation in Mia-PaCa-2 cells, suggesting that Mia-PaCa-2 cells are more susceptible to PDI inhibition.

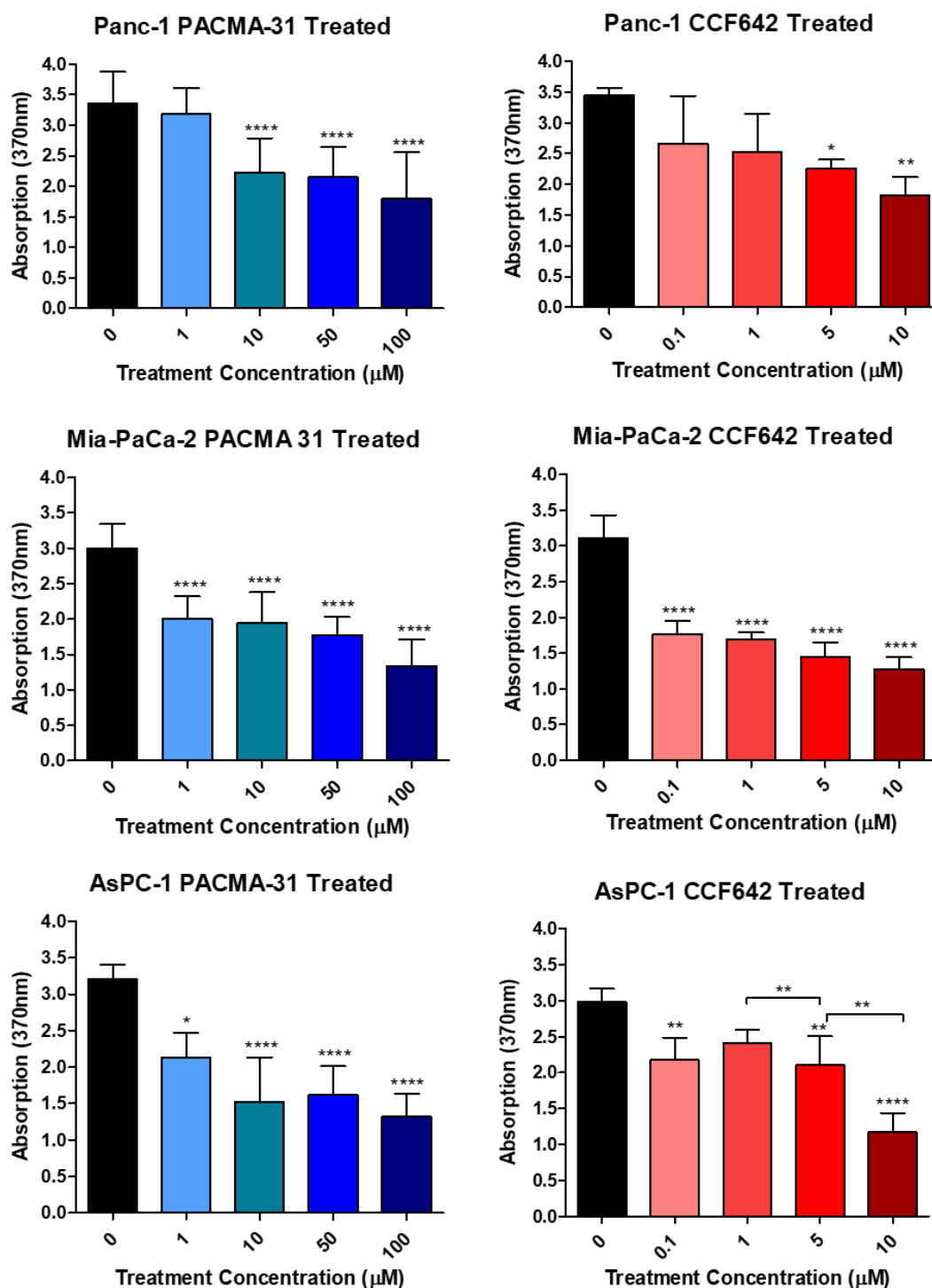


Figure 4.3. Pacma-31 and CCF642 treatment decreases the rate of cell proliferation in pancreatic cancer cell lines.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with PACMA-31 and CCF642 for 48 hours. Absorption (370nm) was measured after 72 hours. Data was collected from 8 wells for each treatment group, with 4 repeats of the experiment. N=4 for each treatment group. Error bars denote standard deviation. The degree of significance in comparison between the control and each treatment group were analysed. Significance is also shown compared between treatment concentrations. **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

4.3.2.3 MTT Proliferation Assay

The MTT assay was also used to determine cell growth with Pacma-31 and CCF462 48 hour treatment in Panc-1, Mia-PaCa-2, and AsPC-1 (Figure 4.4). Optical density was measured at 570nm, proportional to cell number. The MTT assay hence differs from the BrdU proliferation assay, in that it is more proportional to the determination of cell viability rather than rate of cell proliferation. Reflective of results shown in Figure 4.3, a dose dependent decrease in cell viability was shown with both Pacma-31 and CCF642 treatment across all cell lines, with respective 100 μ M and 10 μ M treatments showing the greatest decrease (**** $p < 0.0001$). A more significant decrease was shown across treatments in the MTT assay than in the BrdU assay, which may reflect the fact that the assay measures cell viability rather than proliferation alone, and so results may also account for some cell death.

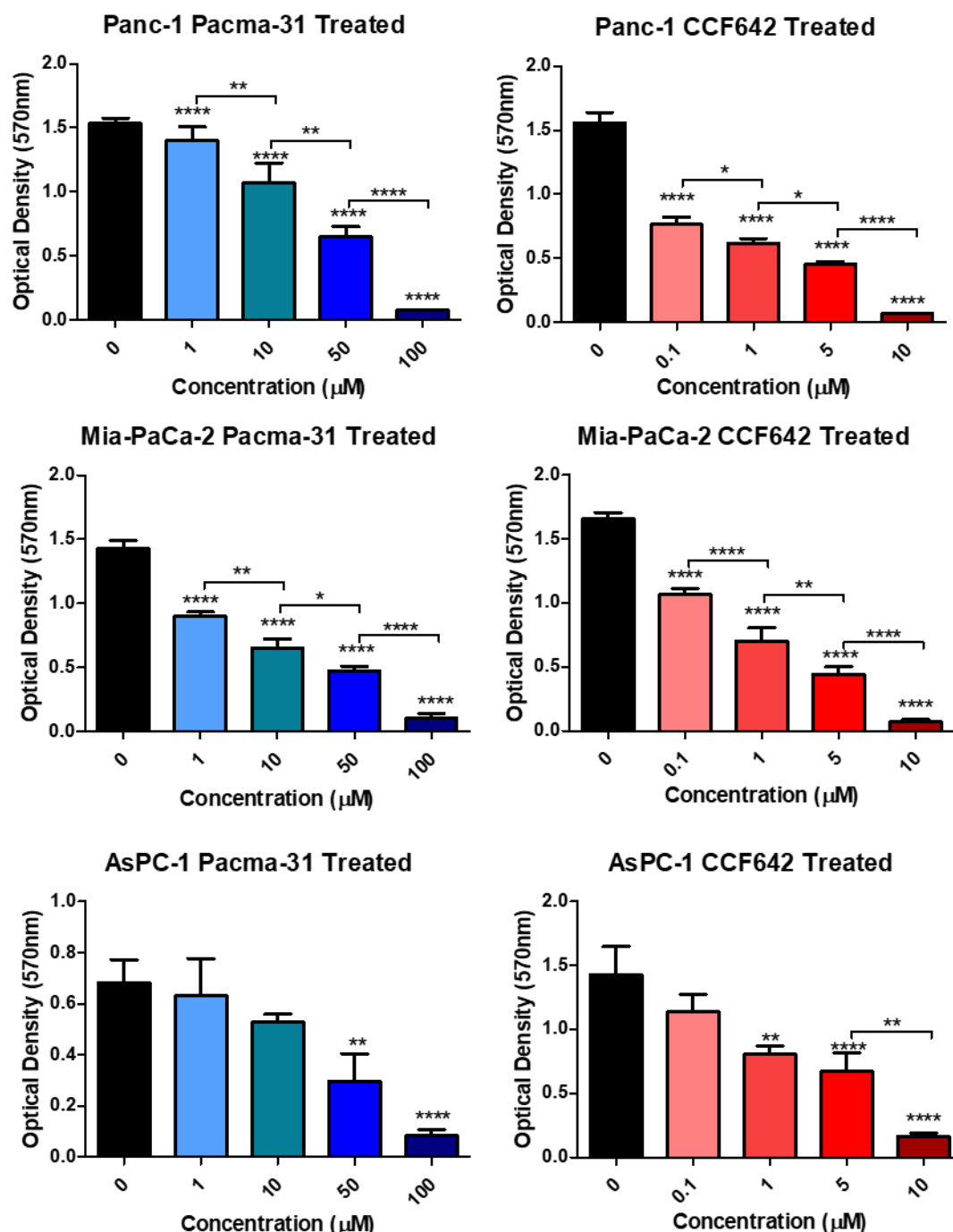


Figure 4.4. Pacma-31 and CCF642 treatment decreases pancreatic cancer cell viability.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with PACMA-31 and CCF642 for 48 hours. Optical Density (570nm) was measured after 72 hours. Data was collected from 6 wells for each treatment group, with 3 repeats of the experiment. N=3 for each treatment group. Error bars denote standard deviation (SD). The degree of significance in comparison between the control and each treatment group were analysed. Significance is also shown compared between treatment concentrations. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

4.3.3 Effects of PDI Inhibitor Treatment on Pancreatic Cancer Cell Migration

A scratch assay was performed to assess the effect of 48 hour Pacma-31 and CCF642 treatment on Panc-1, Mia-PaCa-2 and AsPC-1 cell migration (Figure 4.5). The area of the wound was measured to assess the rate of cell migration, at 0, 24 and 48 hours post treatment. All treatment groups showed a significant increase in migration over 24 hours with the exception of 50 μ M Pacma-31 treated Panc-1 and 10 μ M CCF642 treated AsPC-1. Despite all treatment groups showing a significant increase in migration over 48 hours, treatments in Panc-1 and Mia-PaCa-2 showed a dose dependent decrease in migration, with Pacma-31 and CCF642 treatment showing an inhibited rate of migration compared to untreated cells. This inhibition of cell migration was less significant in AsPC-1 cells. Mia-PaCa-2 cells exhibited the greatest rate of cell migration compared to the other cell lines.

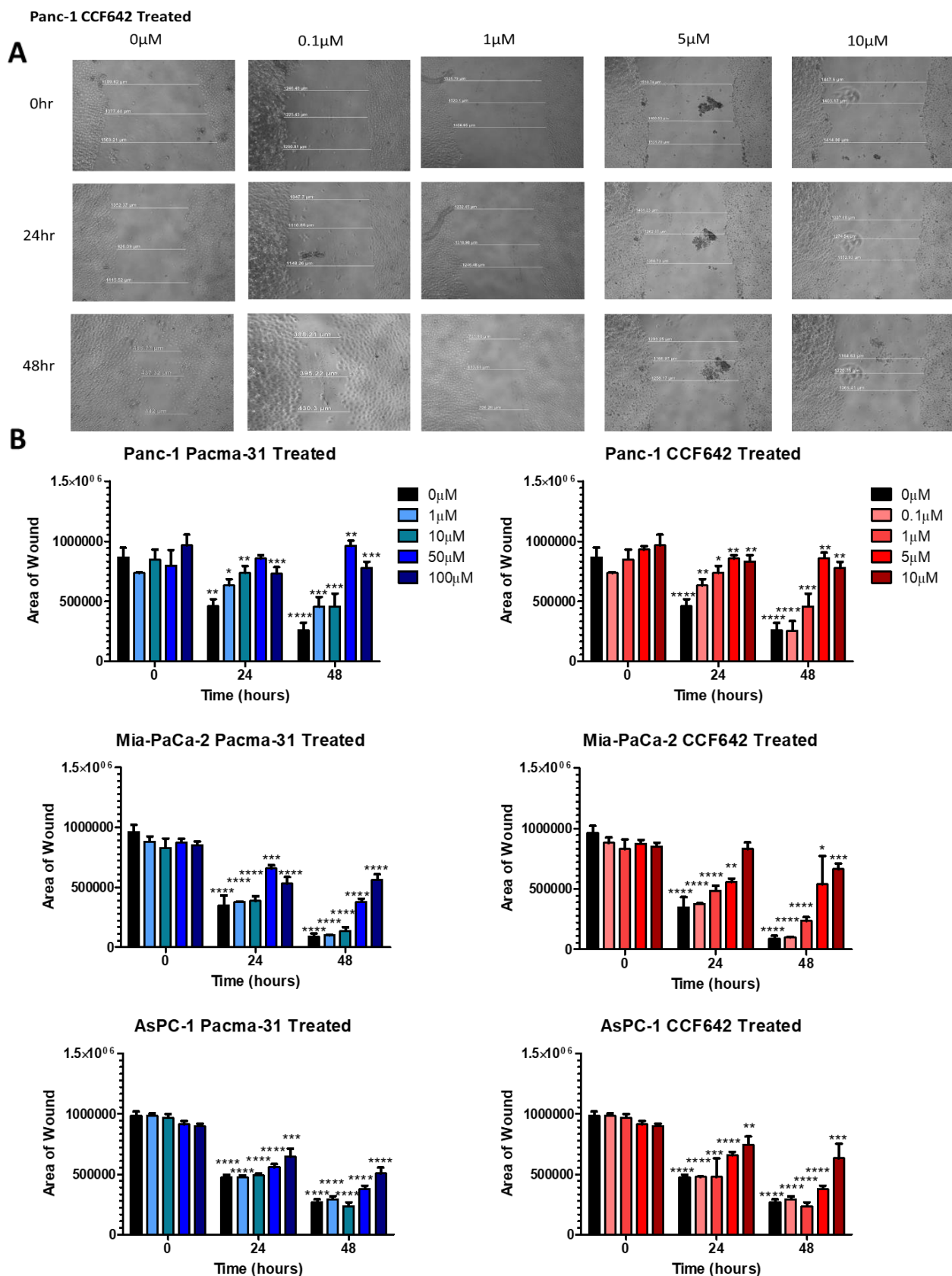


Figure 4.5. Pacma-31 and CCF642 treatment decreases the rate of cell migration.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with PACMA-31 and CCF642 for 48 hours. (A) Representative images of assay, depicting the dose

dependent inhibition of cell migration with PACMA-31 treatment in Panc-1. All wells were photographed using a DMIRB inverted light microscope at 5X magnification. Images were taken at the time points of 0, 24 and 48 hours post scratch. The images also display measurements of the wound width taken using the Image J software measure tool to provide a visual insight of the changes in wound area size over time. (B) The area of the wound was measured using Image J software to assess cell migration. Data was collected from 3 wells for each treatment group, with 3 repeats of the experiment. N=3 for each treatment group. Error bars denote standard deviation (SD). The degree of significance in comparison to the 0 hour measurement in each treatment group were analysed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

4.3.4 Knockdown of PDI Protein Expression by siRNA Transfection

Western blot analysis confirmed no AGR2 protein expression in normal Mia-PaCa-2 cells (Figure 4.6A). This confirmed use of Mia-PaCa-2 as a natural knockout of AGR2 as first outlined in Figure 3.1. Cells were transfected with PDIA1 and AGR2 siRNAs and knockdown was confirmed after 48-hour treatment in all cell lines (Figure 4.6B). Mia-PaCa-2 cells were not transfected with AGR2 siRNA due to showing no AGR2 expression naturally. Cells were also treated with a vector control (cells treated with the same volume of transfection reagent [4 μ l/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and non-targeting scrambled siRNA control (6.25nM) for 48 hours as controls for the study. beta-actin was used as a control for confirmation of protein expression in cells.

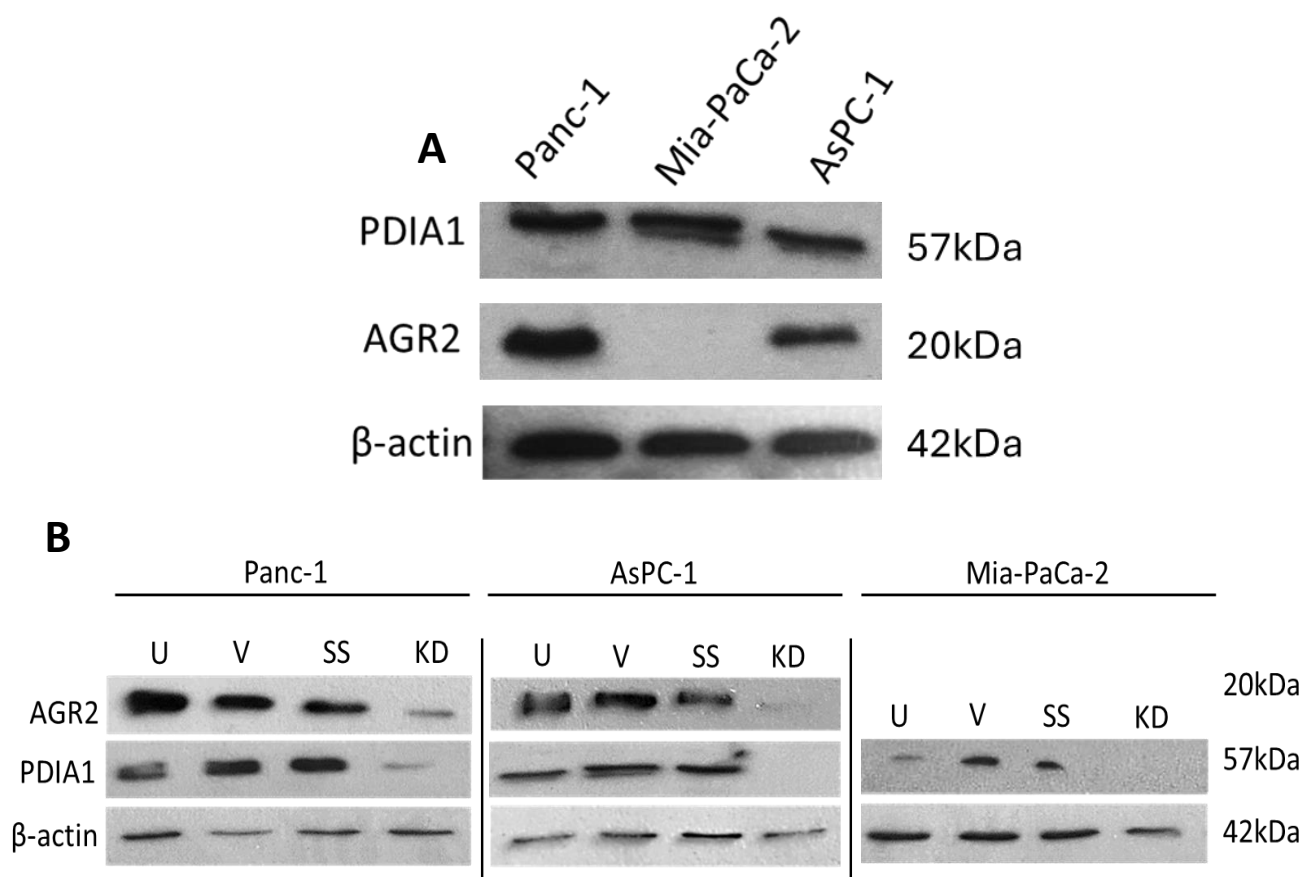


Figure 4.6. AGR2 and PDIA1 protein expression is knocked down after 48 hour siRNA transfection in pancreatic cancer cell lines.

(A) PDIA1 and AGR2 protein expression was measured in normal Panc-1, Mia-PaCa-2 and AsCP-1 cells to show evidence of no AGR2 expression in Mia-PaCa-2 (B) Following optimisation of siRNA concentration and transfection time, cells were transfected with AGR2 and PDIA1 siRNAs for an optimal time of 48 hours at a final concentration of 6.25nM. Control groups included untreated, vector control (cells treated with the same volume of transfection reagent [4μl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and a non-targeting scrambled siRNA transfection (6.25nM). AGR2 and PDIA1 protein levels were determined by Western blot analysis with Beta-actin as a control. N=3 per gene, per cell line. Cells were treated with 6.25nM siRNA transfection for 48 hours. (U) = Untreated, (V) = Vector Control, (SS) = Scrambled siRNA, (KD) = Knockdown.

4.3.5 Knockdown of PDI RNA Expression by siRNA Transfection

PDI mRNA expression was measured for PDIA1 and AGR2 in Panc-1, AsPC-1 and Mia-PaCa-2 cell lines using RT-qPCR. Initial RT-qPCR experiments were conducted to confirm that there was no AGR2 mRNA expression in normal Mia-PaCa-2 cells, aligning with the protein expression results shown in Figure 4.6. Cells were transfected with the siRNA knockdown for 48 hours. After 48 hours the media of cells was changed, and cells were then incubated at 37°C for a further 24 hours. RNA was harvested for RT-qPCR at 24 hours post media change (i.e. 72 hours after initial cell seeding) and harvested at subsequent time points of 48, 72, 96 and 120 hours post media change (Figure 4.7). RNA expression of both PDIA1 and AGR2 across all cell lines were significantly ($***p<0.0001$) knocked down for up to 120 hours post media change. AsPC-1 showed a more delayed knockdown of AGR2 than Panc-1, however showed an almost complete knockdown by 120 hours. These data confirmed that 48 hour siRNA treatment was sufficient for measuring the effects of PDI knockdown in proliferation and migration assays.

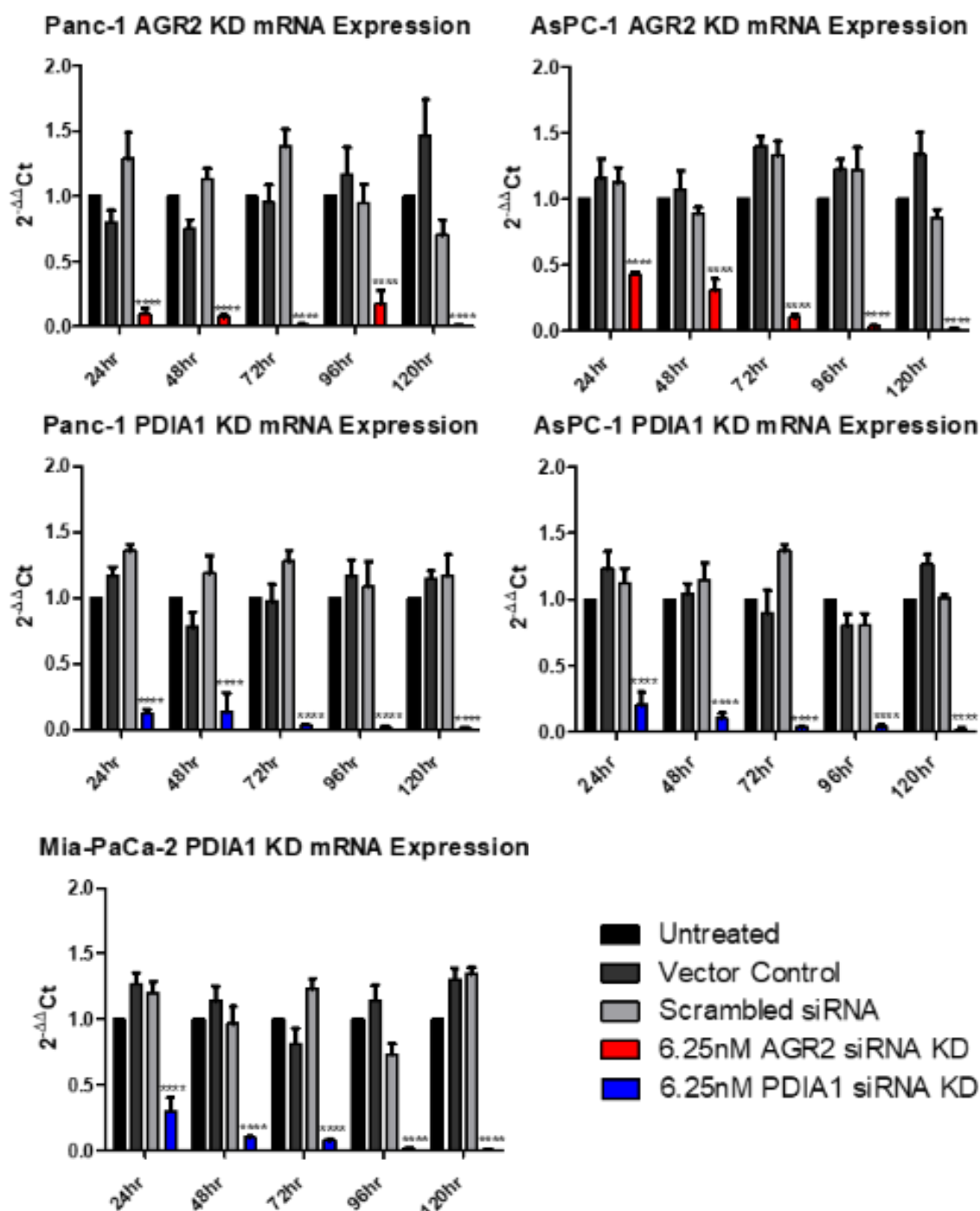


Figure 4.7. AGR2 and PDIA1 mRNA expression is knocked down after 48 hour siRNA transfection in pancreatic cancer cell lines.

Controls groups included untreated, vector control (cells treated with the same volume of transfection reagent [4μl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and a non-targeting scrambled siRNA transfection (6.25nM). Cells were transfected with AGR2 and PDIA1 siRNAs at a final concentration of 6.25nM. AGR2 and PDIA1 mRNA expression was calculated according to the $\Delta\Delta C_t$ method using the reference housekeeping gene RPLP0 from RT-qPCR analysis. N=3 per gene, per cell line. Error bars denote standard deviation. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests. ****p<0.0001.

