MicroRNAs in Pancreatic Ductal Adenocarcinoma: New Approaches For Better Diagnosis And Therapy

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MicroRNAs in Pancreatic Ductal Adenocarcinoma: New Approaches for Better Diagnosis and Therapy

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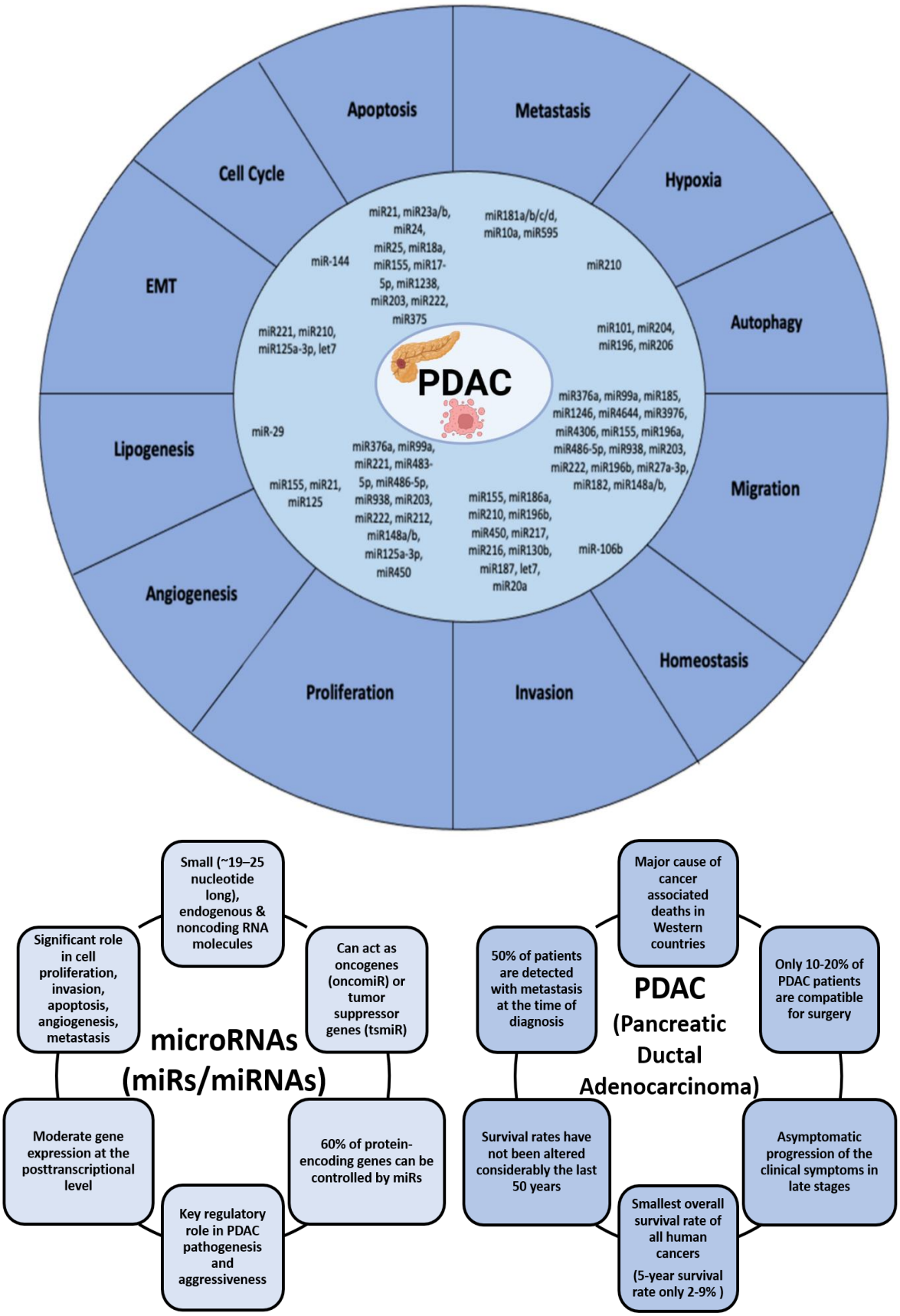
Graphical Abstract



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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies with less than an 8% 5-year survival rate, which has remained unchanged over the last 50 years. Early detection is particularly difficult due to the lack of disease-specific symptoms and reliable diagnostic biomarkers. Multimodality treatment including chemotherapy, radiotherapy (used sparingly) and surgery has become the standard of care for patients with PDAC. Carbohydrate antigen 19-9 (CA 19-9) is the most common diagnostic biomarker; however, it is not specific enough for asymptomatic patients. MicroRNAs (miRs/miRNAs) are small non-encoding RNA molecules, which have been related with PDAC progression and metastasis. In particular, miR-21, miR-221, miR-155 and miR-126 have to date been shown to be highly dysregulated in human malignancies including PDAC and are involved in numerous cancer-related mechanisms such as cell growth, differentiation, metastasis, invasion, and cell death. The aim of this thesis was to examine the mode of action of miR-21, miR-221, miR-155 and miR-126 *in vitro* for improved diagnosis and treatment of PDAC and specifically, investigate the role of the oncogenic miR-21 in cellular proliferation, migration, invasion, apoptosis, cell cycle arrest, senescence, protein content and mitochondrial function by using CRISPR/Cas9 knockouts. The findings provide promising new insights into the metastatic predisposition of PDAC through the evaluation of specific miR signature profiles (*in vitro*). Such miR signatures could prompt a pioneer precision medicine approach for individual PDAC cases and allow a more effective early diagnosis and control of PDAC, facilitating more effective treatment.

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List of Abbreviations

ADAM9	ADAM Metallopeptidase Domain 9
AKT	Ak Strain Transforming
ALDH-1	Aldehyde Dehydrogenase 1
ANXA2	Annexin A2
APAF1	Apoptotic Peptidase Activating Factor 1
ARMTL2	Aryl Hydrocarbon Receptor Nuclear Translocator Like
ATP	Adenosine Triphosphate
BME	Basement Membrane Extract
BPD	Benign Pancreatic/Peri-pancreatic Diseases
BRCA2	Breast Cancer Gene 2
CA 19-9	Carbohydrate Antigen 19-9
Cd	Cadmium
CDC25B	Cell Division Cycle 25B
$CdCl_2$	Cadmium Chloride
CDK	Cyclin-Dependent Kinases
CDK4/6	Cell Division Protein Kinase 4/6
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CEA	Carcinoembryonic Antigen
CSCs	Cancer Stem Cells
CXCR4 00	CX-C Chemokine Receptor Type 4
CycD	D-Cyclins
DMEM	Dulbeccos' Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNMT1	DNA Methyltransferase 1
DNMT3A	DNA Methyltransferase 3A
E2F1	E2F Transcription Factor 1

ECAR	Extracellular Acidification Rate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
EP300	E1A Binding Protein P300
ERK	Extracellular Receptor Kinase
ESCC	Esophageal Squamous Cell Carcinoma
FasL	Fas Ligand
FBS	Foetal Bovine Serum
FBXW7	F-Box and WD Repeat Domain Containing 7
FFPE	Formalin-Fixed Paraffin-Embedded
FOLFIRINOX	5-FU, Irinotecan, Oxaliplatin and Leucovorin
FOXM1	Forkhead Box M1
FOXO	Forkhead Members of the Class O
FOXO1	Forkhead Box Protein 1
5-FU	5-Fluorouracil
FZD4	Frizzled 4
GO	Gene Ontology
HER-2/neu	Human Epidermal Growth Factor Receptor
HGF	Hepatocyte Growth Factor
HH	Hedgehog Signalling Pathway
HIPPO	Protein Kinase Hpo
HIF-1	Hypoxia-Inducible-Factor-1
HOTAIR	Homeobox (HOX) Transcript Antisense RNA
HPDE	Human Pancreatic Ductal Epithelial Cell Line
HR+	Hormone Receptor-Positive
IARC	International Agency for Research on Cancer
IPMN	Intraductal Papillary Mucinous Neoplasm

IRAK-1	Interleukin 1 Receptor Associated Kinase 1
JAK	Janus Kinase
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KOs	Knockouts
K-RAS	Kirsten Rat Sarcoma Viral Homolog
LC-MS/MS	Liquid Chromatography-Mass Spectrometry
LRP-6	Low Density Lipoprotein Receptor
Maml2, 3	Mastermind-Like Coactivators 2, 3
MAPK	Mitogen-Activated Protein Kinase
Mcl-1	Myeloid Cell Leukemia-1
MDSCs	Myeloid-Derived Suppressor Cells
MET	Mesenchymal–Epithelial Transition Factor Gene
MIG6	Mitogen-Inducible Gene 6
MiRs/miRNAs	MicroRNAs
MMP7	Matrix Metallopeptidase 7
MMP12	Matrix Metallopeptidase 12
MORF4	Mortality Factor 4
MRGBP	Mortality Related Gene-Binding Protein
mRNA	Messenger RNAs
mTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUC4	Mucin 4
ncRNAs	Non-coding RNAs
NGS	Next Generation Sequencing
Ni	Nickel
$NiCl_2$	Nickel Chloride
nMDSCs	Neutrophil-like Heterogeneous Myeloid-Derived Suppressor Cells
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells

OCR	Oxygen Consumption Rate
OncomiR	Oncogenic miR
PanIN	Pancreatic Intraepithelial Neoplasia
PARP	Poly-ADP Ribose Polymerase
PBS	Phosphate-Buffered Saline
PCa	Pancreatic Cancer
PCSCs	Pancreatic Cancer Stem Cells
PD-0332991	Palbociclib
PDAC	Pancreatic Ductal Adenocarcinoma
PDGF	Platelet-derived Growth Factor
PDK1	Pyruvate Dehydrogenase Kinase 1
PDX	Patient-Derived Xenografts
PI	Propidium Iodide
PI3K	Phosphoinositide-3-kinase
PKM2	Isoform M2 of Pyruvate Kinase
PNI	Perineural Invasion
pRb	Phosphorylated Retinoblastoma
PRR11	Proline Rich 11
PTCA1	Patched1
PTEN	Phosphatase and Tensin Homolog
PUMA	P53 Upregulated Modulator of Apoptosis
RB	Retinoblastoma
RECK	Reversion-Inducing-Cysteine-rich Protein with Kazal Motifs
RGF	Reduced Growth Factor
RhoA	Ras Homolog Family Member A
ROCK	Rho-Associated Kinase
RPII	RNA Polymerase 2
RREB1	Ras Responsive Element Binding Protein 1

SA- β	Senescence β-Galactosidase
SA-miRs	Senescence-Associated miRs
SD	Standard Deviation
SHC1	Src Homology 2 Domain Containing 1
SIRT1	Sirtuin 1
SMAD4	SMAD Family Number 4
SOCS3	Suppressor of Cytokine Signalling 3
SOCS6	Suppressor of Cytokine Signalling 6
SOD2	Superoxide Dismutase 2
SPRY2	Sprouty Homolog 2
STAT3	Signal Transducer and Activator of Transcription 3
TGF-β	Transformation of Growth Factor β
TNM	Tumour-Node-Metastasis
TP53	Tumour Protein 53
UTRs	Untranslated Regions
VEGF	Vascular Endothelial Growth Factor
YAP1	Yes-Associated Protein 1
ZEB1	Zinc Finger E-box binding homeobox 1
ZNF652	Zinc Finger Protein 652

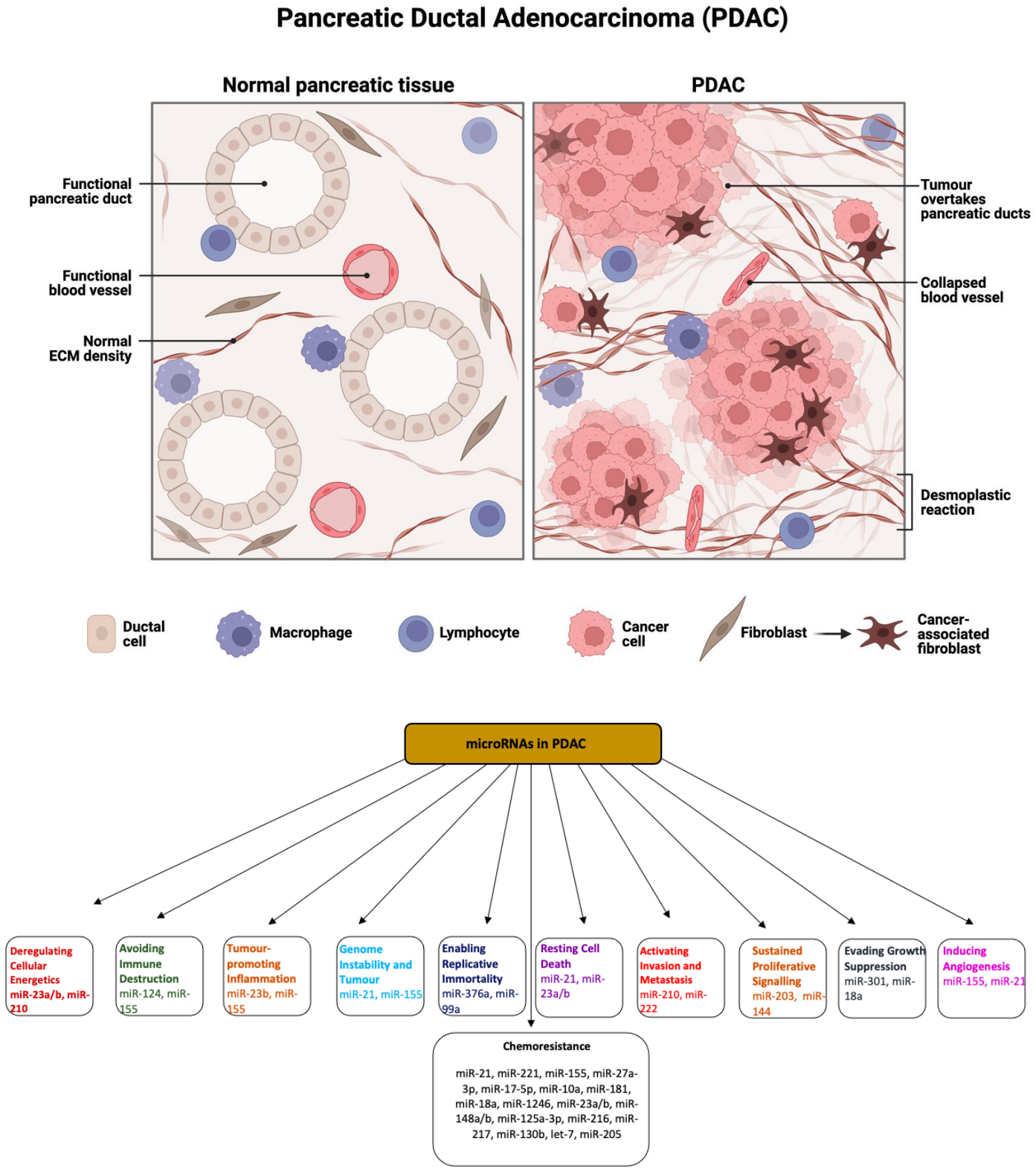
Author's Declaration

I declare that all the material contained in this thesis is my own work. Collaborations for associated publication are declared. All the parts, which have been published are available online in open access journals (European Journal of Cell Biology, Toxics, International Journal of Molecular Science, Archives of Toxicology, Translational Oncology, Stresses and Pancreatology), and have been referenced accordingly in bibliography and throughout this thesis.

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis was carried out by the Cambridge Centre for Proteomics (University of Cambridge, Cambridge, UK), whereas small RNA-sequence for 3D PDAC cell samples carried out by Novogene (Cambridge, UK) (**Chapter 6**).

Chapter 1

Graphical Abstract



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Highlights

- PDAC is one of the most lethal malignancies, which is usually not diagnosed until at an advanced stage.
- Non-coding RNAs (ncRNAs) are RNA molecules, which do not encode proteins and have been recognised to play a central role in PDAC pathogenesis and could be used as diagnostic, prognostic, and therapeutic biomarkers for PDAC.
- Different miRs can act either as oncogenes or tumour suppressors in PDAC.
- MiRs are involved in several key signalling pathways during PDAC pathogenesis.

Keywords: pancreatic cancer; microRNAs; cell signalling pathways; metabolic stress; diagnosis; prognosis; therapeutics

1. General Introduction

PDAC is the eighth primary source of cancer-related deaths globally with a 5-year survival rate of 3–6% (Carrato et al., 2015; Gall et al., 1991; Srivastava et al., 2014; Von Hoff et al., 2009). PDAC is highly lethal and a major cause of cancer-associated deaths in Western countries (Von Hoff et al., 2009), while the poor prognosis of PDAC is often the outcome of late diagnosis of the disease when the tumour is locally advanced or has metastasised (Srivastana et al., 2011). Approximately, 10–20% of PDAC patients are suitable for surgery at the time of diagnosis, and 9.7% of PDAC cases are at a local stage when initially diagnosed (Cartwright et al., 2008). PDAC prognosis is poor, due to enhanced cell proliferation, invasion, and metastatic features in combination with the chemo and radiotherapy resistance of PDAC cells (Srivastana et al., 2014). CA 19-9 has been the common diagnostic biomarker for PDAC during the past thirty years (Scara et al., 2015); however, CA 19-9 is not characterised as a PDAC-specific biomarker, especially for symptomless patients (Chan et al., 2014). Thus, the lack of

specific biomarkers has raised the need of the development of novel biomarkers for the early diagnosis and prognosis of PDAC (Siegel et al., 2014), in addition to developing efficient therapeutic strategies to minimise PDAC-related death rates (Blum and Kloog, 2014). Different stages of PDAC are described in **Figure 1**.

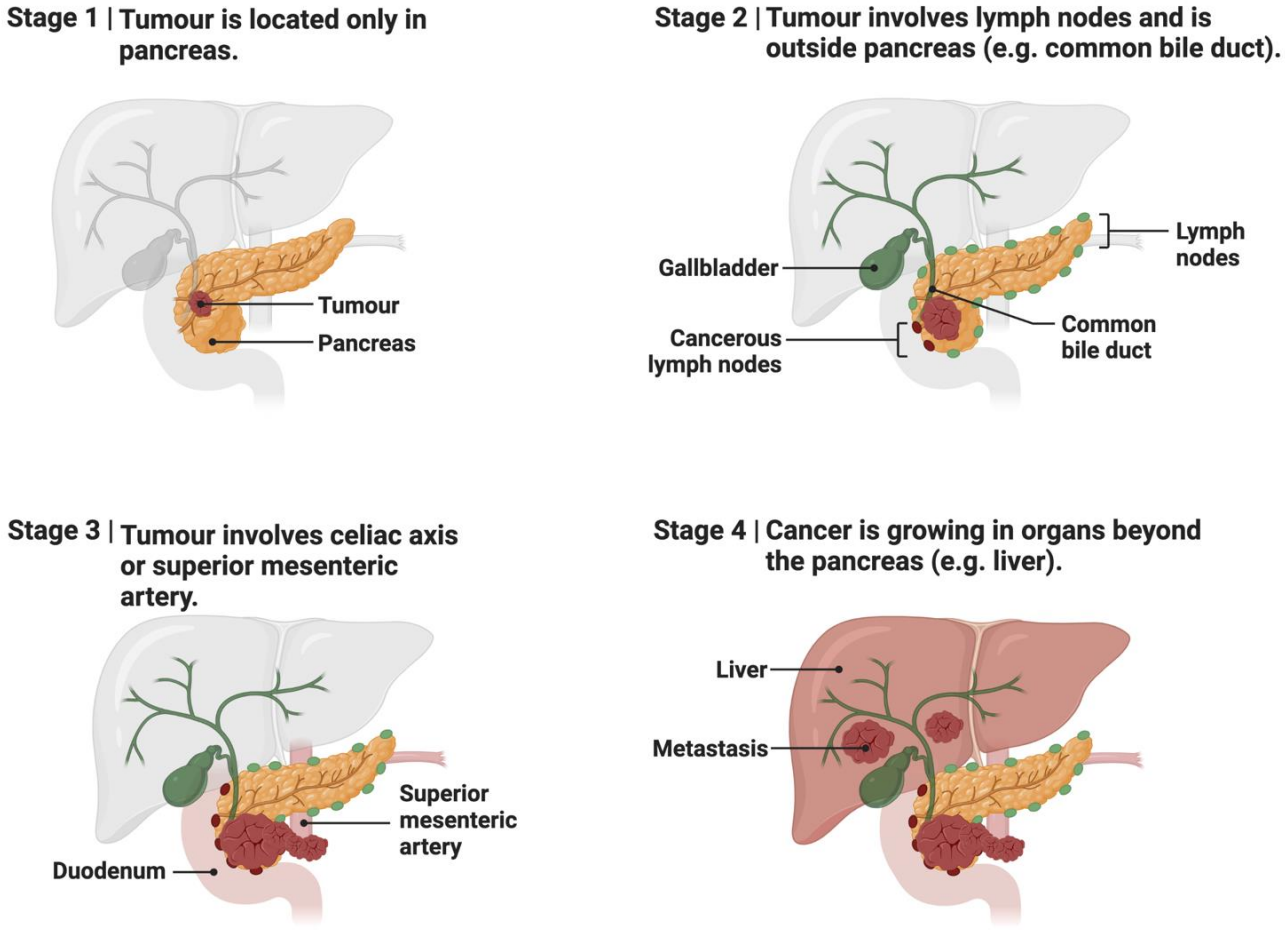


Figure 1: Stages 1-4 of PDAC development. Progression of PDAC can be separated into 4 main stages. In stage 1, the cancer is located in the top layers of pancreatic duct cells and has not invaded in deeper tissues, lymph nodes or distant sites. In stage 2, the cancer is larger than 4cm but is still within the pancreas and has not spread to nearby lymph nodes and distant sites. In stage 3, the cancer is any size within the pancreas and has spread to four or more nearby lymph nodes, while in stage 4, the cancer has spread to distant sites such as the liver, lungs, bones, and peritoneum and might have spread to nearby lymph nodes (Cancer Research, UK). Created with BioRender.com.

In recent years, molecular biology of PDAC in diagnosis has progressed as well as staging and treatment (Abbruzzese et al., 2008). However, since PDAC is one of the highly metastatic malignancies, only a small portion of PDAC patients has noted a significant improvement in overall survival rates with current treatment approaches (Abbruzzese et al., 2008; Neoptolemos et al., 2018). Gemcitabine is the first drug treatment with improved median survival, but only by 5.5% (Olive et al., 2009). Another commonly used chemotherapy agent, FOLFIRINOX (5-FU, irinotecan, oxaliplatin, and leucovorin) combined with gemcitabine has been suggested to elevate the median survival rates to 11.1 months in contrast to 6.8 months in the gemcitabine alone (Conroy et al., 2011). Nevertheless, limitations of the current therapeutic options for PDAC accentuate the importance of a better understanding of the molecular mechanisms and pathways linked to PDAC. Therefore, improved understanding of modified molecular signalling pathways, would facilitate earlier diagnosis in the progression of PDAC and aid development of beneficial therapeutic regimens that could abort the aggressiveness of PDAC (Xu et al., 2016).

PDAC prognosis originates from pre-cancerous lesions in the pancreatic intraepithelial neoplasia (PanIN-I, II, III) progressing to more advanced invasion and metastasis, which is associated both with several oncogene's activation and tumour suppressors inactivation (Maitra et al., 2008). The most common genetic alterations known to date to be linked to PDAC development are mutations of Kirsten rat sarcoma viral homolog (*K-RAS*) and overexpression of human epidermal growth factor receptor (HER-2/neu) (Hezel et al., 2006). At later stages (PanIN III, IV), inactivation of tumour suppressor genes (TS) including cyclin-dependent kinase inhibitor 2A (*CDKN2A*), tumour protein 53 (*TP53)* and SMAD family number 4 (*SMAD4*) have been considered as key regulators of PDAC pathogenesis (Hezel et al., 2006; Iacobuzio-Donahue et al., 2012; Ryan et al., 2014). Moreover, studies have revealed that 12 main signalling pathways comprising KRAS signalling, Hedgehog signalling, apoptosis, control of G1/S phase transition and transforming growth factor

beta (TGF-β) signalling are dysregulated in more than 80% of PDAC patients (Jones et al., 2008; Sun et al., 2015).

A number of identified modifiable risk factors are shown to significantly raise the risk of PDAC development up to 132-fold (Becker et al., 2014). Some of these factors include chronic pancreatitis development, diabetes mellitus, and physical inactivity (Maisonneuve et al., 2015). Additionally, some environmental exposures can induce PDAC development such as inhalation of cigarette smoke (Barone et al., 2016) and exposure to toxic metals including arsenic, nickel (Ni), and cadmium (Cd) (Barone et al., 2016; Buha et al., 2017; Djordjevic et al., 2019; Wallace et al., 2020b).

MiRs are small (18 to 28 nucleotides-long), endogenous, non-coding, evolutionary conserved, single-stranded RNA molecules. They are shown to moderate gene expression at the posttranscriptional level through the binding to the complementary sequences of their target mRNAs at the 3′ untranslated regions (3′ UTRs), which allow them to control the expression levels of several genes and regulate various signalling pathways (Bartel et al., 2004; Friedman et al., 2009; Lin et al., 2015; Turchinovich et al., 2016). Poy et al. (2004) were the first to perform a miR expression profiling study in mouse pancreas. Studies on miRs have exhibited a significant role of these biomolecules also in cell growth and development, as well as in cell differentiation, survival and lipid glucose metabolic pathways in numerous cancers and autoimmune diseases (Meltzer et al., 2005; Słotwiński et al., 2018). MiRs can act either as oncogenes (oncomiR) or tumour suppressor genes (tsmiRNA) (Galasso et al., 2012). Some of the most common oncogenic miRs identified to date are miR-21, miR-221 and miR-155, while miR-34 and miR-146 are characterised as tumour suppressor miRs (Baradaran et al., 2019). Furthermore, Papaconstantinou et al. (2013) have reported an upregulation in the expression of miR-222, miR-21, miR-210, miR-221 and miR-155 in PDAC patient tissue. According to Greither et al. (2010), elevated levels in the expression of miR-155, miR-21, miR-203, miR-210 and miR-222 can result in poor survival of PDAC patients due to the development of fibrogenesis, through *TGF-β.*

Thus, identifying the most expressed miRs with their related signalling pathways is crucial for a better understanding of PDAC pathophysiology.

1.1. MiRs as Diagnostic Biomarkers for PDAC

The aberrant expression of miRs is linked to initiation, proliferation, induction of epithelial-mesenchymal transition (EMT), metastasis and chemoresistance of PDAC cells (Barrera et al., 2023; Turchinovich et al., 2011). Stable miRs expressions were detected in tissues, blood plasma patient and healthy samples and several other body fluids such as serum, urine, and breast milk (Lee et al., 2007). MiR signatures profiles are also detectible in and effective for the identification of PDAC in formalin-fixed paraffin-embedded (FFPE) tissues (Bloomston et al., 2007; Schultz et al., 2014), bile (Zabron et al., 2013), biopsy (Frampton et al., 2013) and faecal samples (Link et al., 2012). MiRs are also referred to as "circulating microRNAs", which can be found either encapsulated in cell-secreted vesicles or are vesicle-free (Calin et al., 2004). Aberrant expression of miRs can be the result of the amplification or deletion in a genomic region, where miRs genes are expressed (Cai et al., 2004). Even though the biological functions of the identified miRs present a considerable ambiguity, the examination of the expression profiles of these biomarkers can provide useful information regarding their regulation and function (Lee et al., 2003). Despite the narrow knowledge of these molecules, a plethora of miRs can be characterised as vital biomarkers not only for the early prognosis and diagnosis of PDAC but also for better management of therapeutics (Friedman et al., 2009).

MiR genes can be located either in independent noncoding DNA loci or in the introns of protein-coding genes (Stanczyk et al., 2008). MiR biogenesis can be separated into three main steps: **1**) in the nucleus, RNA polymerase II transcribes miR genes to form pri-miRs **2**) pri-miRs are trimmed by the Drosha RNase III endonuclease to release a pre-miR hairpin, which is actively transported to cytoplasm by Ran-GTP and exportin-5 **3**) final maturation in the cytoplasm, where the pre-miR is cleaved into a

single-stranded mature miR by Dicer RNase III endonuclease, and miR assembles the RNA-induced silencing complex (RISC) through the binding to the proteins of the Argonaute (Ago) family (Cullen et al., 2004; Lee et al., 2004; Suzuki et al., 2012) (**Figure 2**). It has been estimated that more than 60% of protein-encoding genes can be controlled by miRs (Friedman et al., 2009). There are approximately 1872 annotated human miR precursor genes, which are processed into 2578 mature miR sequences, and more than 940 members of the miR family have been identified in 1-5% of the human genome (Cullen et al., 2004; Stanczyk et al., 2008). The abnormal expression of miRs can be modulated not only through epigenetic modulations and alterations in DNA copy number but also via genetic mutations, chromosomal abnormalities, and DNA methylation (Suzuki et al., 2012). Moreover, miRs downregulate gene expression through translational repression, mRNA cleavage and deadenylation (Lee et al., 2004). Genome investigations have disclosed that several miRs genes are presented in cancer-related genomic regions, which can be a region with loss of heterozygosity, a region with amplification, deletions and translocations, fragile sites, and breakpoint regions (Calin et al., 2004).

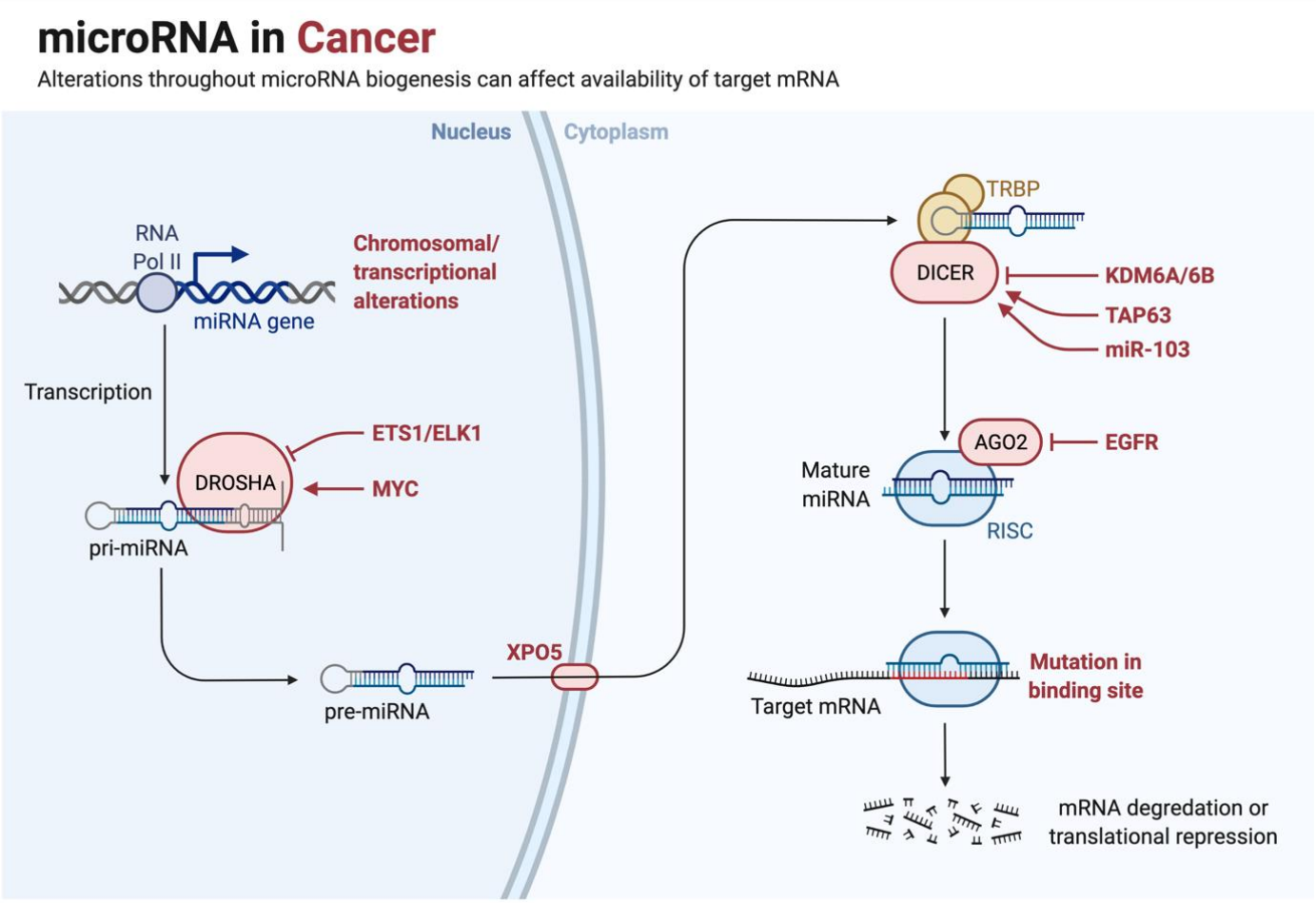


Figure 2: MicroRNA biogenesis. Alterations in miRs biogenesis during cancer development occurs at several steps during miR maturation and include genetic alterations, chromosomal or transcriptional aberrations and mutations in binding sites. Created with BioRender.com.