4.3.6 Effects of PDI knockdown on PDI Enzymatic Activity

PDIA1 and AGR2 knockdown was further confirmed by a significant decrease (**** $p < 0.0001$) in PDI activity across all cell lines (Figure 4.8). PDI activity was significantly decreased after 48-hour siRNA knockdown of AGR2 and PDIA1, vector (cells treated with the same volume of transfection reagent as siRNA knockdown treated cells) and scrambled siRNA treatments also acted as controls. PDI activity was significantly reduced at similar rates across all cell lines and with both AGR2 and PDIA1 knockdowns, confirming siRNA transfection knocked down PDI effectively.

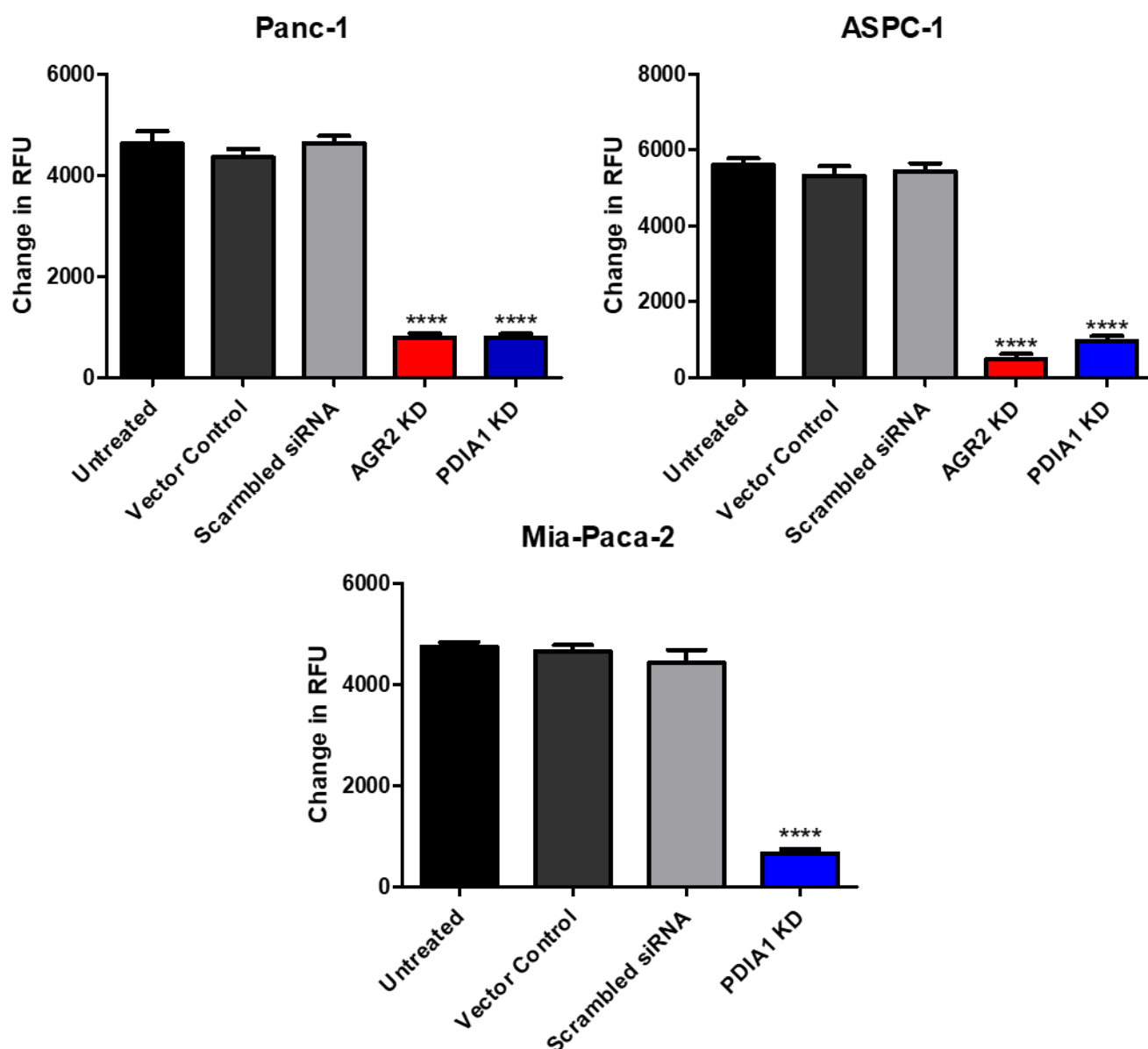


Figure 4.8. AGR2 and PDIA1 siRNA knockdown decreases PDI activity in pancreatic cancer cell lines.

PDI activity was measured following 48-hour siRNA knockdown treatment. Cells were also treated with a vector control (cells treated with the same volume of transfection reagent [4μl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and a scrambled siRNA control for 48 hours. Following substrate addition, fluorescence was measured immediately in kinetic mode for 30 minutes at Ex/Em = 490/580. Change in RFU after 30 minutes was calculated to determine relative PDI activity. N=3 for each treatment group. Error bars denote standard deviation (SD). Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests. ***p<0.001, ****p<0.0001.

4.3.7 Effects of PDI knockdown on Pancreatic Cancer Cell Proliferation

4.3.7.1 BrdU Proliferation Assay

AGR2 and PDIA1 48-hour siRNA knockdown significantly decreased (**** $p < 0.0001$) the rate of cell proliferation across Panc-1, AsPC-1, and Mia-PaCa-2 (Figure 4.9). AGR2 knockdown showed a significantly (**** $p < 0.0001$) greater inhibition on cell proliferation compared with PDIA1 knockdown in Panc-1 and AsPC-1. AGR2 knockdown in AsPC-1 showed the greatest decreased rate of proliferation compared to the untreated control. PDIA1 knockdown was also shown to be most effective in AsPC-1 compared with Panc-1 and Mia-PaCa-2. The decrease in rate of proliferation by AGR2 and PDIA1 knockdown was equivalent to that shown in Figure 4.3 with 100 μ M Pacma-31 and 10 μ M CCF642 treatments.

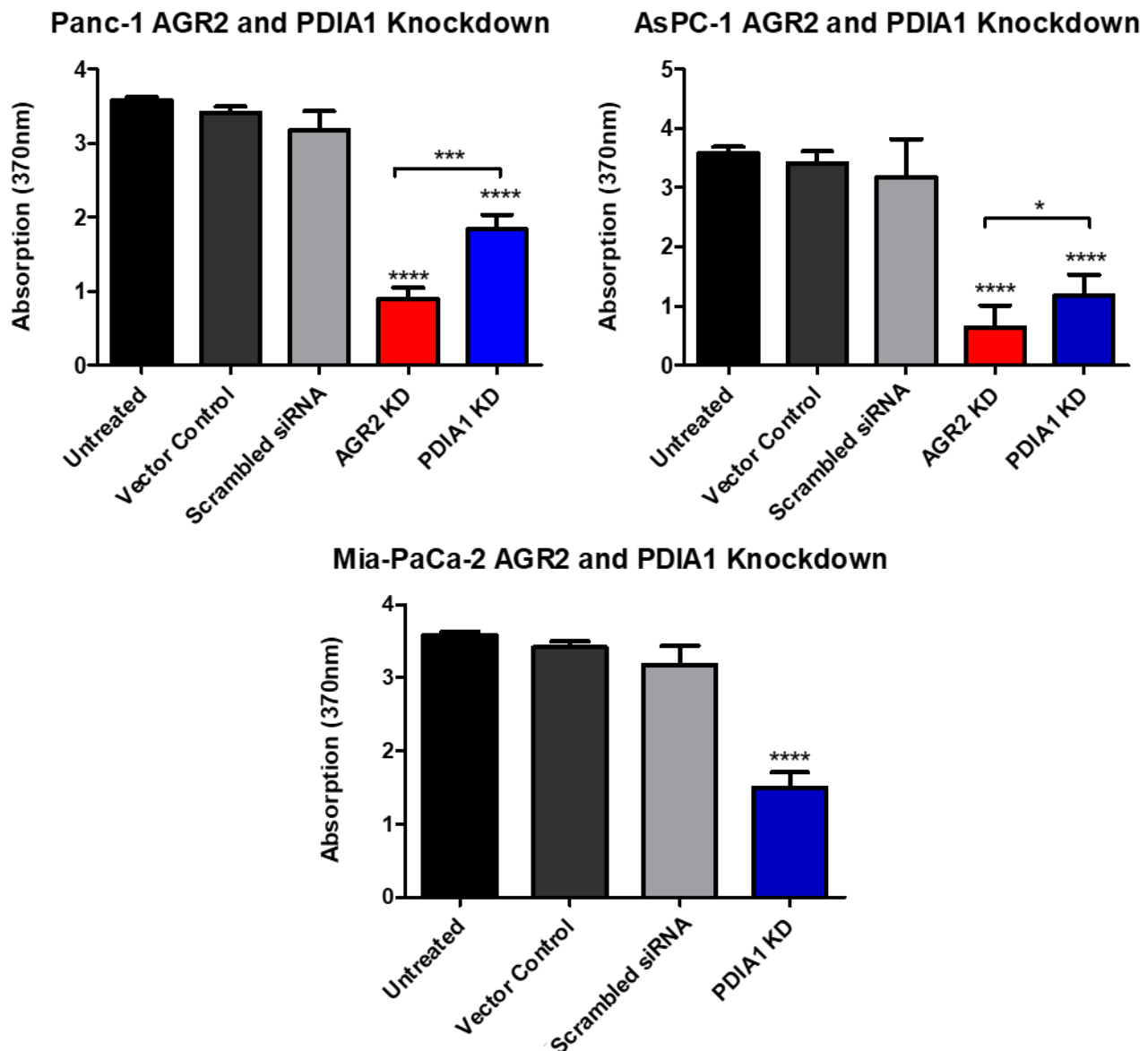


Figure 4.9. AGR2 and PDIA1 siRNA knockdown decreases the rate of cell proliferation in pancreatic cancer cell lines.

Following 48-hour AGR2 and PDIA1 siRNA knockdown treatment, absorption (370nm) was measured after 72 hours post media change. Cells were also treated with a vector control (cells treated with the same volume of transfection reagent [4µl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and scrambled siRNA control for 48 hours. Data was collected from 8 wells for each treatment group, with 4 repeats of the experiment. N=3 for each treatment group. Error bars denote 95% confidence interval (CI). Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests. ****p<0.0001.

4.3.7.2 MTT Proliferation Assay

AGR2 and PDIA1 48-hour siRNA knockdown significantly decreased (**** $p < 0.0001$) Panc-1, AsPC-1 and Mia-PaCa-2 cell viability (Figure 4.10). Results reflected those shown in Figure 4.9 with AGR2 and PDIA1 knockdown in AsPC-1 showing the greatest decrease in cell viability. Overall, these results further confirm that selective inhibition of PDIA1 and AGR2 are as effective in decreasing the viability of pancreatic cancer cells as seen by the highest dose PDI inhibitor treatments.

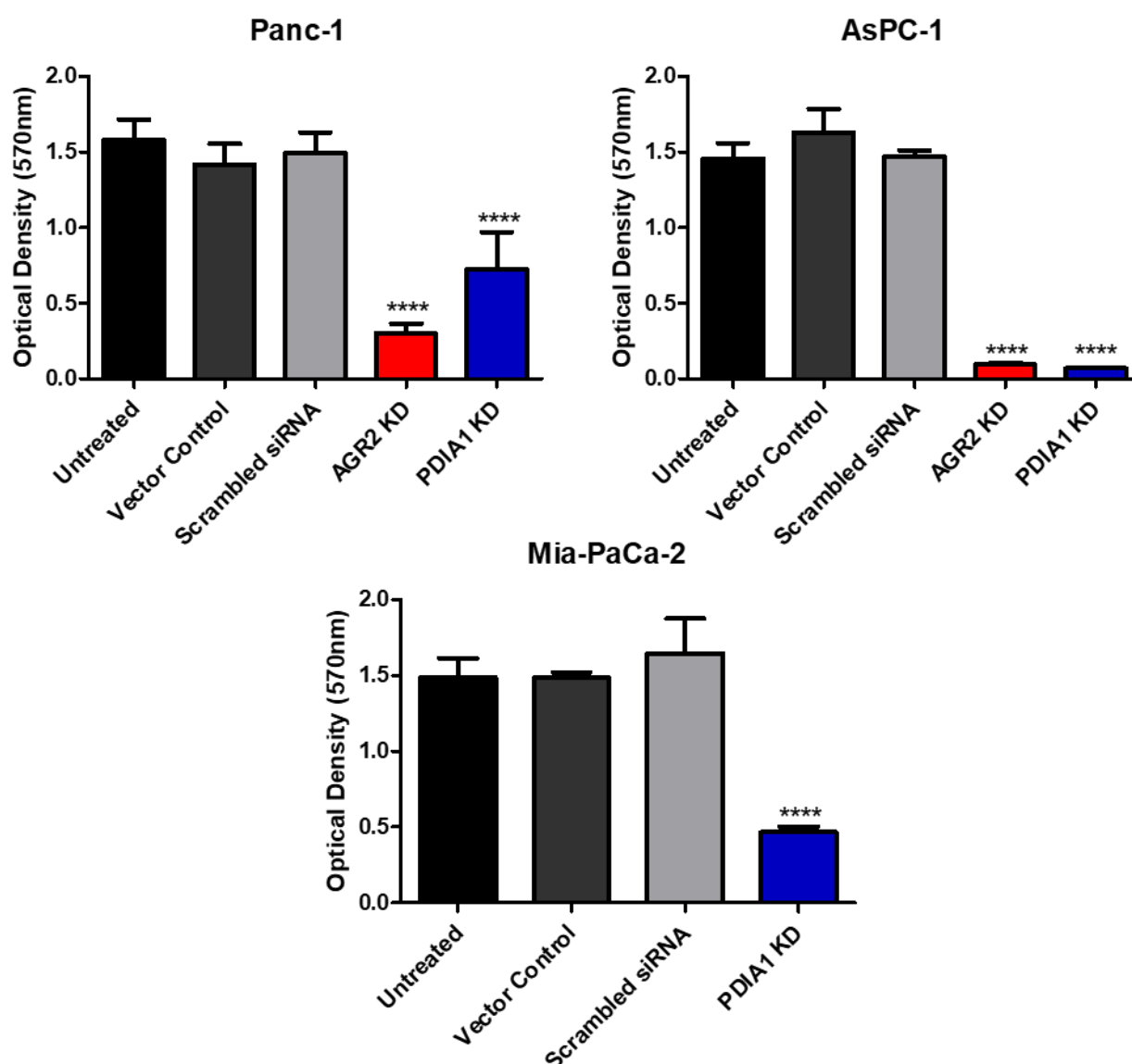


Figure 4.10. AGR2 and PDIA1 siRNA knockdown decreases pancreatic cancer cell viability.

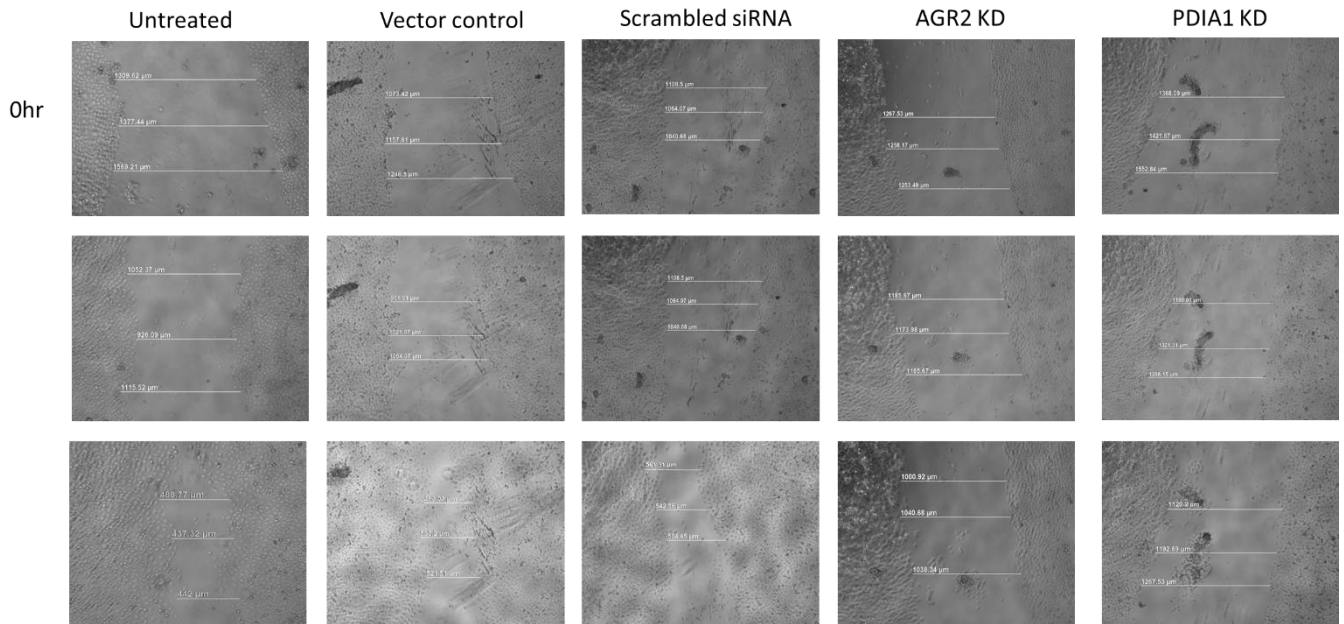
Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with AGR2 and PDIA1 siRNA knockdown for 48 hours. Cells were also treated with vector control (cells treated with the same volume of transfection reagent [4μl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and scrambled siRNA control for 48 hours. Optical Density (570nm) was measured after 72 hours. Data was collected from 3 wells for each treatment group, with 3 repeats of the experiment. N=3 for each treatment group. Error bars denote standard deviation (SD). The degree of significance in comparison between the control and each treatment group were analysed. Significance is also shown compared between treatment concentrations. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

4.3.8 Effects of PDI knockdown on Pancreatic Cancer Cell Migration

AGR2 and PDIA1 48-hour siRNA knockdown was shown to be effective in decreasing the rate of cell migration in Panc-1 (Figure 4.11). Both AGR2 and PDIA1 showed a less significant rate of cell migration (* $p < 0.05$) compared to the untreated, vector and scrambled siRNA controls (**** $p < 0.0001$) at 24 hours. This was also reflected at 48 hours with the rate of cell migration in AGR2 and PDIA1 knockdown less significant (** $p < 0.01$) compared to the controls (**** $p < 0.0001$). This shows that both AGR2 and PDIA1 inhibition slows the rate of cell migration in Panc-1 cells. PDIA1 knockdown in Mia-PaCa-2 also shows a decreased rate of migration (** $p < 0.001$) compared to the controls (**** $p < 0.0001$) at 48 hours. PDIA1 and AGR2 knockdown did not show a significant effect on the rate of cell migration in AsPC-1. This suggests that PDIA1 and AGR2 may not play roles in the promotion of cell migration in AsPC-1 cells. Similar results were shown in Figure 4.5 in which Pacma-31 and CCF642 treatment showed the least significant effect on cell migration in AsPC-1.

A

Panc-1 AGR2 and PDIA1 KD



B

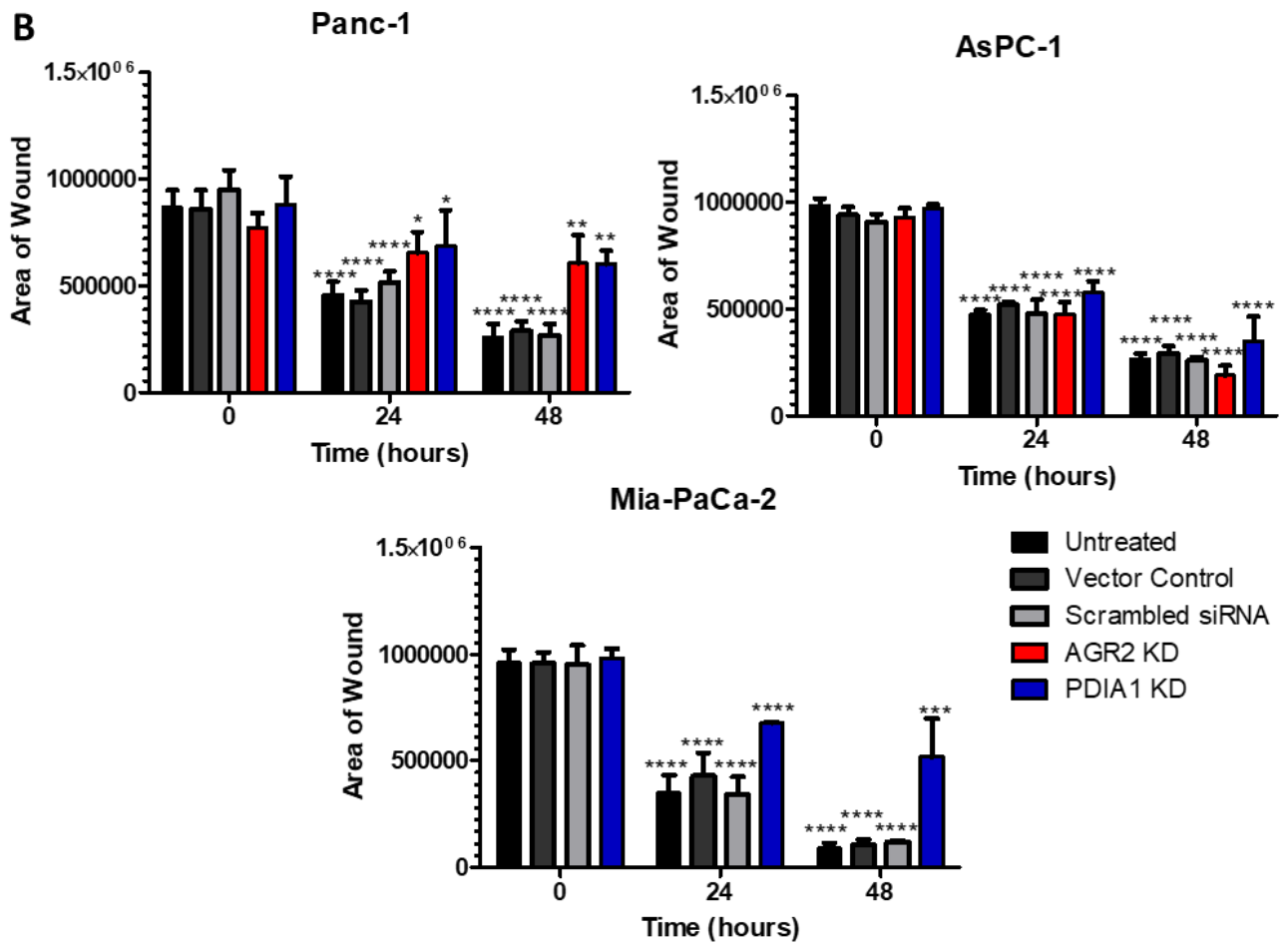


Figure 4.11. AGR2 and PDIA1 siRNA knockdown decreases the rate of cell migration.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with AGR2 and PDIA1 siRNA knockdown for 48 hours. Cells were also treated with a vector control (cells treated with the same volume of transfection reagent [4µl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and scrambled siRNA control for 48 hours. (A) Representative images of assay, depicting inhibition of cell migration with AGR2 and PDIA1 knockdown treatment in Panc-1. All wells were photographed using a DMIRB inverted light microscope at 5X magnification. Images were taken at the time points of 0, 24 and 48 hours post scratch. The images also display measurements of the wound width taken using the Image J software measure tool to provide a visual insight of the changes in wound area size over time. (B) The area of the wounds was measured using Image J software to assess cell migration. Data was collected from 3 wells for each treatment group, with 3 repeats of the experiment. N=3 for each treatment group. Error bars denote standard deviation (SD). The degree of significance in comparison between the control and each treatment group were analysed. Significance is also shown compared between treatment concentrations. *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

4.4 Discussion

4.4.1 Pacma-31 and CCF642 as Treatments for Pancreatic Cancer

With evidence of promising anticancer effects in other cancer types, the effects of PDI inhibitors; Pacma-31 and CCF642 were investigated in pancreatic cancer cell lines: Panc-1, Mia-PaCa-2 and AsPC-1. Pacma-31 and CCF642 both showed a dose-dependent decrease in the rate of proliferation and cell viability (Figures 4.3 and 4.3). Both Pacma-31 and CCF642 treatment showed the greatest inhibition of cell proliferation in the non-AGR2 expressing Mia-PaCa-2 cell line, highlighting AGR2 as a potential influential oncogene in cell survival (Figure 4.3). Whilst this result may reflect the difference in PDI member expression levels within the three cell lines, different PDI members are targeted by each inhibitor. Both inhibitors are non-selective, and it is unclear which PDI members each inhibitor is targeting.

Pacma-31 has been shown to inhibit PDIA1, PDIA3, PDIA4, PDIA6 and TXNDC5 and CCF642 has been shown to inhibit PDIA1, PDIA3 and PDIA4 (Table 1.4). However, both may inhibit other members in addition to these. Both Pacma-31 and CCF642 act as irreversible inhibitors that inhibit PDI through binding to cysteine or lysine residues respectively, in the α or α' active sites to disrupt the canonical CXXC motif (Figure 4.1), and hence PDI activity. This suggests that they would not be effective in inhibiting non-canonical PDI members that do not possess CXXC motifs such as AGR2. Further research is needed to determine the most effective mode of action of inhibition for specific PDI members. With both inhibitors being non-selective it is likely that they also have off-target effects that may be either beneficial or detrimental to tumour progression.