1.1.1. Oncogenic miRs in PDAC

Oncogenic miRs have been identified and related to PDAC in various tissues and body fluids as well as in *in vitro* studies. It has been shown that miR-376a and miR-301 are upregulated in PDAC tissues, while miR-23a and miR-23b over expressions were detected in saliva of PDAC patients (Humeau et al., 2015; Liu et al., 2012b). Moreover, miR-21, is one of the most studied oncogenic miRs, along with miR-20a, miR-24, miR-25, miR-99a, miR-185 and miR-191. Due to their increased expression levels in serum and tumor tissues in PDAC, they were suggested to be useful diagnostic markers (Kunovsky et al., 2007; Liu et al., 2012a; Zhao et al., 2020). Interestingly, miRs that were highlighted as possible biomarkers denoted an accuracy of 83.6% compared to CA 19-9, which is only 56.4% accurate (Madhavan et al., 2015; Morimura et al., 2011; Yu et al., 2020). Another study reported increased expressions of miR-1246, miR-4644, miR-3976 and miR-4306 in serum derived exosomes of PDAC patients (Kawaguchi et al., 2013), whereas miR-221 and miR-18a were also found to be upregulated in plasma of PDAC patients (Komatsu et al., 2014; Ohuchida et al., 2011; Zhang et al., 2014). MiR-221 is associated with distant metastasis, interestingly, miR-221 and miR-18a expression levels were reduced after surgery (Komatsu et al., 2014; Ohuchida et al., 2011; Zhang et al., 2014). Therefore, miR-221 and miR-18a could be effective diagnostic and prognostic biomarkers (Komatsu et al., 2014; Ohuchida et al., 2011; Zhang et al., 2014). Furthermore, miR-194 expression levels were significantly elevated and linked with poor prognosis of PDAC (Lin et al., 2014), while Bloomston et al. (2007) suggested the overexpression of miR-155, miR-181a, b, c, d and miR-196a in PDAC human tissue samples.

Similarly, miR-10a, miR-17-5p and miR-92 expressions were found to be significantly upregulated in PDAC tissue cases (Szafranska et al., 2007), additionally, Lin et al. (2014) showed the overexpression of miR-1238, miR-4290 and miR-483-5p in PDAC serum patient samples (Khan et al., 2021). Moreover, miR-486-5p and miR-938 have been proposed as potential diagnostic serum biomarkers for PDAC (Khan et al., 2021)

and further studies have suggested the upregulation of miR-203, miR-210, miR-222, miR-196b and miR-196a in PDAC tissue samples (Munding et al., 2012). Wang et al. (2009) also showed that the upregulation of miR-27a-3p effectively discriminated PDAC tissue cases from benign pancreatic/peri-pancreatic diseases (BPD). In addition, a study by Khan et al. (2021) revealed that miR-215-5p, miR-122-5p, miR-192-5p and miR-181a-2-3p are significantly upregulated in PDAC tissue and serum samples. Conclusively, miR-135b, which is an oncogenic miR in PDAC, can be characterised as a potential diagnostic biomarker since it presented high sensitivity (92.9%) and specificity (93.4%) for the discrimination of PDAC cases in tissue samples (Munding et al., 2012). An overview of PDAC associated miRs is provided in **Table 1** and **Figure 3** and show the functional involvement of several miRs during PDAC development.

Table 1: Oncogenic miRs associated with PDAC.

miR	Expression in PDAC	Clinical Values	Functional Involvement in PDAC	Detected	Biology Tested	Control	Number of Patients	Group Tested	References
miR-10a	Up	D, T	Increase chemoresistance and metastasis	Tissue +Panc-1, BxPC3, AsPC-1, Capan-1, MIA Paca-2, SW1990, HDPE cell line	Cell lines	HPDE	15 Cell lines	Primary tumours	(Cai et al., 2013; Xiong et al., 2018)
miR-17-5p	Up	D, P, T	Increase cell growth apoptosis, decreased chemosensitivity to gemcitabine	Tissue +Panc-1, BxPC3, AsPC-1, Capan-1, MIA Paca-2, SW1990, HDPE cell line	Cell lines	HPDE	15 Cell lines	Primary tumours	(Wang et al., 2009; Yan et al., 2012; Zhao et al., 2020)
miR-18a	Up	D, P, T	Inhibition of apoptosis, increase cell growth	Plasma, Tissue + Panc-1	Patients, Cell lines	30 Healthy controls	36	Stages I, II, IV	(Morimura et al., 2011)
miR-21	Up	D, P, T	Inhibition of apoptosis, increase gemcitabine resistance, aggressiveness	Saliva, Blood, Tissue	Patients, Animals	4 Healthy controls	7	Locally advanced and unresectable PDAC	(Bloomston et al., 2007; Dillhoff et al., 2008; Humeau et al., 2015; Morimura et al., 2011; Park et al., 2009; Wang et al., 2009; Zhao et al., 2020)

miR-23a	Up	D, T	Inhibition of apoptosis	Saliva	Patients, Animals	4 Healthy controls	7	Locally advanced and unresectable PDAC	(Frampton et al., 2014; Humeau et al., 2015; Ohuchida et al., 2011)
miR-23b	Up	D, T	Inhibition of apoptosis, Radioresistance	Saliva	Patients, Animals	4 Healthy controls	7	Locally advanced and unresectable PDAC	(Humeau et al., 2015; Wang et al., 2013a)
miR-24	Up	D, P	Inhibition of apoptosis	Blood-Serum	Patients	158 Healthy controls	197	Stages I, II, III, IV	(Liu et al., 2012a; Liu et al., 2014b)
miR-25	Up	D, P	Inhibition of apoptosis	Blood-serum	Patients	158 Healthy controls	197	Stages I, II, III, IV	(Liu et al., 2012a; Liu et al., 2014b)
miR-27a-3p	Up	D, T	Increase growth, migration, and colony formation *in vitro*	Blood	Patients	20 Healthy controls	20	Stage IA, IB, IIA, IIB, III, IV	(Frampton et al., 2014; Wang et al., 2013b)
miR-92	Up	D	Increase cell growth, inhibition of cell differentiation	Tissue +Panc-1, BxPC3, AsPC-1, Capan-1, MIA Paca-2, SW1990, HDPE cell line	Cell lines	HPDE	15 Cell lines	Primary tumours	(Ohuchida et al., 2012)
miR-99a	Up	D, P	Increase cell proliferation, invasion, migration	Blood-serum	Patients	158 Healthy controls	197	Stages I, II, III, IV	(Liu et al., 2012a; Liu et al., 2014b)

miR-135b	Up	D, P	Increase tumour growth, promote cell adaptation to metabolic stress, suppress glycolysis	Tissue	Patients	Normal pancreatic tissue	52	-	(Munding et al., 2012)
miR-155	Up	D, P, T	Decrease apoptosis, Increase cell invasion, migration, metastasis, generation of reactive oxygen species	Plasma, Tissue	Patients	Adjacent non-cancerous tissues	65	PDAC patients with surgical resection	(Bloomston et al., 2007; Gironella et al., 2007; Greither et al., 2010; Zhao et al., 2020)
miR-181a, b, c, d	Up	D, T	Increase migration and metastasis	Plasma, Tissue	Patients	Adjacent non-cancerous tissues	65	PDAC patients with surgical resection	(Bloomston et al., 2007; Cai et al., 2013; Schultz et al., 2014)
miR-182	Up	D, P	Increase tumour growth, invasion, and migration	Plasma	Patients	Healthy controls	109	Stages I, II, III, IV	(Chen et al., 2014)
miR-185	Up	D, P	Increase invasion, migration	Blood-serum	Patients	158 Healthy controls	197	Stages I, II, III, IV	(Liu et al., 2012a; Liu et al., 2014b)

miR-191	Up	D, P	Inhibition of cell differentiation	Blood-serum	Patients	158 Healthy controls	197	Stages I, II, III, IV	(Liu et al., 2012a; Liu et al., 2014b)
miR-194	Up	D, P	Increase tumour growth and invasion	Tissue + Panc-1, BxPC3, AsPC-1, Capan-1, MIA Paca-2, SW1990 cell line	Patients, Cell lines	3 Adjacent non-cancerous tissues	9	PDAC patients with surgical resection	(Zhang et al., 2014)
miR-196a	Up	D, P	Increase invasion and migration	Plasma, Tissue	Patients	Adjacent non-cancerous tissues	65	PDAC patients with surgical resection	(Bloomston et al., 2007; Liu et al., 2012b; Schultz et al., 2014)
miR-196b	Up	D, P	Increase invasion and migration	Tissue	Patients	35 Normal pancreatic tissue	165	Stage IA, IB, IIA, IIB	(Calatayud et al., 2017)
miR-203	Up	D, P	Increase cell proliferation, migration, invasion, decrease apoptosis	Tissue	Patients	7 Normal pancreatic tissue	10	Stage III, IV	(Greither et al., 2010; Szafrańska et al., 2007; Zhao et al., 2020)
miR-210	Up	D, P	Promotes invasion and EMT	Plasma, Tissue	Patients	7 Normal pancreatic tissue	10	Stage III, IV	(Greither et al., 2010; Liu et al., 2012; Szafrańska et al., 2007; Zhao et al., 2020)
miR-212	Up	D, P	Increase proliferation	Tissue + MIA Paca-2, AsPC1 cell line	Patients, Cell lines	Normal pancreatic tissue	41	PDAC patients with surgical resection	(Yue et al., 2019)

miR-221	Up	D, P, T	Increase cell proliferation, migration, EMT	Plasma, Tissue +Panc-1 cell line	Patients, Cell lines	30 Healthy volunteers	47	Stages I, II, III, IV	(Bloomston et al., 2007; Kawaguchi et al., 2013)
miR-222	Up	D, P	Increase cell proliferation, migration, invasion, decrease apoptosis	Tissue	Patients	7 Normal pancreatic tissue	10	Stage III, IV	(Greither et al., 2010; Szafrańska et al., 2007; Zhao et al., 2020;)
miR-301	Up	D	Increase cell growth	Tissue +Panc-1, HS766T, MIA PaCa-2, BxPC3, Panc10.05 cell lines	Cells, Patients	6 Normal pancreatic tissue	28	Stage II, III	(Lee et al., 2007)
miR-376a	Up	D	Increase cell proliferation, invasion, migration	Tissue + Panc-1, HS766T, MIA PaCa-2, BxPC3, Panc10.05 cell lines	Cells, Patients	6 Normal pancreatic tissue	28	Stage II, III	(Lee et al., 2007)
miR-483-5p	Up	D	Increase proliferation and colony formation *in vitro*	Serum	Patients	27Matched Healthy controls	49	Stages I, II, III, IV	(Lin et al., 2014)
miR-486-5p	Up	D	Increase cell proliferation,	Plasma	Patients	5 Healthy controls	7	Pre-operative PDAC	(Le Large et al., 2015)

			migration, and invasion						
miR-938	Up	D	Increase cell proliferation, migration, and invasion	Plasma	Patients	5 Healthy controls	7	Pre-operative PDAC	(Le Large et al., 2015)
miR-1238	Up	D	Inhibition of apoptosis	Serum	Patients	27 Matched Healthy controls	49	Stages I, II, III, IV	(Lin et al., 2014)
miR-1246	Up	D, T	Increase chemoresistance, cell invasion and migration	Serum	Patients	12 Healthy controls	131	Stages I, II, III, IV	(Hasegawa et al., 2014; Madhavan et al., 2015a)
miR-3976	Up	D	Increase cell invasion and migration	Serum	Patients	12 Healthy controls	131	Stages I, II, III, IV	(Madhavan et al., 2015a)
miR-4306	Up	D	Increase cell invasion and migration	Serum	Patients	12 Healthy controls	131	Stages I, II, III, IV	(Madhavan et al., 2015a)
miR-4644	Up	D	Increase cell invasion and migration	Serum	Patients	12 Healthy controls	131	Stages I, II, III, IV	(Madhavan et al., 2015a)
miR-4290	Up	D	Inhibition of cell differentiation	Serum	Patients	27 Matched Healthy controls	49	Stages I, II, III, IV	(Lin et al., 2014)

D: diagnostic biomarker, P: prognostic biomarker, T: therapeutic target. Adapted from Mortoglou et al. (2021a).

1.1.2. Tumour Suppressor miRs linked to PDAC

A number of tumour suppressor miRs have been related to PDAC in patient samples and *in vitro* and *in vivo* studies. It has been suggested that the downregulation of miR-148a, b and miR-375 expressions could be used to differentiate PDAC, normal pancreatic and pancreatitis tissues (Bloomston et al., 2007). Another downregulated miR is miR-125a-3p, which was correlated to EMT and gemcitabine resistance in PDAC *in vitro* (Liu et al., 2018; Moriya et al., 2017). Another study has reported that both miR-450 and miR-205 aberrantly expressed in fifteen PDAC cell lines (Ohuchida et al., 2012), while Lin et al. (2014) suggested the downregulation of miR-1280, miR-492, miR-595 and miR-663a in PDAC serum human samples. Specifically, miR-663a is found to be closely correlated with the tumour-node-metastasis (TNM) stages in PDAC (Lin et al., 2014). Thus, these novel non-invasive biomarkers could be utilised for the prognosis of PDAC, while a combined miR panel could be a valuable diagnostic and prognostic tool for PDAC (Lin et al., 2014). MiR-216 and miR-217 were also downregulated in PDAC tissue samples and hence, they could be used as diagnostic biomarkers for PDAC patients (Szafranska et al., 2007). MiR-130b is also considerably downregulated in PDAC tissue cases and was closely related to poor prognosis, elevated tumour size, late TNM stage, lymphatic invasion, and distant metastasis (Zhao et al., 2013a). Moreover, a study by Khan et al. (2021) indicated that miR-30b-5p, miR-216b-5p, miR-320b, and miR-214-5p were downregulated in PDAC tissue and serum samples (**Table 2**).

Table 2: Tumour suppressor miRs identified in PDAC.

miR	Expression in PDAC	Clinical Values	Biological processes involved	Detected	Biology Tested	Control	Number of patients	Group Tested	Reference
let-7	Down	D, P, T	EMT, invasion	Tissue	Patients	Normal Pancreatic tissue	170	Stages IA, IB, IIA, IIB	(Schultz et al., 2012a)
miR-20a	Down	D, P	Proliferation, invasion	Blood-plasma	Patients	Healthy individuals	197	Stages I, II, III, IV	(Humeau e al., 2015; Liu et al., 2012)
miR-125a-3p	Down	D, T, P	Cell proliferation and migration, chemosensitivity, EMT	Blood	Patients, Panc-1, BxPC3, AsPC-1, Capan-2, MIA-PaCa-2 cell lines	Adjacent non-cancerous tissues	421	Advanced PDAC cases	(Kojima et al., 2015; Liu et al., 2018; Moriya et al., 2017)
miR-126	Down	D, P	Cell proliferation, migration, invasion	Tissue	Patients	Normal Pancreatic tissue	455	Stages O, I, II, III, IV	(Zhao et al., 2020)
miR-1280	Down	D, P	Tumour growth	Blood	Patients	27 Healthy controls	49	Stages I, II, III, IV	(Lin et al., 2014)
miR-130b	Down	D, P, T	Cell proliferation, invasion	Tissue	Patients, Panc-1, BxPC3, AsPC-1, SW1990, MIA-PaCa-2 cell lines	Matched Normal Pancreatic tissue	52	Stage I, II III, IV	(Zhao et al., 2013a)

miR-144-3p	Down	D, P	Cell cycle arrest, migration, invasion, metastasis, cell proliferation	Tissue	Patients + Panc-1 cell line	Paired adjacent non-tumour tissues	10	Stages IA, IB, II, III	(Yang et al., 2019)
miR-148a	Down	D, P, T	Cell proliferation, invasion, migration	Plasma, Tissue	Patients	Matched Adjacent non-cancerous tissues	65	PDAC patients with surgical resection	(Bloomston et al., 2007; Schultz et al., 2014; Schultz et al., 2012a)
miR-148b	Down	D, T	Cell proliferation, invasion, migration, inhibition of chemosensitization	Plasma, Tissue	Patients	Matched Adjacent non-cancerous tissues	65	PDAC patients with surgical resection	(Kunovsky et al., 2018; Zhao et al., 2013c)
miR-187	Down	D, P	Invasion, migration	Tissue	Patients	Normal Pancreatic tissue	170	Stages IA, IB, IIA, IIB	(Schultz et al., 2012a)
miR-205	Down	D, T	Chemoresistance	Pancreatic Juice	Patients	19 Non-healthy (NPNH) controls	50	Advanced PDAC cases	(Mittal et al., 2014; Schultz et al., 2014; Singh et al., 2013; Wang et al., 2014)
miR-216	Down	D, T	Increase cell proliferation, invasion	Tissue	Patients, +3 Cell Lines	Normal Pancreatic tissue	10	Stage III, IV	(Szafrańska et al., 2007; Wang et al., 2014)

miR-217	Down	D, T	Increase cell proliferation, migration, invasion, DNA damage, stress responses, genome stability and cell survival	Tissue	Patients, +3 Cell Lines	Normal Pancreatic tissue	10	Stage III, IV	(Singh et al., 2013; Szafrańska et al., 2007)
miR-375	Down	D, P	Tumour growth and apoptosis	Plasma, Tissue	Patients	Matched Adjacent non-cancerous tissues	65	PDAC patients with surgical resection	(Bloomston et al., 2007; Szafrańska et al., 2007)
miR-450	Down	D	Cell differentiation, proliferation, migration, and invasion		Panc-1, BxPC3, AsPC-1, Capan-2, MIA-PaCa-2 cell lines		15 PDAC cell lines	Primary tumours	(Ohuchida et al., 2012)
miR-492	Down	D, P	Tumour growth and stage	Blood	Patients	27 Healthy controls	49	Stages I, II, III, IV	(Lin et al., 2014)
miR-595	Down	D, P	Migration, metastasis	Blood	Patients	27 Healthy controls	49	Stages I, II, III, IV	(Lin et al., 2014)
miR-663a	Down	D, P	Tumour growth and stage	Blood	Patients	27 Healthy controls	49	Stages I, II, III, IV	(Lin et al., 2014)

D: diagnostic biomarker, P: prognostic biomarker, T: therapeutic target. Adapted from Mortoglou et al., (2021a).

Several miRs have been linked to various biological processes in PDAC development such as metastasis, invasion, migration, angiogenesis, promotion of inflammation, resist of cell death, genome instability, proliferation, and immune destruction (Mortoglou et al., 2021a, b). Particularly, two oncogenic miRs, miR-21 and miR-155, have been associated with inflammation, genome instability, invasion, metastasis and resisting of cell death; miR-221 with proliferation, and replicative immortality, while the tumour suppressor miR-126 is related to sustained proliferative signalling and invasion (**Figure 3**) (Mortoglou et al., 2021a, b).

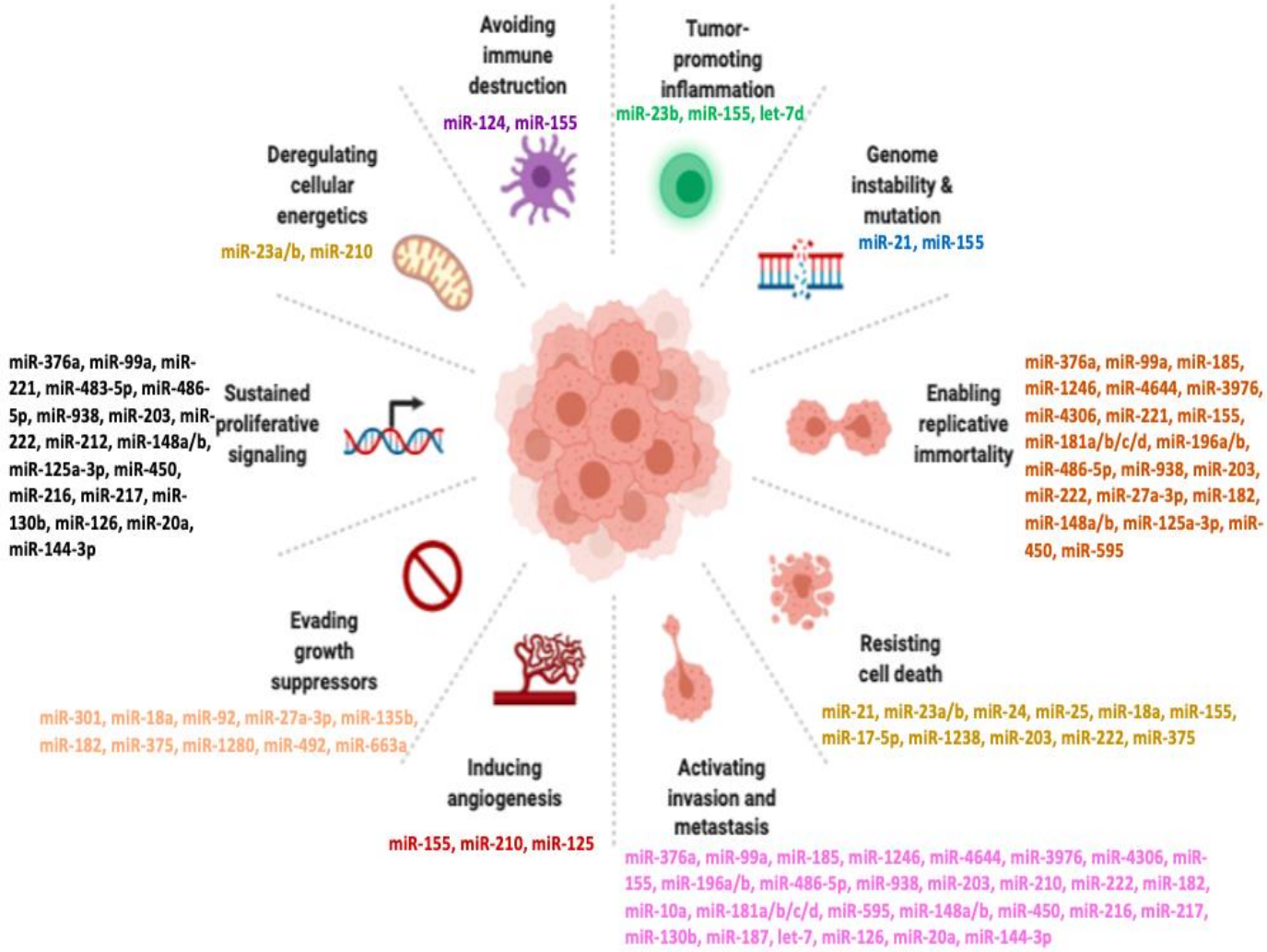


Figure 3: Involvement of miRs in PDAC progression. MiRs can be classified as oncogenes or tumour suppressors by moderating several key downstream gene targets, which control different cellular and biological processes involved in cancer progression. Adapted from Mortoglou et al., (2021a). Created with BioRender.com.

1.2. Involvement of miRs in different PDAC stages

1.2.1. Early Stage of PDAC Associated miRs

In previous studies, it has been found that that miR-1290 could be used to determine stage I PDAC with high sensitivity (88%) and specificity (84%) (Li et al., 2010). MiR-19a-3p was shown to be overexpressed in PDAC tissue samples with stage 2 tumour and could be utilised not only as an early non-invasive diagnostic biomarker for PDAC but also as a prognostic biomarker for patients with poor overall survival rate (Zou et al., 2019). Another study identified the upregulation of miR-29a, miR-29b, miR-103 and miR-320 as early diagnostic predictors of PDAC and therefore, it can be supported that these miRs could also be utilised for the early diagnosis of PDAC (Liu et al., 2012b) (**Table 3, Figure 4**).

1.2.2. Late Stage of PDAC Associated miRs

Both miR-196a and miR-196b were found to be overexpressed in PDAC patients with PanIN-II and III lesions and correlated with poor survival (Slater et al., 2014). Particularly, miR-196a and miR-196b presented 100% sensitivity and specificity in the discrimination of mice PDAC serum samples compared to healthy controls (Slater et al., 2014). Especially, miR-196a is an effective prognostic biomarker because patients with unresectable PDAC (stages III and IV) had considerable higher expression levels of miR-196a in comparison with patients in the early stages of the disease (stages I and II), who presented lower levels (Kong et al., 2011). Moreover, Chen et al. (2014) revealed that miR-182 was overexpressed in PDAC plasma patient samples in correlation to healthy controls and was linked to advanced clinical stages and lymph node metastasis.

Furthermore, overexpression of miR-21 has been also associated with advanced stages of PDAC (Song et al., 2013), metastasis to lymph nodes and liver, increased

gemcitabine-resistant and poorer survival in PDAC plasma human samples (Abue et al., 2015). Previous studies also described that miR-21, miR-29b, miR-146a, miR-182, miR-193a-3p, miR-193b, miR-200a, miR-200b, miR-425, miR-486-3p, miR-708 and miR-874 were significantly dysregulated in PanIN-II and III lesions in comparison to PanIN-I lesions and normal pancreatic tissues (Alemar et al., 2015; Yu et al., 2012). Ryu and colleagues (2010) also found that miR-155 was upregulated in PanIN-II and III in comparison to PanIN-I and healthy controls. Thus, a large body of evidence supported recommendations utilising specific miRs profiles as potential effective early diagnostic and prognostic tools for PDAC subtypes (**Table 3, Figure 4**).

Table 3: Deregulated miRs in PDAC lesions.

PDAC Lesion	Overexpressed miRs	Down Regulated miRs	References
PanIN-I	miR-1290, miR-320, miR-221, miR-210, miR-200a, miR-200b, miR-193b, miR-182, miR-181a, miR-181b, miR-155, miR-145, miR-103, miR-21	miR-296-5p, miR-181c, miR-107	(Lee et al., 2007; Li et al., 2013; Liu et al., 2012; Slater et al., 2014; Zou et al., 2019)
PanIN-II	let-7, miR-874, miR-708, miR-486-3p, miR-425, miR-222, miR-205, miR-200, miR-200a, miR-200b, miR-200c, miR-196a, miR-196b, miR-193a-3p, miR-155, miR-145, miR-29b/a, miR-21, miR-10b	miR-296-5p, miR-217, miR-148	(Abue et al., 2015; Chen et al., 2014; Hernandez et al., 2016; Kong et al., 2011; Song et al., 2013; Yu et al., 2012; Zou et al., 2019)
PanIN-III	let-7, miR-874, miR-708, miR-486-3p, miR-425, miR-338-3p, miR-222, miR-221, miR-205, miR-200, miR-196a, miR-196b, miR-182, miR-155, miR-145, miR-29b, miR-21, miR-18a, miR-10b,	miR-452, miR-296-5p, miR-218, miR-217, miR-148, miR-126, miR-125b	(Abue et al., 2015; Alemar et al., 2015; Chen et al., 2014; Hernandez et al., 2016; Kong et al., 2011; Ryu et al., 2010; Zou et al., 2019)

Adapted from Mortoglou et al.,(2021a).

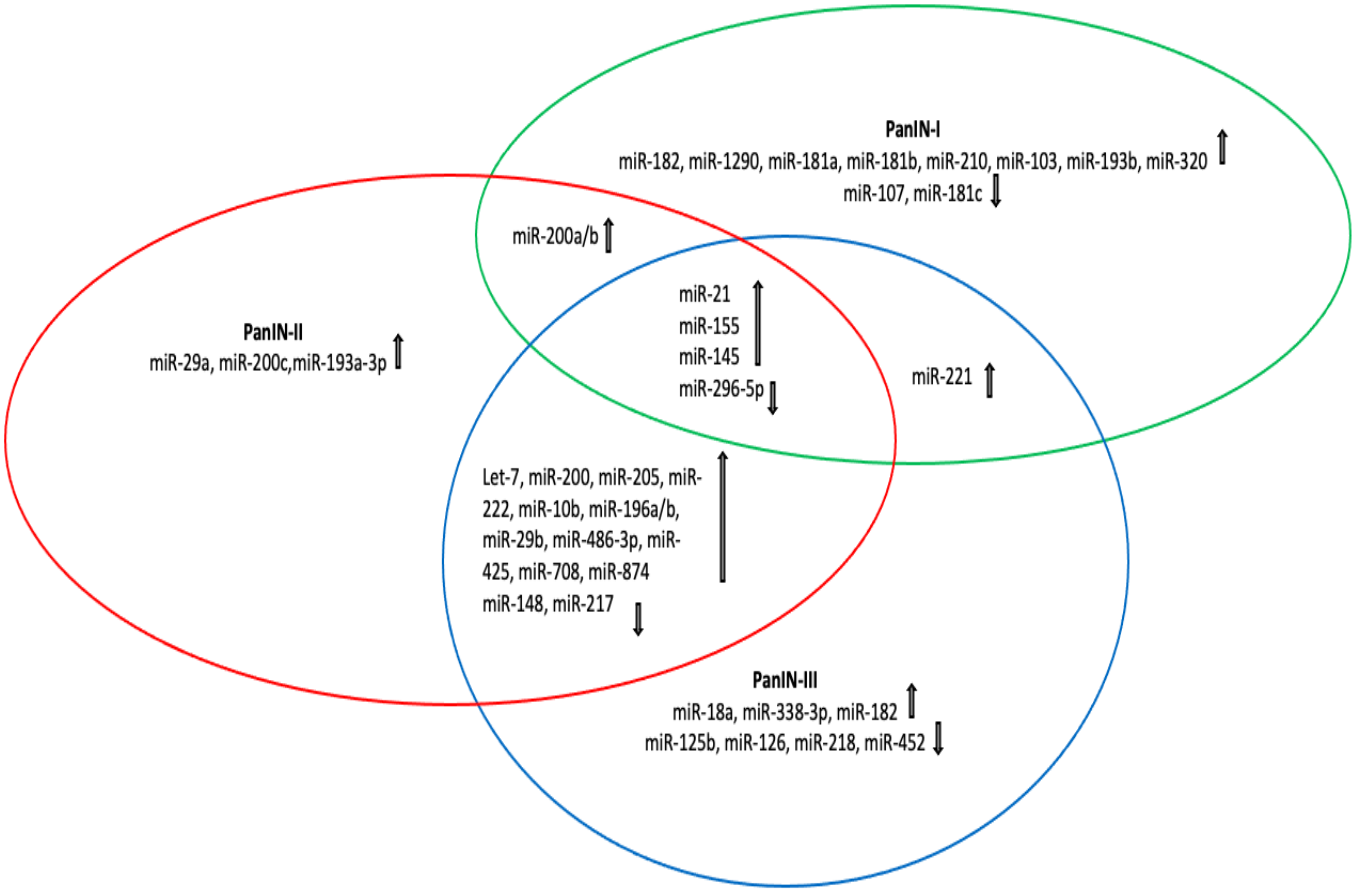


Figure 4: Overlap and differentially expressed miRs between different stages of PDAC. It can be noted that miR-21, miR-155, miR-145 and miR-296-5p are presented in all PanIN lesions, while miR-200a/b in both PanIN-I and II. MiR-221 are detected only in PanIN-I and III. Adapted from Mortoglou et al., (2021a).

1.3. MiRs as Prognostic Biomarkers in PDAC

Upregulated miR-132 (Park et al., 2011), miR-21 (Dillhoff et al., 2008) and downregulated miR-96 (Yu et al., 2010), miR-34a (Ji et al., 2009) have been reported to aberrantly expressed in PDAC tissues in relation to normal adjacent tissue and associated with poor overall survival (Jamieson et al., 2012). A study by Giovannetti et al. (2010) demonstrated that miR-21 overexpression is linked to worse outcomes in PDAC tissue samples, who have undergone gemcitabine therapy, and particularly noted that PDAC cases with miR-21 expression above median present shorter median overall survival (5.5-7.9 months). Moreover, Dillhoff et al. (2008) suggested that miR-21 upregulation is associated with poor survival rates in 79% of PDAC cases. Therefore, there are a substantial number of studies, which suggest that miR profiles could be considered not only as novel diagnostic and prognostic biomarkers for PDAC but also as predictors of treatment efficacy (Vorvis et al., 2016). A previous study by Wang et al. (2014a) noted that PDAC tissues contained hypermethylated miR-124-1, miR-124-2, and miR-124-3, which was related to poor survival rates of PDAC cases.