Both Pacma-31 and CCF642 also have a relatively high IC_{50} of $10\mu M$ and $2.9\mu M$ respectively, showing a lack of potency. This is reflected by the highest dose treatment showing the greatest decrease in PDI activity (Figure 4.2). It should be noted that while this assay is only aimed at measuring PDI reductase activity, results may not necessarily reflect the full impact of PDI inhibition on the level of enzymatic activity. While reductase assays are widely used for PDI activity measurement, some research has suggested that PDI reduction assays might more significantly reflect PDI expression levels rather intrinsic isomerase activity, particularly considering the fact that reduction is just a one step of thiol rearrangement (Watanabe, et al. 2014). Assays to measure PDI isomerase activity, are more complex but have been developed for experimental use. PDI isomerase activity assays are based on gain-of-function of an inactive protein substrate containing unfolded disulfides. In the presence of PDI, disulfide bonds are folded back to a “correct” position, allowing the

measurement of activity of the recovered substrate (Watanabe, et al. 2014). RNase with scrambled thiols can be used as initial substrate for PDI isomerase assay (Watanabe, et al. 2014). This method has been extensively used in mechanistic PDI studies and could be considered for use in further studies for measuring overall isomerase activity of PDI.

Pacma-31 and CFF642 treatment was also shown to effect pancreatic cancer cell migration (Figure 4.5). Panc-1 and Mia-PaCa-2 showed a dose dependent decrease in the rate of migration, suggesting that PDI inhibition in turn inhibits cell migration. This inhibition of cell migration was less significant in AsPC-1 cells. With AsPC-1 possessing high expression levels of AGR2, and the proposed lack of AGR2 inhibiting ability of Pacma-31 and CCF642, this implies that AGR2 may play an important role in promoting cell migration. As previously mentioned, AGR2 has been shown to promote cell migration through activation of cathepsin B/D and mucins (Dumartin et al., 2011). In contrast to this, non-AGR2 expressing Mia-PaCa-2 cells exhibited the greatest rate of cell migration compared to the other cell lines. Again, it should be noted that due to the non-selective nature of PDI inhibitor treatments, off target effects are likely, of which could also contribute to confounding factors of the scratch assay method such as cell proliferation or apoptosis.

Pacma-31 and CCF642 treatment and PDIA1 and AGR2 knockdown studies demonstrate significant inhibition of cell proliferation. This large alteration in cell proliferation could also influence the accuracy of cell migration results, as an inhibition of cell proliferation could also affect the rate of wound closure alongside cell migration. This should therefore be noted as a limitation of the scratch assay

method. A significant change in cell proliferation is a well acknowledged confounding factor that can influence several results, also including gene expression studies. Differences in cell growth rates correlate with the proportion of cells in each phase of the cell cycle (Locard-Paule et al., 2022). Slower-growing cells will have more cells in G1 and G2 phase and fewer in S and M phase, and S and M phase-specific proteins will therefore be less abundant in the lysates, potentially affecting both protein expression and RNA expression experiments (Locard-Paule et al., 2022). The significant change in cell proliferation shown with PDI inhibitor and PDI knockdown studies should be noted as a limitation across all studies, including cell migration and gene expression studies.

4.4.2 PDIA1 and AGR2 as Potential Therapeutic Targets

Through data mining led investigations, PDIA1 and AGR2 were identified as promising therapeutic targets, highlighted as most highly expressed PDI members in pancreatic cancer. Selective inhibition of PDIA1 and AGR2 through siRNA knockdown was confirmed by measurement of both mRNA expression via RT-qPCR (Figure 4.8) and protein expression via Western blotting (Figure 4.6) This was further confirmed by a significant decrease in PDI activity with 48-hour siRNA PDIA1 and AGR2 knockdown (Figure 4.8). Expression of PDIA1 and AGR2 was assessed for up to 120 hours post media change following 48-hour siRNA transfection. This confirmed evidence of a prolonged knockdown and suitability for cell proliferation and migration studies. While the use of siRNA transfection does not provide a complete knockout which could be achieved via the use of CRISPR, results indicated a significant knockdown in both protein and mRNA expression, with mRNA

expression of PDIA1 and AGR2 showing averagely a 90% total knockdown of expression by the post media change time point for proliferation and migration assay measurements.

The successful siRNA knockdown of both PDIA1 and AGR2 and consequential detrimental effects on cell proliferation demonstrates the potential of these PDI members as therapeutic targets for pancreatic cancer. Figure 4.9 shows that PDIA1 knockdown was effective in decreasing the rate of cell proliferation across all three cell lines. AGR2 knockdown however, demonstrated an even greater effect, particularly in AsPC-1 where AGR2 expression is expressed at the highest level. This result was also reflected in measurement of cell viability via MTT assay (Figure 4.10). Previous studies have shown that siRNA knockdown of AGR2 has led to inhibition of the growth signalling ERK/AKT pathway in pancreatic cancer cells (Li et al., 2018). The decreased rate of proliferation with AGR2 knockdown seen in this study, may therefore reflect this. Interestingly, AGR2 is present on the cell surface and can be secreted by cancer cells (Wayner et al. 2012). This is significant as normal AGR2 expressing cells do not show cell surface expression, it is not secreted, and the expression is localized to the cell interior (Ho et al. 2016). AGR2 differs from other PDI members in that it possesses a non-canonical ER retention motif at its C-terminal (KTEL). Despite this structural difference, AGR2 still has the ability to bind to the ER KDEL receptor (Chevet et al. 2012). However, its divergence from the canonical KDEL motif suggests that it possesses a lower affinity for binding; hence contributing to its ability to be diversely trafficked and to exit the ER, particularly when overexpressed (Chevet et al. 2012). AGR2 has been reported to play other intracellular roles outside of its localisation to the ER such as the

regulation of p53 signalling, and hence tumour growth (Pohler et al. 2004). Further research needs to be carried out to assess the tumour growth associated roles of AGR2 both within the cell and as a secretory protein.

PDIA1 knockdown in AsPC-1 showed a greater decrease in cell viability in the MTT assay than the decrease shown in cell proliferation in the BrdU assay. While the MTT assay measures cell viability rather than cell proliferation alone, results may also account for some evidence of apoptosis. With PDIA1 known for its ability to regulate UPR signalling and proposed to attenuate activations of chronic UPR pathways through UPR receptor interactions, it could be implied that PDIA1 knockdown is of more significant detriment to pancreatic cancer cell viability than proliferation alone. This probes further investigations into the influence of PDIA1 inhibition on chronic UPR induced apoptosis.

PDIA1 and AGR2 knockdown were also shown to inhibit the rate of cell migration in Panc-1 (Figure 4.11). PDIA1 knockdown also decreased cell migration rate in Mia-PaCa-2 cells. Despite this, both PDIA1 and AGR2 knockdown showed no significant effect on cell migration in AsPC-1. This therefore contradicts the suggestion from PDI inhibitor treated cells that AGR2 may be driving cell migration in AsPC-1. And instead indicates that decreased rate of migration shown with high dose PDI inhibitor treatments may be due to off target effects or the inhibition of differing PDI family members. With PDIA1 and AGR2 expression exhibiting significant effects on cell migration in Panc-1 and Mia-PaCa-2, this suggest that the roles of PDIA1 and AGR2 in cell migration differ in AsPC-1 cells, potentially not interacting with metastasis-

associated pathways. AsPC-1 cells were shown to be the most greatly inhibited cell line in terms of cell proliferation with PDIA1 and AGR2 knockdown, compared to other cell lines. This therefore combats the previously proposed limitation that suggested that results of inhibited migration are exaggerated by inhibition of cell proliferation.

The greatest inhibition of cell migration is shown in Mia-PaCa-2 cells at 48 hours with PDIA1 knockdown. Results again also confirmed Mia-PaCa-2 as the cell line with the fastest rate of cell migration. As previously described, PDIA1 is known for its roles in the promotion of metastatic tumours in various cancer types, particularly in breast cancer (Bakker et al., 2022). With results suggesting influence of PDIA1 in the promotion of cell migration in pancreatic cancer, it could be proposed that PDIA1-associated metastatic pathways observed in breast cancer may be implicated in pancreatic cancer also. As previously discussed, the Wnt signalling pathway is a well-recognised oncogenic signalling pathway in pancreatic cancer, associated with the induction of EMT and hence increased invasiveness and metastasis (Grant et al., 2018). In breast cancer cells, PDIA1 has been shown to play a role in the regulation of Wnt signalling, known for its role in metastasis (Yang et al., 2022). It could therefore be proposed that PDIA1 plays a similar role in pancreatic cancer cells. This promotes the need for further investigations into the mechanisms by which PDI members may drive pancreatic cancer metastasis, particularly since the presence of progressed metastatic disease is attributed to extremely low survival rates.

While results do not indicate the mechanisms by which PDIA1 and AGR2 drive pancreatic cancer cell proliferation and migration, it is clear that their inhibition is determinantal to tumour progression, emphasising their potential as oncogenic targets. Further investigations now need to be made into the impact of their inhibition within ER stress to further determine the impact on pancreatic cancer cell survival.

CHAPTER 5 – THE EFFECTS OF PDI INHIBITION ON ER STRESS AND APOPTOSIS

Chapter 5 - The effects of PDI inhibition on ER stress and apoptosis

5.1 Introduction

5.1.1 The Therapeutic Potential of the UPR Pathway in Cancer

As an adaptive response to ER stress, the UPR pathway acts to maintain cell homeostasis through activation of molecular chaperones and other transcriptional factors that correct misfolded proteins and promote quality control (Chevet et al., 2015). As previously described, UPR activations are controlled via BiP interactions with three integrated UPR signalling sensors: PERK, ATF6 and IRE1 α (Chevet et al., 2015). The role of the UPR in the promotion of cell survival in cancer cells is well established, however recent research has demonstrated the potential of the UPR as a direct driver of cell transformation (Wang and Kaufman, 2014). UPR activation is recognised as a vital step in oncogenic transformation. Through interactions with tumour-suppressor and oncogene networks, the UPR is activated to promote protein folding and ER expansion for cell division (Wang et al., 2012).

The UPR pathway has been highlighted as a potential target to suppress tumour growth through knockdown of PERK, which has been observed to slow tumour growth through a decreased production of angiogenic mediators in various cancer types (Wang et al., 2012). However, PERK inhibition may not show as favourable effects in the treatment of pancreatic cancer, as although anti-tumour effects have been shown with PERK inhibitor treatment, PERK also plays important roles in pancreatic function (Atkins et al., 2013). Use of a PERK inhibitor in pancreatic cancer treatment therefore needs to be pursued with caution. In conjunction with the

highlighted pro-survival roles of the UPR, prolonged UPR activation leads to chronic ER stress, triggering pathways of apoptosis and consequent anti-cancer effects.

The therapeutic potential of the UPR pathway has been evidenced through combined treatment of proteasome inhibitor, bortezomib and COX-2 inhibitor, celecoxib in glioblastoma (Kardosh et al., 2008). In this study, combined treatment of these two inhibitors triggered an elevated expression of ER stress marker BiP and apoptotic marker CHOP, with further evidence of increased apoptotic cell death (Kardosh et al., 2008). These results show the potential of apoptosis-associated UPR activations as an anti-cancer therapy. Recent studies have suggested that bortezomib may act to inhibit XBP-1 splicing to drive anti-cancer effects through increased apoptosis in myeloma cells (Lee et al., 2003). This highlights XBP-1 as a potential downstream target of the UPR, to further drive exaggerated ER stress and hence cancer cell death.

5.1.2 The Therapeutic Potential of XBP-1 in Cancer

XBP-1 is recognised as a key mediator of the UPR pathway. Transcribed XBP-1 is converted into its activated, spliced form (XBP-1s) via IRE1 α activations (Wang et al., 2011). IRE1 is recognised as the most evolutionary conserved protein of the UPR pathway, through its foremost interactions with XBP-1 (Wang et al., 1998). XBP-1s therefore plays an essential role in the regulation of ER stress. XBP-1 was first discovered as a unique basic-region leucine zipper (bZIP) transcription factor of the cAMP-response element-binding (CREB)/ATF4 family (Chen et al., 2020). Through the process of unconventional splicing by IRE1, XBP-1s acts to induce the expression of various ER protective genes involved in mechanisms of protein folding,

secretion and the clearance of misfolded proteins via ERAD activation (Yoshida, 2007).

Interestingly, due to the essential role of XBP-1 in protein secretion, it is highly expressed in pancreatic β cells, being that their function is dependent on large productions of secretory proteins (Yoshida, 2007). Further to this, XBP-1 has been shown to be required for the expression of PDI family genes in pancreatic β cells, including PDIA1, by binding directly to their promoters (Shi et al., 2019). This highlights the potential significant role XBP-1 may play in promoting cell survival through PDI-associated activation. While the role of XBP-1 in pancreatic cancer is yet to be fully elucidated, its role has been documented in several cancers (Ozel et al., 2022). Many oncogenic signalling pathways including Myc, AP-1 and NF- κ B have been shown to be activated by XBP-1, demonstrating potential tumorigenic influence (Chae et al., 2016).

In breast cancer, recent studies have highlighted XBP-1 as a potential factor in controlling cell survival in oestrogen-receptor α -positive cancer cells through modulation of the NF- κ B pathway (Chevet et al., 2015). Overexpression of XBP-1 in these breast cancer cells was associated with dysregulation of genes responsible for apoptosis, cell-cycle progression, and oestrogen-induced tumour growth (Chevet et al., 2015). XBP-1 was further shown to promote tumorigenesis in triple negative breast cancer (TNBC) whereby, XBP-1 drives tumorigenicity via the regulation of HIF1 α expression, demonstrating a cross-talk between the UPR and HIF1 α in the transcriptional reprogramming of cells toward transformation (Chen et al., 2014). The

recent study further demonstrated that a depletion of XBP-1 in TNBC cells inhibited both tumour growth and relapse, promoting the targeted inhibition of XBP-1 as an alternative treatment strategy (Chen et al., 2014). As previously mentioned, PDIA1 has been evidenced to play a role in the regulation of both NFkB and HIF-1 α signalling in breast cancer (Bakker et al., 2022). This therefore suggests a potential collaborative role between XBP-1s and PDIA1 in the regulation of these oncogenic pathways.

5.1.3 The Therapeutic Potential of BiP in Cancer

The role of ER chaperone, BiP in UPR activations is well described. In unstressed conditions, BiP constitutively binds to IRE1, PERK and ATF6, preventing activation. Upon induction of ER stress, BiP dissociates from the three sensors, allowing autophosphorylation and hence activation (Chevet et al., 2015). While BiP therefore plays a pivotal role in UPR pathway activation and hence ER homeostasis, BiP is also a downstream transcriptional target of ATF6 α where it has been reported to serve as a malignancy marker for cells (Chevet et al., 2015). In ER stress conditions, ATF6 α induces BiP expression where it acts to bind to misfolded proteins and alleviate ER stress (Chevet et al., 2015). While under normal conditions, BiP is located in the ER lumen, an overexpression (observed across several cancer types) enables BiP to be detected on the cell surface (Arap et al., 2004). Recent research suggests that stress response proteins present on the surface of tumour cells may act as molecular targets (Arap et al., 2004). BiP has been established as a functional tumour cell surface target with the potential to suppress tumour growth in prostate and breast cancers (Arap et al., 2004). Recent studies have reported that the

presence of BiP on the surface of metastatic prostate cancer cells may induce proliferation and invasion associated signal pathways (Misra et al., 2006).

The therapeutic implications of BiP are further evidenced in chemoresistant cancer cells, whereby an attempt to target the tumour vasculature with antiangiogenic therapeutic agents drives inadvertent BiP induction (Lee, 2007). These chemoresistant cells correlate with BiP overexpression, in conjunction with the suppression of apoptosis (Lee, 2007). Aside from its role in maintaining ER homeostasis, BiP actively suppresses apoptosis by binding and inhibiting the activation of caspase 7, which is activated upon both ER stress and treatment of cytotoxic drugs (Lee, 2007). Many cytotoxic drugs initiate programmed cell death through executor caspases including caspases 3, 6 and 7, with caspase-3 and caspase-7 being the most structurally similar (Reddy et al., 2003). Therefore, in multiple tumour types, BiP overexpression contributes to the resistance of a range of chemotherapeutic agents (Lee, 2007). In malignant glioma, known to be an extremely chemoresistant tumour type, BiP knockdown was shown to sensitise the cells to cytotoxic drug treatment, highlighting BiP as a novel target (Li & Lee, 2006).

5.1.4 UPR Pathway-induced Apoptosis

As previously stated, overactivation of the UPR pathway drives pathways of apoptosis which could be influential in the development of novel anti-cancer therapies. A pro-apoptotic UPR response can be driven by sustained IRE1 signalling which leads to the recruitment of TRAF2, responsible for the activation of apoptosis signal-regulating kinase (ASK1) and consequent JNK activation (Urano et al., 2000).

The JNK branch of the IRE1 UPR pathway regulates several pathways of apoptosis, driving cell death (Urano et al., 2000). Further evidence is needed to understand intensity of the ER-stress inducing stimulus required to drive an IRE1 led pro-apoptotic response. Allosteric inhibition of IRE1 has demonstrated increased cell survival in pancreatic β -cell destruction-mediated diabetes (Ghosh et al., 2014). As an ER stress-induced disease, these results are consistent with the described apoptotic roles of IRE1. CHOP is another well-known pro-apoptotic component of the UPR pathway. While the PERK/ATF4/CHOP pathway has been widely researched as a driver of cell death, CHOP can also be a transcriptional target of ATF6 and XBP-1 (Ron & Walter, 2007). The mode of action of the ATF4/CHOP apoptotic axis is still unclear, however it has been proposed that the pathway may mediate reactive oxygen species (ROS) (Iurlaro & Muñoz-Pinedo, 2016). CHOP has been shown to induce a hyperoxidising environment through the activation of endoplasmic reticulum oxidoreductin 1 (ERO1 α), driving cell death (Iurlaro & Muñoz-Pinedo, 2016).

UPR-induced apoptosis, whether IRE1 or CHOP mediated, has been widely shown to occur through two main pathways: the extrinsic or death receptor pathway and the mitochondrial pathway (Iurlaro & Muñoz-Pinedo, 2016). In the death-receptor pathway, homo-oligomerization activates caspase-8 which in turn cleaves and activates caspase-3. The mitochondrial pathway differs in that Bcl-2 family proteins allow the release of cytochrome c, activating caspase-9 and in turn, executioner caspases: caspase-7 and caspase-3 (Iurlaro & Muñoz-Pinedo, 2016). The involvement of caspase-3 is therefore highlighted in both apoptotic pathways, highlighting it as a marker of ER stress-induced apoptosis.

5.1.5 Hypothesis and Aims

It is hypothesised that the inhibition of PDI promotes UPR pathway activation and as a result, downstream activation of protective ER stress markers. It is further hypothesised that prolonged UPR activation with ER stress will result in a cellular signalling switch from pro-survival mechanisms to ER stress-induced apoptosis through caspase activations.

In an effort to better elucidate the downstream UPR mechanisms of PDI inhibition in pancreatic cancer, the aims of the current study were as follows:

- To assess the effect of PDI inhibitor (PACMA-31 and CCF642) treatment on XBP-1s and caspase-3 RNA expression by RTq-PCR
- To assess the effect of PDIA1 and AGR2 knockdown on BiP and XBP-1s protein expression by Western blotting
- To assess the effect of PDIA1 and AGR2 knockdown on XBP-1s and caspase-3 mRNA expression by RT-qPCR
- To assess the effect of PACMA-31 and CCF642 treatment on capsase-3 activity
- To assess the effect of PDIA1 and AGR2 knockdown on capsase-3 activity

5.2 Materials and Methods

5.2.1 Cell Culture and Seeding

Panc-1, Mia-PaCa-2 and AsPC-1 pancreatic cancer cell lines were cultured as described in section 2.1. Cell lines were maintained at 37°C in a humidified 5% CO₂ incubator in 75cm² flasks. Cells were seeded into 6 well plates for protein or RNA extraction and 96 well plates for caspase-3 activity assays.

5.2.2 PDI Inhibitor Treatment

PDI inhibitors; Pacma-31 and CCF642 (Bio techne, UK) were prepared as described in table 2.1 and 4.2.2. Cells were treated with Pacma-31 and CCF642 for a total of 48 hours prior to proliferation and caspase-3 activity assays or protein and RNA extraction.

5.2.3 siRNA Transfection

AGR2 and PDIA1 were knocked down following siRNA transfection as described in section 2.3. The details of siRNA products used are shown in table 2.5.

5.2.4 Protein Extraction and Western Blot

Protein extraction and Western blotting was carried out as described in sections 2.7, 2.8, 2.9 and 4.2.4. Cells were transfected with siRNA prior to protein harvesting to detect changes in BiP and XBP-1s protein expression. Beta-actin expression was

used for the normalization of protein loading. Details of antibodies and their dilutions are described in Table 2.4.

Densitometry analysis of Western blot analysis was performed using Image J software (imagej.nih.gov/ij). The intensity of the bands for the control protein, (β -actin), and proteins of interest were analysed using the box tool at a set size. The background intensity of each sample was also measured and subtracted from the band intensities. The proteins of interest were then normalized to beta-actin, which served as a loading control.

5.2.5 RNA Extraction, cDNA synthesis and RT-qPCR

RNA extraction was performed with the RNeasy® Mini RNA extraction kit (Qiagen, UK), following the manufacturer's instructions and as described in section 2.4. cDNA synthesis was then performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) according to the manufacturer's instructions and as described in section 2.5. mRNA levels were determined by real-time PCR using TaqMan gene expression assays (ThermoFisher, UK) for XBP-1s and caspase-3. Relative mRNA levels were normalised to the mean CT value of the housekeeping gene RPLP0 (ThermoFisher, UK). Details of gene expression assay are shown in table 2.3. Reactions were analysed and Δ CT and $\Delta\Delta$ CT were calculated according to the $2^{-\Delta\Delta$ CT method to determine relative mRNA expression.

5.2.6 Caspase-3 Activity Assay

Caspase-3 activity was measured using the caspase-3 assay kit (Colorimetric) (Abcam), according to the manufacturer's instructions and as described in section 2.12. Caspase-3 activity was determined by the detection of chromophore p-nitroaniline (p-NA) absorbance after cleavage from the labelled substrate DEVD-p-NA. Caspase-3 activity was measured following both Pacma-31 and CCF642 PDI inhibitor treatment as described in section 2.2 or PDIA1 and AGR2 siRNA knockdown as described in section 2.3. 200µg of protein in 50µL cell lysis buffer was added per well of a 96 well plate (Corning, UK). A DEVD-p-NA substrate was added to each sample to a final concentration of 200µM. The plate was then incubated for 2 hours at 37°C and absorbance was measured at 405nm on a microplate reader.

5.2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software 5 (GraphPad Software, USA). A one-way ANOVA was used to determine significance and analyse various between independent treatment groups, with post-hoc Tukey's or Dunnett's multiple comparison tests to determine which treatment groups were significantly different if one-way ANOVA test showed significance. Significance is represented by; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3 Results

5.3.1 Effects of PDI Inhibitor Treatment on Caspase-3 Activity

The effects of treatment with PDI inhibitors, Pacma-31 and CCF642 on caspase-3 activity were investigated (Figure 5.1). Panc-1, Mia-PaCa-2 and AsPC-1 were all treated with Pacma-31 and CCF642 for 48 hours at the concentrations detailed in Table 2.1. Caspase-3 activity was significantly induced across all treatments but was shown to be least significant in Pacma-31 treated Panc-1. Caspase-3 activity increased in a dose dependent manner, with the highest treatment doses demonstrating the highest rate of caspase-3 activity. This implies that both Pacma-31 and CCF642 treatment are inducing apoptotic pathways, to activate caspase-3 and contribute to pancreatic cancer cell death. Panc-1 differed from the other cell lines in that normal, untreated cells showed a natural higher level of caspase-3 activity. This may have contributed to why fewer changes were observed with Pacma-31 treatment. The greatest increase in caspase-3 activity was shown in AsPC-1 with 100 μ M Pacma-31 treatment (**** $p < 0.0001$).