Additionally, further examples of miRs with prognostic impact in PDAC blood samples are miR-451a and miR-1290 and tissue miR-10b, miR-17-5p, miR-29c, miR-126, miR-155, miR-203, miR-218, miR-221 and miR-222 (Zhao et al., 2020). Particularly, between these miRs, miR-21, miR-451a, miR-23a, miR-155 and miR-218 presented the highest prognostic impact in PDAC blood samples and linked to poor survival (Zhao et al., 2020). Frampton et al. (2014) also confirmed the prognostic impact of miR-23a and miR-27a in PDAC tissue samples, who have undergone surgical resection and particularly highlighted that high expression of miR-23a and miR-27a is associated with aggressive tumour behaviour. In addition, upregulation of miR-200c was also linked with limited survival in PDAC patients through the reduction in the levels of mucin 4 (*MUC4)* and *MUC16* (Radhakrishnan et al., 2013). Specifically, *MUC4* and

MUC16 regulate tumour microenvironment, metastasis, and invasion in PDAC (Radhakrishnan et al., 2013). Therefore, these miR family members can be considered as valuable prognostic and metastatic predictors for patients with PDAC (Radhakrishnan et al., 2013). Similarly, Greither et al. (2010) also remarked that the overexpression of miR-210 designated poorer survival rates in PDAC patients and hence, these miRs could be used as potential effective prognostic biomarkers for this malignancy in tissue samples.

Recently, it has been reported that downregulated miR-4521 is associated with uncontrolled proliferation of PDAC cells *in vitro* and poor overall survival rate (Li et al., 2016a). Additionally, Yang et al. (2019) identified that matrix metallopeptidase 7 (MMP7) can be targeted with the upregulation of miR-144-3p, which further controls the progression of PDAC development *in vivo*. MMP7 is highly expressed in PDAC tissues and associated with metastasis, while it can be targeted with the upregulation of miR-144-3p, which leads to longer survival rates *in vitro* (Yang et al., 2019). Specifically, PDAC cells with miR-144-3p presented longer survival rates (Yang et al., 2019). Also, patients with upregulated miR-182 presented shorter disease-free survival and overall survival compared to patients with low expression levels (Chen et al., 2014). Bloomston et al. (2007) established that upregulated miR-30a-3p, miR-105, miR-127, miR-187, miR-452, and miR-518a-2 are associated with a better prognosis of PDAC patients, while Schultz et al. (2012b) identified that the upregulation of miR-212 and miR-675 and downregulation of miR-148a, miR-187 and let-7g were linked to worse prognosis in PDAC tissue cases. A further study by Greither et al. (2010) in tissue samples showed that miR-200c and upregulated miR-302 are related to PDAC patients' outcome and poor survival, while Li et al. (2016a) suggested that downregulated miR-506 is also associated with poor prognosis in PDAC blood samples (Li et al., 2016a). A further study revealed that miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p and miR-4476 presented a higher accuracy for the diagnosis of PDAC in correlation to CA 19-9 and

carcinoembryonic antigen (CEA) in serum samples (Kojima et al., 2015). Further examples of miRs associated with better survival rates are the upregulation of miR-142 and miR-204 in PDAC tissue samples (Ohuchida et al., 2011), while downregulated miR-19a-3p presented a better survival impact (Zou et al., 2019). Hence, it can be suggested that miRs could act as effective prognostic biomarkers.

1.4. Signalling Pathways Associated with PDAC

1.4.1. TGF-β and HGF-MET Signalling Pathways

SMADs have been widely associated with PDAC progression and are involved in several biological processes, such as cell proliferation, differentiation, and apoptosis in PDAC pathogenesis through the transduction of the TGF-β signalling pathway (Truty et al., 2007). The TGF-β pathway is regulated by three principal target genes, namely *TGFBR2*, *SMAD3* and *SMAD4*, which are involved in cell growth, differentiation, and cell cycle progression in PDAC (Birchenall-Roberts et al., 2004). Specifically, TGF-β is one of the most critical EMT-inducing factors in several malignancies, including PDAC (Ellenrieder et al., 2001). Moreover, *SMAD4* mutations are common in the TGF-β pathway (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012) and are found in 60% of PDAC cases (Jones et al., 2008), where it is associated with a higher risk of metastasis and poor prognosis (Blackford et al., 2009; Iacobuzio-Donahue et al., 2009). *SMAD4* inactivation is present in the late stages of PDAC development, while normal expression of *SMAD4* is observed in early PanIN lesions (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). Specifically, TGF-β moderates EMT, metastasis, extravasation of colonisation sites, and escape from immune surveillance in advanced and metastatic PDAC cases (Mu et al., 2011). A study by Li et al. (2014b) stated that the loss of *SMAD4* can cause elevated levels of forkhead Box M1 (FOXM1), nuclear localisation of β-catenin, and decreased levels of miR-494, which results not only in elevated cell proliferation, migration, and invasion, but also in increased resistance to gemcitabine in PDAC patients (Garajová et al.,

2014). Further miRs that are also linked to *SMAD4* expression in PDAC are miR-421 and miR-483-3p (Hao et al., 2010; Hao et al., 2011). The mesenchymal–epithelial transition factor gene (MET) can be activated as a response to hepatocyte growth factor (HGF) (Garajová et al., 2014). Specifically, *Src*, MEK–extracellular signal regulated kinases (ERK1/2), phosphoinositide 3-kinases (PI3K)–AKT, nuclear factor kappa-light-chain-enhancer of activated B cells (NK-κB), mammalian target of rapamycin (mTOR), and signal transducer and activator of transcription (*STAT*) are common effector molecules of the HGF-MET signalling cascade (Zhu et al., 2011), and are associated with cell proliferation, migration, and survival (Shah et al., 2007). In PDAC, elevated MET expression levels have been observed and linked to TNM stage (Shah et al., 2007). Furthermore, increased MET expression levels have also been correlated to EMT-like changes and gemcitabine resistance in PDAC (Bao et al., 2012). MiR-26a has been associated with the upregulation of MET in PDAC cancer stem cells (CSC) (Bao et al., 2012). A previous study has revealed that miR-424-5p is overexpressed in PDAC, and that it can also regulate the ERK1/2 signalling pathway through the negative modulation of suppressor of cytokine signalling 6 (*SOCS6*) (Wu et al., 2013).

1.4.2. JAK–STAT Signalling Pathway

Previous studies have shown that mutations in the Janus kinase (*JAK)–STAT* signalling pathway are closely associated with PDAC progression (Garajová et al., 2015). Specifically, downregulation of let-7 has been determined in PDAC and has been associated not only with the decreased phosphorylation/activation of *STAT3* and its downstream signalling events, but also with the downregulated growth and migration of PDAC cells (Patel et al., 2014). The cytoplasmic expression of suppressor of cytokine signalling 3 (*SOCS3*) can be enhanced by let-7 re-expression, which further leads to the blockage of *STAT3* activation by *JAK2* (Patel et al., 2014). Further miRs associated with the JAK–STAT pathway in PDAC are miR-216a, miR-130b, and miR-

155 (Yonemori et al., 2016). MiR-216a is downregulated in PDAC tissue samples and is the direct target of *JAK2* (Hou et al., 2014). Additionally, miR-130b is overexpressed in PDAC cells and tissue samples and binds directly to the 3′-UTR of STAT3 mRNA (Zhao et al., 2013a). Aberrant expression of this miR is linked to poor prognosis, suppression of cell proliferation, and invasion in PDAC via *STAT3* inhibition (Zhao et al., 2013a). MiR-155 is also closely correlated with the *JAK–STAT* signalling pathway through the inhibition of *SOCS1* (Yonemori et al., 2016), which further promotes PDAC cell invasion and migration (Huang et al., 2013).

1.4.3. PI3K–AKT Signalling Pathway

The PI3K–AKT signalling pathway is associated with cell proliferation and the inhibition of apoptosis in PDAC *in vitro* (Ferro, 2014). Specifically, PI3K–AKT–mTOR signalling, which is suppressed by phosphatase and tensin homolog (*PTEN*), can be targeted by miR-21, miR-221, and miR-181a (Arisan et al., 2021; Liu et al., 2014c; Park et al., 2009; Sarkar et al., 2013). MiR-21 inhibits cell cycle arrest, apoptosis, and gemcitabine sensitivity *in vitro*, and thus miR-21 can result in the uncontrolled proliferation and migration of PDAC cells (Park et al., 2009; Sarkar et al., 2013; Uysal-Onganer et al., 2021). MiR-181a also prompts the migration of PDAC cells (Liu et al., 2014c). Controversially, miR-375 and miR-220c are also linked to the PI3K–AKT signalling pathway in PDAC (Yonemori et al., 2016). Pyruvate Dehydrogenase Kinase 1 (*PDK1*) encodes a kinase downstream of *PI3K* and is the direct target of miR-375 (Zhou et al., 2014b). Moreover, the upregulation of miR-200c is also linked to both EMT (Yu et al., 2010) and *MUC4* expression in PDAC tissue samples (Schultz et al., 2012B). Specifically, *MUC4* expression results not only in the stabilisation of *HER2*, but also in the activation of *AKT*, which further leads to the activation of N-cadherin in mouse pancreatic tissue (Kaur et al., 2014; Rachagani et al., 2012).

1.4.4. TP53 Signalling Pathway and Apoptosis

The loss of *TP53* is detected in more than 70% of PDAC tissue samples, particularly in advanced cases, with the most common mutations being missense point mutations (Bardeesy and Depinho, 2002; McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). The inactivation of p53 has consequences for PDAC heterogeneity and chemoresistance (Rozenblum et al., 1997), and results in the dysregulation of several biological processes, such as cell proliferation, migration, invasion, and apoptosis (Nalls et al., 2011). Gain-of-function mutations in *TP53* rise the metastatic predisposition and proliferation properties of tumour cells *in vitro*, consistent with the reduced function of wild-type p53 (Hu et al., 2021), while Morton et al., (2010) reported that mut-p53 ($Trp53^{R172H}$) enabled malignant transformation from premalignant lesions to PDAC. Studies in PDAC tissue samples have shown that some examples of miRs that can target p53 are miR-222 and miR-203 (Greither et al., 2010). Previous studies have demonstrated that *TP53* directly regulates miR-34, which in turn targets NOTCH, which is responsible for the maintenance and survival of PDAC cells (Fulda et al., 2009). Moreover, it has been shown that miR-155 downregulates the *TP53*-induced nuclear protein 1 gene, which promotes PDAC progression *in vitro* (Gironella et al., 2007). Dysregulation of apoptosis is a common cause of chemoresistance in PDAC and can be caused by several stimulatory and inhibitory factors that are associated with a high number of miRs (Fulda et al., 2009). For example, oncogenic miR-21 is observed in several cancer types and controls genes that are necessary for apoptosis (Arlt et al., 2013). Additionally, miR-23a can target apoptotic peptidase activating factor 1 (*APAF1)*, which results in the activation of caspase-9 and prompts apoptosis in several cancer types, including PDAC in Panc-1 cell line (Liu et al., 2015b). Furthermore, in PDAC, a decrease in the expression levels of BIM has been observed through the modulation of miR-24, which inhibits apoptosis and the cell cycle (Liu et al., 2015b).

1.4.5. KRAS Signalling Pathway

K-RAS mutations have been reported in more than 90% of PDAC patients, and also in colorectal cancer, lung adenocarcinomas, and urogenital cancers (Kanda et al., 2012; Timar et al., 2020). The most frequent mutation of *KRAS* in PDAC is detected in codon G12 of exon 2 (G12D (40%) and G12C (33%) (Hu et al., 2021). Point mutations in codon 12 of the *KRAS* oncogene inhibit the conversion from GTP to GDP, which leads to the constitutive activation of downstream signalling pathways that promote carcinogenesis (Buscail et al., 2020; Zeitouni et al., 2016). In recent years, numerous miRs have been found to be key regulators of the *KRAS* signalling pathway in PDAC (Zhao et al., 2010). Specifically, miR-217 acts as a tumour suppressor in PDAC, which directly targets the *KRAS* oncogene (Zhao et al., 2010). MiR-217 causes a decrease in the constitutive phosphorylation of the downstream signal transducer *AKT* in both PDAC tissue samples and PDAC cell lines (Panc-1, MiaPaCa-2, BxPC3, AsPC-1) (Zhao et al., 2010). A study by Yu et al. (2010) also suggested that miR-96 not only targets *KRAS*, but also negatively modulates the phosphorylated AKT signalling pathway downstream of *KRAS*. Further studies have also observed that miR-126 and let-7d target *KRAS* via post-transcriptional upregulation (Jiao et al., 2012). MiR-206 acts as a tumour suppressor in PDAC and was found to inhibit both the *KRAS* and annexin A2 (*ANXA2*) oncogenes as miR-206 can be a negative regulator of oncogenic *KRAS*-induced *NF-κB* transcriptional activity, which leads to reduced proangiogenic and pro-inflammatory factors that further result in tumour growth and poor prognosis (Keklikoglou et al., 2015; Vorvis et al., 2016). MiR-27a has been found to be overexpressed in PDAC cell lines (Panc-1, MiaPaCa-2) and has been linked to reduced cell growth and migration (Yonemori et al., 2016). Specifically, miR-27a is the direct target of *Sprouty2*, which controls *KRAS* expression (Ma et al., 2015a). The overexpression of miR-143/145 is repressed by activated *KRAS*, which promotes the growth of PDAC cells (Kent et al., 2010). Additionally, activated *KRAS* can stimulate downstream signalling components, including mitogen activated protein kinase (*MAP2K1*)/*MEK* and *MAPK1*/*ERK2* (Eser et al., 2014; Furukawa et al., 2015; Ryan et

al., 2014). Collisson et al. (2012) have shown that the MAPK signalling pathway is involved in the genesis of PanIN, especially in the early stages of PDAC development. Therefore, further understanding of the oncogenic role of *KRAS* in PDAC could lead to novel strategies for earlier diagnosis and a more effective targeted therapy for this malignancy.

1.4.6. Epidermal Growth Factor Receptor and HER2/neu Signalling Pathways

The epidermal growth factor receptor (EGFR) family is a group of cytoplasmic receptor tyrosine kinases that contains the human EGF receptor or HER1 (EGFR or ErbB-1), HER2 (EGFR2 or ErbB-2), HER3 (EGFR3 or ErbB-3), and HER4 (EGFR4 or ErbB-4) (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). Specifically, *ErbB-2* is commonly upregulated in PDAC, particularly in PanIN lesions and carcinoma *in situ* lesions and has been linked with invasion (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). Moreover, two common ligands of EGFR, EGF and TGF-α, are significantly upregulated in PDAC tissues (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). TGF-α can activate Notch signalling downstream of EGFR signalling (Miyamoto et al., 2003). Similarly, *ErbB-3* has been also found to be overexpressed in PDAC tissue human samples with advanced tumour stages and limited survival time (Friess et al., 1995). Hence, it has been suggested that targeting *EGFR* could be an effective therapeutic strategy in PDAC patients (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). Subsequently, *ErbB-1* has been linked to both aggressiveness and unfavourable prognosis in PDAC cases (Ueda et al., 2004). Furthermore, *EGFR* upregulation is observed in more than 95% of PDAC cases and is closely associated with the aberrant expression of miRs in PDAC (Ali et al., 2014). Specifically, a study by Du Rieu et al. (2010) indicated that *EGFR* could promote the overexpression of miR-21 in PDAC cells, whereas Ali et al. (2014) showed a correlation between miR-146a and the upregulation of *EGFR* in PDAC cells (MiaPaCa-2, Panc-1, AsPC-1, BxPC3 and Panc-28) and PDAC tissue samples. Another study also demonstrated that miR-200c can

directly target the expression levels of mitogen-inducible gene 6 (*MIG6*), which is a negative modulator of *EGFR* (Izumchenko et al., 2014). The *HER2/neu* signalling pathway plays a key role in PDAC prognosis (Li et al., 2016a), as 50% of PDAC cases show *HER2* upregulation (Aumayr et al., 2014). Recent studies have suggested a correlation between miR-150 and the *HER2/neu* signalling pathway (Garajová et al., 2015); in particular, miR-150 is upregulated in PDAC and results in a considerable reduction in the expression levels of its target gene, *MUC4* (Garajová et al., 2015).

1.4.7. Notch and Hedgehog Signalling Pathways

The Notch signalling pathway, which is crucial for tissue proliferation, organ development, cell differentiation, and apoptosis, has been linked with poor prognosis in PDAC (Cai et al., 2004; Radtke et al., 2003). The oncogenic role of Notch homolog 1 (Notch) activation contributes to stem cell self-renewal, cell proliferation, apoptosis, migration, invasion, metastasis, and angiogenesis (De Lao and Murtaugh, 2009). Elevated expression of Notch pathway genes is present in early PanIN lesions (Miyamoto et al., 2003). It has been demonstrated in PDAC tissue samples that several miRs are associated with the Notch pathway (Jamieson et al., 2012). Specifically, miR-34 can be directly regulated by *TP53*, which further leads to the downstream signalling of Notch targets (Jamieson et al., 2012). Moreover, Notch-1/2 can be downregulated through the restoration of miR-34 expression in PDAC cancer stem cells (CSCs) (Ji et al., 2009). Furthermore, treatment of PDAC stem cells with chromatin-modulating agents can also lead to the suppression of miR-34 targets such as *Bcl-2,* cell division protein kinase 6 (*CDK6), and* sirtuin 1 (*SIRT1)* (Nalls et al., 2011). A further study by Brabletz and colleagues (2011) suggested that miR-200 can inhibit Notch pathway components such as *Jagged1* and the mastermind-like coactivators (Maml2 and Maml3), which further prompt the enhancement of Notch activation through EMT and *ZEB1*. Other studies have also remarked that the upregulation of miR-145 and downregulation of let-7a and miR-200 causes not only a decrease in the expression of EMT-related transcription factors, but also the inhibition of Notch1 via miR-144

(Sureban et al., 2013). The Hedgehog signalling pathway (HH) acts as a regulator of embryonic development but is also responsible for the moderation of CSCs (Atlaba et al., 2002). Specifically, several cases with deregulations in HH signalling have been observed in PDAC (Thayer et al., 2003). Previous studies have shown that the activity of this pathway is upregulated in PanIN lesions, which suggests that HH could be a regulator of the early and late stages of PDAC pathogenesis (Thayer et al., 2003). The HH pathway also plays a crucial role in cell cycle progression and apoptosis in PDAC cellular models (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). Hence, the inhibition of several HH pathway components could be a novel therapeutic strategy for PDAC (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). A study by Dosch et al. (1990) has demonstrated that Hedgehog, which is a secreted ligand, can bind to its receptor, Patched1 (PTCA1). In PDAC, *PTCA1* can be downregulated by miR-212, which is overexpressed in this malignancy. This could lead to unfavourable effects on cell growth, migration, and invasion via the HH pathway (Ma et al., 2014).

1.4.8. Wnt/β-Catenin Signalling Pathway

The WNT/β-catenin signalling pathway plays a crucial role in several biological and cellular processes, such as proliferation, differentiation, invasion, and migration, while it can also affect the PDAC tumour cell compartment (Yonemori et al., 2016). MiRs activate or repress Wnt through the targeting of Wnt ligand/receptor, ligand/receptor proteins, β-catenin interacting complex transcription factors and Wnt pathway components. Besides, Wnt activation elevates expression of miRs via the binding of β-catenin, which binds to promoter regions to activate transcription (Peng et al., 2017). MiR-29c, which is downregulated in PDAC, can be suppressed by *TGF-β* but can also moderate the regulators upstream of WNT, including frizzled 4 (FZD4), FZD5, FRAT2, and low-density lipoprotein receptor (LRP-6) (Jiang et al., 2015b). Similarly, miR-29a expression levels are associated with both the resistance of PDAC

cells to gemcitabine and with the activation of the Wnt/β-catenin signalling pathway *in vitro* (Nagano et al., 2013). Moreover, this pathway can be activated when the cell membrane of the WNT receptor is bound to its ligands, which leads to the release of β-catenin into the cytoplasm (McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). The elevated expression of β-catenin is observed in most PDAC cases, especially in the later stages of PanIN lesion development (Lowy et al., 2003; Zeng et al., 2006).

1.4.9. Cell Cycle Signalling Pathway and p16/CDKN2A Inactivation

Regulation of the cell cycle is critical for homeostasis and plays major roles in the development of cancer. The cell cycle is controlled by the activity of Cdk/cyclins, which regulate several mechanisms in normal cells (Santo et al., 2015). Importantly, the overexpression of cyclin D1 and downregulation of p16 have been identified in several PDAC cases (Chen et al., 2008). Recent studies have shown that a range of miRs are associated with dysregulations in cell cycle signalling (Yonemori et al., 2016). Specifically, cyclin D1-dependent kinase can be targeted by miR-107 to initiate cell cycle progression (Santo et al., 2015), whereas, in PDAC, miR-107 can be silenced through the methylation of CpG islands in its 5′ promoter region (Lee et al., 2009). A study by Zhao et al. (2013b) showed that miR- 192 results in the promotion of cell proliferation, and it can also moderate cycle progression via the G1 to S-phase transition in PDAC cells (Panc-1, MiaPaCa-2, BxPC3, AsPC-1). This study revealed that the overexpression of miR-192 led to an elevated expression of cyclin D1, cyclin D2, CDK4, and CDC2 (Zhao et al., 2013b). Further research denoted that miR-301a is responsible for the promotion of PDAC cell proliferation through the inhibition of the *Bim* gene (Chen et al., 2012), which acts as an initiator of apoptosis through the generation of multidomain pro-apoptotic proteins, including *Bak* and *Bax* (Willis et al., 2005). Additionally, miR-193b can deregulate the action of the *KRAS* pathway in PDAC (Jamieson et al., 2012). MiR-193b acts as a cell cycle brake in PDAC, which induces G1- phase arrest and reduces the fraction of cells in the S phase (Jin et al., 2015). MiR-223 can indirectly target cyclin E2, which further inhibits F-Box and WD

repeat domain containing 7 (FBXW7) (Ma et al., 2015a; Xu et al., 2015). Moreover, cyclin E2 can be also targeted by *p27* and *p57*, which negatively regulate G1/S progression via miR-222 (Park et al., 2009; Zhao et al., 2015a). Conclusively, a previous study has also indicated that miR-148a can target cell division cycle 25B (CDC25B), which controls the activation of distinct CDK/cyclin complexes (Boutros et al., 2007). The p16-Ink4A locus is encoded by the *CDKN2A* tumour suppressor gene (Schutte et al., 1997). Loss of p16 is linked to early PanIN lesions through invasive carcinomas (Wilentz et al., 1998). P16 is a well-described cyclin-dependent kinase inhibitor, which binds to CDK4 and CDK6 (Partensky et al., 2013); it is the product of the *CDKN2A* gene and is connected to PDAC (Okamoto et al., 1994). P16 is involved in the inhibition of the activity of phosphorylated retinoblastoma (pRb), which is responsible for G1/S transition (Partensky et al., 2013). Moreover, certain miRs such as miR-222 can directly target p27 and p57, which are essential cell cycle inhibitors (Medina et al., 2008). A further study has also denoted that dysregulated expression levels of both miR-132 and miR-212 can cause G2/M cell cycle arrest and decreased cell proliferation in PDAC (Park et al., 2011). Nevertheless, these mechanisms have not been fully understood in PDAC and therefore, further investigation of these pathway regulators in PDAC is vital (Zhao et al., 2018a) (**Figure 5**).

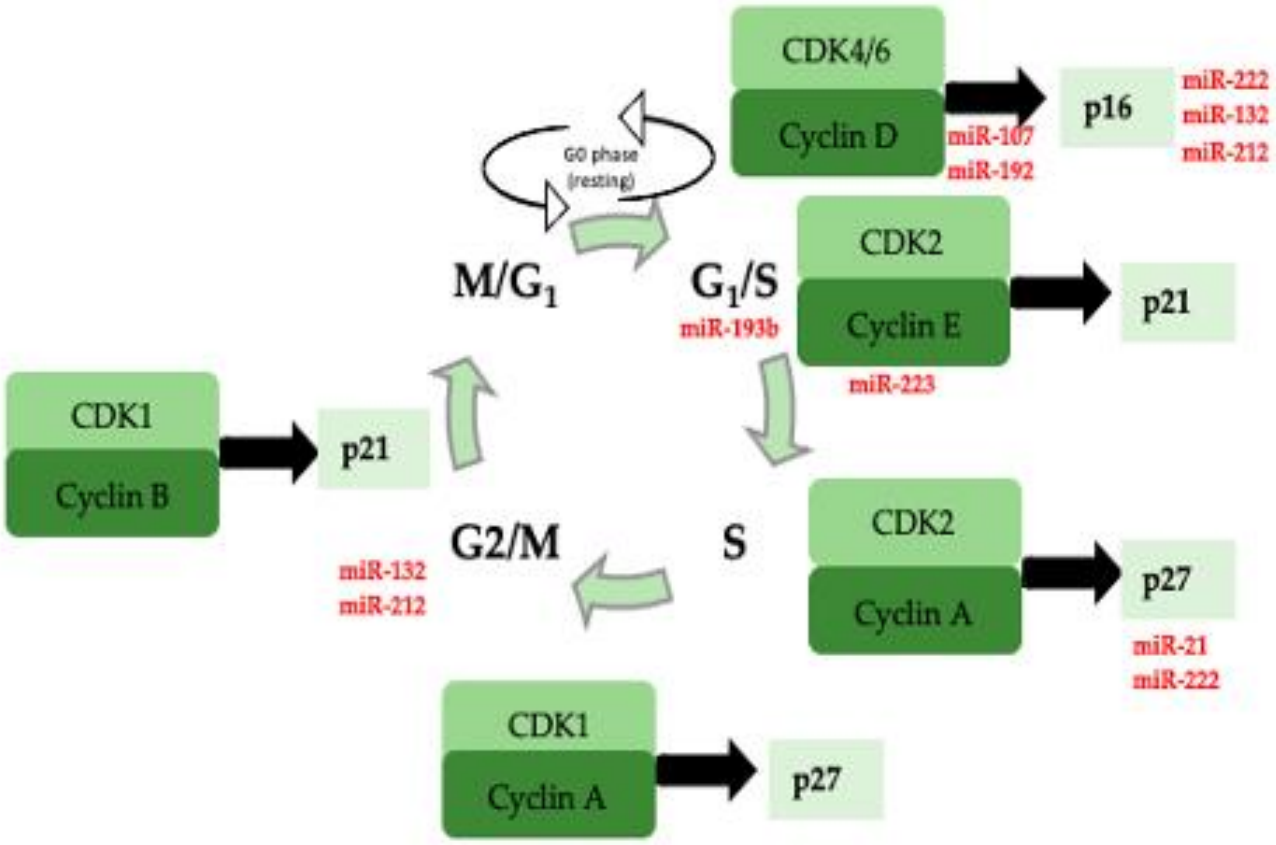


Figure 5: Role of miRs in cell cycle. Several miRs are associated with alterations in cell cycle signalling. Specifically, miR-21 and miR-222 are closely linked to both p27 and p16, while miR-132 with p21, which is a key regulator in cell cycle signalling.

1.4.10. Transcription Factors and DNA Methylation

Transcription factors can affect the downstream gene transcription of signal transduction pathways, which are targeted not only by genetic alterations, but also through epigenetic alterations, which lead to the aggressiveness of PDAC (Nebert et al., 2002). Specifically, both DNA methylation and histone tail alterations are associated with epigenetic modulation through chromatin remodelling (Lomberk et al., 2015). Controversially, forkhead members of the class O (FOXO) transcription factors have been characterised as tumour suppressors in numerous biological processes, such as stress resistance, metabolism, cell cycle, apoptosis, oxidative stress, (Farhan et al., 2017) and DNA repair (Roy et al., 2011). MiR-21 can target forkhead box protein 1 (*FOXO1),* which promotes PDAC growth *in vivo and in vitro* (Song et al., 2015). Similarly, *FOXO3a* can be targeted by miR-155 in PDAC, which further leads to cell proliferation and metastasis through the generation of reactive oxygen species (Wang et al., 2015). In addition, NF-κB is closely related to cellular processes and its expression is attributed to the aggressiveness of PDAC (Fujioka et al., 2003). NF-κB can be downregulated upon the upregulation of miR-146a in PDAC (Li et al., 2010a); NF-κB activity can be also increased via miR-301a in PDAC, which results in increased tumour growth (Lu et al., 2010). Previous studies have also revealed that DNA methylation plays a crucial role during PDAC development (Denis et al., 2011). Specifically, DNA methyltransferase 1 (DNMT1) is commonly upregulated in several cancer types (Dhe-Paganon, et al., 2011), including PDAC, and can be targeted both by miR-148b and miR-152 (Azizi et al., 2014). Moreover, in PDAC, miR-141 can directly target Yes-associated protein 1 (YAP1), which is a main downstream effector of the protein kinase Hpo (HIPPO) pathway. The Hippo pathway controls tissue homeostasis, organ size, regeneration, and tumourigenesis (Moroishi et al., 2015). MiR-217 can directly target SIRT1, which is an NAD-dependent deacetylase that regulates cell proliferation, differentiation, apoptosis, metabolism, DNA damage, stress responses, genome stability, and cell survival (Chen et al., 2013a). Upregulated SIRT1 can also generate EMT in PDAC patients (Deng et al., 2014) (**Table 4, Figure 6**).

Table 4: MiRs related signalling pathways and target genes in PDAC.

miRs	Regulation in PDAC	Signalling Pathways Involved	Target Genes	Functional Involvement in PDAC	References
let-7	Down	JAK-STAT K-RAS	*STAT3, SOCS3, N-cadherin, ZEB1*	Tumour growth, migration	(Patel et al., 2014)
let-7a	Up	NOTCH	*JAK, STAT*	EMT	(Brabletz et al., 2011)
let-7d	Up	K-RAS	*KRAS*	Cell proliferation, migration, invasion, apoptosis	(Jiao et al., 2012)
miR-21	Up	PI3K-AKT K-RAS EGF-R Cell Cycle Apoptosis	*G12D, p27, p57, FOXO1, Bcl-2, FasL, PI3K, AKT, PTEN, PDCDM, RECK, SPRY2*	Cell cycle arrest, apoptosis, gemcitabine resistance, aggressiveness	(Du Rieu et al., 2010; Park et al., 2009; Talotta et al., 2008)
miR-23a	Up	Apoptosis	*APAF1*	Apoptosis	(Liu et al., 2015b)
miR-24	Up	Apoptosis	*BIM*	Apoptosis, cell cycle	(Liu et al., 2015b)
miR-26a-5p	Down	HGF-MET	*ARMTL2, Cyclin E2, MMP12*	Cell proliferation, migration, survival, EMT	(Bao et al., 2012)

miR-27a	Up	K-RAS	*Sprouty2*	Cell proliferation, migration, invasion, apoptosis	(Ma et al., 2010)
miR-29a	Up	Wnt	*MUC1*	Cell proliferation, differentiation, invasion, migration, resistance of PDAC cells in gemcitabine	(Nagano et al., 2013)
miR-29c	Down	Wnt	*ZD4, FZD5, FRAT2, LRP-6*	Cell proliferation, differentiation, invasion, migration	(Jiang et al., 2015b)
miR-34a	Down	Tp53 NOTCH	*NOTCH1/2CDK6, SIRT1*	Cell proliferation, migration, invasion, apoptosis	(Jamieson et al., 2012; Nalls et al., 2011)
miR-96b	Down	K-RAS	*KRAS, AKT*	Cell proliferation, migration, invasion, apoptosis	(Yu et al., 2010)
miR-107	Up	Cell Cycle	*cyclin D1-dependent kinase*	Cell proliferation	(Lee et al., 2009)
miR-126	Down	K-RAS	*KRAS*	Cell proliferation, migration, invasion, apoptosis	(Jiao et al., 2012)
miR-130b	Down	JAK-STAT	*STAT3*	Cell proliferation, invasion	(Yonemori et al., 2016; Zhao et al., 2013a)
miR-132	Up	P16	*Rb1*	G2/M cell cycle arrest, cell proliferation	(Park et al., 2011)
miR-141	Down	DNA Methylation	*YAP1, MAP4K4*	Tissue homeostasis, organ size and regeneration	(Moroishi et al., 2015)
miR-143	Down	K-RAS	*KRAS, GEF1/2, MMP-2, MMP1*	Tumour growth	(Kent et al., 2010)

miR-144	Down	NOTCH	*PRR11*	EMT	(Sureban et al., 2013)
miR-145	Down	K-RAS NOTCH	*KRAS*	Tumour growth, EMT	(Kent et al., 2010; Sureban et al., 2013)
miR-146a	Down	EGF-R	*EGFR, NF-κB, IRAK-1, MTA-2*	Increased tumour growth	(Ali et al., 2014)
miR-148a	Down	Cell Cycle	*CDC25B*	Cell proliferation, differentiation, invasion, migration	(Boutros et al., 2007)
miR-148b	Down	DNA Methylation	*DNMT1, AMPKa1*	Cell proliferation, differentiation, invasion, migration	(Azizi et al., 2014)
miR-150	Up	HER-2/neu	*MUC4, IGF-1R*	Invasion, migration	(Garajová et al., 2015)
miR-152	Down	DNA Methylation	*DNMT1*	Cell proliferation, differentiation, invasion, migration	(Azizi et al., 2014)
miR-155	Up	JAK-STAT Tp53	*SOCS1* *TP53-induced nuclear protein 1 gene, FOXO3a, RHOA, SMAD1/5, ZNF652*	Cell invasion, migration, metastasis, generation of reactive oxygen species	(Gironella et al., 2007; Huang et al., 2013; Yonemori et al., 2016)
miR-181a	Up	PI3K-AKT	*PTEN*	Migration	(Liu et al., 2014c)
miR-192	Up	Cell Cycle	*cyclin D1, cyclin D2, CDK4, CDC2*	Cell proliferation	(Zhao et al., 2013b)
miR-193b	Down	Cell Cycle	*KRAS*	G1-phase arrest	(Willis et al., 2005)

miR-200	Up	NOTCH	*Jagged1, Maml2,Maml3*	EMT	(Brabletz et al., 2011)
miR-200c	Down	EGF-R	*MIG6, EP300*	Invasion, migration	(Izumchenko et al., 2014)
miR-203	Up	Tp53	*p53*	Cell proliferation, migration, invasion, apoptosis	(Greither et al., 2010)
miR-206	Down	K-RAS	*KRAS, ANXA2*	Decrease of proangiogenic and proinflammatory, tumour growth and progression	(Jamieson et al., 2012; Keklikoglou et al., 2015)
miR-212	Up	P16 Hedgehog	*PTCA1, Rb1,*	G2/M cell cycle arrest, cell proliferation	(Ma et al., 2014; Park et al., 2011)
miR-216a	Down	JAK-STAT	*JAK2*	Cell proliferation, invasion	(Hou et al., 2015; Yonemori et al., 2016)
miR-217	Down	K-RAS, DNA Methylation	*KRAS, SIRT1*	Cell proliferation, migration, invasion, apoptosis, metabolism, DNA damage, stress responses, genome stability and cell survival	(Chen et al., 2013a; Zhao et al., 2010)
miR-220c	Up	PI3K-AKT	*MUC4*	EMT	(Yonemori et al., 2016)
miR-221	Up	PI3K-AKT	*Cdk4, p16, E2F, CDKN1B, MMP-2, MMP-9, PUMA, PTEN, MDM2, ICAM-1, P27, BIM, SOD2, STAT5A*	Proliferation, migration	(Sarkar et al., 2013)

miR-222	Up	Tp53 P16 Cell cycle	*p53, p27, p57, MMP2, MMP9, PUMA, PTEN, BIM, MMP1, SOD2, STAT5*	Cell proliferation, migration, invasion, apoptosis	(Greither et al., 2010; Medina et al., 2008; Zhao et al., 2015a)
miR-223	Up	Cell Cycle	*FBXW7, Cyclin E2*	Cell proliferation, differentiation, invasion, migration	(Ma et al., 2015; Xu et al., 2016)
miR-301a	Up	Cell Cycle	*Bim, Bak, Bax, NF-κB*	Cell proliferation	(Chen et al., 2012)
miR-375	Down	PI3K-AKT	*PDK1*	Cell proliferation, invasion, migration	(Yonemori et al., 2016; Zhou et al., 2014b)
miR-421	Up	TGF-β	*SMAD4*	Cell proliferation, migration, invasion	(Hao et al., 2011)
miR-424-5p	Up	HGF-MET	*SOCS6*	Cell proliferation, migration, survival, EMT	(Wu et al., 2013)
miR-483-3p	Up	TGF-β	*SMAD4*	Cell proliferation, migration, invasion	(Hao et al., 2010)
miR-494	Down	TGF-β	*FOXM1*	Cell proliferation, migration, invasion, increased resistance to gemcitabine	(Li et al., 2014c)

ZEB: Zinc Finger E-Box Binding Homeobox 1; PI3K: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; BCL2: Apoptosis Regulator; FasL: Fas Ligand; RECK: Reversion-inducing-cysteine-rich protein with kazal motifs; SPRY2: Sprouty homolog 2; ARMTL2: Aryl Hydrocarbon Receptor Nuclear Translocator Like; MMP12: Matrix Metallopeptidase 12; FRAT2: FRAT Regulator of WNT Signalling Pathway 2; Rb1: RB Transcriptional Corepressor 1; MAP4K4: Mitogen-Activated Protein Kinase 4; PRR11: Proline Rich 11; IRAK-1: Interleukin 1 Receptor Associated Kinase 1; DNMT1 DNA Methyltransferase 1; RHOA: Ras

Homolog Family Member A; ZNF652: Zinc Finger Protein 652; MIG6: Mitogen-inducible gene 6; EP300: E1A Binding Protein P300; E2F1: E2F Transcription Factor 1; PUMA: p53 upregulated modulator of apoptosis; SOD2: Superoxide Dismutase 2. Adapted from Mortoglou et al., (2021b).

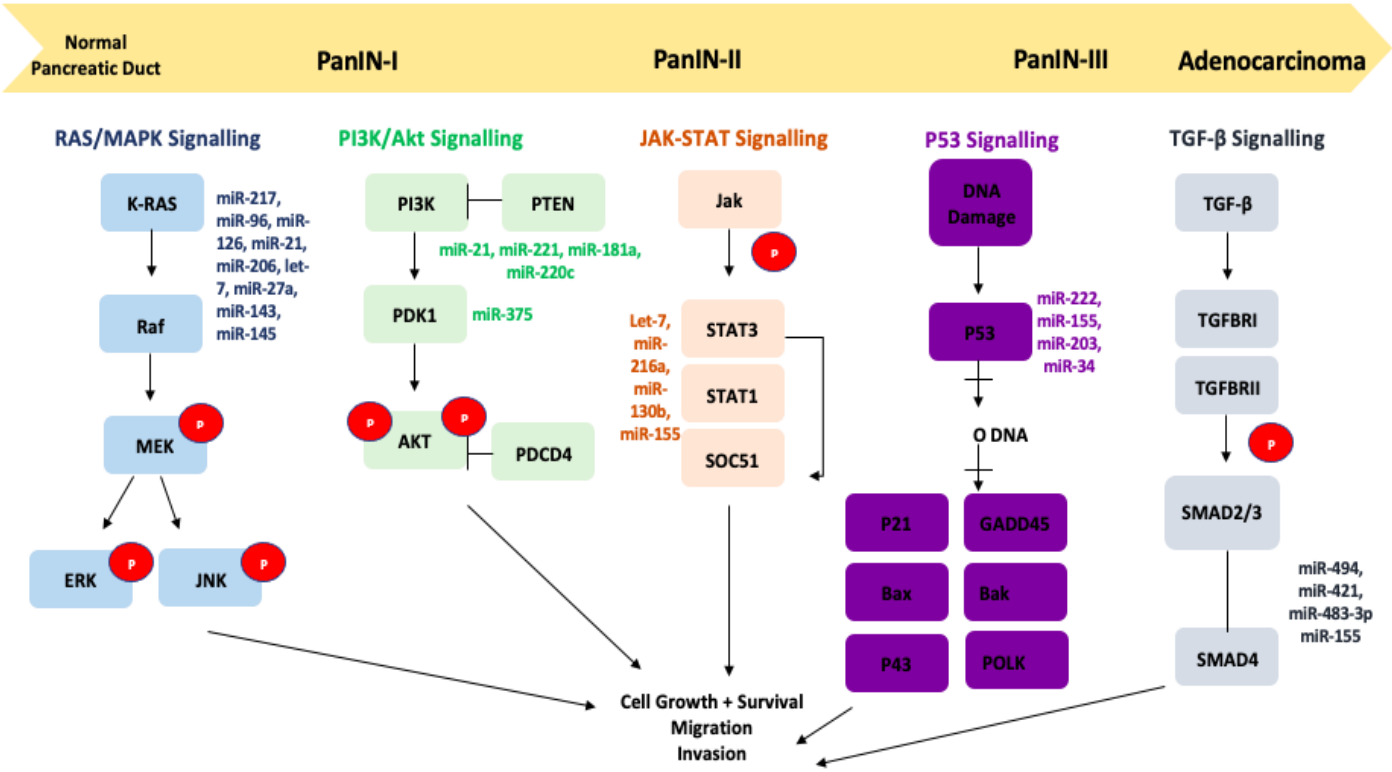


Figure 6: Mechanistic role of miRs in PDAC prognosis and diagnosis. MiRs are involved with main signalling pathways in PDAC. RAS/MAPK, JAK-STAT and PI3K/Akt signalling pathways are associated with earlier stages of PDAC progression, while p53 and TGF-β with more advanced stages. Adapted from Mortoglou et al., (2021b).

1.5. Epigenetic Regulation in PDAC via miRs

Chromatin alterations such as promoter methylation and histone deacetylation are common factors, which can silence numerous tumour suppression genes in several cancer types (Ye et al., 2020). Similarly, miRs can also act as epigenetic regulators in PDAC (Ye et al., 2020); even the research on the impact of epigenetic alterations related to miRs in PDAC is limited (Li et al., 2009a). Specifically, a recent study has suggested the consistent CpG methylation of miR-34a and miR-34-b/c in tumour tissues including mammary, colorectal, and pancreatic tumours, and soft tissue sarcomas (Vogt et al., 2011). Another study has shown that CpG island methylation can also affect downregulation of the tumour suppressor miR-1247 (Yi et al., 2017). Besides, miR-137 can downregulate the expression levels of mortality factor 4 (MORF4)-related gene-binding protein (MRGBP), while the loss of miR-137 can result in the overexpression of MRGBP. MRGBP can bind directly to the histone acetyltransferase complex, and its loss can lead to the suppression of PDAC cell growth and replication, promotion of apoptosis and inhibition of migration and invasion (Ding et al., 2018). The re-expression of miR-137 can significantly inhibit migration and invasion of PDAC cells, therefore, loss of miR-137 can act as a pioneer tumour promoter through the overexpression of MRGBP in PDAC (Ding et al., 2018).

Furthermore, it has been suggested that epigenetically silenced miRs can prompt EMT. A study by Zagorac et al. (2016) revealed that CSCs had elevated levels of DNA methylation due to higher DNMT1 levels, which can result in the suppression of the miR-17-92 cluster. Specifically, targeting DNMT1 blocked both the self-renewal and *in vivo* tumorigenic potential of CSCs via the re-expression of miR-17-92 (Zagorac et al., 2016). Additionally, the restoration of miR-377 can lead to the decrease of DNMT1 expression levels and reactivation of target genes *BNIP3* and *SPARC* through promoter demethylation. This can result in the inhibition of cell proliferation and promotion of apoptosis in PDAC cells (Azizi et al., 2017). Synthetic histone

methylation reversal agents can epigenetically reprogramme both miR-663a and miR-4787-5p, which results in the attenuation of *TGF-β1*–induced EMT features in PDAC (Mody et al., 2016). Expression levels of miR-663b can be suppressed epigenetically through the long noncoding RNA, homeobox (HOX) transcript antisense RNA (HOTAIR), which is responsible for cell cycle regulation, apoptosis, and migration in PDAC cells (Cai et al., 2016). Particularly, upregulation of miR-663b can lead not only to the targeting of cell proliferation, invasion, and migration but also to the induction of apoptosis. The re-expression of mir-663b can block tumour growth by targeting insulin-like growth factor 2 in PDAC (Cai et al., 2016). Therefore, it can be concluded that the validation of the epigenetic alterations related to miRs, can prompt new insights for more effective PDAC therapies.

1.6. Current & Potential Therapy Approaches by using miRs in PDAC

Several researchers have examined the regulation of miR activity for the development of therapeutics strategies for PDAC, while current therapies have limited impact on median overall survival of PDAC patients (Vorvis et al., 2016). MiRs can promote the generation of tailored treatment strategies for individual PDAC cases (Subramani et al., 2015). Consequently, miR-targeting approaches can induce alterations in both chemosensitivity and radiosensitivity of PDAC cells (Vorvis et al., 2016). A handful of studies have indicated that antisense targeting of both miR-21 and miR-221 can promote an improvement in the chemosensitivity of gemcitabine and inhibition of cell proliferation (Park et al., 2009). Moreover, transfection of miR-21 could elevate the activity of *PTEN*, which further results in the enhancement of gemcitabine-induced cell apoptosis (Zhu et al., 2008). Another study suggested that the inhibition of miR-21 using a lentivirus vector can inhibit cell proliferation (Sicard et al., 2013). Additionally, the inhibition of miR-21 led to apoptosis and reduced PDAC cell proliferation (Moriyama et al., 2009), while low miR-21 expression levels correlated

with higher chemosensitivity to 5-FU (Hwang et al., 2010). It has also been suggested that miR-181b inhibition resulted in a high sensitivity to gemcitabine and higher levels of apoptosis (Cai et al., 2013). MiR-17-5p inhibition in PDAC cells causes reduced cell growth, elevated caspase-3 activation and a higher chemosensitivity to gemcitabine via the overexpression of *Bim* (Yan et al., 2012). MiR-205 mimics could promote the restoration of chemosensitivity to gemcitabine and a minimised expression of stem cell markers *OCT3/4* and *CD44* (Singh et al., 2013). Furthermore, expression of tumour suppressor miR-205 decreased PDAC cell invasion and the restoration of gemcitabine chemosensitivity (Mittal et al., 2014).

Elevated expression levels of miR-23b can lead to the inhibition of radiation-induced autophagy and sensitisation of PDAC cells to radiation (Wang et al., 2013a). The restoration of let-7 in PDAC cells showed an inhibition of cell proliferation, *KRAS* expression and *MAPK* activation (Torrisani et al., 2009), while Zhao et al. (2010) showed that miR-127 directly inhibits KRAS as the transfection of PDAC cells with miR-217 resulted in a significant suppression of cell growth through the reduction in K-RAS protein levels and decreased downstream activation of the AKT pathway. Moreover, upregulated miR-148b resulted in the suppression of PDAC cell growth, which further led to the induction of apoptosis and cell-cycle arrest at S phase, inhibition of invasion and enhancement of chemosensitivity of PDAC cells (Zhao et al., 2013c). In addition, miR-137 mimic resulted in reduced cell invasion, tumour growth *in vivo* and an elevated sensitivity to fluorouracil in Panc-1 PDAC cells (Xiao et al., 2014). Similarly, miR-216a upregulation can have as an outcome the targeting of the *JAK2*/*STAT3* signalling pathway and xenograft tumour growth *in vivo* in PDAC cells (Wang et al., 2014b). Another study showed that when miR-218 expression is restored, cell migration and invasion reduced in PDAC (He et al., 2014a). Furthermore, miR-34a and miR-143/145 expressions showed a therapeutic efficacy, inhibition of tumour growth and induction of apoptosis in PDAC subcutaneous and orthotopic xenograft models through the downregulation of *SIRT1*, CD44, aldehyde

dehydrogenase (ALDH), *KRAS*, and RAS responsive element binding protein 1 (*RREB1)* (Pramanik et al., 2011). Particularly, miR-34a nanocomplexes can considerably result in the suppression of PDAC cell growth and in the elevation of apoptosis through the downregulation of *E2F3*, *Bcl-2, c-myc* and cyclin D1 (Hu et al., 2013). Therefore, miR-34a nanocomplexes could be nominated as novel therapeutic strategies for PDAC (Hu et al., 2013).

Further studies revealed that inhibition of miR-155 expression led to the re-expression of TP53INP1 and enhanced apoptosis (Gironella et al., 2007). Moreover, when miR-27a was targeted, reduced cell growth, colony formation and migration were observed in Panc-1 PDAC cell line (Ma et al., 2015a). Specifically, it has been stated that the expression of P-glycoprotein, which is a multidrug efflux pump that has been characterised as mechanism of resistance of cancer cells, is controlled both by miR-27a and miR-451 (Gilles et al., 2018). Anti-miR-371-5p treatment can result in the inhibition of cell proliferation (He et al., 2014c), whereas combined inhibition of miR-21, miR-23a and miR-27a can have as a synergistic result a decreased cell proliferation, which could be utilised for the development of effective PDAC therapies (Frampton et al., 2014). It has been also suggested that miR-506 can result in the enhancement of chemosensitivity in PDAC through the targeting of cell proliferation and the induction of cell cycle arrest at the G_1/S transition (Jung et al., 2016). Specifically, *SPHK1* is downregulated by miR-506 and participates in *Akt*/*NF-kB*–dependent apoptosis, which further induces chemoresistance (Jung et al., 2016). The knockdown of miR-1246 caused gemcitabine sensitivity in gemcitabine-resistant PDAC cell lines (Frampton et al., 2014); upregulation of transcription factor–activating protein 2γ, which is negatively controlled by miR-10a-5p and can lead to the re-sensitisation of PDAC cells to gemcitabine (Xiong et al., 2018).

MiR-34b could be used as an effective therapeutic agent, since it negatively regulates oncogenic *SMAD3* (Liu et al., 2013), while miR-101 expression can promote E-cadherin

and thus, decrease PDAC tumour growth (Qazi et al., 2012). MiR-204 expression can have as an outcome the downregulation of myeloid cell leukemia-1 (*Mcl-1*) as well as apoptosis (Chen et al., 2013b). Furthermore, targeting miR-31 *in vitro* reduces cell proliferation, migration, and invasion (Laurila et al., 2012). Besides, targeting both miR-132 and miR-212 by antisense miR oligonucleotides led to the inhibition of PDAC tumour growth through the action on the retinoblastoma tumour suppressor (*Rb1*) (Hernandez et al., 2016). It has been also proposed that the restoration of miR-150 could inhibit cell growth in PDAC cells (Srivastava et al., 2011). Conclusively, it can be suggested that miR-based therapeutics could be a pioneer and effective therapeutic approach for PDAC (Li et al., 2016b). However, specific issues, including the specificity in the delivery to certain cells of interest and safety, should be also addressed (Sun et al., 2014).

Moreover, a study by Wang et al. (2016) showed that downregulation of miR-320a increased resistance to fluorouracil through its effects on programmed cell death 4. Additionally, it has been reported that miR-365 can induce gemcitabine resistance in PDAC cell lines through the targeting of Src homology 2 domain containing 1 *(SHC1)* and apoptosis promoting protein BAX. Especially the knockdown of both *SHC1* and *BAX* elevated resistance to gemcitabine (Hamada et al., 2014). Recent studies also indicated that the enforced expression of miR-145 or miR-146a resulted in the reduction of PDAC cell growth and invasive capacity by targeting *ITGA11, MAGEA4, SET RPA1, MCM2, ABCC1, SPTBN1, SPTLC1 or EGFR, IRAK-1, MTA-2* (Huang et al., 2015; Li et al., 2010a). Arora and colleagues (2014) also promoted that the use of a poly (D, l-lactide-co glycolide)–based nano-formulation of miR-150 *(*miR-150-NF*)* in PDAC cell lines resulted in the reduction of cellular proliferation. Despite these promising preclinical developments, it is still uncertain how these formulations will be effectively applied in PDAC patients (**Figure 7**).

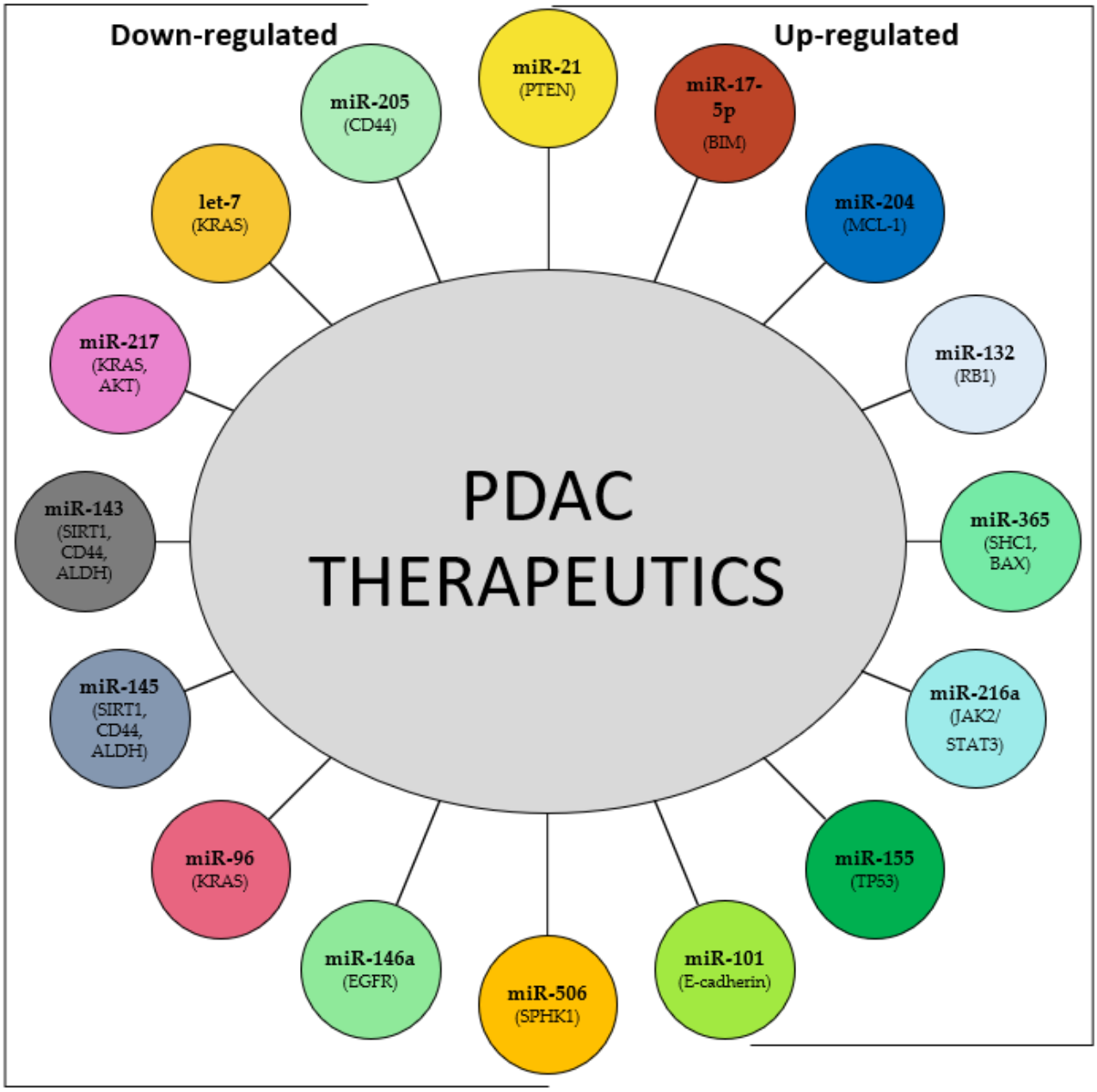


Figure 7: The role of miRs in PDAC therapy. Interactions between miRs and their target genes have prompted novel therapeutic strategies for PDAC. Specifically, miR-155, miR-21 and miR-221 together with their target genes could be used for the prediction of gemcitabine-based chemotherapy efficacy as first-line treatment of PDAC patients (Mortoglou et al., 2021a).

1.7. Aims and Objectives

The main aims of this project were to:

1) Summarise miR-mediated pathways, therapeutic resistance mechanisms and impact of metabolic alterations in the progression of PDAC.

2) Determine the roles and functions of miRs in diagnosis and treatments of PDAC.

3) Examine the expression levels of hsa-miR-21-5p, hsa-miR-221-5p, hsa-miR-155-5p and hsa-miR-126-p *in vitro* in three PDAC cell lines (Panc-1, MiaPaCa-2, BxPC3) compared to the normal pancreatic cell line (HPDE).

4) Generate hsa-miR-21-5p knockouts in Panc-1 and MiaPaCa-2 PDAC cell lines by using CRISPR/Cas9 technology.

5) Evaluate the role of hsa-miR-21-5p in PDAC stemness and EMT using CRISPR-mediated knockouts (KOs) approaches *in vitro*.

6) Study the role of hsa-miR-221-5p, hsa-miR-155-5p and hsa-miR-126-5p in response to key environmental toxins: cadmium chloride and nickel chloride in Panc-1 and MiaPaCa-2 PDAC cell lines.

7) Assess the role of cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors mediated therapeutic responses in Panc-1 and MiaPaCa-2 PDAC cell lines.

8) Generate 3D models in Panc-1 and MiaPaCa-2 PDAC cell lines and their miR-21 KOs and examine miRs signature profiles by using small RNA-sequencing.

Chapter 2: Methodology

2.1. Pancreatic Cell Lines & Cell Cultures and Reagents

The cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Three PDAC cell lines: Panc-1 (ATCC® CRL-1469™), MiaPaCa-2 (ATCC® CRL-x1420™), BxPC-3 (ATCC® CRL-1687™), and one non-tumorigenic human pancreatic ductal epithelial cell line (HPDE; H6c7, ATCC® CRL-4023) cell lines were cultured according to ATCC's recommendations, to 80% confluence in 75 cm^2 flasks in complete Dulbecco's Modified Eagle's Medium (DMEM), with 10% foetal bovine serum (FBS) at 37 °C with 5% CO_2. Unless otherwise specified, cells were grown in their defined optimum growth media. PDAC cell lines were selected based on their genetic profile as following: (Panc-1 and BxPC3: TP53, CDKN2A, MAP2K4, SMAD4 mutations; MiaPaCa-2: TP53, CDKN2A, MAP2K4, KRAS mutations).

PD-0332991 was purchased from Selleck Chemicals (Houston, TX, USA) and was dissolved in dimethyl sulfoxide (DMSO) at an initial stock concentration of 10 mM and stored as aliquots at −20 °C. For the PD-0332991 experiments (**Chapter 6**), Panc-1 and MiaPaCa-2 cells were treated with 2 μM for 24 h PD-0332991 based on previously published studies by Rencuzogullari et al. (2020) and Sevgin et al. (2021). Specifically, these studies have shown that significant changes in cellular viability, proliferation and apoptosis were observed when Panc-1 and MiaPaCa-2 cells were treated with 2 μM for 24 h compared to doses 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 7.5 and 10 μM for 24 h.

2.2. CRISPR/Cas9 Assay

The lentiviral CRISPR/Cas9-mediated miR-21 gene editing vectors encoding four different gRNAs, eGFP (control), and Cas9 protein was kindly provided by Dr. Junming Yue, University of Tennessee Health Science Center, USA, and produced based on the recommendations of previously published studies (Huo et al., 2017; Livak et al., 2001; Yue et al., 2010). Specifically, stable cell lines were generated by

transducing the MiaPaca-2 and Panc-1 cells with the lentiviral CRISPR/Cas9 miR-21 gene editing vectors and selection in puromycin (1–10 μg/mL). For the validation of the efficiency of the CRISPR/Cas9 gRNAs, a genomic cleavage assay using the GeneArt™ genomic cleavage detection kit (Invitrogen, Loughborough, UK) was carried out. Genomic DNA was extracted from PDAC Panc-1 and MiaPaCa-2 cells transduced with miR-21 lentiviral CRISPR/Cas9 and four different gRNAs or control vectors (GFP empty and wildtype). Genomic cleavage assay was performed following the manufacturer's protocol. Initial PCR was performed by amplifying the gRNAs targeting region using the primers pri-miR-21F (5′-GGGGATTTCTTGGTTTGTGAA-3′) and pri-miR-21R (5′-ATACAGCTAGAAAAGTCCCTGAAAA-3′) and applying a PCR annealing temperature of 55 °C. 3 μL of each PCR product was run on a 2% agarose gel for amplification check-up, and the remaining amount was used in the cleavage assay. PCR samples were cleaved using a detection enzyme, and cleavage products were run and analysed on a 4150 TapeStation system using a high sensitivity D1000 ScreenTape and reagents (Agilent, UK). Nondigested samples were included as cleavage control. The total fraction cleaved was represented by two electrophoresis bands/peaks. The sum of the percentage of the integrated area of the two cleaved bands corresponded to the total fraction cleaved. The following equation was used to calculate the cleavage efficiency of each clone: Cleavage Efficiency= 1– [(1–fraction cleaved). The clone/gRNA presenting the highest cleavage (CRISPR/Cas9) efficiency (KO2, KO4) was selected for further functional studies. The clone/gRNA performance data are presented in **Supplementary Figures 1-2.** CRISPR miR-21 KOs were used in **Chapter 3, and 6.**

2.3. RNA Extraction and RT-qPCR

Total RNA was isolated from Panc-1, MiaPaCa-2, BxPC-3, and HPDE (stored at –80 °C) using RNAzol® RT (Sigma, Hertfordshire, UK). Specifically, cells were isolated by centrifugation at 500 × g for 5 min, then lysed in 0.5 mL of RNAzol and allowed to stand for 15 min at room temperature. Then lysed cells were centrifuged at 12,000 × g

for 15 min at room temperature; the supernatant was mixed with an equal volume of 100% isopropanol to precipitate RNA, let stand for 10 min, and then centrifuged at 12,000 × g for 10 min at room temperature. DNA digestion was performed by using a RNAse-free DNase set (Qiagen, Manchester, UK), according to the manufacturers' instructions to effectively prevent DNA contamination. Briefly, 10 μL DNAse I stock solution was added to 70 μL Buffer RDD, after mixing with pipetted 80 μL DNAse I and incubating at room temperature for 15 min. Then, RNA pellets were washed twice with 0.5 mL 75% ethanol (v/v) per 1 mL of supernatant and centrifuged at 8,000 × g for 3 min at room temperature. The alcohol solution was removed with a micropipette, the RNA pellet was solubilised in RNAse-free water, and samples were vortexed at room temperature for 3 min. The RNA concentration was measured using the NanoDrop Spectrophotometer (ThermoFisher Scientific, Hemel Hempstead, UK.) at 260 nm and 280 nm absorbance.

Reverse transcription of RNA to cDNA was carried out using the miRCURYâLNAâ RT Kit (Qiagen, UK) with incubations at 25 °C for 5 min, 42 °C for 60 min and 95 °C for 5 min. The miRCURYâ LNAâ miRNA SYBRâ Green (Qiagen, UK) was used in conjunction with MystiCq microRNA qPCR primers for miR-21 (has-miR-21-5p), miR-155 (hsa-miR-155-5p), miR-221 (hsa-miR-221-5p) and miR-126 (hsa-miR-126-5p), which were all obtained from Sigma (UK) (**Table 5**). The conditions for thermocycling were heat activation at 95 °C for 2 min, followed by 40 cycles at denaturation at 95 °C for 10 s and combined annealing/extension at 56 °C for 60 s. The miR-21, miR-155, miR-221 and miR-126 expression levels were normalised to that of RNU6 using the CT/2^$^{\Delta\Delta CT}$ method (Livak et al., 2001). These miRs were examined *in vitro* n **Chapter 3.**

Table 5: Mature sequences for miRs primers.

miRs	Mature Sequence
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGU
hsa-miR-221-5p	ACCUGGCAUACAAUGUAGAUUU
hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG

Mature sequences have been given from miRBase database (https://www.mirbase.org/) (Manchester, UK).

cDNAs to assess the mRNA expression levels of E-cadherin, Vimentin, Snail, Wnt-11, Zeb1, CD44, CD133, CXCR4, and ALDH1 were synthesised by using qScript™ cDNA Supermix (Quantabio, Lutterworth, UK) as follows incubations at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. Precision®Plus qPCR Master Mix (Primer Design, Chandler's Ford, UK) was used for RT-qPCR synthesis for the assessed EMT and CSCs markers with the following thermocycling conditions for 40 cycles: 95 °C for 2 min, 95 °C for 10 s, and 60 °C for 60 s. The primers for Snail, Wnt-11, and E-cadherin, were designed and purchased from Sigma (Paisley, UK), Vimentin, and Zeb1 from Integrated DNA Technologies (IDT) (Leuven, Belgium), while CD133, CD24, CD44, CXCR4, and ALDH1, from ThermoFisher Scientific (UK). Primer sequences are presented in **Table 6**. Relative levels of mRNA expression were calculated as described before (Arisan et al., 2021) by using the comparative $CT/2^{-\Delta\Delta Ct}$ method (Livak et al., 2001) with RNA polymerase II (RPII) as the reference gene for the in-cell-line-based studies. CD133, CD24, CD44, CXCR4 and ALDH1 CSCs were examined in **Chapter 3**, whereas Snail, Wnt-11, E-cadherin, Vimentin and Zeb1 in **Chapters 3, 4**.