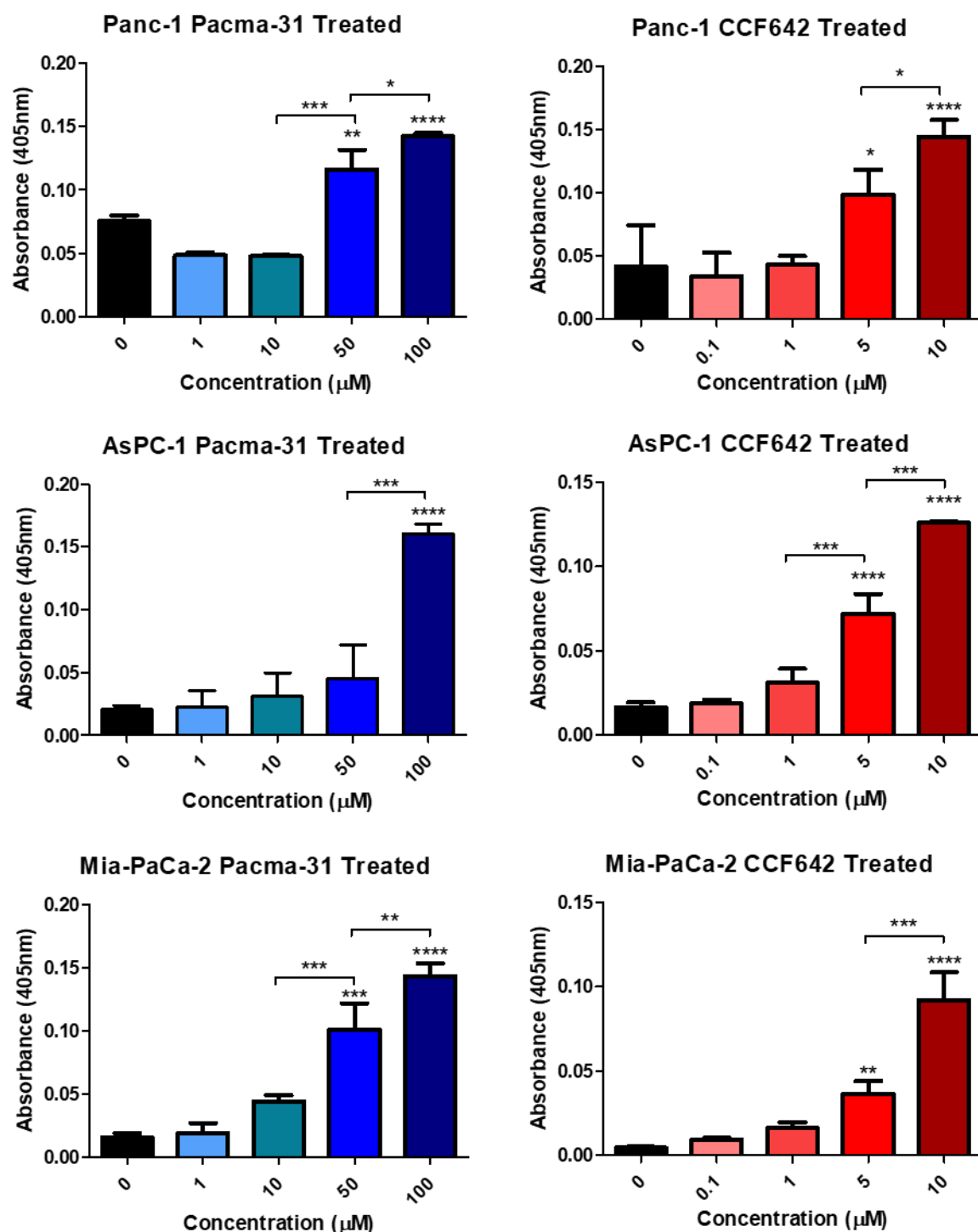


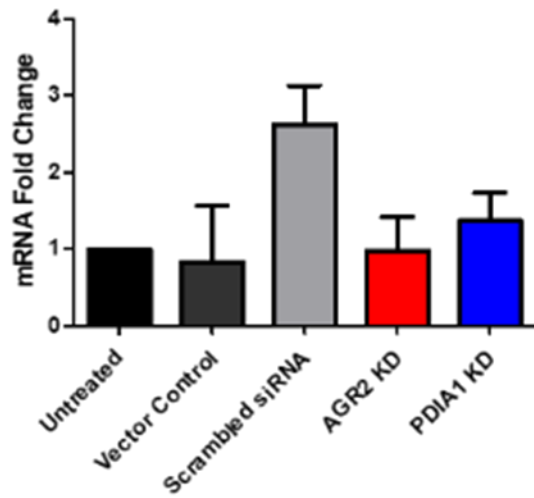
Figure 5.1. Pacma-31 and CCF642 treatment induces caspase-3 activity in pancreatic cancer cell lines.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with Pacma-31 at concentrations 1 μ M, 10 μ M, 50 μ M and 100 μ M and CCF642 at concentrations 0.1 μ M, 1 μ M, 5 μ M and 10 μ M for 48 hours. Absorbance (405nm) was measured following substrate addition to determine relative caspase-3 activity. N=3, error bars denote SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

5.3.4 Effects of PDI Knockdown on XBP-1s mRNA Expression

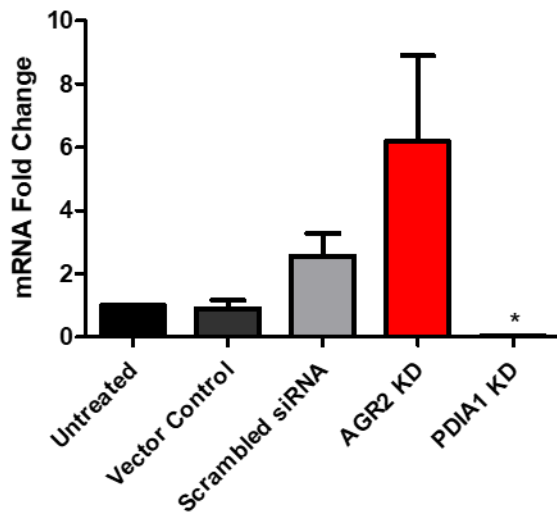
XBP-1s mRNA expression was measured in Panc-1, AsPC-1 and Mia-PaCa-2 cell lines using RT-qPCR following 48hr PDIA1 and AGR2 siRNA knockdown (Figure 5.2). XBP-1s expression was significantly decreased in AsPC-1 in PDIA1 knockdown cells (* $p < 0.05$). This was the only significant change in XBP-1s expression observed across all three cell lines. This therefore indicates that PDIA1 may show interaction with XBP-1s as another protective downstream UPR gene.

Panc-1 XBP-1s Expression



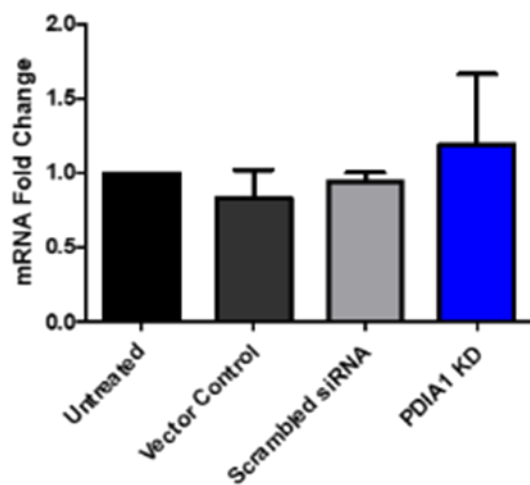
Panc-1	RPLPO					XBP-1s				
	Ct1	Ct2	Ct3	Ct4	Ct5	Ct1	Ct2	Ct3	Ct4	Ct5
Untreated	19.18	18.59	17.32	18.84	17.59	27.16	26.05	27.55	25.81	25.47
Vector Control	20.47	19.87	19.45	22.66	22.65	30.68	30.74	27.78	34.15	33.98
Scrambled siRNA	20.86	20.31	19.82	18.86	18.97	27.24	26.08	28.34	26.53	25.27
AGR2 KD	20.04	19.54	19.44	20.21	20.26	30.03	28.86	28.59	28.81	27.19
PDIA1 KD	19.05	18.53	19.04	18.24	17.71	27.05	25.62	27.83	26.25	25.15

AsPC-1 XBP-1s Expression



AsPC-1	RPLPO			XBP-1s		
	Ct1	Ct2	Ct3	Ct1	Ct2	Ct3
Untreated	19.47	19.11	19.19	27.98	28.56	27.24
Vector Control	19.46	18.41	17.46	27.46	28.27	26.37
Scrambled siRNA	18.60	18.00	18.30	25.44	25.69	26.18
AGR2 KD	19.31	18.59	18.20	24.31	25.72	25.13
PDIA1 KD	21.26	19.93	17.61	33.95	34.70	33.53

Mia-PaCa-2 XBP-1s Expression



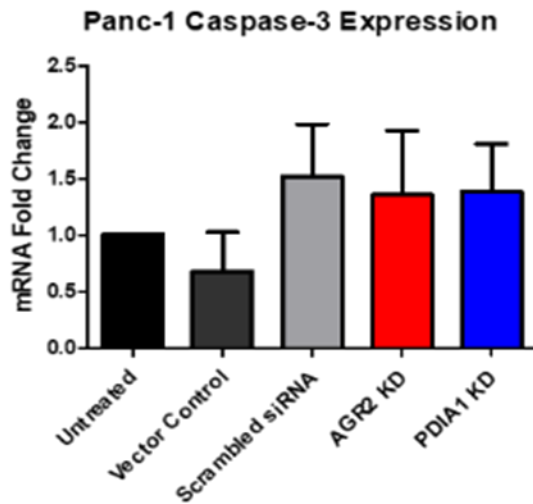
Mia-PaCa-2	RPLPO					XBP-1s				
	Ct1	Ct2	Ct3	Ct4	Ct5	Ct1	Ct2	Ct3	Ct4	Ct5
Untreated	18.91	18.40	19.66	18.47	18.47	25.33	24.69	27.21	26.20	26.20
Vector Control	19.27	18.69	19.52	19.02	19.02	26.30	26.60	27.11	27.14	26.21
Scrambled siRNA	20.01	19.47	21.15	18.41	18.41	26.29	25.77	28.67	26.45	26.49
PDIA1 KD	18.78	18.07	18.56	19.96	19.96	28.78	28.69	24.95	27.00	26.71

Figure 5.2. PDIA1 and AGR2 siRNA knockdown treatment influences mRNA expression of XBP-1s in pancreatic cancer cell lines.

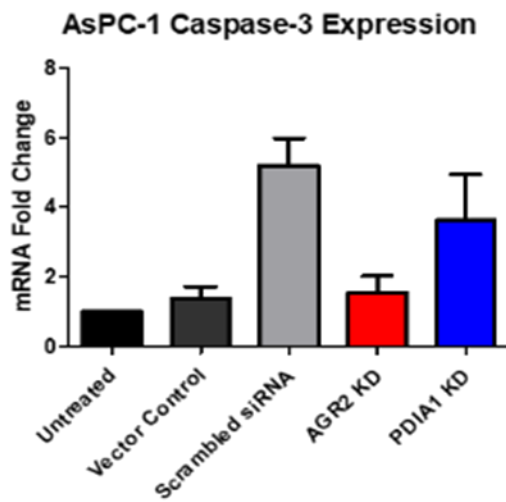
Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were transfected with AGR2 and PDIA1 siRNAs at a final concentration of 6.25nM 48 hours. Controls groups included untreated, a vector control (cells treated with the same volume of transfection reagent [4µl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and a non-targeting scrambled siRNA control (6.25nM). XBP-1s mRNA expression was calculated according to the Δ CT method using the reference housekeeping gene RPLP0 from RT-qPCR analysis. N=5 for Panc-1 and Mia-PaCa-2 per gene; N=3 for AsPC-1 per gene. Average CT values (from 3 technical replicates) for 5 or 3 repeats of each experiment are shown in tables adjacent to respective cell lines and treatment groups. Error bars denote standard deviation. Error bars denote standard deviation. *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

5.3.5 Effects of PDI Knockdown on Caspase-3 mRNA Expression

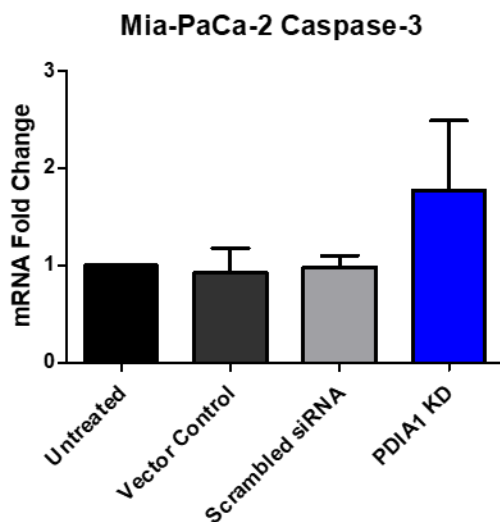
Caspase-3 mRNA expression was measured in Panc-1, AsPC-1 and Mia-PaCa-2 cell lines using RT-qPCR following 48hr PDIA1 and AGR2 siRNA knockdown (Figure 5.3). No significant changes in caspase-3 expression were shown with either PDIA1 or AGR2 knockdown across all three cell lines. Although an increase in expression was shown with PDIA1 knockdown in Mia-PaCa-2, this was not found significant due to variations, shown by error bars denoting standard deviation. While AGR2 and PDIA1 do not appear to influence caspase-3 expression, this does not necessarily correlate with the activation of apoptosis. Caspase-3 activity was therefore also assessed.



Panc-1	RPLPO					Caspase-3				
	Ct1	Ct2	Ct3	Ct4	Ct5	Ct1	Ct2	Ct3	Ct4	Ct5
Untreated	19.18	18.59	17.38	18.37	17.61	25.52	22.94	25.40	24.34	22.83
Vector Control	20.47	19.87	19.45	22.73	22.71	30.66	29.04	26.67	30.54	27.61
Scrambled siRNA	20.86	20.31	19.87	18.87	19.02	25.80	23.33	27.73	26.87	24.12
AGR2 KD	20.04	19.54	19.40	20.40	20.27	27.64	25.00	26.63	26.85	23.72
PDIA1 KD	19.05	18.53	19.04	18.29	17.56	25.37	22.92	25.44	24.29	22.97



AsPC-1	RPLPO			Caspase-3		
	Ct1	Ct2	Ct3	Ct1	Ct2	Ct3
Untreated	19.89	19.11	19.19	26.22	26.51	26.48
Vector Control	19.70	18.41	17.46	26.45	24.90	24.13
Scrambled siRNA	18.82	18.00	18.30	22.53	23.54	23.06
AGR2 KD	19.10	18.59	18.20	26.23	24.93	24.51
PDIA1 KD	21.26	19.93	17.61	25.62	24.80	24.67



Mia-PaCa-2	RPLPO					Caspase-3				
	Ct1	Ct2	Ct3	Ct4	Ct5	Ct1	Ct2	Ct3	Ct4	Ct5
Untreated	18.86	18.28	19.61	18.62	18.06	25.69	21.60	26.52	25.83	24.63
Vector Control	19.24	18.70	19.65	18.67	17.98	27.38	23.26	26.55	25.06	24.50
Scrambled siRNA	19.98	19.41	21.22	18.45	18.11	26.56	22.60	29.01	25.47	24.76
PDIA1 KD	18.84	18.10	18.69	20.11	19.19	28.45	23.62	23.85	25.55	24.98

Figure 5.3. PDIA1 and AGR2 siRNA knockdown treatment influences mRNA expression of Caspase-3 in pancreatic cancer cell lines.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were transfected with AGR2 and PDIA1 siRNAs at a final concentration of 6.25nM 48 hours. Controls groups included untreated, a vector control (cells treated with the same volume of transfection

reagent [4µl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and a non-targeting scrambled siRNA control (6.25nM). Caspase-3 mRNA expression was calculated according to the Δ CT method using the reference housekeeping gene RPLP0 from RT-qPCR analysis. N=5 for Panc-1 and Mia-PaCa-2 per gene; N=3 for AsPC-1 per gene. Average CT values (from 3 technical replicates) for 5 or 3 repeats of each experiment are shown in tables adjacent to respective cell lines and treatment groups. Error bars denote standard deviation. T Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

5.3.6 Effects of PDI Knockdown on Caspase-3 activity

The effects of PDIA1 and AGR2 siRNA knockdown on Caspase-3 activity were investigated (Figure 5.4). Despite no significant changes observed in caspase-3 expression in Figure 5.3, AGR2 knockdown in Panc-1 (*p<0.05) demonstrated a significantly increased rate in caspase-3 activity compared to the untreated control. Whilst PDIA1 demonstrated no significant change. This highlights AGR2 as a potential therapeutic target in triggering ER stress and apoptosis via caspase-3 activation in pancreatic cancer. No significant increase in caspase-3 activity was observed with PDIA1 knockdown in Mia-PaCa-2.

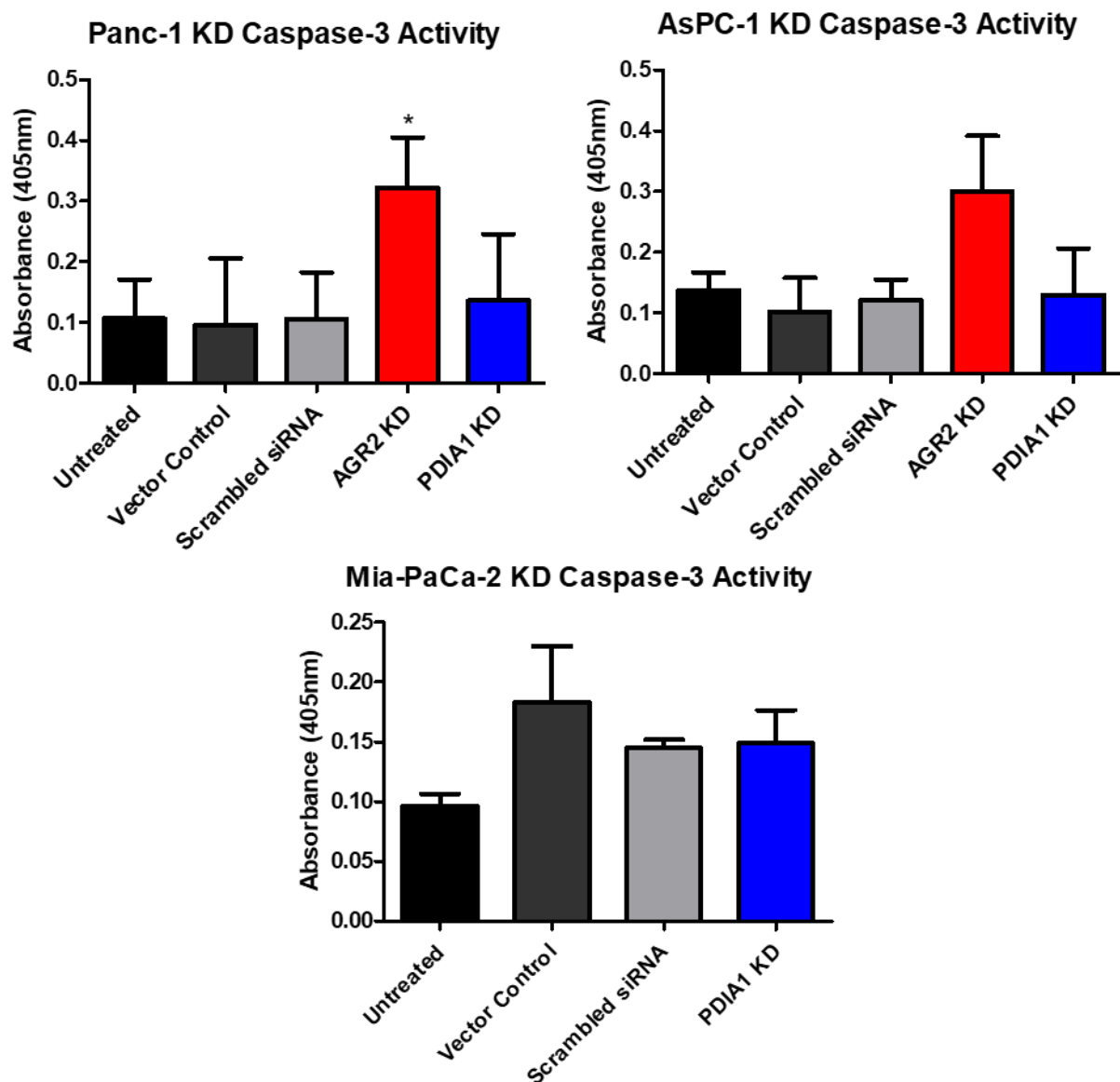


Figure 5.4. PDIA1 and AGR2 siRNA knockdown induces caspase-3 activity in pancreatic cancer cell lines.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were transfected with AGR2 and PDIA1 siRNAs at a final concentration of 6.25nM 48 hours. Controls groups included untreated, vector control (cells treated with the same volume of transfection reagent [4μl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) and a non-targeting scrambled siRNA control (6.25nM). Absorbance (405nm) was measured following substrate addition to determine relative caspase-3 activity. N=3, error bars denote SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

5.3.7 Effects of PDI Knockdown on XBP-1s and BiP Protein Expression

The effects of PDIA1 and AGR2 siRNA knockdown on BiP and XBP-1s protein expression were observed in Panc-1, AsPC-1, and Mia-PaCa-2 by Western blotting (Figure 5.5A) beta-actin was used as a control for confirmation of protein expression in cells. The significance of changes in protein expression was also analysed by semi-quantitative Western blot analysis (Figure 5.5B). Results of XBP-1s expression correlated with that of mRNA expression shown in Figure 5.2, in that western blot results also showed decreased XBP-1s protein expression in PDIA1 knockdown cells AsPC-1 (* $p < 0.001$). Although a decrease in XBP-1s expression was also shown with PDIA1 knockdown in Panc-1 cells, this was not found to be significant. No significant change in XBP-1s expression was shown with AGR2 knockdown or with PDIA1 knockdown in Mia-PaCa-2 or with AGR2 knockdown. AGR2 and PDIA1 knockdown also did not demonstrate a significant change in BiP protein expression.

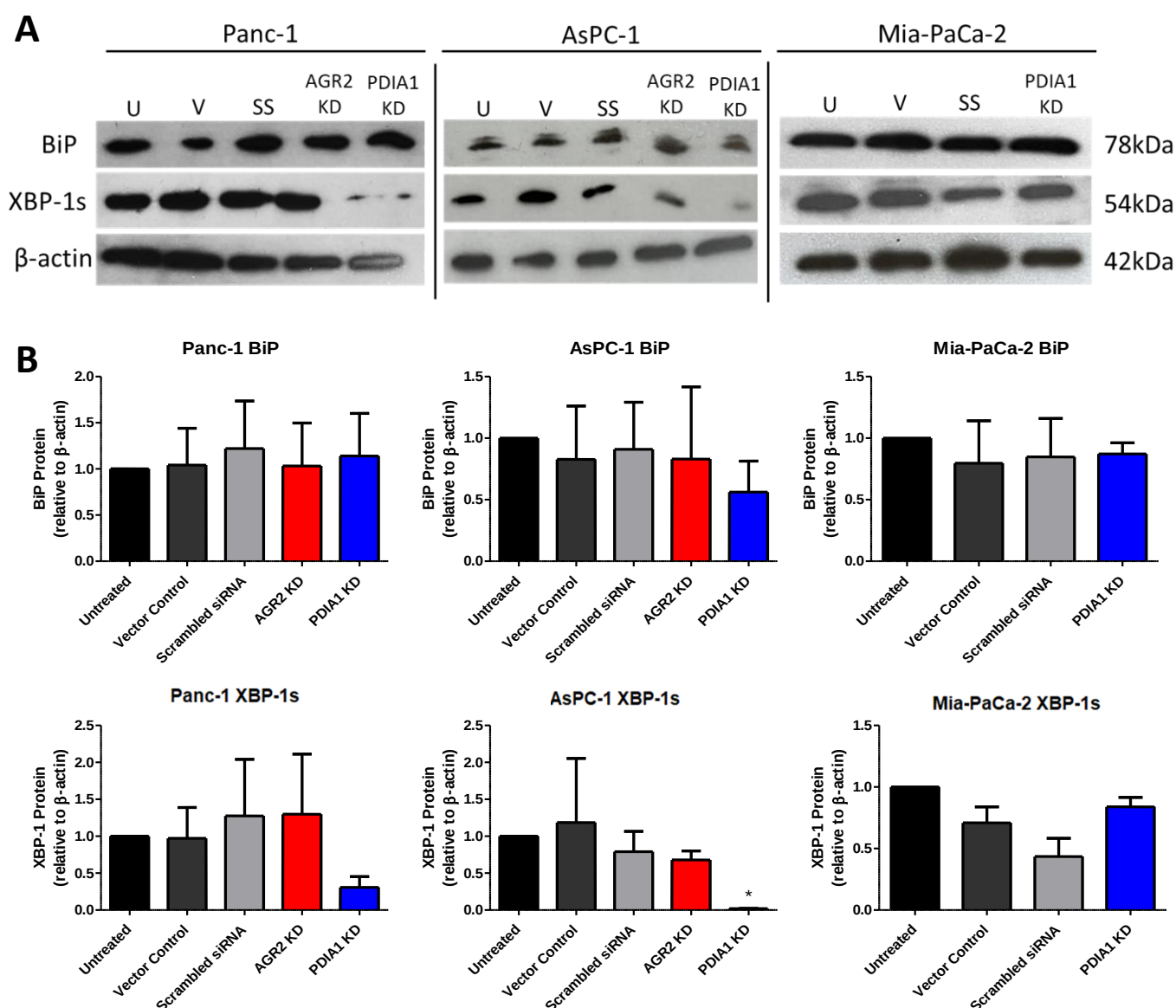


Figure 5.5. PDIA1 and AGR2 siRNA knockdown treatment influences protein expression of XBP-1s and BiP in pancreatic cancer cell lines.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were transfected with AGR2 and PDIA1 siRNAs at a final concentration of 6.25nM for 48 hours. Control groups included untreated, vector control (cells treated with the same volume of transfection reagent [4μl/well DharmaFECT Reagent 1] as siRNA knockdown treated cells) a non-targeting scrambled siRNA *control* (6.25nM). (A) BiP and XBP-1s protein levels were determined by Western blot analysis with Beta-actin as a control. N=3 per protein, per cell line. (B) Semi-quantitative Western blot analysis of the data in panel A, Beta-actin expression was used for the normalization of protein loading. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Dunnett's multiple comparison tests. (U) = Untreated, (V) = Vector Control, (SS) = Scrambled siRNA, (KD) = Knockdown.

5.4 Discussion

Pancreatic cancer cells frequently experience ER stress, triggering UPR pathway activation to maintain ER homeostasis (Dumartin et al., 2017). PDI, induced by UPR pathway activation, further participates in controlling ER homeostasis and promoting cancer cell survival. This study aimed to inhibit PDI to achieve prolonged UPR pathway activation, resulting in a cellular signalling switch from pro-survival mechanisms to ER stress induced apoptosis.