Table 6: Sequences of primers used in the study.

Primer	Forward Sequence (5′–3′)	Reverse Sequence (5′–3′)
E-cadherin	AAGAAGCTGGCTGACATGTACGGA	CCACCAGCAACGTGATTTCTGCAT
Wnt-11	GTGAAGGACTCGGAACTCGT	CTTCTGTTCCTGGTGGCTTC
Snail	TTTCTGGTTCTGTGTCCTCTGCCT	TGAGTCTGTCAGCCTTTGTCCTGT
Vimentin	TACAGGAAGCTGCTGGAAGG	ACCAGAGGGAGTGAATCCAG
Zeb1	GGGAGGAGCAGTGAAAGAGA	TTTCTTGCCCTTCCTTTCTG
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
RPII	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA
CD133	AAGCATTGGCATCTTCTATGG	AAGCACAGAGGGTCATTGAGA
CD24	GAAAACTGAATCTCCATTCCACAA	TGAAGAACATGTGAGAGGTTTGAC
CD44	CCAGAAGGAACAGTGGTTTGGC	ACTGTCCTCTGGGCTTGGTGTT
ALDH1	ATCAAAGAAGCTGCCGGGAA	TCTTAGCCCGCTCAACACTC
CXCR4	GCCAACCATGATGTGCTGAAAC	GCCAACGTCAGTGAGGCAGA

2.4. Flow Cytometry Analysis and Cell Cycle Analysis

Cells (seeding density 2×10^5 in each well/6-well plate) were pelleted by centrifugation for 5 min at 500 × g. The supernatant was removed, and cells were resuspended in 10 µL of CD133 (APC Mouse Anti-Human CD133), CD13 (CD13 PE), and CD24 (Alexa Fluor® 700 Rat Anti-Mouse CD24) antibodies (BD, Berkshire, UK) and incubated for 30 min on ice in the dark. Then, cells were washed twice with 1 × phosphate-buffered saline (PBS) for 5 min at 500 × g and resuspended in 200 µL of 1 × PBS.

For PD-0332991 study, Panc-1 and MiaPaCa-2 wt and their miR-21 KOs were seeded at 5 × 10^4 in 6-well plates and exposed to PD-0332991 (2 μM, for 24 h). Following trypsinisation, the cells were centrifuged at 500 × g for 5 min. The cells were then fixed with 70% ethanol and incubated at 4°C until the analysis (Rencuzogulları et al., 2020). Later, the cells were stained with propidium iodide (PI)/RNase staining buffer for 30 min and analysed by a flow cytometer (BD Accuri Bioscience, Franklin Lakes, NJ, USA). Results for both experiments were analysed using BD LSRFortessa X-20 (BD Bioscience, UK). PI results were shown in **Chapter 6**.

2.5. Immunostaining

Panc-1 wt (Control) and miR-21 KO cells were seeded into 6-well plates and allowed to settle overnight; the cells were washed with 1 × PBS and fixed with 4% formaldehyde for 20 min at room temperature. Cells were washed with 1× PBS twice, following blocking with 5% w/v bovine serum albumin (Invitrogen, Loughborough, UK) for 30 min at room temperature. The wells were washed with PBS twice and primary antibodies E-cadherin, Snail (20C8) (Invitrogen, Waltham, MA, USA), and Wnt-11 (GeneTex; CA, USA) (1/1000) were added and incubated for 1 h. After washing the cells, either goat anti-rabbit IgG Alexa Fluor or anti-mouse IgG (1/500) (ThermoFisher, Oxford, UK) was added and incubated for 1 h. Following another washing step with 1 × PBS, Ribonuclease A 100 mg/mL (Sigma–Poole, Dorset, UK) was added and incubated, gently rocking for 20 min. For counterstaining, 5 μL/mL of 1 nM To-Pro-3 (ThermoFisher Scientific, Oxford, UK) was dispensed into each well and set gently rocking, and then washed twice with PBS for 5 min gently rocking. The results generated were taken from the three biological and technical repetitions. Three to four millilitres of 1 × PBS was added to each well, Leica TCS SP2 (Leica Microsystems; Milton Keynes, UK) confocal microscope was used to analyse the cells. Immunostaining was assessed in **Chapter 3**.

2.6. Matrigel Invasion

Matrigel cell invasion assay was performed as described previously (Uysal-Onganer et al., 2021; Uysal-Onganer et al., 2020; Yi et al., 2013). Briefly, 5×10^5 cells were plated on 24-well Matrigel-coated transwell filters (Matrigel™ Invasion Chamber, Corning; BD Biosciences, Wokingham, Berkshire, UK) in a chemotactic gradient of 1:10% FBS. After 16 h, cells on the lower surface fixed with methanol and counted with the use of a light microscope (**Chapter 3**).

2.7. Colony Formation

Briefly, both MiaPaCa-2 and Panc-1 cells transduced with lentiviral miR-21 gRNA and control vectors were seeded at 1×10^4 density in 6-well plates and incubated for 12 days. After the medium was removed and cells were washed with 1 × PBS solution, cells were fixed with methanol: acetic acid (3:1) for 20 min at room temperature. After removing the fixing agent, cells were stained with 0.5% w/v crystal violet in methanol for 30 min, cells were washed with distilled water, and cells were imaged using an EVOS FL Auto 2 Imaging System (ThermoFisher, UK) (**Chapter 3**).

For PD-0332991 study (**Chapter 6**), Panc-1 and MiaPaCa-2 wt and their miR-21 KOs were seeded at 3×10^3 cells/well in 6-well plates and allowed to adhere for 24 h. After attachment, the cells were treated with PD-0332991 (2 μM, for 24 h). After 24 h, drug-containing media were removed, and the cells were allowed to form colonies in complete media for 10 days, and then colonies were stained as described above. The colony numbers were quantified by using Image J (ImageJ (nih.gov)).

2.8. Cadmium Chloride and Nickel Chloride Treatment

Panc-1 and MiaPaCa-2 cells were split in 6-well plates at 5-day intervals depending on confluence and cells were grown to 80% confluency in preparation for 14 days treatment with cadmium chloride (**Chapter 4**) and nickel chloride (**Chapter 5**) ($CdCl_2$, $NiCl_2$; 50 μM). The selection of the specific concentration (50 μM) for both $CdCl_2$ and

$NiCl_2$ is based on previously published data by Djordjevic et al., (2019), who showed that the lethal concentration (LC_{50}) value in PDAC cells (AsPC-1, BxPC3, Panc-1, Panc-10.5 and MiaPaCa-2) was 50 μM.

2.9. Cell Viability Assay

Panc-1 and MiaPaCa-2 cells and their miR-21 KOs were plated (1×10^4 cells per well in 96-well plates) and incubated for 16 h to determine the cell proliferation by a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. After 16 h incubation, the total number of invaded cells was determined by a MTT assay (Abcam, Cambridge, UK). Absorbance was measured using a CLARIOstar plate reader (BMG Labtech, Aylesbury, UK) at 540–590 nm and normalised according to the control. The experiments were performed three times from different biological samples with 3 technical repeats (**Chapters 3**).

The effects of the CDK4/6 inhibitor PD-0332991 on Panc-1 and MiaPaC-2 cell lines wt and their KOs were determined by a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cells were seeded at a density of 1×10^4 cells per well in 96-well plates and treated with PD-0332991 (2 μM, for 24 h) as previously described (Mortoglou et al., 2022; Rencuzogullari et al., 2020). Then, 10 μL of MTT dye (5 mg/mL in PBS, Sigma; St. Louis, MO, USA) was added to the culture medium and incubated at 37 °C for 4 h. To solubilise the formazan crystals converted from MTT by the mitochondrial enzymes, 100 μl DMSO (Sigma; St. Louis, MO, USA) was added. The absorbance of the suspension at 570 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA) (**Chapters 6)**.

2.10. Caspase activity assay

Measurements of caspase activities in Panc-1 and MiaPaCa-2 wt and their miR-21 KOs were performed using the commercially available Caspase-Glo 3/7 Assay (Promega, Madison, WI), according to the manufacturer's instructions. Cells were harvested after

24 h incubation with PD-0332991 (2 μM) and subjected to caspase-3 activity detection. Specifically, Caspase-Glo® 3/7 Assay reagent (100μl) was prepared according to the Caspase-Glo® 3/7 Assay Technical Bulletin TB323 and added to each well. Plates were briefly shaken and incubated at room temperature for 1 h. Caspase-Glo® 3/7 luminescent signal was read on the BMG FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany). Background signal (media-only wells) was subtracted from all values and mean ± standard deviation (SD) were calculated from triplicates (**Chapter 6**).

2.11. Senescence β-Galactosidase Staining

Panc-1 and MiaPaCa-2 wt and their miR-21 KOs were seeded as 3×10^5 cells in 6-well plates and were treated with PD-0332991 (2 μM, for 24 h). After 24 h of PD-0332991 (2 μM) exposure, the medium was removed, and the cells were washed twice with 1 × PBS. Cells were fixed according to the protocol indicated by Senescence β-Galactosidase (SA-β) Staining Kit #9860 (CST, Danvers, MA, USA). Then, cells were microscopically examined after β-gal administration using EVOS FL Auto 2 Imaging System (ThermoFisher, UK) (Dhir et al., 2019; Yuedi et al., 2020). The colony numbers were quantified by using Image J (**Chapter 6**).

2.12. Wound Healing Assay

Panc-1 and MiaPaCa-2 wt and their miR-21 KOs were seeded at 80% density cells/well in 24-well plates until cells reached monolayer confluence and then were exposed to PD-0332991 (2 μM, for 24 h). A straight scratch on the cell monolayers was created using a sterile 10 μL pipette tip to obtain a wound in the monolayer. Detached cells were washed with 1 × PBS gently, and cells were then supplemented with a renewed medium containing 5%/*v*/*v* FBS and incubated at 37 °C. Wound closure was observed every 24 h until the wound closed using EVOS FL Auto 2 Imaging System (ThermoFisher, UK) (×10). The program was used for analysing the wound area at

different time points and presented by bar graphics using GraphPad software (v.9) (**Chapter 6**).

2.13. XF Cell Mito Stress Test

Oxygen consumption rate (OCR) was measured using the Seahorse XF Cell Mito Stress Test Kit (Aglient, UK) according to the manufacturer's instructions. Briefly, Panc-1 and MiaPaCa-2 wt and miR-21 KOs cells were seeded in XF24 cell culture microplates at 4×10^4 cells/well density until cells reached monolayer confluence. After the treatment of the cells with PD-0332991 (2 μM, for 24 h), the culture medium was replaced with Seahorse XF Base Medium (Agilent, UK), 2 mM glutamine, 1 mM sodium pyruvate and 10 mM glucose, pH 7.4) and the plate was pre-incubated for 1h at 37°C in a non-CO_2 incubator. OCR was measured under basal conditions, and then after sequential injections of different reagents, where their concentrations were optimised for each cell line as: 1.5 μM oligomycin (respiratory Complex V inhibitor that allows for the calculation of adenosine triphosphate (ATP) production by mitochondria), 2.5 μM (Panc-1 cells) and 3 μM (MiaPaCa-2 cells) carbonyl cyanide-p-trifluoromethoxy-phenyl-hydrazon (FCCP; an uncoupling agent allowing determination of the maximal respiration and the spare capacity), and finally a combination of 1 μM rotenone and 1 μM antimycin A (Complex I and III inhibitors, respectively) to stop mitochondrial respiration enabling the calculation of the background (i.e., non-mitochondrial respiration driven by processes outside the mitochondria). Wave Software (version 2.6.3) was used to design, run, and view results from the MitoStress assay. OCR measurements were normalised to protein content using the Bradford Assay carried out as follows: after the measurements, cells were collected, lysed in RIPA (Sigma-Aldrich, Gillingham, UK, containing 10% protease inhibitor cocktail, Sigma-Aldrich), proteins from cells were extracted as described in section 2.14 and quantified by Bradford Assay. For Bradford assay, five different dilutions of a protein standard was prepared (0-2 mg/ml), then 10 μl of each standard and sample solution was added in 96-wells plate and 200 μl of Bradford dye

reagent (Bio-Rad, UK) was added. Plate was incubated at room temperature for 5 min and absorbance was measured at 595 nm with a microplate reader (Bio-Rad, Hercules, CA, USA) (**Chapter 6**).

2.14. Protein Isolation and Liquid Chromatography-Mass Spectrometry (LC-MS/MS) Proteomic Analysis

Total protein was extracted from treated and Panc-1 and MiaPaCa-2 wt cells and their miR-21 KO2 by using RIPA + buffer (Sigma, UK), which was supplemented with 10% protease inhibitor complex (Sigma, UK). Samples were pipetting gently at regular intervals while continuously shaking the cell preparation on ice for 2 h. Then, the cell preparations were centrifuged at 16,000 × g at 4 °C for 30 min to collect the supernatants containing the extracted proteins. In-gel digestion was used for LC-MS/MS analysis, carried out by the Cambridge Centre for Proteomics (University of Cambridge, Cambridge, UK). The samples were prepared 1:1 in reducing Laemmli sample buffer (BioRad; containing 5% β-mercaptoethanol, Sigma-Aldrich, UK), boiled and run 0.5 cm into a 10% TGX gel (BioRad, UK), and then cut out as one whole band per sample in both Panc-1 and MiaPaCa-2 PDAC cell lines (treated/non-treated wt control cells, treated/non-treated miR-21 KO2 cells). Proteomic analysis was carried out by the Cambridge Centre for Proteomics (Cambridge, UK) according to previously described methods (Pamenter et al., 2019). Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits; individual ion scores > 30 indicated identity or extensive homology ($p < 0.05$). An R-script was written to analyse the proteins expressed under four different conditions (treated/non-treated wt control cells, treated/non-treated miR-21 KO2 cells) for the two PDAC cell lines (Panc-1 and MiaPaCa-2). For each cell line, the proteins that are uniquely expressed for each of these conditions were identified and the ones that were expressed under all the conditions. This R-script is open access (GPU v.3 licence) and can be downloaded from https://github.com/dsengupt/ProteomicProfileComparison.git (**Chapter 6**).

2.15. Protein Interaction Network Analysis

To identify local network clusters (STRING), gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways for proteins from treated with PD-0332991 and non-treated Panc-1 and MiaPaCa-2 wt and their miR-21 KO2s, STRING analysis was used (https://string-db.org/, accessed in October 2022). Predicted protein interaction networks were built based on hits identified from the LC-MS/MS analysis using the protein IDs and organism choice *Homo Sapiens* in the STRING software. For protein lists, "multiple proteins" was selected, confidence set at "medium," and network interaction connecting lines were based on known and predicted interactions. Protein networks were annotated for pathway analysis and data were exported as labelled network images for KEGG and/or Excel files for KEGG and Reactome pathways and STRING network clusters (**Chapter 6**).

2.16. Pancreatic 3D cell culture and Small RNA-sequencing

For the formation of 3D pancreatic culture cells, Cultrex™UltiMatrix Reduced Growth Factor (RGF) Basement Membrane Extract (BME) (Bio-techne, UK) was used. Specifically, Cultrex UltiMatrix RGF BME was thawed overnight at 4°C to prevent untimely gelling. Panc-1 and MiaPaCa-2 wt cells and miR-21 KO2 were cultured according to ATCC's recommendations, to 80% confluence in 75 cm^2 flasks in DMEM, with 10% FBS at 37 °C with 5% CO_2. After trypsinisation, cells were centrifuged at 500 × g for 3 min and medium was aspirated. Cell pellets were resuspended in Cultrex UltiMatrix RGF BME, and 50 μL of the Cultrex UltiMatrix RGF BME/organoid suspension was dispensed in the center of 6-well plates by using a pre-warmed (37°C) tissue culture plate. Then, inverted culture plates containing the domes were placed into a 37^0C incubator for 30 min and 1mL of organoid growth culture medium (DMEM) was added and changed every 3-4 days. After 14 days, cell culture media was removed, each well was washed with 1mL of ice-cold PBS and 1mL of Cultrex™ Organoid Harvesting Solution (Bio-techne, UK) was added in each well. 6-well plates were incubated at 4^0C for 1 h with moderate shaking and once matrix depolymerised,

content was centrifuged at 500 × g for 5 min at 4^0C. The cells pellet was washed with 1mL ice cold PBS and centrifuged again at 500 × g for 5 min at 4^0C. Isolated 3D culture cells were used for RNA extraction and small RNA-sequencing (**Chapter 6**).

RNA extraction was conducted by using the miRNeasy Micro Kit (Qiagen, UK) according to manufacturer's protocol. The RNA concentration was measured using the NanoDrop Spectrophotometer (ThermoFisher Scientific, Hemel Hempstead, UK) at 260 nm and 280 nm absorbance. Quality control, library preparation and small RNA sequencing was conducted by Novogene (Cambridge, UK) by using Illumina Sequencing (SE50). For the library preparation, NEB Next® Multiplex Small RNA Library Prep Set for Illumina kit was used. Quality control and electrophoresis results for RNA degradation are presented in **Supplementary Table 1 and Supplementary Figure 3**, respectively.

2.17. Small RNA-sequencing analysis

The unpurified raw data are 5' primer contains, no insert tags, oversized insertion, low quality reads, poly A tags and small tags. Contaminant reads was removed from the raw files and clean reads was filtered by using the following steps: (**1**) Exclude reads of which more than 50% bases has a base quality score no more than 5; (**2**) Exclude reads containing N > 10%; (**3**) Exclude reads with 5' primer contaminants; (**4**) Exclude reads without 3' primer and reads without the insert tag; (**5**) Trim 3' primer sequence; (**6**) Exclude reads with polyA/T/G/C. The small RNA adapted sequences, which were used were RNA 5′ Adapter (RA5), part: 5'-GTTCAGAGTTCTACAGTCCGACGATC-3' and RNA 3' Adapter (RA3), part: 5'-AGATCGGAAGAGCACACGTCT-3'. Homo Sapiens GRCh38/hg38 was used as a reference genome. Clean reads were further analysed by using Galaxy Software version 23.1.rc1 (https://usegalaxy.org/). MiR-related signalling pathways were

analysed by using DIANA tools mirPath (version 3) ((DIANA TOOLS - mirPath v.3 (uth.gr)) (Vlachos et al., 2015).

2.18. Statistical Analysis

GraphPad Prism version 9.01 (GraphPad Software, San Diego, USA) was used for statistical analysis and preparation of graphs. The expression levels of different miRs, proteins, CSCs, MTT, cell viability, colony formation, caspase 3/7, PI, wound assay and seahorse results in PDAC cell lines were examined using ANOVA and Bonferroni multiple comparisons tests followed by Tukey's post-hoc analysis. All data have been checked for normal distribution before any analysis. For the nickel chloride and cadmium chloride studies, statistical t-tests were used to assess the expression levels of selected miRs, and proteins compared with the controls. Experiments were carried out in technical triplicates. Statistical significance was conducted as Tukey at $p \leq 0.05$, while all the results were presented as mean ± SD.

2.19. Health and Safety

All the chemical safety regulations have been completed before the experiments (Chemical Hazards COSHH form).

2.20. Ethics

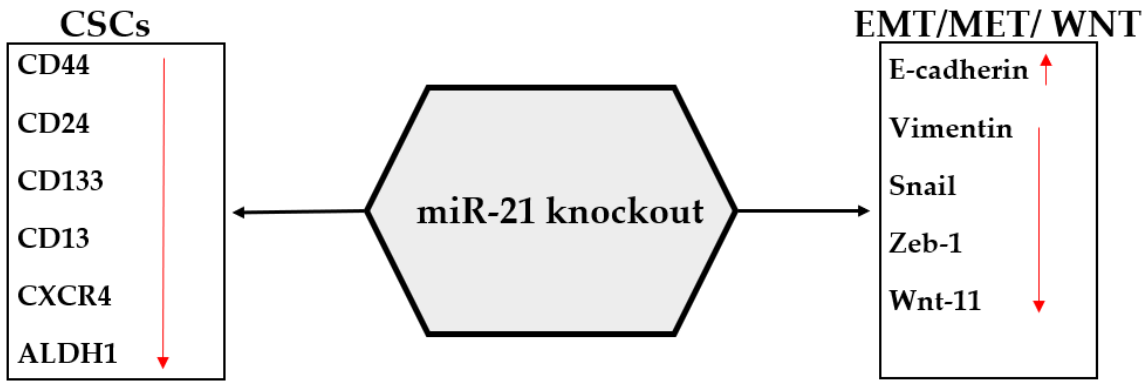
All the ethics have been considered and followed according to the University of Westminster Ethics Committee and UK regulations.

2.21. Funding

This work was supported by my University of Westminster SLS1 PhD studentship (September 2020-September 2023).

Chapter 3: MicroRNA-21 Regulates Stemness in Pancreatic Ductal Adenocarcinoma Cells

Graphical Abstract



Highlights

- MiR-21 knockout suppressed cellular invasion and proliferation of Panc-1 and MiaPaCa-2 PDAC cells.
- MiR-21 knockout reversed the expressions of EMT and CSCs markers.

3.1. Abstract

PDAC is the most common and aggressive type of pancreatic cancer (PCa) with a low survival rate. MiRs have been associated with the chemoresistance and metastasis of PDAC and the presence of a subpopulation of highly plastic "stem"-like cells within the tumour, known as CSCs. In this part of the study, the role of miR-21 was investigated, which is highly expressed in Panc-1 and MiaPaCa-2 PDAC cells in association with CSCs. Following miR-21 knockouts (KO) from both MiaPaCa-2 and Panc-1 cell lines, reversed expressions of EMT and CSCs markers were observed. The expression patterns of key CSC markers, including CD44, CD133, CX-C chemokine receptor type 4 (CXCR4), and ALDH1, were changed depending on miR-21 status.

CRISPR miR-21 (KO) suppressed cellular invasion of Panc-1 and MiaPaCa-2 cells, as well as the cellular proliferation of MiaPaCa-2 cells. The data of this current study suggest that miR-21 is involved in the stemness of PDAC cells, may play roles in mesenchymal transition, and that miR-21 poses as a novel, functional biomarker for PDAC aggressiveness.

Keywords: pancreatic ductal adenocarcinoma; microRNAs; non-coding RNAs; cancer stem cells; metastasis; epithelial–mesenchymal transition

3.2. Introduction

MiR-21 is one of the most oncogenic miRs in PDAC and has been shown to closely associated with cancer stemness. Specifically, in PDAC, miR-21 among with miR-155, and miR-221 have been found to act as oncomiRs, while miR-126 and miR-375 were shown to act as tumour suppressors miRs (Galasso et al., 2012; Hussain et al., 2016). MiR-21, miR-221, and miR-155 can distinguish cases of PDAC from healthy individuals with a sensitivity of approximately 64% and a specificity of 89% (Kosaka et al., 2010; Wang et al., 2009). Importantly, miRs present a higher diagnostic efficiency than the current diagnostic marker CA 19-9, especially for the early diagnosis of PDAC (Chan et al., 2014; Kojima et al., 2015; Sawabu et al., 2004; Schultz et al., 2014).

CSCs are involved in chemoresistance and play critical roles in the metastasis of several cancers, including PDAC (Kim et al., 2011; Klonisch et al., 2008; Li et al., 2007; Li, et al., 2009; Marcato et al., 2011; Miranda-Lorenzo et al., 2014; Niess et al., 2015; Valle et al., 2018). CSCs contribute to elevated expression levels of anti-apoptotic proteins, ABC transporters, and multidrug resistance genes, high autophagic flux that leads to microenvironment stresses (Cojoc et al., 2015; Dawood et al., 2014; Lei et al., 2017; Plaks et al., 2015; Santamaria et al., 2017). Pancreatic CSCs (PCSCs) are less than 1% of all pancreatic cancer cells and are critical mediators of PDAC tumour growth, maintenance, metastasis, and chemoresistance (Dalla Pozza et al., 2015).

EMT is characterised as a critical mechanism of the metastatic cascade, which includes the loss of cell-cell adhesion, elevated cell motility, the repression of E-cadherin, and the upregulation of mesenchymal markers, such as Vimentin, N-cadherin, Snail, and Zeb1 (Kalluri et al., 2009). E-cadherin downregulation is associated with poor prognosis, differentiation, and chemoresistance in PDAC (Felipe Lima et al., 2016; Iacobuzio-Donahue et al., 2009; Lamouille et al., 2014; Winter et al., 2008). Transcription marker Zeb1 suppresses E-cadherin through the repression of both miR-203 (an inhibitor of stemness) and miR-200 family members, which control the expression levels of stem cell factors (Renz et al., 2017). Zeb1 overexpression is linked to advanced PDAC stages and poor malignancy outcome, migration, and invasion in response to nuclear factor (NF)-κB signalling (Arumugam et al., 2009; Buck et al., 2007; Maier et al., 2010). Non-canonical Wnt-11 overexpression is associated with poor prognosis and TNM staging in PDAC (Arisan et al., 2020; Guo et al., 2009; Uysal-Onganer et al., 2010; Wang et al., 2016; Wang et al., 2018a).

Higher expression levels of Snail, a potent EMT-inducing transcription factor, are related to 80% of PDAC cases, E-cadherin downregulation, lymph node invasion, higher tumour grade, and poorly differentiated PDAC cells (Hotz et al., 2007). EMT is regulated via molecular pathways linked to oncogenic and tumour suppressor ncRNAs, chromatin remodelling, epigenetic and posttranslational modifications, alternative splicing events, and protein stability (De Craene and Berx, 2013; Fedele et al., 2017). Vimentin is an essential marker of EMT and associated with Notch and miR-200 expression levels and it can affect treatment response *in vitro*, including elevated gemcitabine-resistance in PDAC (Klymkowsky et al., 2009; Traub et al., 1985). Overexpression of Vimentin is linked to metastasis and poor overall survival in PDAC (Javle et al., 2007). Furthermore, the association between EMT and CSCs has been extensively evaluated, for instance, PDAC cells, which have undergone EMT, and express epithelial markers, such as E-cadherin and mesenchymal markers, such as Zinc Finger E-Box Binding Homeobox 1 (Zeb1), and Snail exhibit stem cell properties

(Rhim et al., 2012; Shibue et al., 2017). The main markers of PCSCs are CD133, CD24, CD44, ESA/EpCAM (epithelial-specific antigen), c-Met, ALDH1, DclK1, CXCR4, and Lgr5 (Rao et al., 2015; Santamaria et al., 2017). Importantly, EMT and autophagy processes are also closely linked with CSCs markers during PDAC development (Hruban et al., 2006; Kure et al., 2012). Recent reports stated that CD24, CD44, CXCR4, ESA, and nestin are upregulated in advanced pancreatic intraepithelial neoplasia (PanIN) grades (Oshima et al., 2007), while others have shown that the expression of $cMet^{+}$ $CD133^{+}$ $CD34^{+}$ $CD45^{-}$ $Ter119^{-}$, Pdx1, CD9, CD24, CD44, CD13, and CD133, are linked to poor prognosis of PDAC (Hruban et al., 2006; Kure et al., 2012; Lee et al., 2014; Li et al., 2015; Liu et al., 2018; Oshima et al., 2007; Wang et al., 2019a).

MiR-21 is related to PDAC prognosis; overexpression of miR-21 was detected in PDAC patients and correlated with poor prognosis and overall survival according to the TCGA dataset (**Supplementary Figure 4**).

3.3. Results

In summary, using three different PDAC cell lines (MiaPaca-2, Panc-1, and BxPC3), it was found that miR-21, miR-221, miR-155, and miR-126 expressions were significantly altered in PDAC cell lines compared with normal pancreatic ductal epithelial cell lines (HPDE). Following the knockout of miR-21 in Panc-1 and MiaPaca-2 cells using CRISPR/Cas9, reversed expressions of E-cadherin, Vimentin, Snail, Wnt-11, and Zeb1 were detected, suggesting that these markers are targets of miR-21. Expression levels of the key CSC markers, CD13, CD133, CD44, CD24, CXCR4, and ALDH1 were significantly downregulated depending on miR-21 status. KO of miR-21 led to a significant reduction in cellular invasiveness of Panc-1 and MiaPaCa-2 cells and a significant decrease in cellular proliferation of MiaPaCa-2 cells. Overall, these *in vitro* data suggest that miR-21 is strongly involved in the pathophysiology of PDAC.

3.3.1. Expression Profiles of miR-21, miR-221, miR-155, and miR-126 in PDAC *In Vitro*

PDAC associated miR-21, miR-155, miR-221, miR-155, and miR-126 expression levels were quantified using RT-qPCR in three different PDAC cell lines (MiaPaCa-2, Panc-1, and BxPC3), compared with human pancreatic ductal epithelial (HPDE), which served as the control cell line. The HPDE cells had the most epithelial properties expressed relatively, with the least miR-21-5p, miR-155-5p, and miR-221-5p and this was used as a 'baseline' (**Figure 8A–C**). Out of the cell lines tested, Panc-1 cells expressed the highest levels of miR-21-5p (117-fold elevation; $n = 3$; $p < 0.0001$; **Figure 8A**), miR-155-5p (105-fold increase; $n = 3$; $p < 0.0001$; **Figure 8B**), and miR-221-5p (32-fold upregulation; $n = 3$; $p < 0.0001$; **Figure 8C**) relatively compared with HPDE cells. Similarly, MiaPaCa-2 cells presented a 43-fold increase in the miR-21-5p ($n = 3$; $p < 0.05$; **Figure 8A**), a 51-fold overexpression in miR-155-5p ($n = 3$; $p < 0.01$; **Figure 8B**) and a 21-fold significant rise in miR-221-5p ($n = 3$; $p < 0.001$; **Figure 8C**). BxPC3 cells did not show significant changes in both miR-21-5p and miR-221-5p expressions (16-fold; 3-fold, respectively; $n = 3$; $p > 0.05$ for both; **Figure 8A,C**), however, they showed a 36-fold significant upregulation in miR-155-5p expression ($n = 3$; $p < 0.05$; **Figure 8B**). The expression levels of miR-126-5p indicated a 57-fold downregulation in BxPC3 cells ($n = 3$; $p < 0.01$; **Figure 8D**), a 81-fold decrease in the MiaPaCa-2 cell line ($n = 3$; $p < 0.001$; **Figure 8D**), and a 94-fold reduction in Panc-1 cells ($n = 3$; $p < 0.0001$; **Figure 8D**) in comparison with normal HPDE cells.

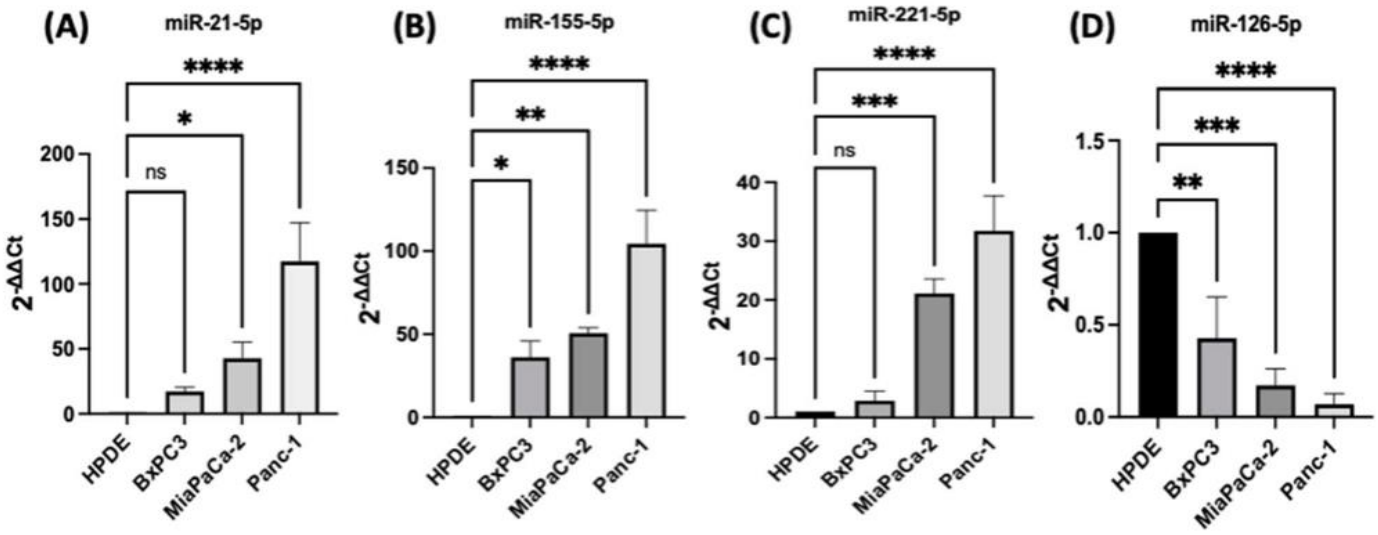


Figure 8: RT-qPCR analysis of miRs levels miR-21, miR-221, miR-155, and miR-126 in PDAC cell lines (BxPC3, MiaPaCa-2, Panc-1, compared with HPDE). (**A**) miR-21-5p relative expression was significantly increased in MiaPaCa-2 and Panc-1 but not in BxPC3 PDAC cell lines; (**B**) miR-155-5p relative expression was significantly overexpressed in BxPC3, MiaPaCa-2, and Panc-1 PDAC cell lines; (**C**) miR-221-5p relative expression was significantly upregulated in MiaPaCa-2 and Panc-1 but not in BxPC3; (**D**) miR-126-5p relative expression was significantly reduced in PDAC cell lines. The column graphs represent the average of three replicates of RNA isolated from each cell line. Data normalised according to RNU6 expression by fold analysis ($n = 3$, $p < 0.05$ for all). Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2022a).

3.3.2. Development of miR-21 KO MiaPaca-2 and Panc-1 Cell Lines

The qRT-PCR data showed that miR-21 expression was the most elevated in the Panc-1 and MiaPaCa-2 PDAC cell lines correlating with *in vivo* data, which was obtained from TCGA database (**Supplementary Figure 4**). Therefore, both Panc-1 and MiaPaca-2 were used to further investigate the effect of knocking out miR-21, and cells were transduced with four different miR-21 gRNAs, as well as a control vector. MiR-21 expression analysis showed that miR-21 expression was significantly reduced by 65 and 73-fold in Panc-1 knockout (KO) clones 2 (KO2) and 4 (KO4), respectively, compared with Panc-1 vector alone ($n = 3$; $p < 0.0001$ for all; **Figure 9A**). Similarly, expression levels of miR-21 were also significantly decreased by 83-fold in KO2 and 97-fold in KO4 ($n = 3$; $p < 0.01$ for all; **Figure 9B**) in MiaPaCa-2. The knockout miR-21 Panc-1 and MiaPaCa-2 PDAC cell lines were further assessed for EMT, and CSC markers and cellular invasion.

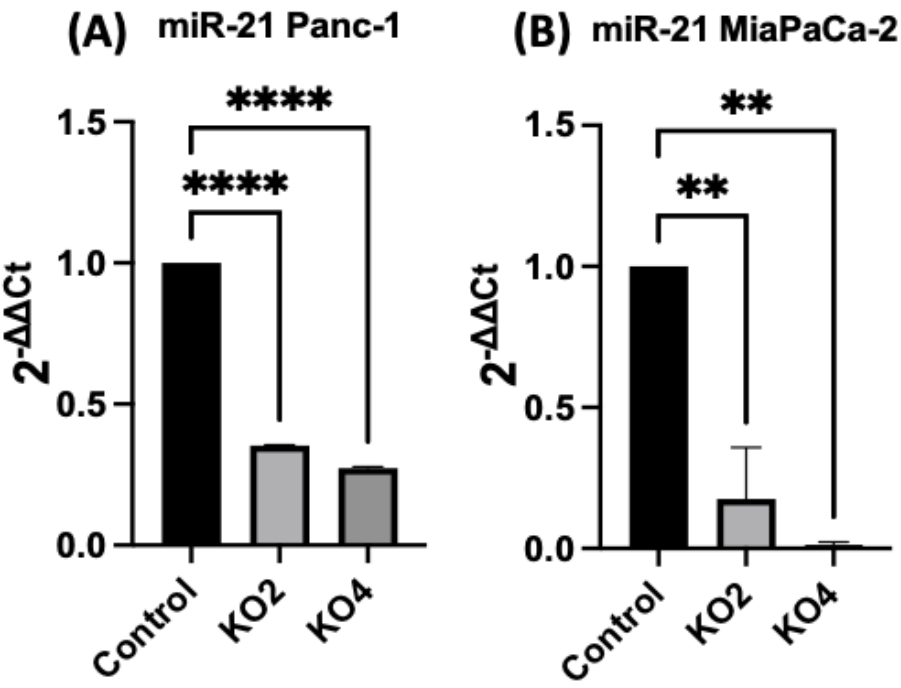


Figure 9: MiR-21 expression levels are significantly reduced in Panc-1 and MiaPaCa-2 KOs. (**A**) Relative expression levels of miR-21 in miR-21 KO Panc-1 cells. MiR-21 expression levels of miR-21 KO2 and miR-21 KO4 cell were downregulated significantly compared to the control Panc-1 cells ($n = 3$; $p < 0.0001$ for all). (**B**) Relative expression levels of miR-21 in miR-21 KO MiaPaCa-2 cells. MiR-21 expression levels of miR-21 KO2 and miR-21 KO4 cell were reduced significantly compared to the control MiaPaCa-2 cells ($n = 3$; $p < 0.01$ for all). The column graphic represents the average of three replicates of RNA isolated from Panc-1 and MiaPaCa-2 control and their KOs. Data normalised according to RNU6 expression by fold analysis ($n = 3$, $p < 0.05$). Exact p-values are indicated (** $p \leq 0.01$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2022a).

3.3.3. MiR-21 KOs in PDAC cells Reduce Expression Levels of EMT-Related Markers

The effects of miR-21 KO on regulation of key EMT was assessed using miR-21 KO in Panc-1 and MiaPaCa-2 PDAC cells. E-cadherin mRNA expression levels were found to be significantly overexpressed by 932-fold in Panc-1 miR-21 KO2 and by 906-fold in KO4 ($n = 3$; $p < 0.05$ and 0.01, respectively; **Figure 10A**). Vimentin mRNA levels decreased by 96-fold in KO2 and 99-fold in KO4 ($n = 3$; $p < 0.0001$ for all; **Figure 10A**), whereas Snail mRNA levels were 100-fold reduced in both KO2 and KO4 ($n = 3$; $p < 0.0001$ for all; **Figure 10A**). Zeb1 mRNA expression was reduced by 40-fold in KO2 and by 46-fold in KO4 ($n = 3$; $p < 0.01$ for all; **Figure 10A**) in comparison to the Panc-1 vector alone. The Wnt-11 mRNA expression level was significantly reduced in Panc-1 miR-21 KO4 (58-fold; $n = 3$; $p < 0.05$; **Figure 10A**), but there was not a significant reduction in KO2 (35-fold, $n = 3$; $p > 0.05$; **Figure 10A**). Similarly, in the MiaPaca-2, E-cadherin mRNA levels were significantly upregulated by 349-fold in KO2 ($n = 3$; $p < 0.01$; **Figure 10B**) and by 476-fold in KO4 ($n = 3$; $p < 0.001$; **Figure 10B**). Additionally, Vimentin mRNA levels were decreased by 71-fold in KO2 and by 99-fold in KO4 ($n = 3$; $p < 0.01$ and 0.001, respectively; **Figure 10B**), while Snail mRNA levels presented a significant decrease in KO2 (69-fold) and KO4 (80-fold) ($n = 3$; $p < 0.05$ for all; **Figure 10B**). Zeb1 mRNA levels were suppressed significantly by 49-fold and 77-fold in KO2 and KO4, respectively ($n = 3$; $p < 0.01$ and 0.001, respectively; **Figure 10B**), and Wnt-11 mRNA expression levels reduced in both KO2 and KO4 (57-fold and 49-fold, respectively; $n = 3$; $p < 0.01$ for all; **Figure 10B**). According to immunofluorescence results, E-cadherin was upregulated; Snail and Wnt-11 were downregulated in miR-21 KO clones when compared to control ($n = 3$; **Figure 10C**). Overall, the data of this study suggest that miR-21 is involved in EMT through suppressing epithelial characteristics in the cells.

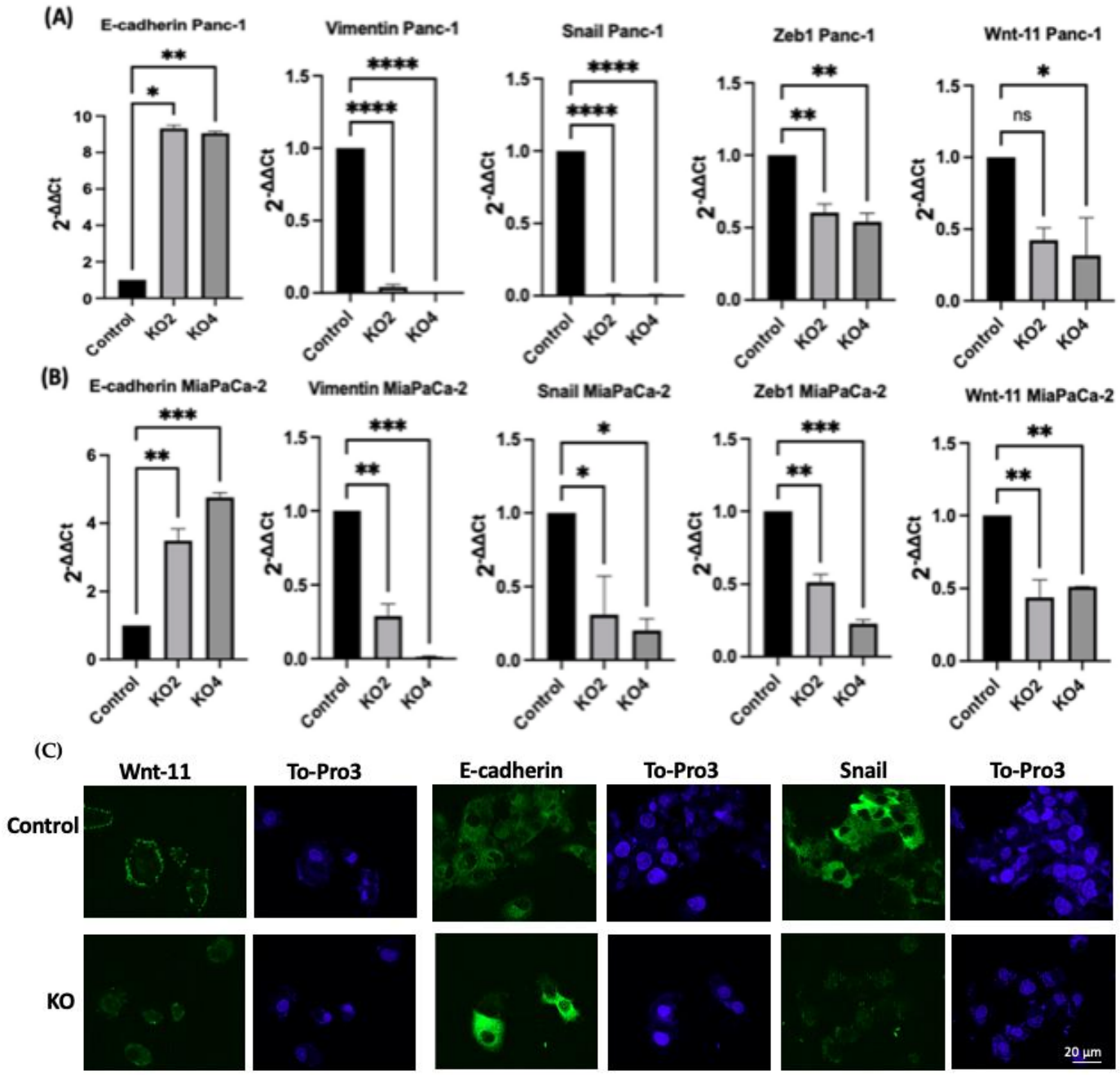


Figure 10: The silencing of miR-21 reduced the mesenchymal phenotype of PDAC cells. (**A**) E-cadherin, Vimentin, Snail, Wnt-11, and Zeb1 in the Panc-1 cell line relative mRNA expression levels. Relative mRNA expression levels of Vimentin, Snail, and Zeb1 were significantly downregulated in miR-21 KO2 and KO4 in the Panc-1 cell line, while E-cadherin was significantly upregulated. Relative mRNA expression levels of Wnt-11 were significantly reduced in miR-21 KO4 but not in miR-21 KO2 in the Panc-1 cell line. (**B**) Relative mRNA expression levels of E-cadherin, Vimentin, Snail, Wnt-

11, and Zeb1 in the MiaPaCa-2 cell line. Relative mRNA expression levels of Vimentin, Snail, Zeb1, and Wnt-11 significantly decreased in miR-21 KO2 and KO4 in the MiaPaCa-2 cell line, while E-cadherin significantly increased. The column graphic represents the average of three replicates of RNA isolated from Panc-1 and MiaPaCa-2 control and their KOs. Data normalised according to RPII expression by fold analysis ($n = 3$, $p < 0.05$). Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. (**C**) Immunofluorescence assays were performed to show Wnt-11, E-cadherin, Snail protein levels, and protein localisation (green) in Panc-1 wt (Control) and miR-21 KO cells. To-Pro3 (blue) was used for staining nuclei. KO2 immunofluorescence results are shown as a representative to compare to control ($n = 3$), the scale bar represents 20 μm in all images. Adapted from Mortoglou et al., (2022a).

3.3.4. MiR-21 KOs Diminish Expressions of CSC Markers in PDAC

The expression levels of specific selected CSCs, which are involved in PDAC progression were assessed in the miR-21 PDAC KO cell lines generated from both Panc-2 and MiaPaCa-2; this included CSC markers CD44, CD24, CD133, CXCR4, and ALDH1. In Panc-1 miR-21 KO clones 2 and 4, the mRNA expression levels of CD44 were decreased by 97% ($n = 3$; $p < 0.01$ for all; **Figure 11A**), whereas CD133 was downregulated by 76% in KO2 and by 52% in KO4 ($n = 3$; $p < 0.01$ and $p < 0.05$, respectively; **Figure 11A**). CXCR4 was reduced by 86% in KO2 and by 100% in KO4 ($n = 3$; $p < 0.0001$ for all; **Figure 11A**), whereas ALDH1 expression levels were reduced by 77% in KO2 and by 95% in KO4 ($n = 3$; $p < 0.01$ for all; **Figure 11A**). The mRNA expression levels of CD24 were reduced by 77% in KO2 and by 88% in KO4 ($n = 3$; $p < 0.05$ and $p < 0.05$, respectively; **Figure 11A**), compared with the control Panc-1 vector alone. In the MiaPaCa-2 miR-21 knockout cells, CD44 mRNA expression levels decreased significantly by 75% in KO2 ($n = 3$; $p < 0.001$; **Figure 11B**) and by 40% in KO4 ($n = 3$; $p < 0.01$; **Figure 11B**), whereas CD133 mRNA levels were decreased by 50% in KO2 and by 42% in KO4 ($n = 3$; $p < 0.05$ for all; **Figure 11B**). Similarly, CXCR4 mRNA expression levels were reduced by 51% in KO2 and by 69% in KO4 ($n = 3$; $p < 0.01$ for all; **Figure 11B**), while ALDH1 mRNA levels decreased by 66% in KO2 and 46% in KO4 ($n = 3$; $p < 0.001$ for all; **Figure 11B**). The mRNA expression levels of CD24 were significantly reduced by 73% in KO2 ($n = 3$; $p < 0.01$; **Figure 11B**) and by 53% in KO4 ($n = 3$; $p < 0.05$; **Figure 11B**). Hence, the findings show that through the knocking out of miR-21 in two PDAC cell lines, the stemness markers CD44, CD133, CD24, CXCR4, and ALHD1 were significantly reduced in both cell lines assessed.

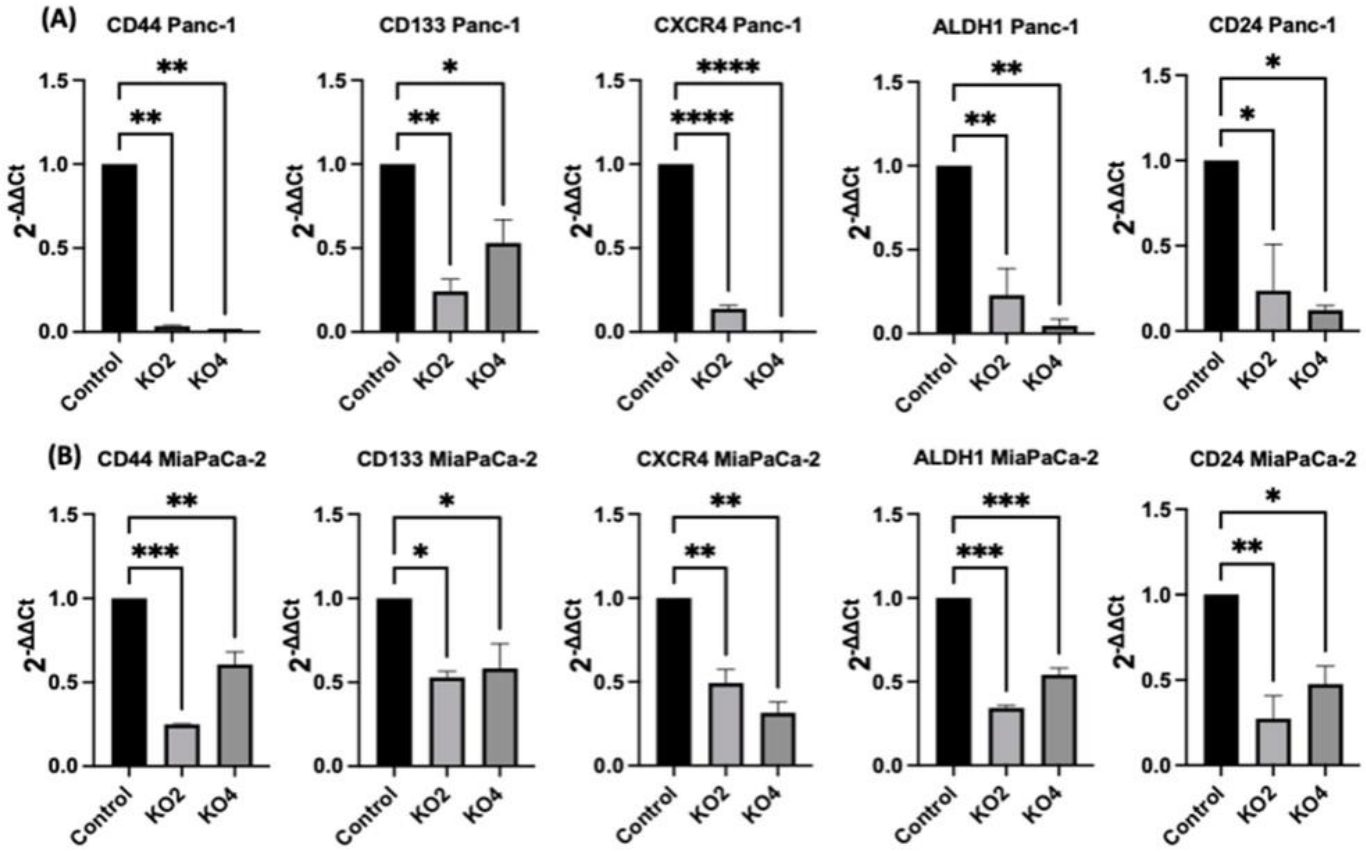


Figure 11: Expression of CSCs markers in PDAC cell lines following miR-21 knockout. (**A**) mRNA expression levels of CD44, CD133, CXCR4, ALDH1, and CD24 in the Panc-1 miR-21 KO cell line, compared with the Panc-1 vector alone. Relative mRNA expression levels of CD44, CD133, CXCR4, ALDH1, and CD24 are significantly reduced in miR-21 KO Panc-1 cells. (**B**) mRNA expression levels of CD44, CD133, CXCR4, ALDH1, and CD24 in the miR-21 KO MiaPaCa-2 cell line, compared with MiaPaCa-2 vector alone. Relative mRNA expression levels of CD44, CD133, CXCR4, ALDH1, and CD24 are significantly reduced in miR-21 KO MiaPaCa-2 cells. The column graphic represents the average of three replicates of RNA isolated from Panc-1 and MiaPaCa-2 control and their KOs. Data normalised according to RPII expression by fold analysis ($n = 3$, $p < 0.05$). Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2022a).

3.3.5. Flow Cytometry Analysis of PCSCs Expression in PDAC miR-21 KO Cells

Several key CSCs associated with PDAC, including CD24, CD133, and CD13 were assessed by flow cytometry in the miR-21 KO cells and compared with their expression in control cells. Results showed that CD133 was expressed at higher levels in Panc-1 control cells (96.9%; n = 3; **Figure 12A**), compared with Panc-1 KO2 (18.2%; 74.4-fold reduction; n = 3; $p < 0.001$; **Figure 12B**) and KO4 (21.8%; 69.3-fold reduction; n = 3; $p < 0.001$; **Figure 12C,G**). Similarly, in MiaPaCa-2 miR-21 KOs, the expression levels of CD133 were significantly higher in MiaPaCa-2 control (68.6%; n = 3; **Figure 12D,H**), compared with MiaPaCa-2 KO2 (17.9%; 52.3-fold decrease; n = 3; $p < 0.001$; **Figure 12E,H**) and KO4 (22.9%; 47.5-fold decrease; n = 3; $p < 0.001$; **Figure 12F,H**). Additionally, in the Panc-1 miR-21 KOs, the positive population for CD13 and CD24 presented a 100-fold and 95.7-fold reduction, respectively, in Panc-1 KO2 (0% and 4.3%, respectively; n = 3; **Figure1 3B**) and 100-fold reduction in KO4 (0.2% and 0.1%, respectively; n = 3; **Figure 13C**), compared with what was observed in the Panc-1 control, where the positive population for both CD13 and CD24 was 97.4% (n = 3; **Figure 13A**). In the MiaPaCa-2 cell line, control cells were positive for CD24 (81.9%; n = 3; **Figure 13D**) and negative for CD13 (0%; n = 3; **Figure 13D**), whereas the percentage of negative cell population for both CD13 and CD24 PCSCs in the MiaPaCa-2 KO2 was 96.4% (90.1-fold; n = 3; **Figure 13E**) and 97.7% in KO4 (91.4-fold; n = 3; **Figure 13F**; **Table 7**). These results indicate that the depletion of miR-21 from both PDAC cell lines results in a decrease in the stemness markers CD133, CD24 and CD133.

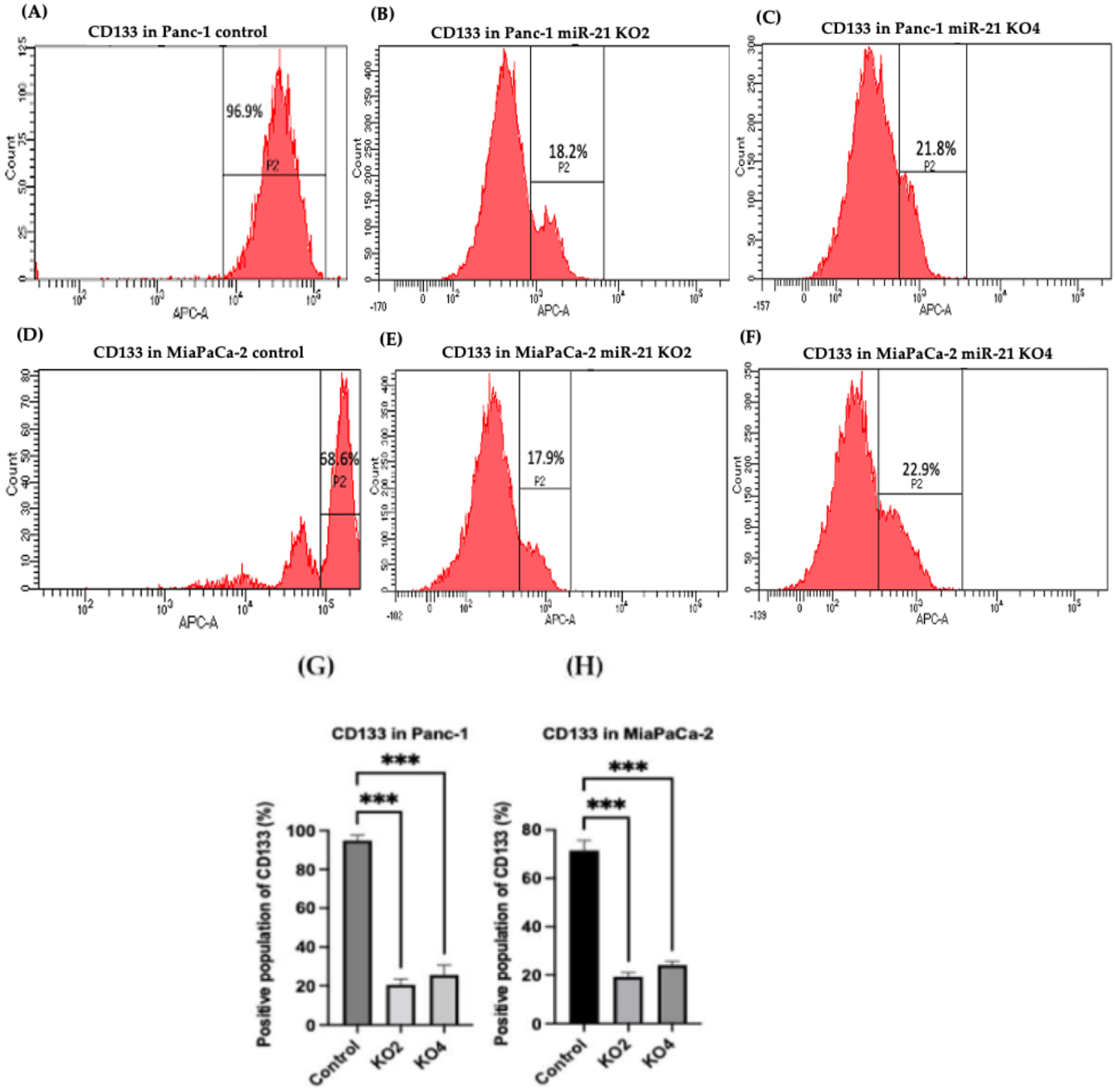


Figure 12: Flow cytometry analysis of CD133 in PDAC control and miR-21 KO cell lines. (**A**–**C**): Expression of CD133 in the Panc-1 miR-21 KOs and control cells: (**A**) Panc-1 vector alone (control cells); (**B**) miR-21 KO2; (**C**) miR-21 KO4. (**D**–**F**): Expression of CD133 in MiaPaCa-2 miR-21 KOs and control cells. (**D**) MiaPaCa-2 vector alone (control cells); (**E**) miR-21 KO2; (**F**) miR-21 KO4. (**G**) Percentage of the CD133 positive population in Panc-1 cells and their miR-21 KOs. CD133 expression was significantly increased in the Panc-1 vector alone compared with the miR-21 KOs in the Panc-1 cell line. (**H**) Percentage of the CD133 positive population in MiaPaCa-2 cells and their

miR-21 KOs. CD133 expression was significantly increased in MiaPaCa-2 control cells compared with their miR-21 KOs. Numbers in the gated areas mark the percentages of cells that were positive for this specific marker. Exact p-values are indicated (*** $p \leq 0.001$); error bars indicate SD. Adapted from Mortoglou et al., (2022a).

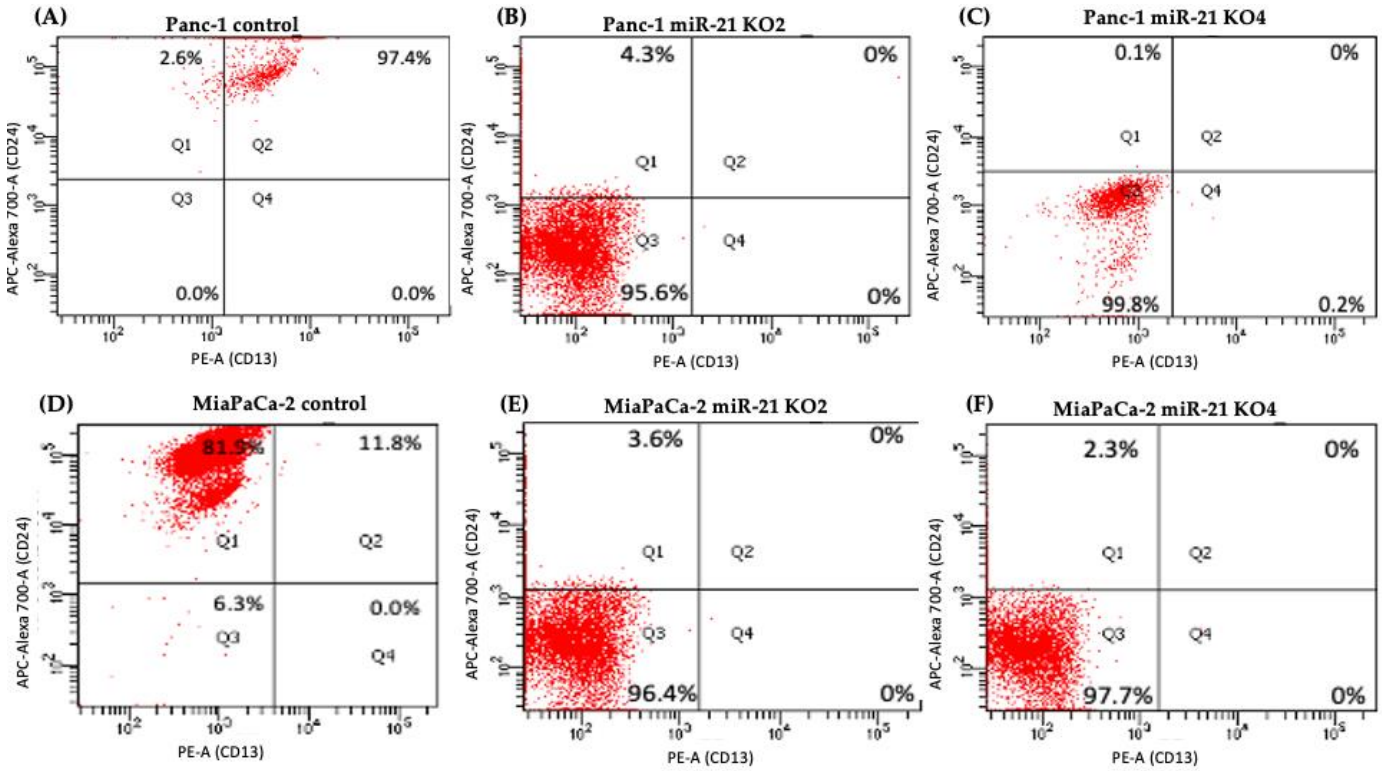


Figure 13: Flow cytometry analysis of CD24 and CD13 in miR-21 KO cell lines. (**A–C**) Expression of CD13 and CD24 in the Panc-1 KOs and control: (**A**) Panc-1 vector alone (control cells); (**B**) miR-21 KO2; (**C**) miR-21 KO4. Expression levels of both CD13 and CD24 were significantly reduced in Panc-1 miR-21 KO2 and KO4, compared with the Panc-1 control cells. (**D–F**): Expression of CD13 and CD24 in MiaPaCa-2 KOs and control: (**D**) MiaPaCa-2 vector alone (control cells); (**E**) miR-21 KO2; (**F**) miR-21 KO4. Expression levels of CD24 were significantly increased in MiaPaCa-2 control cells, while CD13 was significantly reduced not only in MiaPaCa-2 control cells but also in MiaPaCa-2 miR-21 KO2 and miR-21 KO4. APC-Alexa 700 symbolises the CD24 marker, while PE stands for CD13. Numbers in the gated areas mark the percentages of cells that were positive for this specific marker. Adapted from Mortoglou et al., (2022a).