5.4.1 The Effects of PDIA1 Inhibition on XBP-1s Expression

To investigate the potential downstream mechanisms of ER stress through UPR activation, following PDI inhibition, changes in both mRNA and protein expression of XBP-1s were measured. The study showed that PDIA1 knockdown, significantly decreased XBP-1 mRNA and protein expression in AsPC-1 cells (Figures 5.2 and 5.5). In contrast, AGR2 knockdown did not significantly influence XBP-1s in any pancreatic cancer cell line. XBP-1s is recognised as a key pro-survival gene, activated to alleviate ER stress in the UPR. A reduction in XBP-1s expression with PDIA1 knockdown suggests a potential disruption in the activation of the IRE1 α pathway. The phosphorylation of PDIA1 has been demonstrated to alter the activity of the IRE1 α pathway (Yu et al., 2020). This phosphorylation of PDIA1 allows it to bind to IRE1 α , where it acts to attenuate chronic UPR signalling (Yu et al., 2020). An inhibition in PDIA1 would therefore prevent this attenuation of chronic UPR signalling, disrupting normal UPR mechanisms. Under chronic UPR activation, IRE1 α activates the JNK pathway, further activating caspase-3 to induce apoptosis (Hetz & Mollereau, 2014). The significant reduction in XBP-1s expression shown with

PDIA1 knockdown in AsPC-1 may be due to prolonged ER stress resulting in an activation of apoptosis associated chronic UPR pathways, leading to cell death. A study of PDI inhibitor treatment in multiple myeloma cells, suggested that XBP-1s may be degraded by apoptotic enzymes under conditions of acute induction of the IRE1 pathway with chronic UPR activation (Vatolin et al, 2016). This proposed degradation of XBP-1s highlights a compromise in the defence on pancreatic cancer cells against ER stress, highlighting PDI inhibition as a potential effective treatment strategy. The potential influence of PDIA1 inhibition on the UPR pathway is shown in Figure 5.6.

5.4.2 The Potential Influence of AGR2 inhibition on Caspase-3 Activation

While AGR2 does not appear to show interactions or influence XBP-1s expression, results do show influence in caspase-3 activity (Figure 5.4). Both caspase-3 mRNA expression and relative activity were measured to assess the potential effects of PDI inhibition on the induction of caspase-3-induced apoptosis. Caspase-3 has been highlighted as a driver of apoptosis, downstream of chronic UPR activations (Iurlaro & Muñoz-Pinedo, 2016). AGR2 knockdown in Panc-1 was shown to significantly increase caspase-3 activity compared to the control (Figure 5.4). This suggests that AGR2 inhibition in these cell lines trigger a prolonged UPR activation, resulting in apoptosis through caspase-3 activation. However, results showed that caspase-3 mRNA expression levels were not significantly affected by AGR2 knockdown (Figure 5.3). This suggests that whilst AGR2 inhibition may not influence caspase-3 expression levels, AGR2 may play a role in attenuating the extrinsic or death receptor pathway or the mitochondrial pathway, preventing downstream caspase-3

activation. AGR2 could also play a role in attenuating the activations of chronic UPR pathways which would lead to caspase-3 activations. A recent study has found that an overexpression AGR2 in Panc-1 cells significantly reduced CHOP expression (Hong et al., 2021). Whilst it is not clear which chronic UPR pathway AGR2 inhibition activates to induce caspase-3 led apoptosis, one can therefore postulate that the activation of CHOP may be involved. CHOP-induced ER oxidoreductase (ERO)1 α acts to oxidise PDI, generating intracellular hydrogen peroxide (H₂O₂) in the process (Rao et al., 2015). With a depletion in AGR2 expression and increasing ER stress levels, ERO1a may be overexpressed, through the CHOP-associated chronic UPR pathway arm, in attempt to oxidise and restore PDIs to contribute to proper protein folding. This however would also lead to an elevated production of H₂O₂, which has the potential to leak into the cytoplasm and consequently inducing apoptosis through caspase-3 activation. While the limitations of reductase PDI activity assays have been discussed, it should also be noted that results in a relatively short time period do not account for the influence of additional enzymes such as ERO1 α . ERO1 α has the ability restore the oxidative potential of PDI to re-enable protein folding, therefore potentially combatting PDI inhibition efforts (Vatolin et al., 2016). These data highlight the potential association between CHOP activation and AGR2 inhibition, highlighting the need for further investigation.

5.4.3 The Potential Role of AGR2 in the UPR

Increased AGR2 expression has been associated with activation of IRE1 and ATF6, which are both pillars of the UPR pathway (Alsereihi et al., 2019). AGR2 is also likely

to play a key role in the activation UPR pathway itself. The dimerization of AGR2 allows for its interaction with BiP, which in combination trigger UPR activation to reduce ER stress (Moidu et al., 2020). This suggests that while AGR2 knockdown did not influence BiP protein expression, it still may prevent an interaction, hence limiting UPR activation. While under normal conditions, AGR2 mainly exists in homodimers, allowing for BiP interaction, in ER stress AGR2 dimers dissociate and form functional complexes with ERAD to isolate misfolded proteins, highlighting its pro-survival role in the ER (Maurel et al., 2019). The potential influence of AGR2 inhibition in the UPR pathway is shown in Figure 5.6. An imbalance between AGR2 dimer and monomer ratio in the ER leads to the activation of pro-inflammatory signals and the release of AGR2 from the ER into the extracellular environment (Zhang et al., 2023). This may occur due to a protein imbalance in the ER due to ER stress, and the saturation of ER receptor sites due to an overexpression of AGR2 in cancer. It is crucial to also note that whilst AGR2 is present on the cell surface and can be secreted by cancer cells, normal AGR2 expressing cells do not show cell surface expression, it is not secreted, and the expression is localized to the cell interior (Ho et al., 2016). AGR2 has been reported to play other intracellular roles outside of its localisation to the ER such as the regulation of p53 signalling, again highlighting its potential as a therapeutic target to limit ER secretion and consequent tumorigenesis (Pohler et al., 2004).

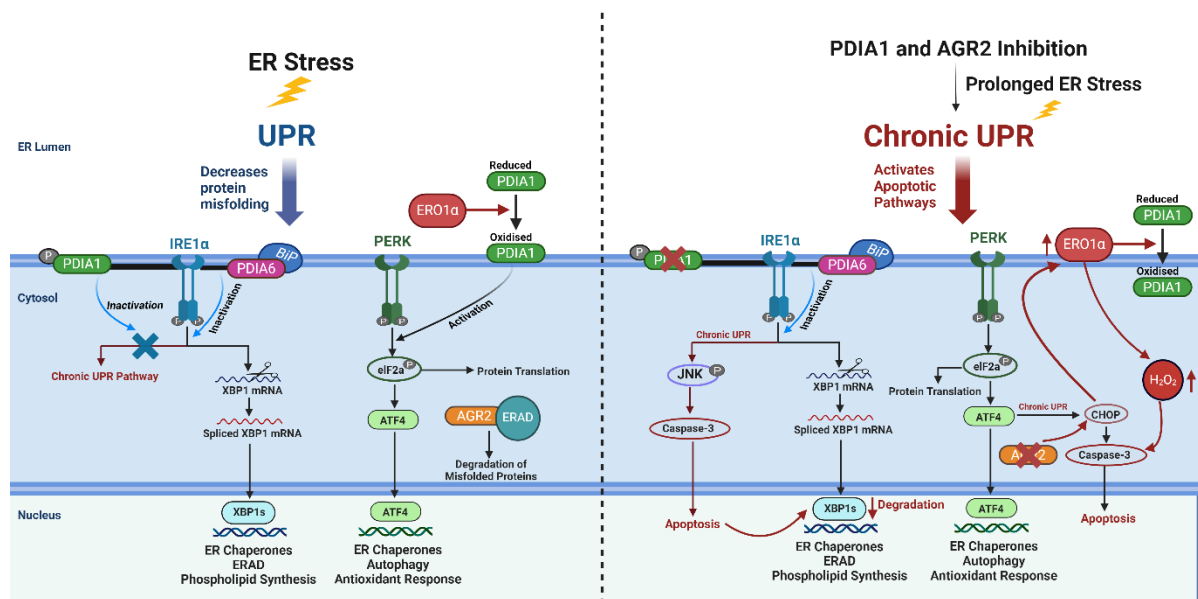


Figure 5.6. The proposed effects of PDIA1 and AGR2 inhibition on the UPR pathway. (Adapted from Powell & Foster, 2021).

Normal UPR pathway activations are shown on the left-side diagram, while the proposed effects of PDIA1 and AGR2 inhibition on the UPR pathway are shown on the right-side diagram. The mechanism of action of PDIA1 and AGR2 in the UPR pathway show the pro-survival role of PDI in ER stress. PDIA1 and AGR2 contribute to decreasing protein misfolding and maintaining ER homeostasis. PDIA1 and AGR2 inhibition triggers chronic a UPR due to prolonged ER stress, consequently activating apoptotic pathways. The potential mechanisms by which PDIA1 and AGR2 inhibition contributes to induced apoptosis and degradation of XBP-1s are proposed as described in text.

In summary, this study demonstrates various potential ways in which PDIA1 and AGR2 are acting as upstream regulators of the UPR pathway, demonstrating their protective properties and potential as therapeutic targets. The study further highlights how AGR2 inhibition could trigger a pro-apoptotic response through chronic UPR signalling and caspase-3 activation. It is further suggested that PDIA1 inhibition may act to repress pro-survival gene XBP-1s. Further research needs to be carried out to fully understand how inhibition of each PDI member alters the activation of specific UPR pathways and downstream pathways of apoptosis. With variation in results among the three cell lines, research is needed to further discern which combination of PDI members or UPR pathway genes would prove to be the most effective target

in developing pancreatic cancer treatment strategies. pancreatic cancer cell growth and contributing to cell death.

CHAPTER 6 – THE THERAPEUTIC POTENTIAL OF PDI INHIBITION AND GEMCITABINE COMBINATION TREATMENT IN PANCREATIC CANCER

Chapter 6 - The therapeutic potential of PDI inhibition and gemcitabine combination treatment

6.1 Introduction

6.1.1 Gemcitabine Therapy in Pancreatic Cancer

Gemcitabine remains a cornerstone of current pancreatic cancer therapies (Beutel and Halbrook, 2023). First proposed as treatment for patients with unresectable pancreatic cancer, gemcitabine has now become a standard chemotherapy drug for pancreatic cancer therapy (Zhang et al., 2022). Compared with other chemotherapy options, gemcitabine is considered a tolerable compound for most pancreatic cancer patients, with clinically manageable adverse effects (Beutel and Halbrook, 2023).

Despite being a standard of care treatment option, substantial efficacy of gemcitabine in pancreatic cancer is not evident in short-term treatments. Complete or partial remission rate is also relatively low at 10–30% (Zhang et al., 2022). However, gemcitabine has been shown to have a significant effect on clinical benefit rate, including degree of pain, painkiller dosage, and weight gain, improving the quality of life of pancreatic cancer patients (Inal et al., 2012).

To improve the therapeutic effect of gemcitabine, it has often been used as a combination therapy with platinum- or fluorouracil-based drugs. These combination therapies have demonstrated a significant effect on both the overall survival (OS) and progression-free survival (PFS) of patients (Zhang et al., 2022). Median OS with gemcitabine monotherapy has been shown to range from 5.6 to 6.8 months (Fernández et al., 2018). Two emerging gemcitabine combination regimens, FOLFIRINOX and nab-paclitaxel have shown significant OS improvement, with a median OS of 11.1 months and 8.5 months, respectively (Fernández et al., 2018).

Both FOLFIRINOX and nab-paclitaxel in combination with gemcitabine are now recommended as first-line treatments for metastatic pancreatic cancer (Fernández et al., 2018). The combination regimen of nab-paclitaxel plus gemcitabine is currently the preferred treatment approach for patients with pancreatic cancer due to a reduced drug toxicity compared with FOLFIRINOX (Muranaka et al., 2017).

Despite the increase in survival observed in metastatic pancreatic cancer patients with nab-paclitaxel plus gemcitabine treatment, the 5-year survival rates of patients remain devastatingly low (Fernández et al., 2018, Siegel et al., 2022). A lack of durable responses to standard-of-care chemotherapy regimens, highlights an urgent need for novel treatment strategies to improve rates of survival.

6.1.2 Chemoresistance in Pancreatic Cancer

Resistance to chemotherapy agents including gemcitabine, FOLFIRINOX, and nab-paclitaxel further complicate pancreatic cancer treatment (Sarvepalli et al., 2019). Several factors can facilitate the development of chemoresistance to aid the survival of cancer cells including tumour heterogeneity, stromal density, and inflammatory mediators (Sarvepalli et al., 2019). While pancreatic cancer is most susceptible to gemcitabine therapy compared with other antitumour drugs, chemoresistance is observed in patients within weeks of starting treatment (Sarvepalli et al., 2019). Long-term use of gemcitabine has been shown to extensively alter the tumour microenvironment in pancreatic cancer, leading to chemoresistance (Long et al., 2021).

The tumour microenvironment of pancreatic cancer is characterised by extensive desmoplasia (Zhao et al., 2022). Desmoplasia is the formation of dense fibrotic

tissue within and surrounding the tumour, generated by activated fibroblasts, myofibroblasts, pancreatic stellate cells and tumour cells, it is also characterised by an overabundance of extracellular matrix components (Ebelt et al., 2020). The fibrotic nature of the pancreatic tumour microenvironment has been shown to contribute to both tumour growth and the induction of gemcitabine resistance (Zhao et al., 2022). Several key tumour microenvironment components have been shown to contribute to chemoresistance in pancreatic cancer cells, including pancreatic stellate cells (PSCs), cancer-associated fibroblasts (CAFs), and CSCs (Sarvepalli et al., 2019). Stromal cells are a key player in the tumour microenvironment and interact with pancreatic cancer cells through the ECM protein (paracrine) pathways to drive gemcitabine resistance (Zhao et al., 2022).

In vitro studies have shown that PSCs activate the Notch signalling pathway which in turn promotes EMT and induction of CSCs, contributing to gemcitabine resistance (Zhao et al., 2022). The majority of CAFs are derived from the activation of PSCs (Nair et al., 2017). Activated CAFs are considered naturally gemcitabine resistant, responsible for enhancing proliferation, stemness and the metastatic capacity of cancer cells, they strongly contribute to chemoresistance. (Huang and Brekken, 2020). CSCs have recently been highlighted as a potential critical component of gemcitabine resistance in pancreatic cancer cells, with in vitro studies demonstrating that CSCs may escape the cytotoxic effect of gemcitabine through EMT (Yin et al., 2011). Gemcitabine resistance is a crucial determinant of chemotherapy efficacy and a pivotal challenge in treatment, driving poor prognosis in pancreatic cancer patients.

6.1.3 Gemcitabine-induced ER Stress

Research has shown that the induction of the ER stress response in pancreatic cancer cells may prove to be a promising therapeutic strategy in combatting issues of gemcitabine chemoresistance (Siegelin, 2012). A recent study demonstrated that gemcitabine treatment in Panc-1 CSCs inactivated the IRE1 branch and activated the PERK branch of the UPR pathway, indicating a chronic UPR pathway switch from cell death to survival (Wang et al., 2017). Gemcitabine was shown to alter the expression of downstream UPR components, reducing XBP-1s expression and increasing spliced ATF6 expression, activating BiP, and ultimately leading to the attenuation of apoptosis in Panc-1 CSCs (Wang et al., 2017). This therefore demonstrates how gemcitabine has the potential to drive chemoresistance through pro-survival UPR pathway mechanisms, combined with CSC induction. Preventing this pro-survival UPR pathway switch, through UPR pathway inhibition, may drive pro-apoptotic activations and help to combat gemcitabine resistance.

A recent study demonstrated a synergistic effect with gemcitabine and fatty acid synthase (FASN) inhibitor Orlistat in pancreatic cancer, demonstrating increased apoptosis and decreased chemoresistance (Tadros et al., 2017). The study hypothesised that Orlistat and gemcitabine combination therapy diminished stemness by inducing apoptosis through ER stress, with treatment showing increased ER stress marker expression including, BiP, XBP-1s, ATF4 and CHOP and increased caspase 3/7 activity (Tadros et al., 2017).

It is therefore proposed that the use of inhibitors of the UPR pathway in combination with gemcitabine can enhance the chemosensitivity of pancreatic cancer cells, highlighting a new therapeutic strategy to be further explored. As a key driver of UPR

pathway activation and ER stress, it can therefore also be proposed that PDI inhibition with gemcitabine combination may be an effective treatment strategy to combat chemoresistance in pancreatic cancer.

6.1.4 The Role of PDI in Chemoresistance

Several studies have shown that PDI plays a role in mediating chemoresistance in various cancer types (Rhaman et al., 2022). These chemoresistant roles also have been shown to differ between PDI family members. For instance, in epithelial ovarian cancer, PDIA3 has been shown to contribute to paclitaxel-resistance (Cicchillitti et al., 2010), while AGR3 has been shown to mediate cisplatin resistance (Gray et al., 2012). Cisplatin resistance has also been shown to be mediated by PDIA5 and PDIA6 in lung adenocarcinoma (Tufo et al., 2014). Tufo et al further showed that inactivation of PDIA4 restored a classical mitochondrial apoptosis pathway, promoting investigations into PDI as a target to combat chemoresistance.

Knockdown of PDIA3 in HCT116 colon cancer cells was also shown to enhance an apoptotic response when in combination with chemotherapy treatment (Hussmann et al., 2015). While these studies suggest the role of various PDI family members in chemoresistance, little is known about their involvement in pancreatic cancer.

With relevance to gemcitabine-resistance, inhibition of PDIA1 via bacitracin treatment has been shown to increase the sensitivity of bladder cancer cells to gemcitabine treatment (Wang et al., 2020). Wang et al showed that PDIA1 inhibition enhanced gemcitabine-induced anticancer effects through activation of the PERK/ATF4/CHOP pathway, with increased levels of caspase-3 activity in the PDIA1 and gemcitabine combination treatment group. PDIA1 is upregulated and highly

expressed in bladder cancer tissue (Wang et al., 2020). These results may therefore be comparable in pancreatic cancer studies, where TCGA analysis has shown PDIA1 to be the most highly expressed PDI family member. These results further highlight the potential role of PDIA1 inhibition and gemcitabine combination treatment on the induction of apoptotic UPR pathways.

In terms of pancreatic cancer, AGR2 has been found to be overexpressed in pancreatic CSCs, suggesting its influence in promoting a chemoresistant tumour environment through the maintenance of cell stemness (Dumartin et al., 2017). A recent study explored the role of AGR2 in the survival of Panc-1 cells and its contribution to drug sensitivity (Hong et al., 2021). The study suggested that AGR2 attenuates tunicamycin-induced ER stress and gemcitabine-induced apoptosis in Panc-1 cells, therefore promoting cell survival (Hong et al., 2021). This study highlights the need for further research into the potential of AGR2 inhibition and gemcitabine combination treatment in further pancreatic cancer cell lines, and to elucidate the effects of treatment on apoptotic pathway activations.

6.1.5 Hypothesis and Aims

It is hypothesised that the combination treatment of PDI inhibition and gemcitabine promotes chronic UPR pathway activation to drive increased apoptosis and to combat gemcitabine resistance. It is further hypothesised that PDI inhibition will enhance pancreatic cancer chemosensitivity to gemcitabine, driving a greater inhibition of cell proliferation with combination treatment.

To assess the effects of PDI inhibition and gemcitabine combination treatment on pancreatic cell survival, the aims of the current study were as follows:

- To measure the effect of PDI inhibitor (PACMA-31 and CCF642) with gemcitabine combination treatment on Panc-1, Mia-PaCa-2 and AsPC-1 cell proliferation and cell viability
- To measure the effect of PDIA1 and AGR2 knockdown with gemcitabine combination treatment on Panc-1, Mia-PaCa-2 and AsPC-1 cell proliferation and cell viability
- To determine, following measurements of cell proliferation, the most effective and clinically relevant PDI inhibition and gemcitabine combination treatment
- To assess the effect of the potentially clinically relevant PDI inhibition and gemcitabine combination treatment on caspase-3, XBP-1s and CHOP mRNA expression by RT-qPCR
- To assess the effect of the potentially clinically relevant PDI inhibition and gemcitabine combination treatment on XBP-1s protein expression by Western blotting
- To assess the effect of the potentially clinically relevant PDI inhibition and gemcitabine combination treatment on caspase-3 activity

6.2 Materials and Methods

6.2.1 Cell Culture and Seeding

Panc-1, Mia-PaCa-2 and AsPC-1 pancreatic cancer cell lines were cultured as described in section 2.1. Cell lines were maintained at 37°C in a humidified 5% CO₂ incubator in 75cm² flasks. Cells were seeded into 6 well plates for protein or RNA extraction and 96 well plates for BrdU proliferation, MTT cell viability and caspase-3 activity assays.

6.2.2 PDI Inhibitor Treatment

PDI inhibitors; Pacma-31 and CCF642 (Bio techne, UK) were prepared as described in table 2.1 and 4.2.2. Cells were treated with Pacma-31 and CCF642 for a total of 48 hours prior to proliferation and caspase-3 activity assays or protein and RNA extraction.

6.2.3 siRNA Transfection

AGR2 and PDIA1 were knocked down following siRNA transfection as described in section 2.3. The details of siRNA products used are shown in Table 2.5.

6.2.4 Gemcitabine combination treatment

Gemcitabine (Thermo Fisher, UK) was prepared as described Table 2.1. Panc-1 Mia-PaCa-2 and AsPC-1 cells were treated with gemcitabine alone at three incremental concentrations based on the IC₅₀ for 48 hours (Table 2.1). In combination treatments,

cells were simultaneously treated with gemcitabine at the lowest dose (0.5 μ M for Panc-1 and 0.05 μ M for Mia-PaCa-2 and AsPC-1) and PACMA-31 and CCF642 for 48 hours as described in section 2.2 or with PDIA1 and AGR2 siRNA knockdown as described in section 2.3.

6.2.5 Brdu Proliferation Assay

Proliferation was measured using Cell Proliferation ELISA, BrdU (Colorimetric) kit (Roche, UK) following the manufacturer's protocol and as described in section 2.10.2. Cell seeding density was optimised for the 72 hour assay, to seed 4000 cells per well of a 96 well plate. Cell proliferation was measured following simultaneous treatment of gemcitabine with PDI inhibitor treatments as described in section 2.2 or PDIA1 and AGR2 siRNA knockdown as described in section 2.3.

6.2.6 MTT Proliferation Assay

Cell viability was measured using the MTT cell viability assay kit (Biotum, UK) according to the manufacturer's instructions and as described in section 2.10.3. Cell viability was assessed based on the ability of metabolically active cells to cleave the yellow tetrazolium salt (MTT) to produce a purple formazan crystal. Optical density of the colorimetric signal (proportional to cell number) was measured to determine cell viability following simultaneous treatment of gemcitabine with PDI inhibitor treatments as described in section 2.2 or PDIA1 and AGR2 siRNA knockdown as described in section 2.3.

6.2.7 Caspase-3/7 activity assay

Caspase 3/7 activity was measured using the Amplite® Colorimetric caspase 3/7 assay kit (AAT Bioquest, UK) according to the manufacturer's instructions and as described in section 2.12. In this live-cell assay, (Z-DEVD)2R110 is used as a chromogenic indicator for caspase 3/7 activity. Following simultaneous treatment of gemcitabine with PDI inhibitor treatments as described in section 2.2 or PDIA1 and AGR2 siRNA knockdown as described in section 2.3, absorbance of R110 was measured at 490nm, to proportionally indicate the level of caspase 3/7 activity.

6.2.8 Protein extraction and Western Blot

Protein extraction and Western blotting was carried out as described in sections 2.7, 2.8, 2.9 and 4.2.4. Cells were transfected with siRNA prior to protein harvesting to detect changes in XBP-1s protein expression. Semi-quantitative Western blot analysis was conducted using Image J software. Beta-actin expression was used for the normalization of protein loading. Details of antibodies and their dilutions are described in Table 2.4.

6.2.9 RNA Extraction, cDNA synthesis and RT-qPCR

RNA extraction was performed with the RNeasy® Mini RNA extraction kit (Qiagen, UK), following the manufacturer's instructions and as described in section 2.4. cDNA synthesis was then performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) according to the manufacturer's instructions and as described in section 2.5. mRNA levels were determined by real-time PCR using

TaqMan gene expression assays (ThermoFisher, UK) for XBP-1s, CHOP and caspase-3. Relative mRNA levels were normalised to the mean CT value of the housekeeping gene RPLP0 (ThermoFisher, UK). Details of gene expression assay are shown in table 2.3. Reactions were analysed and Δ CT and $\Delta\Delta$ CT were calculated according to the $2^{-\Delta\Delta$ CT method to determine relative mRNA expression.

6.2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software 5 (GraphPad Software, USA). A one-way ANOVA was used to determine significance and analyse various between independent treatment groups, with post-hoc Tukey's or Dunnett's multiple comparison tests to determine which treatment groups were significantly different if one-way ANOVA test showed significance. Significance is represented by; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.3 Results

6.3.1 Effects of PDI Inhibitor and Gemcitabine Combination Treatment on Pancreatic Cancer Cell Proliferation

6.3.1.1 BrdU Assay

Panc-1, Mia-PaCa-2 and AsPC-1 were treated with gemcitabine, PACMA-31, CCF642 as monotherapy treatments for 48 hours. Cells were then also treated with gemcitabine and PACMA-31/CCF642 in combination for 48 hours. Absorption was measured to reflect the rate of cell proliferation, through BrdU incorporation into proliferating cells (Figure 6.1). All cell lines exhibited a significant ($***p<0.001$) dose dependent decrease in cell proliferation rate with gemcitabine treatment, compared to the control. However, resistance to gemcitabine was evident across cell lines with the highest dose of both PACMA-31 and CCF642 treatments demonstrating a greater decrease in rate of cell proliferation than the highest dose of gemcitabine treatment. The most significant ($****p<0.0001$) decrease in rate of proliferation was shown with combination treatment of $1\mu\text{M}$ PACMA-31 and $0.05\mu\text{M}$ gemcitabine in Mia-PaCa-2 cells. Treatment of $0.05\mu\text{M}$ gemcitabine alone did not show a significant inhibition of Mia-PaCa-2 cell proliferation. This result suggests that the lowest dose ($1\mu\text{M}$) of PACMA-31 in combination with $0.05\mu\text{M}$ gemcitabine treatment, had a potential synergistic effect in increasing Mia-PaCa-2 cell sensitivity to gemcitabine and driving significant inhibition of cell proliferation. This result was comparable to the substantial decrease in rate of cell proliferation shown with $100\mu\text{M}$ PACMA-31 treatment alone. The combination treatment of $1\mu\text{M}$ PACMA-31 and the lowest dose of gemcitabine was also shown to have a significant effect in Panc-1 ($***p<0.001$) and AsPC-1 ($***p<0.001$) cells. The combination of the $0.1\mu\text{M}$ CCF642 and the lowest dose gemcitabine also demonstrated a significant decrease in proliferation rate

across cell lines, also suggesting a potential synergistic effect, however these were of lesser significance compared with the PACMA-31 and gemcitabine combination in Mia-PaCa-2.

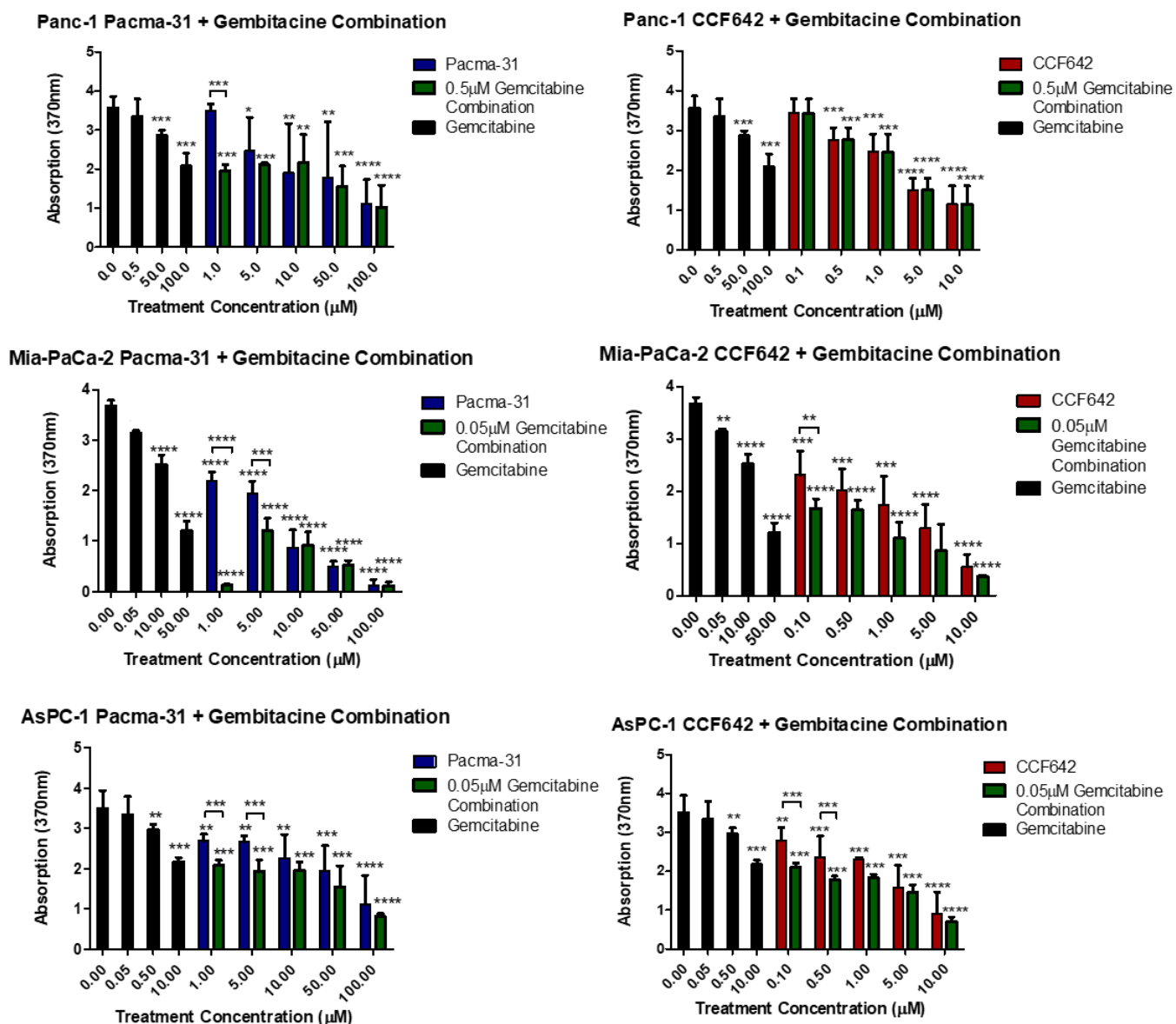


Figure 6.1. PACMA-31 and CCF642 treatment in combination gemcitabine decreased the rate of cell proliferation in pancreatic cancer cell lines.

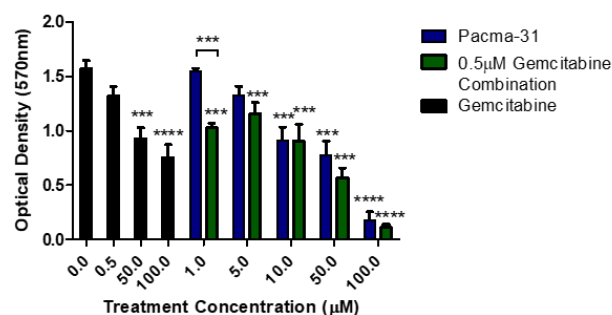
Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with PACMA-31, CCF642 and gemcitabine for 48 hours both as monotherapies and combination therapies. In combination treatment the lowest dose of gemcitabine was combined with PACMA-31 or CCF642 for simultaneous treatment. Absorption (370nm) was measured after 72 hours. Data was collected from 6 wells for each treatment group, with 3 repeats of the experiment. N=3 for each treatment group. Error bars denote 95% confidence interval (CI). The degree of significance between gemcitabine treatments in comparison with the control and between each PDI inhibitor and gemcitabine

combination treatment group were analysed. Significance is also shown compared between treatment concentrations. $**p<0.01$, $***p<0.001$, $****p<0.0001$. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

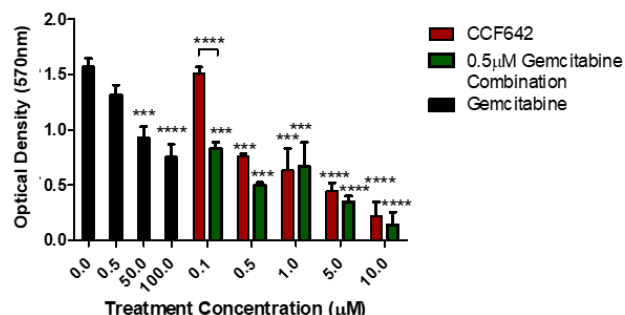
6.3.1.2 MTT Assay

The MTT assay was used to determine cell viability in Panc-1, Mia-PaCa-2 and AsPC-1 with gemcitabine, PACMA-31, CCF642 as monotherapy treatments for 48 hours. Cells were then also treated with gemcitabine and PACMA-31/CCF642 in combination for 48 hours (Figure 6.2). Results were reflective of BrdU proliferation results shown in Figure 6.1, with the most significant ($****p<0.0001$) decrease in cell viability shown with combination treatment of $1\mu\text{M}$ PACMA-31 and $0.05\mu\text{M}$ gemcitabine in Mia-PaCa-2 cells. A significant decrease was also shown with the lowest dose gemcitabine and $1\mu\text{M}$ PACMA-31 combination treatment in Panc-1 ($***p<0.001$). A more significant decrease was shown with gemcitabine treatments alone in the MTT assay compared with results of the BrdU assay. This result may reflect the fact that the MTT assay measures cell viability rather than proliferation alone, and so results may also account for some cell death. A significant decrease in cell viability was also shown with the lowest dose gemcitabine and $0.1\mu\text{M}$ CCF642 combination treatment in Panc-1 and Mia-PaCa-2 cells. While a potential synergistic effect is evident across these combination treatments, this result suggests that the lowest dose ($1\mu\text{M}$) of PACMA-31 in combination with $0.05\mu\text{M}$ gemcitabine treatment, had the greatest potential synergistic effect in Mia-PaCa-2 cells, enhancing chemosensitivity.

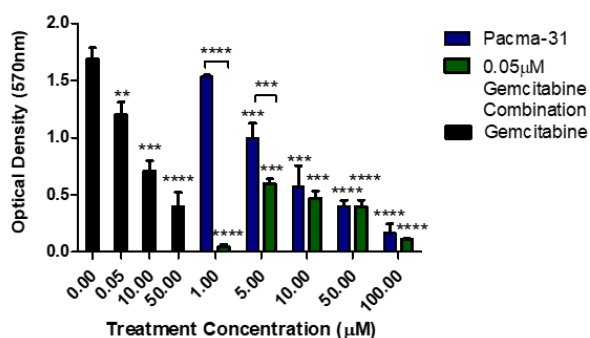
Panc-1 Pacma-31 + Gemcitabine Combination



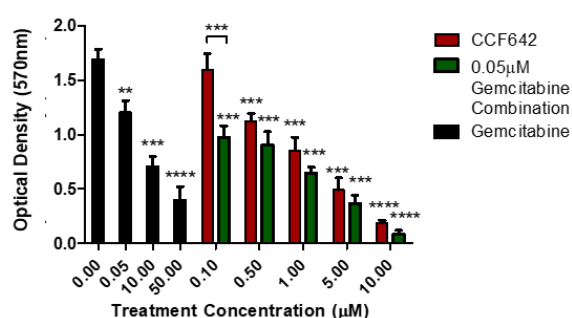
Panc-1 CCF642 + Gemcitabine Combination



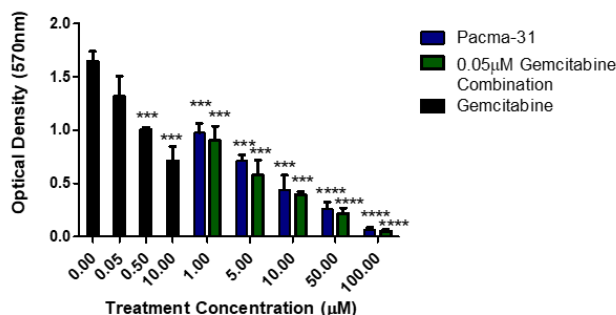
Mia-PaCa-2 Pacma-31 + Gemcitabine Combination



Mia-PaCa-2 CCF642 + Gemcitabine Combination



AsPC-1 Pacma-31 + Gemcitabine Combination



AsPC-1 CCF642 + Gemcitabine Combination

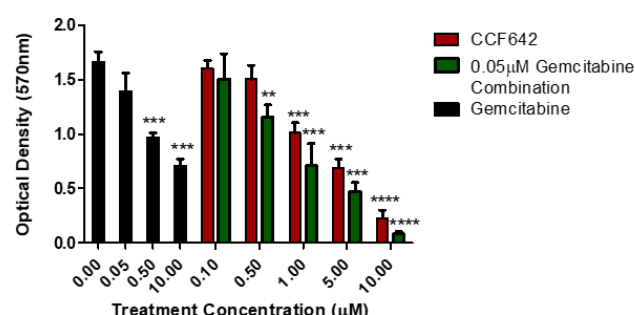


Figure 6.2. PACMA-31 and CCF642 treatment in combination gemcitabine decreased pancreatic cancer cell viability.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with PACMA-31, CCF642 and gemcitabine for 48 hours both as monotherapies and combination therapies. In combination treatment the lowest dose of gemcitabine was combined with PACMA-31 or CCF642 for simultaneous treatment. Optical Density (570nm) was measured after 72 hours. Data was collected from 6 wells for each treatment group, with 3 repeats of the experiment. N=3 for each treatment group. Error bars denote standard deviation (SD). The degree of significance between gemcitabine treatments in comparison with the control and between each PDI inhibitor and gemcitabine combination treatment group were analysed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

6.3.3 Effects of PDI Knockdown and Gemcitabine Combination Treatment on Pancreatic Cancer Cell Proliferation

6.3.3.1 BrdU Assay

Panc-1, Mia-PaCa-2 and AsPC-1 were treated with gemcitabine or PDIA1/AGR2 siRNA knockdown as monotherapy treatments for 48 hours. Cells were then also treated with gemcitabine and PDIA1/AGR2 siRNA knockdown in combination for 48 hours (Figure 6.3). Results showed a significant ($***p<0.001$) dose dependent decrease in rate of cell proliferation with gemcitabine treatment, reflective of those shown in Figure 6.1, with Mia-PaCa-2 demonstrating the highest sensitivity to treatment ($****p<0.0001$). However, while PDIA1 and AGR2 knockdown in combination with 0.05 μ M gemcitabine treatment was shown to be significant ($****p<0.0001$) against the control across all cell lines, results were not significantly different from PDIA1 and AGR2 knockdown treatments alone. This therefore does not reflect the suggested synergistic result shown in Figure 6.1 with PACMA-31 treatment.

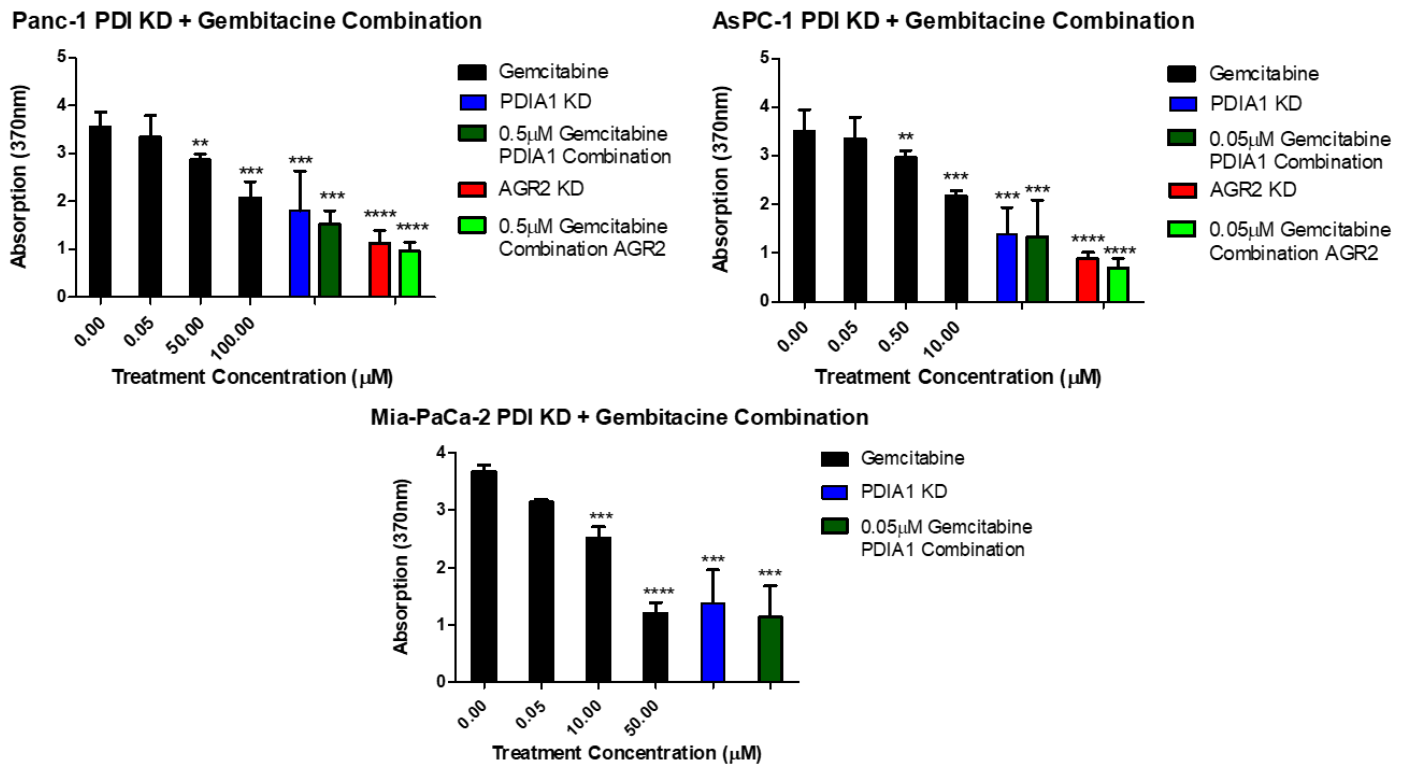


Figure 6.3. PDIA1 and AGR2 siRNA knockdown in combination gemcitabine decreased the rate of cell proliferation in pancreatic cancer cell lines.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with gemcitabine, PDIA1, and AGR2 siRNA knockdown for 48 hours both as monotherapies and combination therapies. In combination treatment, the lowest dose of gemcitabine was combined with PDIA1 or AGR2 knockdown for simultaneous treatment. Absorption (370nm) was measured after 72 hours. Data was collected from 6 wells for each treatment group, with 3 repeats of the experiment. N=3 for each treatment group. Error bars denote 95% confidence interval (CI). The degree of significance between gemcitabine treatments, siRNA knockdown treatments and combination treatments in comparison with the control were analysed. Significance is also shown compared between treatment concentrations. **p=<0.01, ***p=<0.001, ****p=<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

6.3.3.2 MTT Assay

Cell viability was measured in Panc-1, Mia-PaCa-2 and AsPC-1 following gemcitabine treatment, PDIA1 or AGR2 siRNA knockdown as monotherapy treatments for 48 hours. Cells were then also treated with gemcitabine and PDIA1/AGR2 siRNA knockdown in combination for 48 hours (Figure 6.4). Results of gemcitabine and PDIA1/AGR2 knockdown combination treatments were reflective of those shown in Figure 6.3. Though cell viability was significantly ($***p<0.001$) decreased across gemcitabine combination treatments in all cell lines, none showed a significant difference in comparison with PDIA1 or AGR2 knockdown alone.

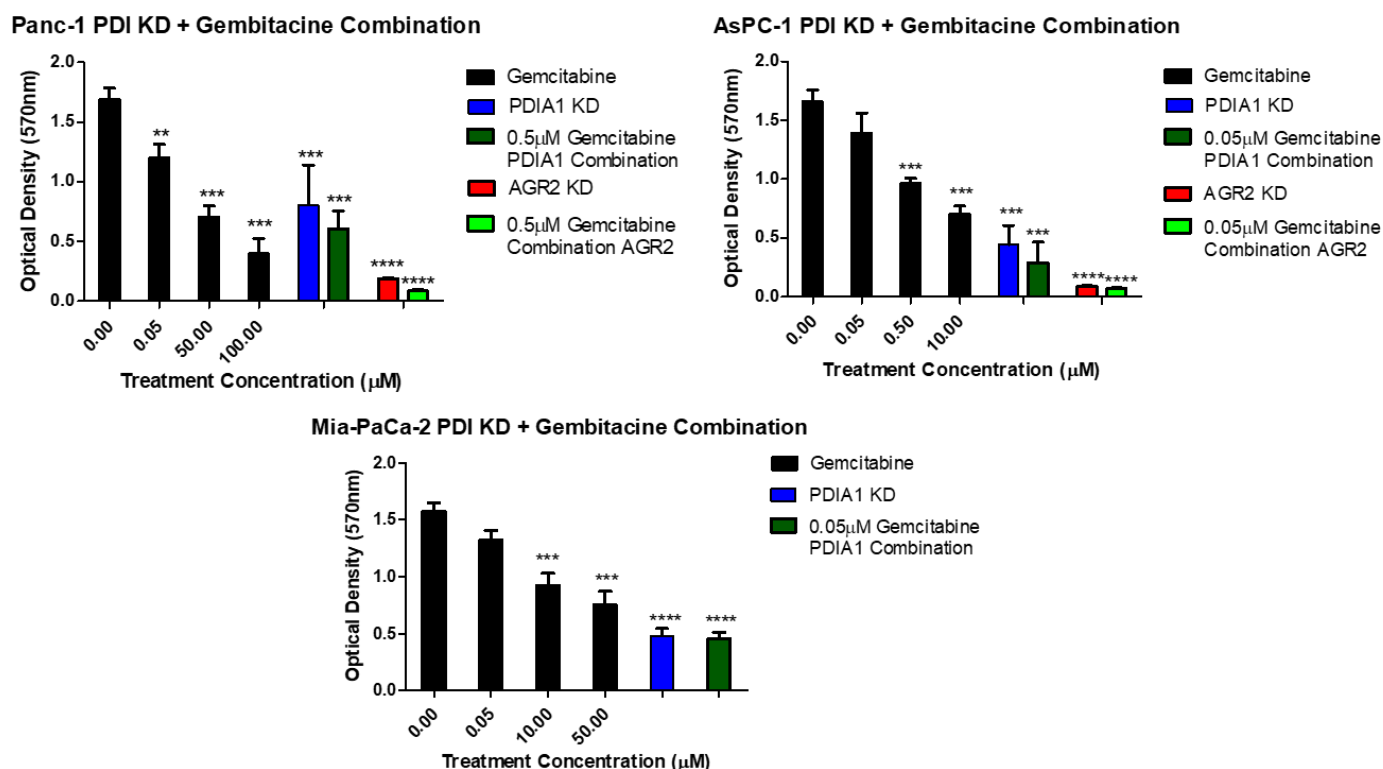


Figure 6.4. PDIA1 and AGR2 siRNA knockdown in combination gemcitabine decreased pancreatic cancer cell viability.

Cell lines Panc-1, AsPC-1 and Mia-PaCa-2 were treated with gemcitabine, PDIA1, and AGR2 siRNA knockdown for 48 hours both as monotherapies and combination therapies. In combination treatment, the lowest dose of gemcitabine was combined with PDIA1 or AGR2 knockdown for simultaneous treatment. Optical Density (570nm) was measured after 72 hours. Data was collected from 6 wells for each treatment group, with 3 repeats of the experiment. N=3 for each treatment group. Error bars denote standard deviation (SD). The degree of significance between gemcitabine treatments, siRNA knockdown treatments and combination treatments in comparison with the control were analysed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

6.3.5 Effects of PACMA-31 and Gemcitabine Combination Treatment on XBP-1s, Caspase-3 and CHOP RNA Expression

Based on results in Figure 6.1 and 6.2, 1 μ M PACMA-31 in combination with 0.05 μ M gemcitabine was identified as the greatest potential synergistic and most effective combination treatment for combatting resistance to gemcitabine in Mia-PaCa-2 cells. The effects of PACMA-31 and gemcitabine combination treatment was therefore further assessed through the measurement of XBP-1s, caspase-3 and CHOP mRNA expression in Mia-PaCa-2 using RT-qPCR (Figure 6.5). Significantly increased XBP-1s expression (* $p < 0.05$) and Caspase 3 expression (** $p < 0.01$) was shown with PACMA-31 and gemcitabine combination treatment in Mia-PaCa-2. While CHOP expression was increased with combination treatment, this was not found to be significant compared with the control. A significant reduction in XBP-1s expression was shown with 0.05 μ M gemcitabine (* $p < 0.05$) and 1 μ M PACMA-31 treatments (* $p < 0.05$). No significant changes in Caspase-3 or CHOP expression were shown with gemcitabine or PACMA-31 as single dose treatments. While these results show significant changes in XBP-1s and Capsase-3 mRNA expression, these results should be interpreted with caution as the Ct values of the housekeeping gene (RPLP0) did show considerable variation across the treatment groups. Further repeat experiments would be required to improve the reliability of this data.