Table 7: Percentage of expression of CD24 and CD13 in PDAC Cell Lines.

Cancer Stem Cell Markers	Panc-1 Control	Panc-1 KO2	Panc-1 KO4	MiaPaCa-2 Control	MiaPaCa-2 KO2	MiaPaCa-2 KO4
$CD24^+$ $CD13^+$	97.4%	0%	0%	11.8%	0%	0%
$CD24^-$ $CD13^-$	0%	95.6%	99.8%	6.3%	96.4%	97.7%
$CD24^+$ $CD13^-$	2.6%	4.3%	0.1%	81.9%	3.6%	2.3%
$CD24^-$ $CD13^+$	0%	0%	0.2%	0%	0%	0%

Adapted from Mortoglou et al., (2022a).

3.3.6. MiR-21 KOs Reduce Cellular Invasion in Panc-1 and MiaPaca-2 Cells

The invasiveness of both Panc-1 and MiaPaca-2 cells was studied by using Boyden chambers with Matrigel over a 16 h period. KO miR-21 resulted in a significant suppression of invasiveness by 31% (KO2) and 22 (KO4) % in Panc-1; 27% (KO2) and 16% (KO4) in MiaPaca-2 cells ($n = 3$; $p < 0.0001$ and 0.001; $p < 0.0001$, respectively; **Figure 14A**). There was no significant change in the cell number over the 16 h in Panc-1 cells; however, a significant 3% reduction in MiaPaCa-2 cellular proliferation was detected in miR-21 KOs ($n = 3$; $p > 0.05$; $p < 0.01$, respectively; **Figure 14B**). The results show that miR-21 KO reduces invasiveness. The Panc-1 miR-21 KO cells did not exhibit a decrease in clonogenicity compared to control cells after 12 days, however, the MiaPaCa-2 miR-21 KO cells presented a reduction in proliferation compared to MiaPaCa-2 control (**Figure 14C**).

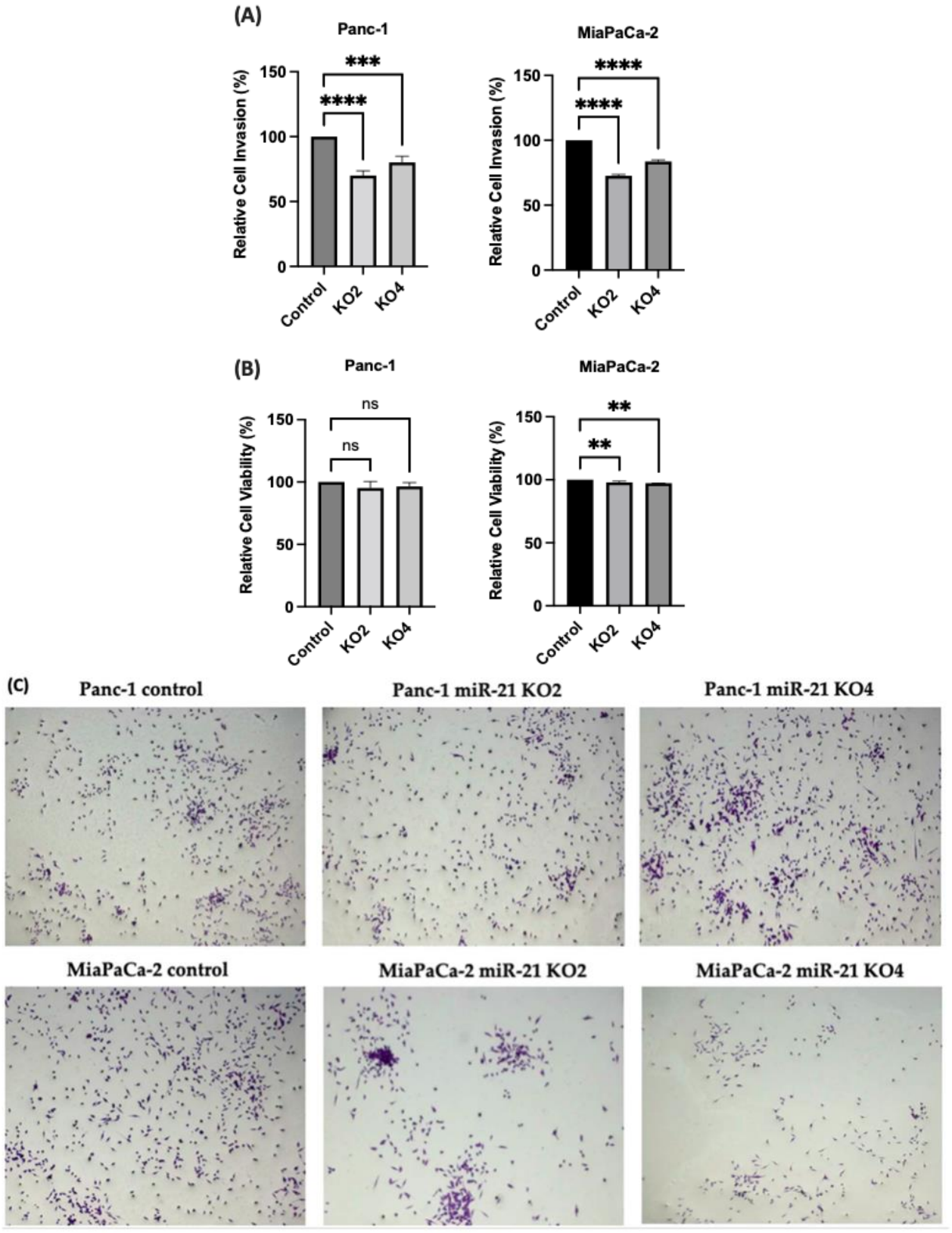


Figure 14: MiR-21 reduced cellular invasion of Panc-1 and MiaPaca-2 cells. (**A**) Control and miR-21 KO cells were plated on Matrigel-coated transwell filters and the extent of invasion was determined after 16 h. The results are plotted as Relative Cell

Invasion (%), which is the percentage of invaded cells compared to the total number of cells seeded. (**B**) The total cell number/proliferation did not change during the course of the experiment in the Panc-1 miR-21 KOs ($n = 3$; $p > 0.05$ for all), however, a small significant reduction was detected in the MiaPaCa-2 miR-21 KOs ($n = 3$; $p < 0.01$ for all). (**C**) Colony formation assay for Panc-1 and MiaPaCa-2 control and miR-21 KO cells. The colonies were observed with crystal violet staining of cells following 12 days. Images were obtained by using EVOS FL Auto 2 Imaging System (ThermoFisher, UK) with 10x magnification. Exact p-values are indicated (** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2022a).

3.4. Discussion

The main results of this study were that: **1**) The expression of oncomiRs miR-21, miR-155, miR-221, and tumour suppressor miR-126 is dysregulated in Panc-1 and MiaPaCa-2 PDAC cell lines compared to normal human ductal epithelial cells; **2**) miR-21 moderates mRNA expression levels of key EMT markers, Wnt-11 expression, and cancer stem-like markers; **3**) miR-21 KO significantly reduces cellular invasion capability of the two PDAC cell lines studied (Panc-1, MiaPaCa-2), indicating a role for miR-21 in cellular invasion capacity of PDAC *in vitro*. In this study, PDAC cell lines (BxPC3, Panc-1 and MiaPaCa-2) were selected based on their genetic profile as following: (Panc-1 and BxPC3: TP53, CDKN2A, MAP2K4, SMAD4 mutations; MiaPaCa-2: TP53, CDKN2A, MAP2K4, KRAS mutations) (Deer et al., 2018). It has been suggested previously that Panc-1 and MiaPaCa-2 cells present mesenchymal characteristics (Shichi et al., 2022); have low E-cadherin and high Vimentin mRNA levels, higher ATP concentrations and present highest migratory and invasive ability between 6 different PDAC cell lines and normal pancreatic cell line (HPDE6) (Minami et al., 2021). Specifically, it has been illustrated that Panc-1 exhibited the highest invasive and migratory abilities between MiaPaCa-2 and BxPC3 PDAC cell lines (Deer et al., 2018). Furthermore, it has been observed that CD24, CD44 and ALDH1 were highly expressed in Panc-1 and MiaPaCa-2 PDAC cell lines (Sasaki et al., 2019). Therefore, Panc-1 and MiaPaCa-2 PDAC cell lines were used to assess the role of miR-21 deletion in cellular proliferation, invasion, EMT and cancer stemness.

PDAC remains one of the main fatal malignancies with no specific biomarker for early diagnosis to date (He et al., 2023; Yi et al., 2013). However, in the last few years, several reports have suggested that miRs expression levels can be used as biomarkers to screen for PDAC diagnosis and prognosis (Deng et al., 2008). High levels of miR-21 expression have been detected in numerous cancers including PDAC (Qu et al., 2017; Zhou et al., 2014a). This part of the study showed that miR-21 expression was

significantly dysregulated in two of the PDAC cell lines, namely Panc-1 and MiaPaCa-2, compared to normal HPDE cells. However, miR-21 expression levels did not significantly change in the PDAC cell line, BxPC3. Overexpression of miR-221 has been shown to play a significant role in platelet-derived growth factor (PDGF)-mediated EMT phenotype, migration, metastasis, and uncontrolled proliferation of PDAC cells (Su et al., 2013). Importantly, when assessing the expression levels of different miRs in the current study, miR-221 was identified as being upregulated in both Panc-1 and MiaPaca-2 PDAC cells. In addition, miR-221 has been found to lead to the minimisation of stem cell repopulating activity in cord blood $CD34^+$ cells by targeting *KIT*, while they also act as inhibitors in the proliferation process of erythroleukemia cell lines (Felli et al., 2005).

Increased levels of miR-155 have previously been reported in PDAC patients compared with normal pancreatic tissues and have been shown to suppress the pro-apoptotic gene p53 (*TP53INP1*), which plays a crucial role in p53 function, in inducing growth inhibition and autophagic cell death, in the repression of tumour cell migration, in cell growth arrest and apoptosis (Shahbazi et al., 2013). Furthermore, previous reports have also shown that overexpression of miR-155 is linked to the clinical stage (especially PanIN-II and PanIN-III), lymph node metastasis, and prognosis in PDAC patients (Han et al., 2012; Ryu et al., 2010; Sasi et al., 2010; Zhang et al., 2012). In this current study, elevated expression of miR-155 was found in all three PDAC cell lines assessed, and this aligns with previous findings in PDAC patients highlighted above.

The downregulation of miR-126 in PDAC has been reported in previous study, and this correlates with the current findings, which noted reduced miR-126 expression in the PDAC cell lines assessed (Sasi et al., 2010). In particular, it was found that miR-126-5p was strongly decreased in BxPC3, MiaPaCa-2, and Panc-1 cells by 57%, 81%, and 94%, respectively, indicating a strong correlation between miR-126 and PDAC

development. MiR-126 is known to inhibit CXCR4, which suppresses cell proliferation, migration, invasion, cell apoptosis, and arrests the cell cycle at the G_0/G_1 transition of PDAC cells (Liu et al., 2014a; Yuan et al., 2016). CXCR4 is a putative mediator between miR-126 and the RhoA/ROCK signalling pathway (Li et al., 2013; Yuan et al., 2016), promoting MAPK p42/44 phosphorylation and activation of the PI3K/AKT pathway (Hu et al., 2019; Li et al., 2013; Wang et al., 2010), which are further linked to lymph node metastasis and the unfavourable overall survival of PDAC patients (Yuan et al., 2016).

Moreover, in the current study, the EMT-associated markers E-cadherin, Vimentin, Snail, Zeb1, and Wnt-11 were found to be controlled by miR-21 and their mRNA expression levels were significantly affected by miR-21 knockouts in the PDAC cells (**Figure 15**). Furthermore, immunofluorescence results indicated that *E-cadherin* expression was gained, while *Snail* and *Wnt-11* expressions were reduced in Panc-1 miR-21 KO2 clones when compared to wt/control (**Figure 10**C). Several studies have disseminated that some miRs can promote EMT and cancer stemness in PDAC (Cioffi et al., 2015; Hasegawa et al., 2014; Jiang et al., 2015a; Jiang et al., 2015b; Lee et al., 2008; Li et al., 2014a; Lu et al., 2014; Ma et al., 2015b; Tsukasa et al., 2016; Zhao et al., 2015b; Zhou et al., 2015). The current study provides some new pilot insights into the role of miR-21 depletion in cancer stemness, EMT, and the Wnt-11 pathway.

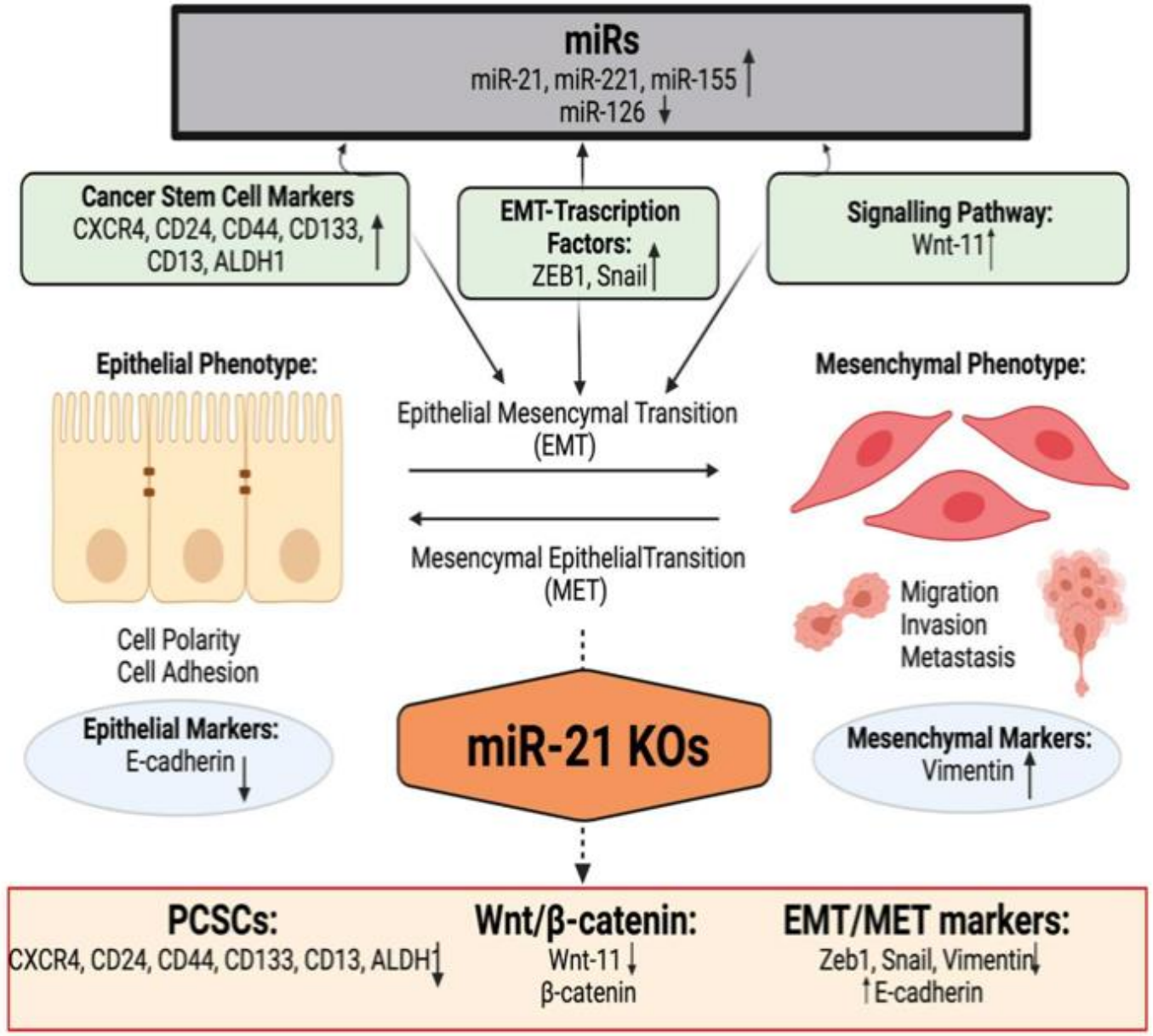


Figure 15: Overview of the involvement of miR-21 in cancer stemness and EMT in PDAC. In the current study, miR-21 affected cancer stemness, EMT, and the Wnt-11 pathway. Adapted from Mortoglou et al., (2022a)**.** Created with BioRender.com.

In this study, it was detected that key CSC markers were dependent on miR-21 status in PDAC cell lines. PCSCs, such as CD24, CD44, CD133, EpCAM, ESA, tyrosine-protein kinase Met (c-met), ALDH1, leucine-rich repeat-containing receptor (lgr5), and serine/threonine-protein kinase (Dclk1), are upregulated during PDAC progression (Ercan et al., 2017; Zhan et al., 2015). The results of the current study showed that miR-21 regulates not only EMT pathways but also seems to play an essential role in CSCs expression, including CD44, CD24, CD133, CD13, ALDH1, and CXCR4. This is consistent with previous studies that have revealed that the capability of these CSCs for self-renewal can be affected by several miRs, including miR-99a, miR-100, miR-125b, miR-192, and miR-429 (Singh et al., 2013; Tesfaye et al., 2019). Additional research by other groups has highlighted interactions between 210 miRs and 258 stem cell-related mRNAs, commonly dysregulated in the PCSCs (Jung et al., 2011). Furthermore, the loss of miR-34 has been observed in CSCs, while its restoration can suppress the spheroid-forming ability via the repression of *Notch* and *Bcl-2,* and restoration of *p53* (Ji et al., 2009). Importantly, recent studies have shown that PCSCs have contributed to crosstalk with the PDAC parenchymal cells through a symbiotic association, leading to early PDAC infiltration and metastasis (Biondani et al., 2018). On other reports also showed that cell-surface markers, including CD133, CXCR4, EpCAM, CD24, CD44, ABCG2, and c-Met, are upregulated in PDAC cases, and specifically, $CD44^{+}$ $CD24^{+}$ $EpCAM^{+}$ cells were associated with a 100-fold increase in tumorigenic potential compared to CD44− $CD24^{-}$ $EpCAM^{-}$ cells (Hermann et al., 2007; Huang et al., 2008; Ikenaga et al., 2010; Li et al., 2007; Li et al., 2011; Marechal et al., 2009; Olempska et al., 2007).

CD24 contributes to cell adhesion and in the development of organs, such as the brain and kidneys (Droz et al., 1990; Poncet et al., 1996), while also being closely associated with PDAC (Gzil et al., 2019). In previous studies, the absence of CD24 has been observed in normal PDAC tissue, while elevated expression levels were observed in the progression from normal ductal epithelium to invasive intraductal papillary

mucinous neoplasm (IPMN) (Jacob et al., 2004). The current data here showed that CD24 was highly overexpressed in the MiaPaCa-2 and Panc-1 cell lines and decreased in miR-21 KOs, which suggests that miR-21 can affect stemness. Moreover, $CD24^+$ population is associated with higher tumour stage, nodal metastasis, higher-grade tumours, microscopic lymphatic, venous and neural invasion in PDAC (Ikenaga et al., 2010), and reduction in CD24 expression, as observed here, in response to miR-21 KO in PDAC cells, may be of significant importance.

CD133 is a transmembrane protein present in lipid rafts, which has been found to play a crucial role in PDAC tumourigenesis (Boivin et al., 2009; Giebel et al., 2004; Röper et al., 2000; Taïeb et al., 2009). Recent studies have shown that Wnt/β-catenin signalling is involved in modulating PDAC progression and in promoting self-renewal of $CD133^+$ cancer cells (Chen et al., 2019; Jiang et al., 2013; Zhan et al., 2017). Importantly, these findings appear to be well supported by another study, which revealed that CD133 expression levels are considerably decreased in normal pancreatic tissue (only 0.01% of cancer cells), compared to PDAC, where the population of this CSC was 0.5–1% of $CD133^+$ cells in less aggressive cell lines to more than 9% of $CD133^+$ cells in clones with high migration (Banerjee et al., 2014; Hou et al., 2014; Xin et al., 2016). Furthermore, a correlation between EMT and PDAC development has been described through the regulation of the NF-κB signalling pathway, which is activated by CD133 (Huber et al., 2004; Nomura et al., 2015). CD133 can therefore induce EMT, while high expression of this marker is correlated to increased proliferation, metastasis of lymph nodes, reduced apoptosis, and tumourigenesis with chemotherapeutic resistance in PDAC cells (Hashimoto et al., 2011). Moreover, the expression of CD133 showed to be associated with reconstitution of PDAC growth and differentiation in 11 primary human PDAC samples and PDAC cell lines (Hermann et al., 2007). Based on this information, the results of the current study, showed a significant reduction in CD133 in response to miR-21 KO in both PDAC cell lines assessed, which indicates the importance of miR-21 in the regulation of this key tumorigenic PDAC protein.

CD44 contributes to cellular adhesion, angiogenesis, and the release of cytokines during PDAC progression (Siegel et al., 2014). Elevated CD44 is linked to EMT-related mesenchymal cancer cell phenotypes (Chen et al., 2018; Cho et al., 2012; Ponta et al., 2003) and to increased levels of several mesenchymal markers, as well as high grade of PDAC, including via the activation Akt pathway, which targets E-cadherin expression and thus generates EMT (Brown et al., 2011). CD44 overexpression furthermore induces expression levels of transcription factors, including Nanog, Sox2, and Oct4, which further promotes miR-302 and miR-21 upregulation and regulates cell growth/self-renewal elevation in $CD44^{high}$ PDAC cells (Bourguignon et al., 2009; Bourguignon et al., 2012a; Bourguignon et al., 2012b; Shiina et al., 2015). Recent studies have suggested that the interaction between CD44 and hyaluronan results in the promotion of miR-21 expression, which further leads to the elevated expression of anti-apoptotic protein Bcl-2 (Cheng et al., 2012; Grzywa et al., 2020). In the current study, it was found that miR-21 moderated the expression levels of several CSCs, including CD44. CD44 indicates reduced stemness in Panc-1 and MiaPaCa-2 cell lines following miR-21 KO. Upregulation of CXCR4 is accepted to be indicative of shorter overall survival and related to an elevated risk of developing lymph node and liver metastasis via the interaction with CXCL12, which can further promote angiogenesis and the formation of new blood and lymphatic vessels (Marechal et al., 2009). Previously, it has been reported that CXCR4 is involved in PDAC pathogenesis (Gao et al., 2010; Koshiba et al., 2000; Marchesi et al., 2004). This correlates with the current data, highlighting that the overexpression of CXCR4 in PDAC cell lines was associated with miR-21 and CXCR4 was significantly reduced in both PDAC cell lines upon miR-21 KO.

ALDH-1, a CSCs marker, is correlated to tumorigenic cells in PDAC (Deng et al., 2010; Jimeno et al., 2009; Kim et al., 2011; Rausch et al., 2010). Here, it was found that the high levels of ALDH-1 expressed in control PDAC cells were significantly reduced in

response to miR-21 KO in both Panc-1 and MiaPaCa-2 cells. CD13 was another PCSCs marker assessed in the miR-21 KO PDAC cells. PDAC patients with more $CD13^{high}$ neutrophil-like heterogeneous myeloid-derived suppressor cells (nMDSCs) have presented a shorter overall survival than those with fewer $CD13^{high}$ nMDSCs (Gabrilovich et al., 2012; Khaled et al., 2013; Zhang et al., 2017a). Moreover, numbers of $CD13^{high}$ nMDSCs decreased after tumour resection of PDAC cases, whereas $CD13^{low}$ nMDSCs were elevated (Gabrilovich et al., 2012; Khaled et al., 2013). CD13 MDSCs could be attributed to perineural invasion (PNI) of PDAC, whereas it was also noted that $CD13^{high}$ nMDSCs revealed increased expression levels of Arg1 compared to $CD13^{low}$ nMDSCs, which resulted in more vigorous immunosuppressive activity (Gabrilovich et al., 2012; Khaled et al., 2013). This underlines the significance of the results of the current study, which indicated that the $CD13^{+}$ population was higher in Panc-1 control cells compared with the miR-21 KOs, while in MiaPaCa-2 cells, CD13 levels were low both in the control and KOs. This is of considerable interest also as Panc-1 is considered the more aggressive PDAC cell line of these two. Therefore, CD13 could be potentially used both for PDAC diagnosis and targeted PDAC treatment, the expression levels of CD13 were upregulated only in the metastatic Panc-1 cell line and not in the MiaPaCa-2 cell line and its two KOs clones.

The results of the current study showed that the miR-21 KOs significantly reduced cell invasion in both Panc-1 and MiaPaca-2 cells. MiR-21 KOs led a small, however significant, reduction in MiaPaCa-2 cell proliferation over a 16 h. These findings are consistent with previous *in vitro* studies, which have identified that Wnt-11 is closely associated with cellular invasion of Panc-1 cells (Dart et al., 2019) and that miR-21 regulates Wnt-11 expression levels not only in PDAC but also in triple-negative MDA-MB-231 breast cancer cells (Arisan et al., 2021). Similarly, previous reports have indicated that transfection with miR-21 precursors can stimulate invasion, extravasation, and metastasis in cellular models of PDAC (Moriyama et al., 2009). According to the TCGA database, the survival ratio of the low expression cohort

(median 22.2 months) was longer than in the high expression cohort (median 19.77 months), based on data from 178 patients. The high expression profile of miR-21 (113 people with high expression of miR-21, compared with 65 people with low expression of miR-21) was significantly correlated with overall survival (**Supplementary Figure 4**).

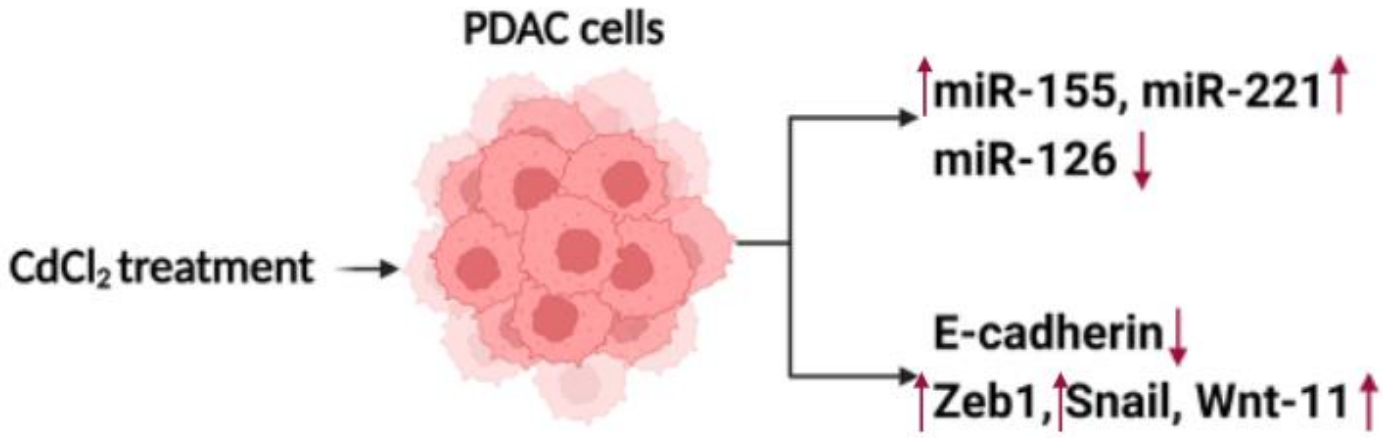
3.5. Conclusions

In conclusion, the current data from this part of the study indicates that miR-21 regulates key CSC markers and affects EMT markers in PDAC. The EMT and Wnt-11 pathway were found to be modulated by miR-21 knockout, highlighting the importance of miR-21 as a potential target of cancer stemness. While further and in-depth studies will be needed to identify all related mechanisms for the role of miR-21 in the poor prognosis and metastasis of PDAC; the data presented in this study provide novel insights into roles for miR-21. Furthermore, this data supports previous findings that show the importance of miR-21 and its potential as a therapeutic target for PDAC.

Chapter 4: Role of miRs in response to Cadmium Chloride in Pancreatic Ductal Adenocarcinoma

Note for the reader: This Chapter includes part of the results of the research paper published by Mortoglou et al., (2022b). The experiments that have been developed by my collaborators are not included in this chapter and these refer to the lethal concentration (LC50) values of $CdCl_2$ in different PDAC cell lines (AsPC-1, BxPC-3, Panc-1, Panc-10.5) and control pancreatic cell line (HPNE) as well as the effects of $CdCl_2$ in cell function and apoptotic associated proteins such as phospho-Akt, PTEN, poly-ADP ribose polymerase (PARP), β-catenin, FOXO1, total p53 and caspase 3/7 activity (see further details on this in Mortoglou et al., 2022b).

Graphical Abstract



Created with BioRender.com

Highlights

- $CdCl_2$ treatment increased the expression levels of the two oncogenic miRs miR-221 and miR-155 and decreased the expression levels of the tumour suppressor miR-126 in Panc-1 and MiaPaCa-2 PDAC cell lines.
- EMT dysregulation was observed following $CdCl_2$ exposure in Panc-1 and MiaPaCa-2 PDAC cells.

4.1. Abstract

Alterations of miR expressions have been associated with apoptosis, angiogenesis, and metastasis during PDAC progression, as well as the role of environmental pollutants such as cadmium (Cd); however, its specific role remains to be fully understood. This part of the study therefore focused on the role of miRs (miR-221, miR-155, miR-126) in response to cadmium chloride ($CdCl_2$) exposure on PDAC *in vitro*. Following treatment with $CdCl_2$, miR-221 and miR-155 were found to be significantly overexpressed, whereas miR-126 was downregulated. An increase in EMT via the dysregulation of mesenchymal markers such as Wnt-11, E-cadherin, Snail and Zeb1 were also observed. Hence, this study provides evidence suggesting that the environmental pollutant Cd can have a significant role in the development of PDAC, pointing to a significant correlation between miRs and Cd exposure during PDAC progression. These findings form the basis for the further in-depth investigations into the roles of miRs in environmental pollution and PDAC.

Keywords: pancreatic ductal adenocarcinoma; non-coding RNAs; microRNAs; environmental toxins; cadmium; epithelial–mesenchymal transition

4.2. Introduction

Interest in environmental toxins and particularly the role of Cd as a toxic metal ubiquitously present in the environment, has risen during the last decades. This is of considerable interest with the reference to its possible role in various human diseases, especially carcinomas (Anđelković et al., 2021; Buha et al., 2018; Buha et al., 2019; Buha et al., 2020). Cadmium's possible role in PDAC has been recently reviewed by Buha et al. (2017) suggesting multiple mechanisms responsible for Cd actions leading to the development of PDAC. The three predominant toxic mechanisms proposed are: **1**) the alterations in the redox status of the cell; **2**) changes in the apoptotic pathways; **3**) and epigenetic changes. Further research, which was a synergy of human observational,

experimental, and *in vitro* studies showed Cd deposition in pancreatic tissue and revealed Cd-induced disturbances in intrinsic pathway of apoptotic activity and elevated oxidative stress in PDAC cells (Djordjevic et al., 2019). Further studies conducted on PDAC cell lines indicated that the mitochondria may be a site of action for Cd in promoting tumour development (Wallace et al., 2019). Such findings clearly point towards Cd exposure as an important risk factor for PDAC development. Hence, this part of the current study is focused on investigating a link between selected miRs (miR-221, miR-155 and miR-126), Cd exposure and PDAC.

Preliminary studies suggested a correlation between aberrant expression levels of numerous miRs and PDAC (Yu et al., 2012), which can have as an outcome inhibition of apoptosis, induce angiogenesis and metastasis (Amirkhah et at., 2015). MiR-221 is one of the most oncogenic miRs in PDAC alongside miR-21 (Bloomston et al., 2007; Uysal-Onganer et al., 2021; Yang et al., 2016; Zhang et al., 2008), while the overexpression of miR-221 has been correlated to a number of malignancies, such as hepatocellular carcinoma, prostate adenocarcinoma, and colorectal carcinoma (Liu et al., 2014a; Tao et al., 2014; Yau et al., 2014; Zheng et al., 2014). Enhanced expression levels of miR-221 are closely associated with PDGF-mediated epidermal-mesenchymal transition phenotype, migration, metastasis, and uncontrolled proliferation of PDAC cells through the inhibition of both MAPK and TGF-β signalling pathways (Masamune et al., 2014; Mortoglou et al., 2021a, b; 2013; Su et al., 2013).