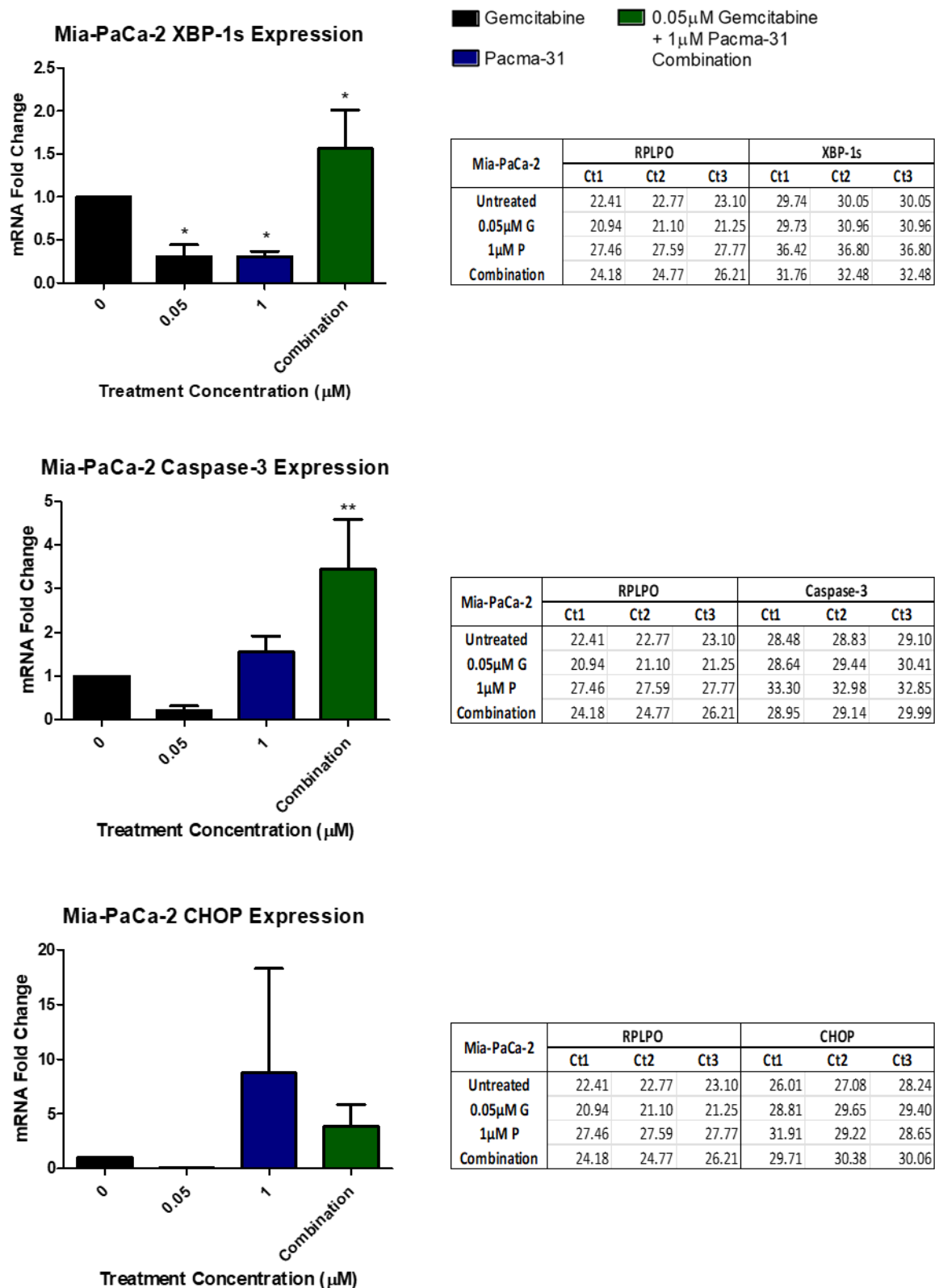


Figure 6.5. Gemcitabine in combination with PACMA-31 treatment influences mRNA expression of Caspase-3, CHOP and XBP-1s in Mia-PaCa-2

Mia-PaCa-2 was treated with PACMA-31 and gemcitabine for 48 hours both as monotherapies and combination therapies. In combination treatment 0.05 μM

gemcitabine combined with 1 μ M PACMA-31 for simultaneous treatment. Controls group included untreated, and the lowest dose of both gemcitabine (0.05 μ M) and PACMA-31 (1 μ M). Caspase-3, CHOP and XBP-1s mRNA expression was calculated according to the Δ CT method using the reference housekeeping gene RPLP0 from RT-qPCR analysis. N=3 per gene, per cell line. Error bars denote standard deviation. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

6.3.6 Effects of PACMA-31 and Gemcitabine Combination Treatment on XBP-1s Protein Expression

The effects of PACMA-31 and gemcitabine combination treatment on XBP-1s protein expression were observed in Mia-PaCa-2 by Western blotting (Figure 6.6A). Beta-actin was used as a control for confirmation of protein expression in cells. The significance of changes in protein expression was also analysed by semi-quantitative Western blot analysis (Figure 6.6B). Results of XBP-1s expression correlated with that of mRNA expression shown in Figure 6.5, in that Western blot results also showed increased XBP-1s protein expression in Mia-PaCa-2 with PACMA-31 and gemcitabine combination treatment, however this was not shown to be significant. The only significant change in XBP-1s protein expression was shown with 0.05 μ M gemcitabine single dose treatment, which showed a significant (* p <0.05) reduction in XBP-1s expression.

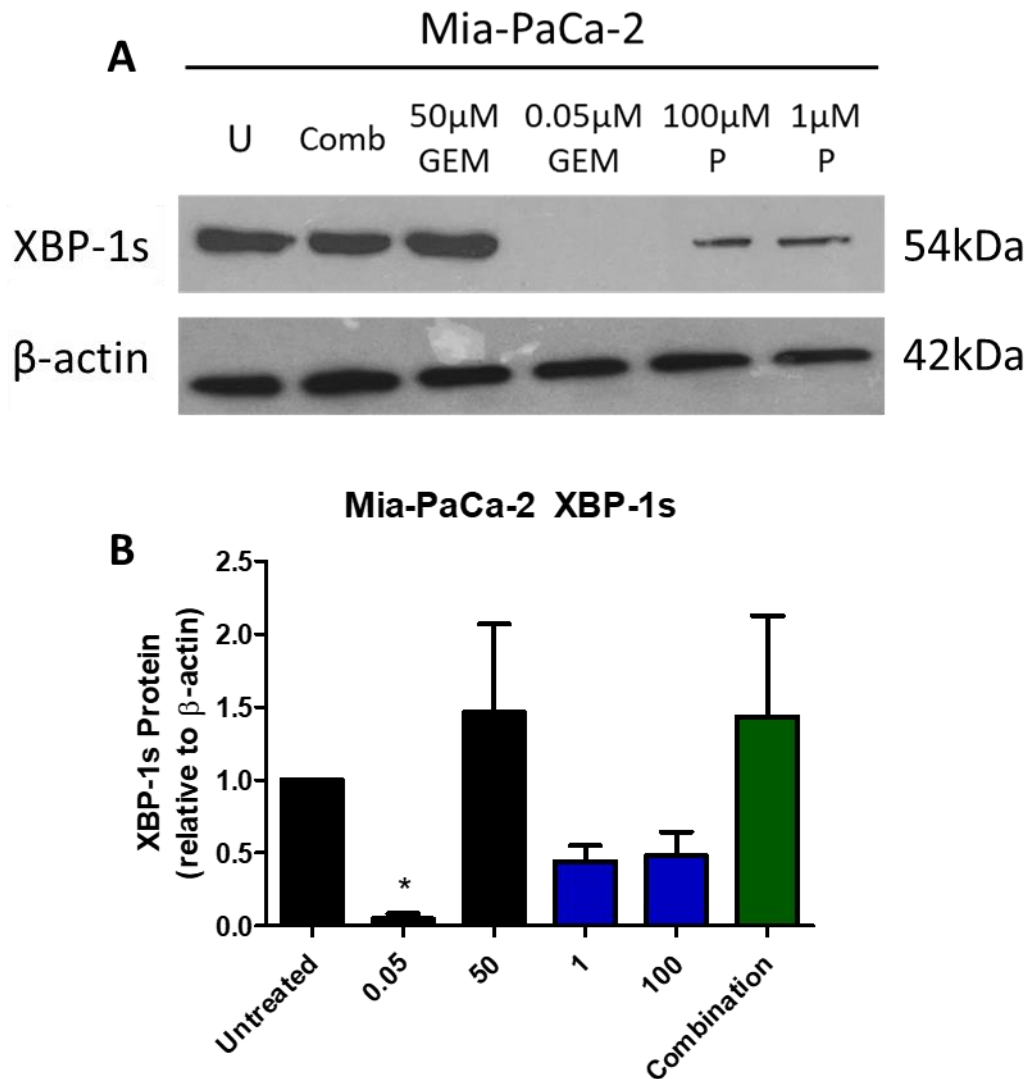


Figure 6.6. Gemcitabine in combination with PACMA-31 treatment demonstrates no significant change in the protein expression of XBP-1s in Mia-PaCa-2.

Mia-PaCa-2 was treated with PACMA-31 and gemcitabine for 48 hours both as monotherapies and combination therapies. In combination treatment 0.05μM gemcitabine combined with 1μM PACMA-31 for simultaneous treatment. Controls group included untreated, and the lowest and highest doses of both gemcitabine (0.05μM and 50μM) and PACMA-31 (1μM and 100μM). (A) XBP-1s protein levels were determined by Western blot analysis with Beta-actin as a control. N=3 per gene, per cell line. (B) Semi-quantitative western blot analysis of the data in panel A, Beta-actin expression was used for the normalization of protein loading. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Dunnett's multiple comparison tests.

6.3.7 Effects of PACMA-31 and Gemcitabine Combination Treatment on Caspase-3/7 Activity

The effects of PACMA-31 and gemcitabine combination treatment on caspase-3/7 activity were investigated in Mia-PaCa-2 (Figure 6.7). Results showed caspase-3/7 activity to not be significantly different with PACMA-31 and gemcitabine combination treatment compared with the control. PACMA-31 and gemcitabine monotherapy treatments also showed no significant difference in caspase-3/7 activity.

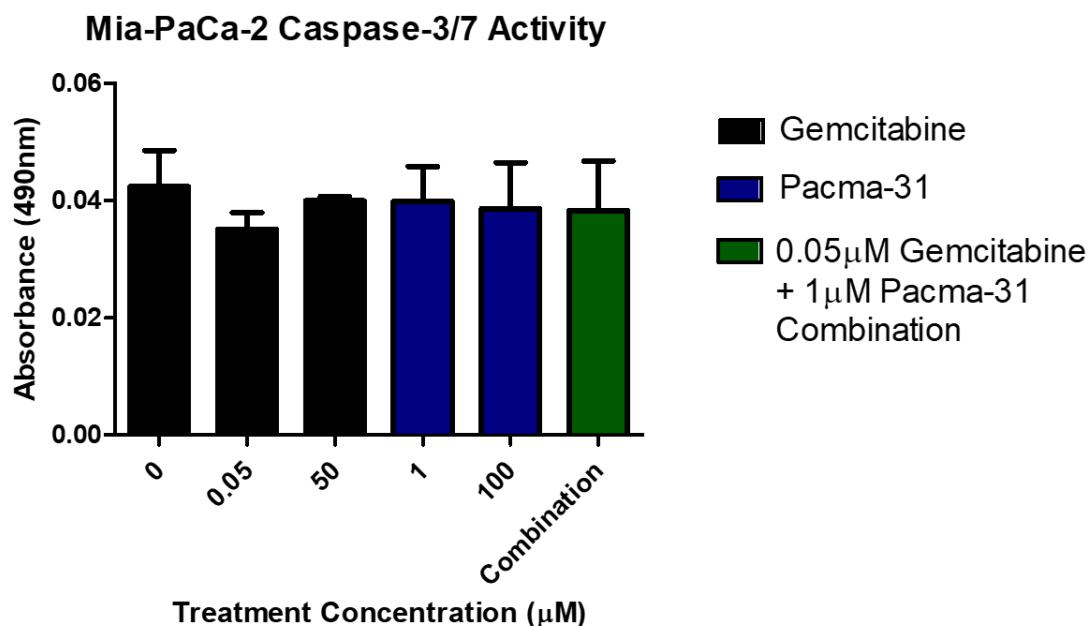


Figure 6.7. Gemcitabine in combination with PACMA-31 treatment induces caspase-3/7 activity in Mia-PaCa-2.

Mia-PaCa-2 was treated with PACMA-31 and gemcitabine for 48 hours both as monotherapies and combination therapies. In combination treatment 0.05µM gemcitabine combined with 1µM PACMA-31 for simultaneous treatment. Controls group included untreated, and the lowest and highest doses of both gemcitabine (0.05µM and 50µM) and PACMA-31 (1µM and 100µM). Absorbance was measured at 490nm to determine relative caspase 3/7 activity. N=3, error bars denote SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison tests.

6.4 Discussion

Pancreatic cancer patients face issues of resistance within weeks of starting gemcitabine treatment (Sarvepalli et al., 2019). PDI has been shown to play roles in promoting cancer cell survival by enhancing chemoresistance. PDI is therefore a potential target for chemotherapy combination treatment as a novel therapeutic strategy. This study aims to identify a potential PDI inhibition and gemcitabine combination treatment strategy to combat pancreatic cancer cell gemcitabine resistance.

6.4.1 The therapeutic potential of PDI inhibition on gemcitabine resistance

Gemcitabine resistance is evident across Panc-1, Mia-PaCa-2 and AsPC-1 cell lines in Figures 6.1 and 6.2. The highest dose gemcitabine treatment was shown to be less effective than the highest dose PDI inhibitor treatment (for both PACMA-31 and CCF642) in driving the inhibition of cell proliferation. These results suggest that while gemcitabine treatment alone does significantly ($***p<0.001$) inhibit pancreatic cancer cell proliferation at a high dose, the effect is not substantial enough for it to be a reliable treatment option, especially when considering the associated toxicity and adverse effects of high dose treatments in patients. Furthermore, long-term use of gemcitabine has been shown to further enhance chemoresistance, suggesting that efficacy will only decrease over time (Long et al., 2021). These results therefore support investigations into potential combination treatment strategies to enhance gemcitabine sensitivity in pancreatic cancer cells.

The current study aimed to determine the most effective PDI inhibition and gemcitabine combination treatment through comparisons of differing PACMA-31 and CCF642 concentrations and PDIA1 and AGR2 knockdowns. Cell proliferation (Figure

6.1) and cell viability (Figure 6.2) results implied that 1 μ M of PACMA-31 in combination with 0.05 μ M gemcitabine treatment had the greatest potential synergistic effect in increasing Mia-PaCa-2 cell sensitivity to gemcitabine and driving significant (**** $p < 0.0001$) inhibition of cell proliferation. This result has potential clinical relevance as a greater inhibition of cell proliferation is shown with combination treatment compared with single dose gemcitabine, despite both PACMA-31 and gemcitabine treatments being at a low dose. Research has shown that the use of synergistic drug pairs can lower the required dose, reduce toxicity, and improve the efficacy of combination chemotherapy (Vogus et al., 2018). While AGR2 and PDIA1 knockdown with gemcitabine combination treatments did show significant (**** $p < 0.0001$) inhibition of cell proliferation (Figure 6.3 and Figure 6.4), the results were not significantly different from knockdown treatments alone. These results therefore did not warrant further investigation into AGR2 and PDIA1 knockdown with gemcitabine combination treatments, as with no significant improvement in efficacy, combination treatment would only contribute to further drug toxicity in patients.

While it can be proposed that PACMA-31 may have off-target effects that act to enhance gemcitabine sensitivity, it should also be noted that PACMA-31 targets multiple PDI family members which may contribute to its effectiveness in chemotherapy combination treatment. Studies have shown that PACMA-31 acts to inhibit several PDI members including, PDIA1, PDIA3, PDIA4, PDIA6, and TXNDC5 (Robinson et al., 2019; Xu et al., 2012). Interestingly, PACMA-31 and gemcitabine combination treatment showed a potential synergistic effect across all cell lines but had the greatest effect in Mia-PaCa-2 cells, which does not naturally express AGR2.

A combined inhibition of several PDI members including both PDIA1 and AGR2 may therefore enhance pancreatic cancer cell sensitivity to gemcitabine.

The demonstration of potential synergy between PACMA-31 and gemcitabine indicates that they have different sites of action that co-ordinately inhibit pancreatic cancer cell proliferation and potentially enhance cell death by apoptosis. The effect of PACMA-31 and gemcitabine combination treatment on apoptosis was assessed by measurement of caspase-3 mRNA expression (Figure 6.5) and caspase-3/7 activity (Figure 6.7). Results showed caspase-3 mRNA expression to be significantly ($**p<0.001$) increased with PACMA-31 and gemcitabine combination treatment compared with the control, suggesting a potential apoptotic effect of the combination treatment. However, the combination treatment did not have a significant effect on caspase-3/7 activity.

6.4.2 The potential influence of PACMA-31 and gemcitabine combination on UPR pathway activations

Both PDI inhibition and gemcitabine are known to exhibit influence on ER stress and UPR pathway activation in pancreatic cancer (Robinson et al., 2021; Wang et al., 2017). The demonstration of potential synergy between PACMA-31 and gemcitabine indicates that their different sites of action may act to further dysregulate UPR pathway activation when in combination. Analysis of XBP-1s expression in Figure 6.6 and show that XBP-1s protein expression is significantly decreased with 0.05 μ M gemcitabine monotherapy ($*p<0.05$). A significant decrease was also demonstrated with 0.05 μ M gemcitabine monotherapy in XBP-1s mRNA expression ($*p<0.05$) (Figure 6.5). This result could be somewhat expected, as gemcitabine has been shown to decrease IRE1 activation and therefore decrease downstream XBP-1s

expression in pancreatic cancer cells (Wang et al., 2017). In contrast to gemcitabine, PDI inhibition has been shown to increase IRE1 pathway activation, as previously hypothesised in Chapter 5, a reduction in XBP-1s expression may suggest potential XBP-1s degradation by apoptotic enzymes under conditions of acute induction of the IRE1a pathway with chronic UPR activation (Vatolin et al., 2016).

Interestingly, XBP-1s mRNA expression is significantly ($*p<0.05$) increased with PACMA-31 and gemcitabine combination treatment (Figure 6.5). This result demonstrates how combination treatment may drive differing UPR activation. While these results demonstrate the potential impact of combination treatment on XBP-1s expression, it should be noted that Figure 6.6 did not show a significant increase in XBP-1 protein expression. However, XBP-1s protein expression was numerically higher with the combination treatment compared with the control and the both results show a significant decrease in XBP-1s expression with $0.05\mu\text{M}$ gemcitabine monotherapy ($*p<0.05$). It should also be noted that the mRNA XBP-1s expression data presents some limitations in that there was variability among the Ct values of the housekeeping control (RPLP0) which could affect the reliability of the results. Further mRNA and protein XBP-1s expression would be required to improve the reliability of these results.

While the PACMA-31 and gemcitabine combination treatment acts to show a potential synergistic effect in the inhibition of cell proliferation, results do not show the same effect in analysis of apoptosis. Whilst caspase-3 mRNA expression was significantly increased with combination treatment, caspase 3/7 activity and CHOP mRNA expressions was not significantly altered with combination treatment. This might therefore suggest that combination treatment could trigger UPR pathway survival mechanisms, including the upregulation of XBP-1s to attenuate apoptosis.

PACMA-31 acts to inhibit both PDIA1 and PDIA6, both of which have been insinuated to attenuate IRE1 activations (Yu et al., 2020; Eletto et al., 2014) (Figure 1.10). Inhibition of PDIA6 has been shown to cause the hyper-activation and sustained autophosphorylation of IRE1 in response to ER stress and hence increased splicing of XBP-1s mRNA (Eletto et al., 2014). The combination of PACMA-31 with gemcitabine may act to exaggerate these effects of PDIA1 and PDIA6 inhibition, driving increased XBP-1s expression and hence pro-survival effects including gemcitabine resistance.

Recent research has indicated that XBP-1s acts to regulate the expression of the anti-apoptotic gene BCL2, as well as other genes involved in the control of cell cycle arrest and apoptosis (Shi et al., 2019). BCL2 acts to prevent pathways of intrinsic apoptosis by inhibiting Bax or Bad, which would otherwise function to drive mitochondrial membrane permeabilization and consequent increased caspase activity (Gomez et al., 2007). The upregulation of XBP-1s in MCF-7 cells was shown to perturb intrinsic apoptosis through BCL2 activations, which ultimately contributed to antioestrogen resistance (Gomez et al., 2007). XBP-1s may act through a similar mechanism in Mia-PaCa-2 cells to attenuate apoptosis and contribute to drug-resistance. XBP-1s may therefore be a useful therapeutic target in combination with PDI inhibitor or gemcitabine treatment to combat issues of resistance. Proposed mechanisms of PACMA-31 and gemcitabine combination on the IRE1/XBP-1 signalling arm of the UPR pathway are shown in Figure 6.8. It should be noted that these mechanisms shown are hypothesised and further investigation is required to confirm the impact of PACMA-31 and gemcitabine combination therapy on XBP-1s expression.

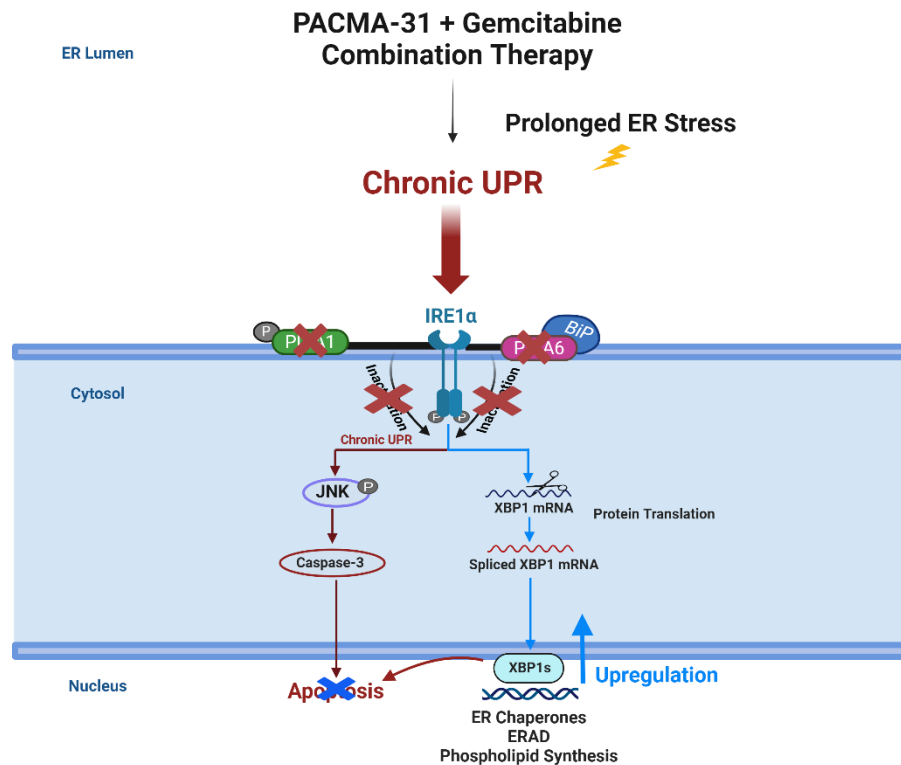


Figure 6.8. The proposed effects of PACMA-31 and gemcitabine combination treatment on the IRE1a signalling arm of the UPR pathway. (Adapted from Powell & Foster, 2021).

The diagram describes the proposed effects of 1 μ M PACMA-31 and 0.05 μ M gemcitabine combination treatment on IRE1 pathway activation. PACMA-31 is proposed to act to inhibit PDIA1 and PDIA6, hence inhibiting their actions on IRE1 receptor inactivation. While increased IRE1 signalling drives a chronic UPR, XBP-1s splicing is also increased, driving an upregulation in XBP-1s which acts to attenuate apoptosis and promote cell survival. These mechanisms highlight how XBP-1s may act to drive gemcitabine resistance, highlighting it as a potential therapeutic target in pancreatic cancer. The mechanisms by which PACMA-31 and gemcitabine combination treatment may contribute to XBP-1s upregulation are proposed as described in text.

CHAPTER 7 – FINAL CONCLUSIONS AND FUTURE DIRECTIONS

Chapter 7 – Final conclusions and future directions

The research work described in this thesis investigated the roles of specific PDI family members and their relationship with the UPR pathway in the progression and survival of pancreatic cancer. Research has shown that the UPR is frequently activated in pancreatic cancer to regulate ER homeostasis under conditions of ER stress. The UPR is thought to therefore contribute to the chemoresistant nature of the pancreatic tumour microenvironment. The chemoresistant nature of pancreatic cancer is synonymous with the lack of effective treatments in advanced and metastatic disease, including prominent issues of resistance to long-term gemcitabine use. PDI acts as a vital component of the UPR, relieving ER stress through its protein folding and chaperone abilities, promoting cell survival. Hence PDI is highlighted as a potential therapeutic target.

The specific impact of each PDI family member on pancreatic cancer progression remains poorly understood. Nor is it understood how these PDI members interact with specific UPR pathway genes to promote pancreatic cancer cell survival. The research work in this thesis investigated how PDI expression is dysregulated in pancreatic cancer and identified PDIA1 and AGR2 as potential key therapeutic targets. The current study further investigated how PDI inhibitor treatment of PACMA-31 and CCF642 and PDIA1 and AGR2 siRNA knockdown inhibited pancreatic cancer cell proliferation, viability, and migration. The study also provides an insight into the downstream UPR mechanisms of PDI inhibition through measurement of ER stress and apoptotic marker (XBP-1s, BiP, Caspase-3, CHOP) expressions and caspase 3/7 activity. Furthermore, the research work provides new knowledge into the effect of PDI inhibition and gemcitabine combination treatment on pancreatic cancer cell proliferation, apoptosis and potential UPR mechanisms.

7.1 AGR2 and PDIA1 are Identified as a Potential Therapeutic Targets

In the comparison of PDI family member expression, data mining identified a significantly greater overexpression of AGR2 in pancreatic cancer tumours compared with normal pancreas tissue. The current study describes how AGR2 has been shown to be expressed across all stages of pancreatic cancer progression. These investigations highlighted AGR2 as both a potential biomarker for pancreatic cancer and therapeutic target. The current study therefore supports research that suggests that AGR2 could be overexpressed prior to KRAS-activating mutations, therefore serving as an early-stage biomarker (Dumartin et al., 2017). With data mining results also demonstrating a significantly high expression of AGR2 in stage IV tumours, the study also supports recent research that shows an overexpression of AGR2 in PanIN 3 lesions, consequently implying the potential role of AGR2 in the development of advanced, progressive disease (Norris et al., 2013).

The distinction of AGR2 from other PDI family members was also observed structurally, with AGR2 shown to be evolutionarily diverse from other PDI members. AGR2 is evolutionarily divergent from the classic CXXC active site motif, it instead possesses a CXXS motif. A CXXS motif may contribute to specialised ER functions such as retrograde transport of secretory proteins out of the ER which have been implicated to contribute to ER homeostasis and cell survival (Norgaard et al., 2001; Higa et al., 2011).

PDIA1 was also chosen as a comparative PDI member to AGR2 for inhibition investigations, as the most highly expressed member in pancreatic cancer. PDIA1 is the archetypal and most well-recognised PDI member and has shown influence across many stages of carcinogenesis and various cancer types (Bakker et al.,

2022). However, little investigation has been done into the roles of PDIA1 in pancreatic cancer. As a canonical CXXC motif-possessing PDI member, most PDI inhibitors have been developed to target PDIA1, including both PACMA-31 and CCF642, further highlighting its potential for use in PDI inhibitor experimental work.

7.2 AGR2 and PDIA1 Inhibition Decrease Pancreatic Cell Proliferation and Migration

The effects of PDI inhibitor (PACMA-31 and CCF642) treatment were investigated in pancreatic cancer cell lines: Panc-1, Mia-PaCa-2, and AsPC-1. Pacma-31 and CCF642 both showed a dose-dependent decrease in the rate of proliferation and cell viability across cell lines, with greatest inhibition of cell proliferation shown in the non-AGR2 expressing Mia-PaCa-2 cell line. PACMA-31 has been shown to inhibit PDIA1, PDIA3, PDIA4, PDIA6 and TXNDC5, and CCF642 has been shown to inhibit PDIA1, PDIA3 and PDIA4. However, both may inhibit other members in addition to these. Both PACMA-31 and CCF642 act as irreversible inhibitors that inhibit PDI through disruption of the canonical CXXC motif. PACMA-31 and CCF642 therefore may not be effective in inhibiting non-canonical PDI members such as AGR2. With both inhibitors also being non-selective it is likely that they also have off-target effects.

To combat this, selective inhibition of PDIA1 and AGR2 through siRNA knockdown was also investigated and confirmed by measurement of mRNA and protein expression and PDI activity. PDIA1 and AGR2 knockdown was effective in decreasing the rate of cell proliferation and viability across all three cell lines. AGR2 knockdown however, demonstrated a greater effect in comparison to PDIA1 knockdown, particularly in AsPC-1 where AGR2 expression is expressed at the highest level. AGR2 differs from PDIA1 in that it can be diversely trafficked and to

exit the ER, particularly when overexpressed (Chevet et al. 2012). AGR2 has been reported to play other intracellular roles outside of its localisation to the ER such as the regulation of p53 signalling, and hence tumour growth (Pohler et al. 2004). Further research needs to be carried out to assess the tumour growth associated roles of AGR2 both within the cell and as a secretory protein.

PACMA-31 and CFF642 treatment also demonstrated a dose dependent decrease in the rate of migration in Panc-1 and Mia-PaCa-2 cell lines. AGR2 knockdown was shown to inhibit the rate of cell migration in Panc-1 and PDIA1 knockdown decreased cell migration rate in both Panc-1 and Mia-PaCa-2 cells. The greatest inhibition of cell migration was shown in Mia-PaCa-2 cells at 48 hours with PDIA1 knockdown. These results suggest influence of PDIA1 in the promotion of cell migration in pancreatic cancer. In breast cancer cells, PDIA1 has been shown to play a role in the regulation of Wnt signalling, known for its role in metastasis (Yang et al., 2022). It could therefore be proposed that PDIA1 plays a similar role in pancreatic cancer cells. This promotes the need for further investigations into the mechanisms by which PDI members may drive pancreatic cancer metastasis, particularly since the presence of metastatic disease is attributed to extremely low survival rates.

These results provide clear indications of PDIA1 and AGR2 influence in driving pancreatic cancer cell proliferation and migration, emphasising the therapeutic potential of PDI inhibition to disrupt tumour progression.

7.3 PDIA1 Inhibition Decreases XBP-1s Expression in the UPR

XBP-1s is recognised as a key mediator of the UPR pathway. Through the process of UPR pathway activation and unconventional splicing by IRE1, XBP-1s acts to

induce the expression of various ER pro-protective genes involved in mechanisms of protein folding, secretion, and the clearance of misfolded proteins via ERAD activations (Yoshida, 2007). The current study showed that PDIA1 knockdown, significantly decreased mRNA and protein XBP-1s expression in AsPC-1 cells. A reduction in XBP-1s expression with PDIA1 knockdown suggests a potential disruption in the activation of the IRE1 α pathway. This phosphorylation of PDIA1 allows it to bind to IRE1 α , where it acts to attenuate chronic UPR signalling (Yu et al., 2020). An inhibition of PDIA1 would therefore prevent this attenuation of chronic UPR signalling, disrupting normal UPR mechanisms. Recent research has suggested that XBP-1s may be degraded by apoptotic enzymes under conditions of acute induction of the IRE1 pathway with chronic UPR activation (Vatolin et al, 2016). This proposed degradation of XBP-1s highlights a compromise in the defence on pancreatic cancer cells against ER stress, highlighting PDIA1 inhibition as a potential effective treatment strategy.

7.4 AGR2 Inhibition Increases Caspase 3 Activity in the UPR

Caspase-3 has been highlighted as a driver of apoptosis, downstream of chronic UPR activations (Iurlaro & Muñoz-Pinedo, 2016). The current study demonstrated that AGR2 knockdown in Panc-1 and AsPC-1 significantly increased caspase-3 activity. This suggests that AGR2 inhibition in these cell lines triggers prolonged UPR activation, resulting in apoptosis through caspase-3 activation. AGR2 could therefore play a role in attenuating the activations of chronic UPR pathways which would lead to caspase-3 activation. Whilst it is not clear which chronic UPR pathway AGR2 inhibition activates to induce caspase-3 led apoptosis, it can be proposed that the

activation of CHOP may be involved as a key apoptotic marker of the UPR pathway and an overexpressed gene in pancreatic cancer. While it can be proposed that AGR2 inhibition triggers chronic UPR signalling and the activation of the CHOP/caspase 3 apoptotic pathway, CHOP can also contribute to oxidative ER stress through the activation of (ERO)1 α which acts to oxidise PDI, generating H₂O₂ (Rao et al., 2015). With AGR2 inhibition implied to trigger chronic UPR signalling, ERO1 α may be overexpressed. While ERO1 α can act to oxidise and restore PDIs to contribute to proper protein folding to combat ER stress, this leads to an elevated production of H₂O₂ in the process, which has the potential to leak into the cytoplasm and signalling apoptosis through caspase-3 activation (Iurlaro & Muñoz-Pinedo, 2016).

The current study highlights how AGR2 inhibition could trigger a pro-apoptotic response through caspase-3 activation and how PDIA1 inhibition could repress expression of the pro-survival gene XBP-1s. Further research needs to be carried out to measure the effect of PDIA1 and AGR2 inhibition on other key components of the UPR pathway to fully discern the mechanisms of action.

7.5 PACMA-31 and Gemcitabine Combination Demonstrates a Synergistic Inhibition of Pancreatic Cancer Cell Proliferation

Gemcitabine remains a cornerstone of current pancreatic cancer therapies (Beutel and Halbrook, 2023). However, resistance to gemcitabine treatment is observed in pancreatic cancer patients within weeks of starting treatment (Sarvepalli et al., 2019). Long-term use of gemcitabine has been shown to extensively alter the tumour microenvironment in pancreatic cancer, leading to chemoresistance (Long et al., 2021). Recent research has suggested that use of inhibitors of UPR pathway in

combination with gemcitabine can enhance the chemosensitivity of pancreatic cancer cells (Tadros et al., 2017). Several studies have shown that PDI plays a role in mediating chemoresistance in various cancer types (Rhaman et al., 2022). The current study aimed to determine the most effective PDI inhibition and gemcitabine combination treatment through investigation of differing PACMA-31 and CCF642 concentrations and PDIA1 and AGR2 knockdowns.

Cell proliferation and cell viability results implied that small dose PACMA-31/CCF642 and gemcitabine combination treatments had a potential synergistic effect in driving the inhibition of cell proliferation across pancreatic cancer cell lines. The combination treatment of 1 μ M PACMA-31 with 0.05 μ M gemcitabine treatment had the greatest potential synergistic effect in increasing Mia-PaCa-2 cell sensitivity to gemcitabine and driving significant inhibition of cell proliferation, despite both PACMA-31 and gemcitabine treatments being at a low dose. This result has potential beneficial clinical implications as low dose combination treatments are preferential to reduce drug toxicity and associated adverse effects. Interestingly, PACMA-31 and gemcitabine combination treatment showed the greatest synergistic effect in Mia-PaCa-2 cells, which does not naturally express AGR2. A combined inhibition of several PDI members through PACMA-31 treatment including both PDIA1 and AGR2 may therefore enhance pancreatic cancer cell sensitivity to gemcitabine.

7.6 PACMA-31 and Gemcitabine Combination Increases XBP-1s mRNA Expression in the UPR

Both PDI inhibition and gemcitabine are known to exhibit influence on ER stress and UPR pathway activations (Robinson et al., 2021; Wang et al., 2017). Interestingly, XBP-1s mRNA expression was significantly increased with PACMA-31 and

gemcitabine combination treatment. This result demonstrates how combination treatment may drive differing UPR activations. While these results demonstrate the potential impact of combination treatment on XBP-1s expression, it should be noted that a significant increase in XBP-1s protein expression was not shown.

While the PACMA-31 and gemcitabine combination treatment acts to show a potential synergistic effect in the inhibition of cell proliferation, results do not show the same effect in the analysis of apoptosis. Whilst caspase-3 mRNA expression was significantly increased with combination treatment, CHOP mRNA expression was not significantly altered with combination treatment and caspase-3/7 activity was not significantly different to 1 μ M PACMA-31 treatment alone. This might therefore suggest that combination treatment triggers UPR pathway survival mechanisms, including the upregulation of XBP-1s to attenuate apoptosis.

It could be proposed that the combination of PACMA-31 with gemcitabine may act to exaggerate these effects of PACMA-3-driven PDIA1 and PDIA6 inhibition, which both play roles in the attenuation of IRE1 activation (Yu et al., 2020; Eletto et al., 2014).

This suggests that enhanced PDIA1 and PDIA6 inhibition could drive increased activation of IRE1 and hence downstream XBP-1s expression. Recent research has indicated that spliced XBP-1s acts to regulate the expression of the anti-apoptotic gene BCL2 (Shi et al., 2019), which acts to prevent pathways of intrinsic apoptosis by inhibiting Bax or Bad and consequent caspase activity (Gomez et al., 2007). This research describes how an upregulation of XBP-1s with combination treatment may act to attenuate apoptosis and promote cell survival. However while these potential mechanisms have been proposed, further experiments are required to confirm the influence of PACMA-31 and gemcitabine combination therapy on XBP-1s expression

and to investigate the potential of XBP-1s as a therapeutic target in combination with PDI inhibitor or gemcitabine to combat issues of resistance in pancreatic cancer.

7.7 Future Aims

While the current study highlights AGR2 and PDIA1 as key potential therapeutic targets for pancreatic cancer, future research would benefit from investigations into the roles of additional PDI family members. These could include those members of which were also identified as potential targets through data mining: PDIA3, PDIA4 and PDIA6. PDIA6 in particular was highlighted in discussion of combination treatment effects, being that it is a target of PACMA-31 and has been shown to act to attenuate IRE1 signalling (Yu et al., 2020; Eletto et al., 2014). PDIA6 may therefore play a crucial role in maintaining ER homeostasis by limiting IRE1 chronic activations. PDIA3 has been described to have implications in UPR mechanisms of ER homeostasis through its interactions with PDIA1, where it has been shown to form a complex with PDIA1, helping to maintain PDIA1 in its reduced state and prevent PERK activation (Appenzeller-Herzog et al. 2008). The absence of PDIA3 leads to an accumulation of oxidised PDIA1 which triggers PERK activation, suggesting that PDIA3 plays an important role in preventing chronic UPR activation. (Kranz et al. 2017). PDIA3 has also been implicated to play potential roles in chemoresistance in various cancer types (Cicchillitti et al., 2010; Hussmann et al., 2015). In addition to this, both PDIA4 and PDIA6 have been shown to mediate resistance to cisplatin-induced apoptosis (Tufo et al. 2014). While these PDI members have been inhibited in this study via PACMA-31 and CCF642 treatment,

selective inhibition of these individual members would contribute to knowledge on their individual influence in UPR activations and hence pro-survival mechanisms.

While the current study shows significant selective inhibition of PDIA1 and AGR2 via siRNA transfection, future studies could benefit from use of the CRISPR-Cas9 system as an effective method for total gene knockout. The current study controlled for potential off-target effects of siRNA transfection through comparisons with scrambled siRNA and benefitted from the efficient and effective approach of siRNA transfection. However, use of CRISPR does eliminate any confounding effects from remnant low levels of protein expression post knockdown and so should be a consideration for future research.

Although the selective inhibition of individual PDI members is a suitable approach for discerning the potential roles of PDI in UPR pathway mechanisms, combination treatment results suggest that future research would also benefit from further investigations into the concomitant inhibition of multiple PDI members. With PACMA-31 and gemcitabine combination therapy demonstrating a potential synergistic effect, perhaps the simultaneous inhibition of multiple PDI members would prove the most effective treatment method. The pan-style PDI inhibitor E64FC26, which unfortunately could not be sourced for this study, could provide potential useful insights in combination with gemcitabine. E64FC6 is known to target the same PDI members as PACMA-31 (PDIA1, PDIA3, PDIA4, PDIA6, and TXNDC5) but has a significantly lower IC_{50} (1.9 μ M) compared with PACMA-31 (10 μ M) (Robinson et al., 2019). If found to be effective at a low dose in combination treatment with low dose gemcitabine, drug toxicity effects would be even more reduced than those potentially observed with PACMA-31 gemcitabine combination therapy. E64FC26 has been shown to induce apoptosis and cytotoxic effects in multiple myeloma cells (Robinson

et al., 2019), whereas PACMA-31 is recognised for its role in inhibition of cell growth (Xu et al., 2012). E64FC26 may therefore be enhance gemcitabine in combination treatment to elicit apoptosis in pancreatic cancer and combat issues of resistance.

While the current study provides insight into the potential roles of PDIA1 and AGR2 in the UPR pathway in pancreatic cancer through measurement of XBP-1s, BiP, caspase-3 and CHOP expressions, there are further UPR components that have not been investigated. These include the three upstream UPR pathway receptors: IRE1, PERK and ATF6. Simultaneous measurement of PERK, ATF4 and CHOP expression together would also give further insight into the effects of PDI inhibition on chronic UPR signalling, with the PERK/ATF4/CHOP pathway being widely recognised as a driver of cell death (Ron & Walter, 2007). Investigations into JAK expression would also help to discern which chronic UPR pathways are activated under PDI inhibitor-induced ER stress conditions. It is also noted that the current study faced some limitations in that CHOP and caspase-3 were only measured for mRNA by RT-qPCR and not for protein expression. This was due to issues with poor antibody quality, affecting consistency of results. However, the key ER stress marker of interest, XBP-1s, was measured for both mRNA expression by RT-qPCR and protein expression by Western blotting. Caspase 3 expression measurements were also supplemented by caspase- 3 activity assays to provide a better insight into potential apoptosis. The current study highlighted XBP-1s as a potential therapeutic target. Further studies are needed explore the effects XBP-1s inhibition, potentially in combination with PDI inhibitor or gemcitabine treatment, on apoptosis and cell survival.

It would also be beneficial to carry out future comparative hypoxia studies to give a better representation of the pancreatic tumour environment. Hypoxia is one of the key components that affect the cellular expression and lead to therapy resistance in

tumour cells (Jing et al., 2019). Recent research has further suggested that, as an extrinsic stress factor, hypoxia is a potential driver of UPR activation and consequent tumour progression in pancreatic cancer (Long et al., 2021). Furthermore, research has shown that PDIA1 plays a role in the regulation of HIF-1 α signalling in breast cancer (Bakker et al., 2022). HIF-1 α is a major regulator of cellular response for the adaptation of tumour cells to hypoxia (Jin et al., 2020). Numerous studies have shown that HIF-1 α plays a crucial role in the progression of pancreatic cancer (Jin et al., 2020). XBP-1s has also been shown to drive tumorigenicity via the regulation of HIF1 α expression (Chen et al., 2014). Further hypoxia-based studies could therefore investigate the cross-talk between the PDI, the UPR and HIF1 α in the transcriptional reprogramming of pancreatic cancer cells toward transformation.

In vivo studies would provide further accuracy for investigations of PDI influence in the tumour environment. With additional biological variables at play, in vivo studies are necessary for future investigations to be able to extrapolate a clinical effect from the implications of PDI inhibition in vitro and to progress potential drug development possibilities. The efficacy and toxicity of PDI inhibition could be examined in severe combined immunodeficient (SCID) mice bearing human pancreatic cancer xenografts as an appropriate in vivo model for pre-clinical drug development.

7.8 Conclusions

The research work in this thesis has identified both AGR2 and PDIA1 as potential therapeutic targets for pancreatic cancer. The findings of this study suggest that PDIA1 and AGR2 aid pancreatic cancer survival through their pro-protective roles in the UPR. These demonstrate how the inhibition of PDI can suppress pancreatic

cancer cell growth and migration as well as induce mechanisms of apoptosis. In this work, new insights are provided into the effects of PDI inhibition on mechanisms of UPR pathway activations, particularly through the dysregulation of XBP-1s expression. The study highlights XBP-1s as a potential novel therapeutic target and its potential role in gemcitabine resistance. The study further highlighted a potential novel synergistic combination treatment of PACMA-31 and gemcitabine to inhibit pancreatic cancer cell growth. These data in turn promote further investigations into the use of PDI or UPR pathway inhibitors with gemcitabine as a potentially effective combination therapy to enhance pancreatic cancer cell chemosensitivity.

CHAPTER 8 – REFERENCES

Chapter 8 – References

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List of publications relating to this thesis

Powell, L. E., & Foster, P. A. (2021). Protein disulphide isomerase inhibition as a potential cancer therapeutic strategy. *Cancer medicine*, 10(8), 2812–2825.

List of conference presentations relating to this thesis

Poster at European Association for Cancer Research 2021 Congress

Poster at European Association for Cancer Research 2022 Congress

Poster at Medical Research Council Meeting 2022, University of Birmingham

CHAPTER 9 – APPENDIX

9.1 Western Blot Images for 3 Repeats

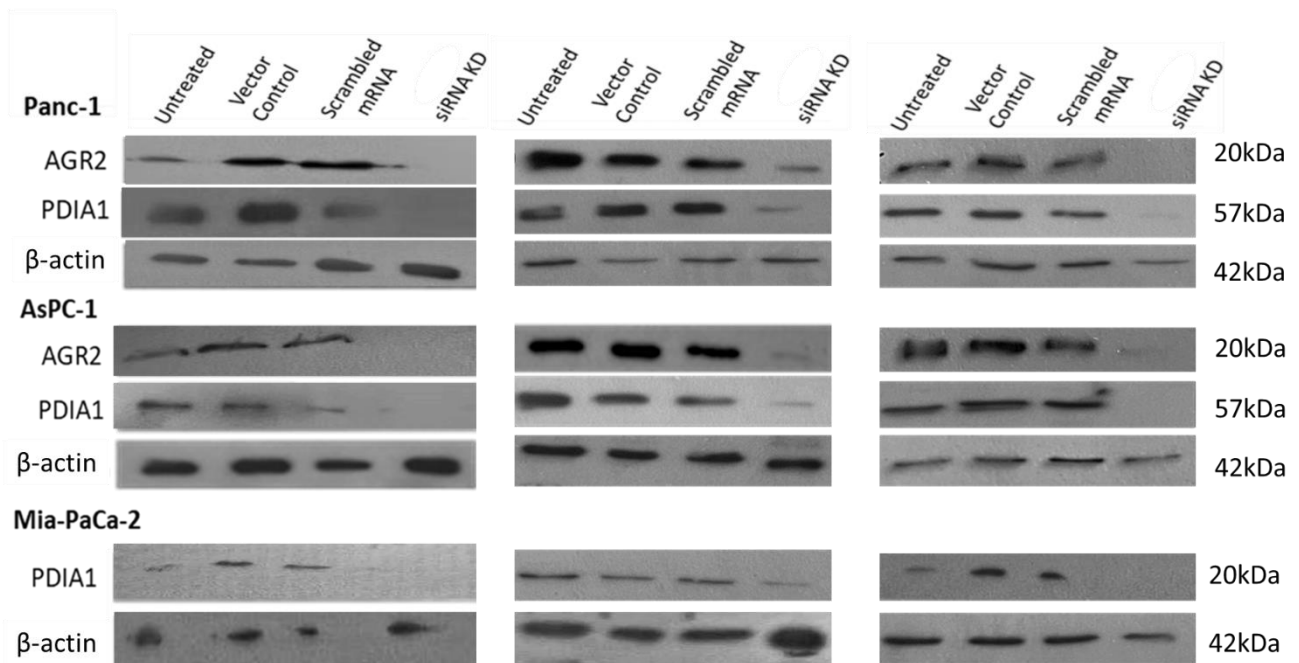


Figure 9.1. AGR2 and PDIA1 protein expression is knocked down after 48 hour siRNA transfection in pancreatic cancer cell lines (n=3)

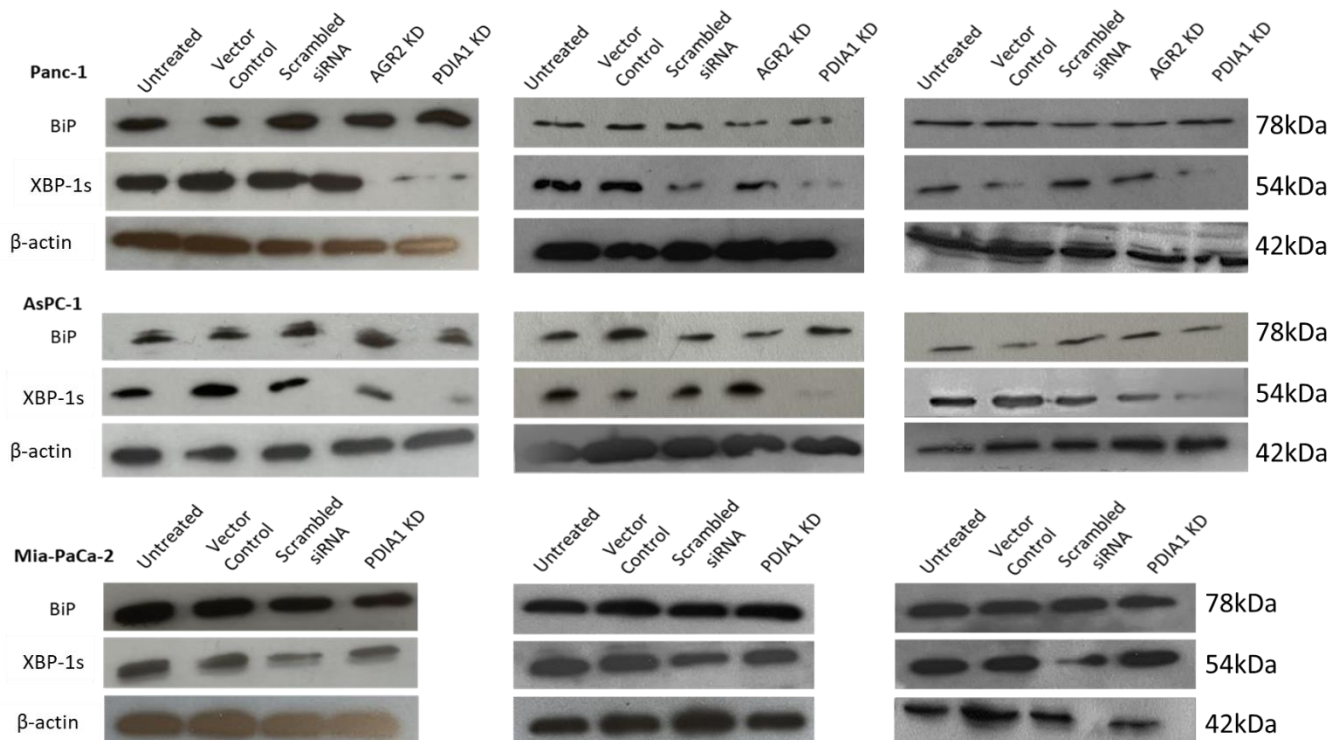


Figure 9.2. PDIA1 and AGR2 siRNA knockdown treatment influences protein expression of XBP-1s and BiP in pancreatic cancer cell lines (n=3)

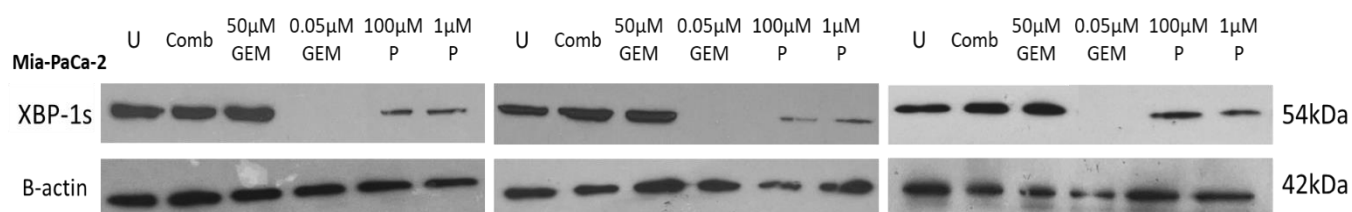


Figure 9.3. Gemcitabine in combination with PACMA-31 treatment influences protein expression of XBP-1s in Mia-PaCa-2 (n=3)