EMT can be characterised as a key component of the metastatic cascade, which includes the repression of E-cadherin and the activation of genes related to motility and invasion (Kalluri and Weinberg, 2009). Therefore, the examination of the most commonly expressed miRs with their related signalling pathways and their associated target genes is crucial for a better understanding of PDAC pathophysiology. Furthermore, upregulation in the expression of miR-155 has been also identified in

PDAC tissue samples (Papaconstantinou et al., 2013). Elevated levels in the expression of miR-155 can result in poor survival in PDAC patients due to the development of fibrogenesis, through TGF-β (Greither et al., 2010; Mortoglou et al., 2021a, b). On the other hand, miR-126 has been linked to PDAC progression, through the post-transcriptional upregulation of *KRAS* (Jiao et al., 2012) and *HER2* (Garajová et al., 2014).

In metastatic process, EMT is responsible for the loss of epithelial cell polarity and cell-cell adhesion (Brabletz et al., 2018; Ye et al., 2017), while studies have shown that miRs are linked to the moderation of EMT, stem cell-like differentiation, invasiveness, migration, and proliferation (Mortoglou et al., 2022a; Piasecka et al., 2018). Wnt-11 is one of the noncanonical Wnt family members, which are activated during carcinogenesis and correlated to a poor prognosis of various cancer types including PDAC (Arisan et al., 2020; Guo et al., 2009; Uysal-Onganer et al., 2010). During EMT, overexpression of the mesenchymal marker Snail and the decrease of the epithelial marker E-cadherin has been noticed (Lin et al., 2010; Nakamura et al., 2011). Particularly, E-cadherin is a protein, which is necessary for the normal epithelial cell maintenance, whereas transcriptional factors including Zeb1, and Snail are highly expressed through the binding in E-cadherin promoter (Felipe Lima et al., 2016; Lamouille et al., 2014; Seton-Rogers, 2016; Singh and Settleman, 2010). Therefore, transcription factors such as Snail and Zeb1 are key players of EMT (Stemmler et al., 2019). Wnt-11 overexpression has been observed in PDAC tissues compared to normal adjacent tissues and is associated with TNM staging (Wang et al., 2018a). Especially, higher levels of Wnt-11 are correlated to stages II, III and IV (Wang et al., 2016). On the other hand, Zeb1 upregulation is correlated to advanced PDAC stages and poor malignancy outcome (Arumugam et al., 2009; Buck et al., 2007; Maier et al., 2010). Zeb1 is also responsible not only for the acquisition of an EMT phenotype but also for migration and invasion in response to NF-κβ signalling in PDAC cells (Maier et al., 2010). Furthermore, downregulation of E-cadherin is associated with poor prognosis

and differentiation in PDAC (Iacobuzio-Donahue et al., 2009; Winter et al., 2008), while elevated expression levels of Snail was found to be related to 80% of PDAC cases (Hotz et al., 2007). Besides, Snail overexpression is connected to reduced E-cadherin expression, higher tumour grade and poorly differentiated PDAC cell lines (Hotz et al., 2007).

Recently, mounting evidence has shown that heavy metals, including Cd, might exert their toxicity through miRs (Wallace et al., 2020a). Hence, this study aimed to investigate miR expressions in response to Cd in metastatic PDAC cells. Subsequently, due to the fact that EMT markers could be proven a novel target for anticancer therapy, the expression levels of Wnt-11, E-cadherin, Snail and Zeb1 were examined following Cd exposure in PDAC *in vitro*.

4.3. Results

In summary, this part of the current study determined the expression levels of specific miRs in response to $CdCl_2$ exposure. Results showed that miR-221 and miR-155 were upregulated in metastatic PDAC cell lines treated with $CdCl_2$, while miR-126 was downregulated. It was also noted that $CdCl_2$ altered the expression levels of EMT markers such as Wnt-11, E-cadherin, Snail and Zeb1. Particularly, Wnt-11, Snail and Zeb1 were considerably upregulated following $CdCl_2$ exposure, while E-cadherin expression level was decreased in PDAC cell lines treated with $CdCl_2$ compared to non-treated cells.

4.3.1 MiRs Expression Levels are Differently Modulated in Response to Cadmium Treatments in PDAC Cells

Based on my previous and other's studies, specific miRs including miR-221, miR-155 and miR-126 are closely associated with the development of PDAC in different stages (Greither et al., 2010; Komoto et al., 2009; Mortoglou et al., 2022a; Su et al., 2013; Uysal-Onganer et al., 2021). Therefore, when assessing miRs expression levels (miR-155,

miR-221, miR-126), significant expression changes were observed in response to $CdCl_2$ treatment. This current study showed that in the Panc-1 cell line, $CdCl_2$ treatment significantly upregulated the expression levels of miR-221 by 65% ($n = 3$; $p < 0.001$; **Figure 16A**) and miR-155 expression levels by 30% ($n = 3$; $p < 0.05$; **Figure 16B**) compared to non-treated PDAC cells (control cells). Expression levels of miR-126 significantly downregulated by 100% ($n = 3$; $p < 0.0001$; **Figure 16C**) in the same PDAC cell line. Similarly, it was found that in the MiaPaCa-2 cell line, expression levels of miR-221 ($n = 3$; $p < 0.05$; **Figure 16D**) and miR-155 ($n = 3$; $p < 0.05$; **Figure 16E**) were significantly increased by 33.5% and by 17%, respectively following $CdCl_2$ exposure, while expression levels of miR-126 were significantly reduced by 84% ($n = 3$; $p < 0.001$; **Figure 16F**).

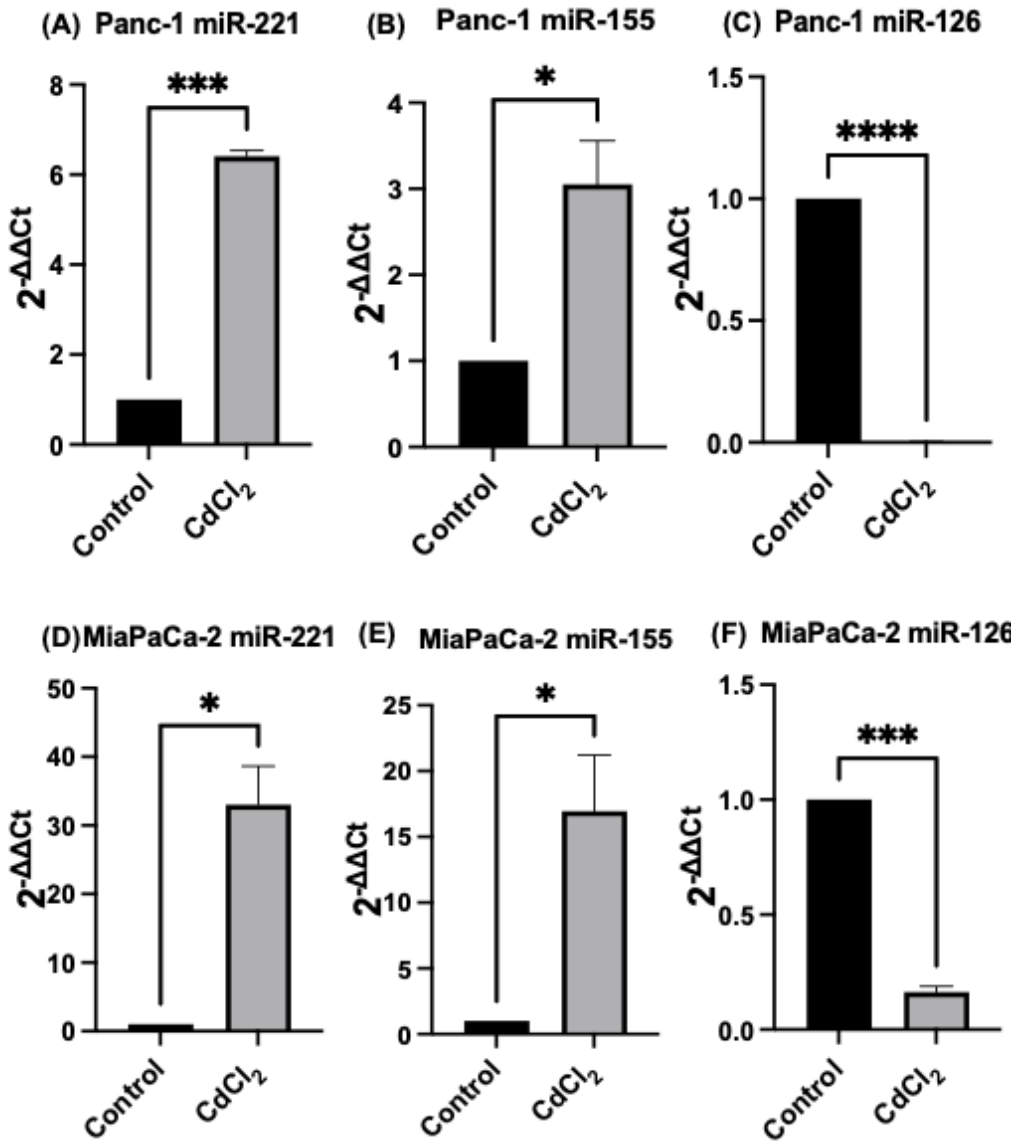


Figure 16: CdCl2 treatment mediated effects on miRs expression levels in PDAC cells. (A–C): Effects of $CdCl_2$ exposure in the Panc-1 cell line: (**A**) miR-221 expression levels; (**B**) miR-155 expression levels; (**C**) miR-126 expression levels. (**D–F**): Effects of $CdCl_2$ exposure in the MiaPaCa-2 cells: (**D**) miR-221 expression levels; (**E**) miR-155 expression levels; (**F**) miR-126 expression levels. The column graphic represents the average of three replicates of RNA isolated from each cell line. Data normalised according to RNU6 expression by fold analysis ($n = 3$, $p < 0.05$). Statistical t-test was used to examine the expression levels of the selected miRs following $CdCl_2$ treatment compared with the non-treated PDAC cells (control cells). Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2022b).

4.3.2. Cadmium Treatment Differently Affect Wnt-11, E-cadherin, Snail and Zeb1 Protein Levels in Panc-1 and MiaPaCa-2 Cells, Following 14 days Treatment

According to my previous and others' reports, EMT markers play a significant role in different biological processes such as invasion and metastasis during PDAC development (Arumugam et al., 2009; Dart et al., 2019; Hotz et al., 2007; Iacobuzio-Donahue et al., 2009; Mortoglou et al., 2022a; Wang et al., 2018b). Therefore, based on my previous study, both Panc-1 and MiaPaCa-2 PDAC cell lines were extensively exposed to $CdCl_2$ for 14 days to further assess the protein levels of Wnt-11, E-cadherin, Snail and Zeb1 by RT-qPCR (Djordjevic et al., 2019; Mortoglou et al., 2022a). In the current study, it was found that in both PDAC cell lines, $CdCl_2$ significantly upregulated Wnt-11, Snail and Zeb1 expression levels and considerably reduced E-cadherin protein levels compared to non-treated PDAC cell lines (**Figure 17**). Specifically, these results indicated that in the Panc-1 cell line, the mRNA expression levels of Wnt-11 ($n = 3$; $p < 0.001$; **Figure 17A**), Snail ($n = 3$; $p < 0.001$; **Figure 17C**) and Zeb1 ($n = 3$; $p < 0.0001$; **Figure 17D**) were significantly increased by 47%, 38% and 42%, respectively following $CdCl_2$ exposure compared with the non-treated Panc-1 cells (Panc-1 control cells), while mRNA expression levels of E-cadherin ($n = 3$; $p < 0.0001$; **Figure 17B**) were significantly decreased by 62% in the same PDAC cell line. Similarly, in the MiaPaCa-2 cell line, the mRNA expression levels of Wnt-11 ($n = 3$; $p < 0.01$; **Figure 17E**), Snail ($n = 3$; $p < 0.01$; **Figure 17G**) and Zeb1 ($n = 3$; $p < 0.0001$; **Figure 17H**) were significantly overexpressed by 50%, 28% and 29%, respectively following $CdCl_2$ exposure compared with the non-treated MiaPaCa-2 cells (MiaPaCa-2 control cells), while mRNA expression levels of E-cadherin ($n = 3$; $p < 0.0001$; **Figure 17F**) were significantly reduced by 47% in the same PDAC cell line.

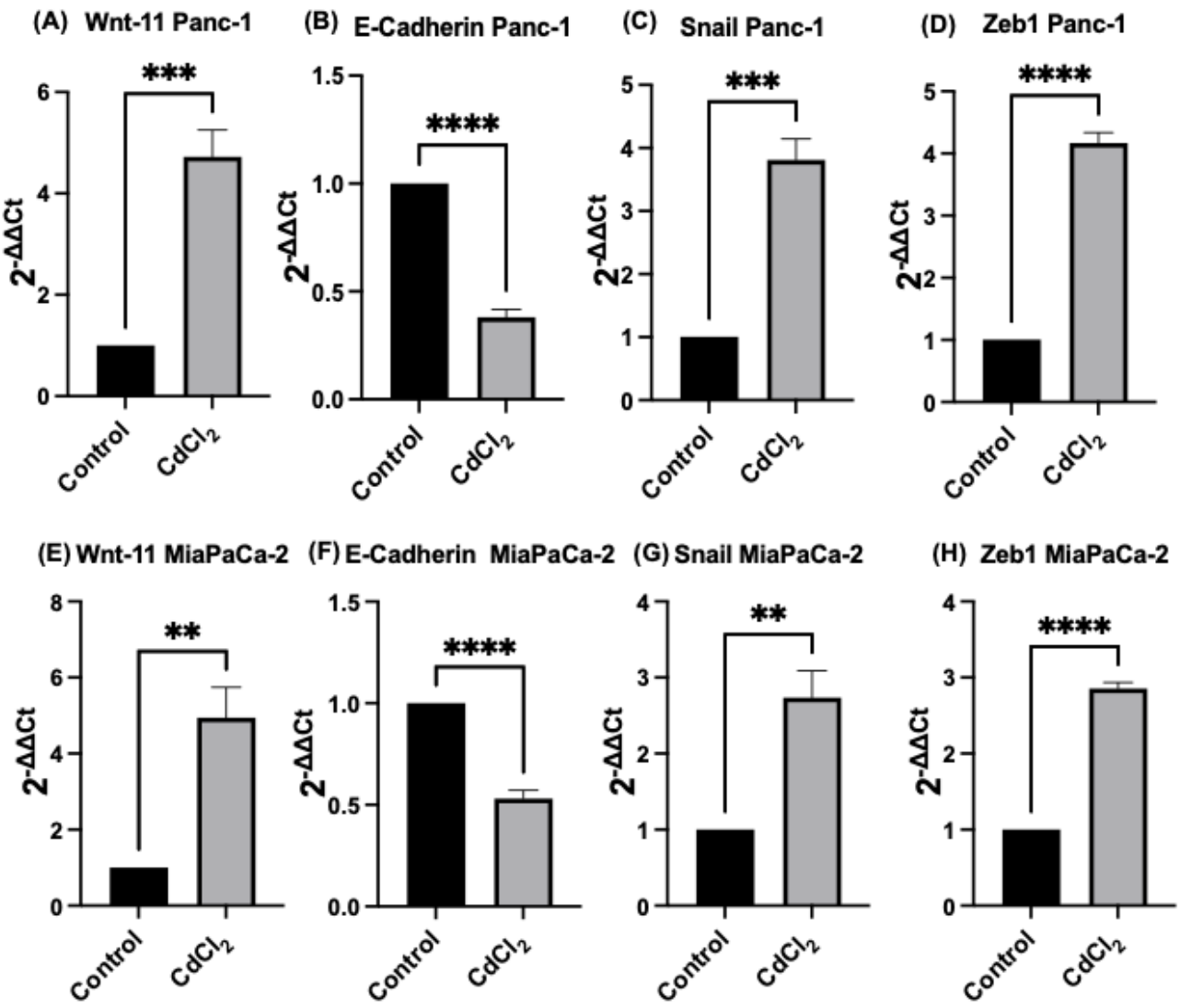


Figure 17: CdCl2 treatment mediated effects on protein mRNA expression levels in PDAC cells. (**A–D)**: Effects of $CdCl_2$ exposure in the Panc-1 cell line: (**A**) Wnt-11 mRNA expression levels; (**B**) E-cadherin mRNA expression levels; (**C**) Snail mRNA expression levels; (**D**) Zeb1 mRNA expression levels. (**E–G**): Effects of $CdCl_2$ exposure in the MiaPaCa-2 cells: (**E**) Wnt-11 mRNA expression levels; (**F**) E-cadherin mRNA expression levels; (**G**) Snail mRNA expression levels; (**H**) Zeb1 mRNA expression levels. The column graphic represents the average of three replicates of RNA isolated from each cell line. Data normalised according to RPII expression by fold analysis ($n = 3$, $p < 0.05$). Statistical t-tests were used to examine the mRNA expression levels of the selected proteins following $CdCl_2$ treatment compared with the non-treated PDAC cells (control cells). Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2022b).

4.4. Discussion

The main outcome of this part of the study is that there is a strong correlation between $CdCl_2$ exposure and selected miR expression during PDAC development.

The miRs response in humans after Cd exposure is very complex, especially considering Cd's ability to bioaccumulate in the body. In studies so far, changes in miRs in terms of either up- or downregulation, were identified in ovarian granulosa cells (Wang et al., 2018b), kidneys (Pellegrini et al., 2016), prostate epithelial cells (Ngalame et al., 2016), hepatic cell lines (Urani et al., 2014), and bronchial epithelial cells (Liu et al., 2015a). In this current study, miRs expression levels were found to be significantly altered in Panc-1 and MiaPaCa-2 PDAC cells lines treated with Cd from control cells. MiR-221 and miR-155 were considerably overexpressed in both Panc-1 and MiaPaca-2 PDAC cell lines treated with $CdCl_2$, while miR-126 expression was significantly reduced.

As an oncogenic miR, miR-221 has been related to several cancers such as glioma (Lu et al., 2009; Siegel et al., 2014; Zhang et al., 2009; Zhang et al., 2012), prostate carcinoma (Mercatelli et al., 2008), hepatocellular cancer (Gramantieri et al., 2009) and lung cancer (Garofalo et al., 2008). Other studies have highlighted that miR-221 not only upregulated in the MiaPaCa-2 cell line compared to normal human pancreatic ductal epithelium (Zhang et al., 2008), but also alterations in its expression levels can promote the metastatic propensity of PDAC cell lines (Xu et al., 2015). According to previous findings, upregulation of miR-221 is common in PDAC tissue samples compared to normal controls and therefore, the expression of miR-221 in PDAC could be used for the discrimination of PDAC from benign PDAC tissue with specificity 93% (Bloomston et al., 2007). Importantly, when assessing the expression levels of miR-221 in the current study, miR-221 was identified to be significantly upregulated more in PDAC cells treated with $CdCl_2$ compared with the non-treated PDAC cell, which

highlighting that $CdCl_2$ exposure increased the oncogenic property of miR-221 in PDAC cells.

MiR-155 is highly upregulated in several solid malignancies such as breast, colon, and thyroid cancers (Babar et al., 2011; Bakirtzi et al., 2011; Jiao et al., 2012; Nikiforova et al., 2008). Recent studies have indicated that miR-155 is linked to poor prognosis of PDAC (Bloomston et al., 2007; Greither et al., 2010; Szafranska et al., 2007). MiR-155 upregulation was noticed in 80% of early pancreatic lesions (Ryu et al., 2010). In the current study, upregulated expression of miR-155 was found in both Panc-1 and MiaPaCa-2 cells treated with $CdCl_2$ compared with the control cells, which indicates a correlation between $CdCl_2$ exposure and the oncogenic capability of miR-155.

It has been also reported that the tumour suppressor miR-126 is correlated to numerous malignancies including lung, gastric, breast and PDAC cancer (Feng et al., 2010; Sempere et al., 2010; Zhou et al., 2016). Specifically, reduced expression levels of miR-126 can lead to cellular migration and invasion through the inhibition of ADAM metallopeptidase domain 9 (*ADAM9*) target gene, which is commonly expressed in PDAC (Grutzmann, 2004). Therefore, the reduced expression of miR-126 in PDAC has been reported in previous studies, whereas in the current study, the expression levels of miR-126 was significantly decreased following $CdCl_2$ exposure in the Panc-1 and MiaPaCa-2 cell lines.

In the current study, it was also found that Cd regulates EMT and dysregulates the expression levels of mesenchymal markers including Wnt-11, E-cadherin, Snail and Zeb1. Specifically, expression of Wnt-11, Snail and Zeb1 was significantly overexpressed following the treatment with $CdCl_2$, whereas E-cadherin was reduced in PDAC cell lines treated with $CdCl_2$. MiRs can promote not only proliferation and tumourigenesis in PDAC, but also to affect tumour microenvironment (Fathi et al., 2021). Specifically, hypoxia and dysregulations in the expression levels of EMT

markers can be altered by several miRs (Lu et al., 2017). Consequently, carcinogenesis can be modulated by certain miRs and signalling cascades including Hedgehog, PTEN/Akt, Wnt, Signal transducer and activator of transcription 3 (STAT3), ERK, JNK, TGF-β and NF-κ (Fathi et al., 2021).

EMT is regulated by numerous complex modulatory networks such as epigenetic alterations, transcriptional control, which comprise EMT-inducing transcription factors (EMT-TFs) as Snail (Zinc finger protein SNAIL), Zeb (Zinc finger E-box-binding homeobox 1), and transcription regulators including miRs (Wang et al., 2017). Especially, miRs are considered as main regulators of EMT in several cancer types but also as novel early biomarkers in PDAC (Ali et al., 2015; Ballehaninna and Calatayud et al., 2017; Chamberlain, 2013; Chang et al., 2017; Dhayat et al., 2015; Gayral et al., 2014; Sethi et al., 2018; Winter et al., 2013). MiRs can regulate EMT through the control of their target messenger RNAs (mRNA) such as the E-cadherin transcriptional repressor ZEB-1 (Brabletz et al., 2011; Krebs et al., 2017; Li et al., 2010b; Tang et al., 2016). For example, miR-200, miR-141, miR-200a, miR-200b, miR-200c, miR-429 (Gregory et al., 2008; Humphries et al., 2015; Mongroo et al., 2010), miR-34a (Ahn et al., 2012; Alemar et al., 2016; Tang et al., 2017), miR-148a (Feng et al., 2016; Peng et al., 2017; Tan et al., 2017; Tan et al., 2018), miR-203a (Jiang et al., 2017; McCubrey et al., 2016; Yang et al., 2017) and miR-655 (Harazono et al., 2013) act as EMT suppressors and negative regulators of metastatic predisposition of PDAC cells (Gregory et al., 2008; Peter et al., 2009), while miR-10b (Ouyang et al., 2013) and miR-197 (Hamada et al., 2013) strongly promotes EMT. Particularly, miR-200 can inhibit the main regulators of EMT such as Zeb1 and Snail, which further results in the overexpression of E-cadherin levels (Gregory et al., 2008; Peter et al., 2009). Moreover, upregulation of miR-200c is linked not only to E-cadherin expression in resected human pancreatic tumour samples but also in better survival rates in comparison to patients with reduced miR-200c levels (Yu et al., 2010). Furthermore, Zeb1 can also suppress miR-200c and miR-141 transcription, which further contributes to the differentiation state

of PDAC cells (Burk et al., 2008). Furthermore, miR-200 family can also inhibit Notch signalling, which is responsible for tissue homeostasis and is closely linked to EMT through the overexpression of Notch signalling and Zeb1 and decrease of miR-200 family in PDAC (Brabletz et al., 2011). A previous study showed that Zeb1 is a key target of miR-655, which is an EMT-suppressor miR that is correlated to favourable overall survival in PDAC patients (Harazono et al., 2013). Ahn et al. (2012) also indicated that miR-34a acts as a target of ZEB-1, which leads to the reduction of invasion and metastasis.

The loss of E-cadherin expression is detected in 43% of PDAC patients and associated with poor outcome and increased invasion and aggressiveness (Hong et al., 2011). The results of this study showed that the mRNA expression levels of E-cadherin were significantly reduced following the exposure of PDAC cells to CdCl2, which points out the importance of assessing the role of CdCl2 in EMT pathways during PDAC progression. Importantly, recent studies have suggested that Zeb1 and Snail overexpression is closely associated with inhibition of apoptosis and chemoresistance against gemcitabine in PDAC (Li et al., 2009b; Vega et al., 2004; Yin et al., 2007). Wnt-11 downregulation can promote not only EMT but also the expression levels of neuronal and stemness markers, which are linked to metastasis (Dart et al., 2019; Wang et al., 2016; Wang et al., 2018b). In the current study, specific findings were that the mRNA expression levels of Wnt-11 were significantly overexpressed in Panc-1 and MiaPaCa-2 cells treated with $CdCl_2$ compared to the control cells, which promote pilot insights into the role of $CdCl_2$ in the Wnt-11 pathways. Based on this information, the results of the current study, which showed a significant dysregulation of these EMT markers following $CdCl_2$ exposure in PDAC cells, indicates the importance of $CdCl_2$ in the regulation of these key tumorigenic PDAC EMT proteins.

The existing evidence is clear, exposure to $CdCl_2$ results in cellular damage that can lead to the development of PDAC. Normal pancreatic function is maintained by

normal functioning of various miRs. Multiple miRs have important roles in normal insulin function and sensitivity, diabetes and PDAC development (Chakraborty et al., 2013). Therefore, examining the different pathways and potential overlap between cellular signalling mechanisms, relationships between miRs/gene/protein expressions are of importance.

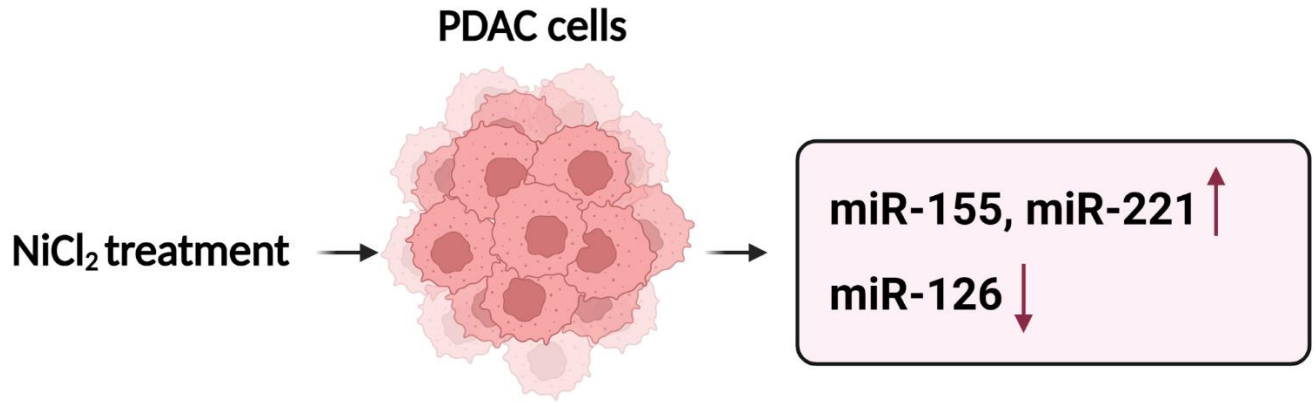
4.5. Conclusions

PDAC is the most fatal malignancy and therefore it is crucial to develop a significant body of knowledge in the field of PDAC carcinogenesis. The role of some significant environmental pollutants, such as Cd in PDAC has been suggested but remain to be fully understood. This is the first research to indicate the role of miRs in response to Cd in PDAC, suggesting a hitherto unrecognised pathway and significant correlation between miRs and Cd exposure during PDAC progression. Further studies are needed to investigate the precise role of miRs in PDAC progression as well as the role of Cd and other environmental pollutants in such processes. Furthermore, epigenetic data, including miR related effects, should be incorporated into risk assessments for Cd exposures improving our ability to predict outcomes and define more efficient prevention measures.

Chapter 5: Nickel's role in Pancreatic Ductal Adenocarcinoma: Potential Involvement of miRs

Note for the reader: This Chapter includes part of the results of the research paper published by Mortoglou et al., (2022c). The experiments were developed by my collaborators have not been included in this chapter and refer to the Ni concentration in PDAC tissue samples compared to healthy controls, to the LC50 values of $NiCl_2$ in different PDAC cell lines (AsPC-1, BxPC-3, Panc-1, Panc-10.5, MiaPaCa-2) and control pancreatic cell line (HPNE) as well as the effects of $NiCl_2$ in cell function and apoptotic associated proteins such as phospho-Akt, PTEN, PARP, β-catenin, FOXO1, total p53 and caspase 3/7 activity (for details please see Mortoglou et al., 2022c).

Graphical Abstract



Created with BioRender.com

Highlights

- $NiCl_2$ treatment of Panc-1 and MiaPaCa-2 increased the expression levels of the oncogenic miR-221 and miR-155 and decreased the expression levels of the tumour suppressor miR-126.

5.1. Abstract

Nickel (Ni), as well as some of their compounds, is classified as human carcinogens by the International Agency for Research on Cancer (IARC). Recent studies have described the role of environmental pollutants such as Ni in PDAC, but the mechanisms of Ni-mediated toxicity in cancer are still not completely understood. Specifically, nickel chloride ($NiCl_2$) has been found to alter the expression and function of miRs in several malignancies, leading to changes in target gene expression. In this part of the study, it was established that the expression levels of miR-221 and miR-155 were significantly upregulated, while the expression levels of miR-126 were significantly decreased in Panc-1 and MiaPaCa-2 PDAC cells exposed to Ni. Hence, this study provides pilot insights indicating that the environmental pollutant Ni plays an important role in the progression of PDAC. The identified miRs, individually or as regulatory networks, may reveal a mechanistic association between Ni exposure and pathophysiological changes in PDAC cases.

Keywords: pancreatic ductal adenocarcinoma; non-coding
RNAs; microRNAs; environmental toxins; nickel

5.2. Introduction

Roles for environmental pollutants in PDAC are of considerable interest, while the topic still remains understudied and the identification of novel contributing pathways, including miRs, requires investigation. Ni is a naturally occurring element, which is found in water, soils, air, and sediments, while IARC has classified its compounds as a "group 1" human carcinogen (IARC, 2018; Oller, et al., 1997). The extensive use of Ni in industrial settings complicates human health effects with the most scrutinised effect being carcinogenesis (Zhu et al., 2019). Moreover, the molecular mechanisms of Ni-induced carcinogenesis comprise hypoxia-inducible factor pathways and the generation of oxidative stress, which further lead to DNA damage and the targeting of DNA repair pathways (Cameron, Buchner and

Tchounwou, 2011; Zhu et al., 2019). A previous study has suggested a link between Ni content and PDAC (Gómez-Tomás et al., 2019), while increasing evidence has promoted the Ni involvement in several pancreas-related dysfunctions (Wallace et al., 2020c).

Roles for miR remain to be investigated and may be of major importance due to the multifaceted roles of miRs in key biological processes including various malignancies and importantly also PDAC (Amirkhah et al., 2015; Arisan et al., 2021; Dart et al., 2019; Lanning et al., 2021; Lin and Gregory, 2015; Meltzer, 2005; Mortoglou et al., 2021a, b; Mortoglou et al., 2022b; Słotwiński et al., 2018; Uysal-Onganer et al., 2020; Uysal-Onganer et al., 2021; Wallace et al., 2020b; Wallace et al., 2020bc). Toxic metals such as Ni have not been studied extensively and therefore, only a small amount of miRs associated with Ni exposure have been characterised (Wallace et al., 2020b; Wallace et al., 2020bc), while their functions and mechanisms in carcinogenesis remain to be elucidated. Hence, the aim of this part of the study was to investigate the role of miRs in response to $NiCl_2$ exposure. The results identified a new role played by miRs in Ni-induced PDAC carcinogenesis.

5.3. Results

5.3.1. Changes of miRs Expression Levels in Response to Nickel Chloride Treatments in PDAC Cells

Effect of $NiCl_2$ exposure was assessed on miR expressions by using Panc-1 and MiaPaCa-2 PDAC cell lines, based on previously published studies (Mortoglou et al., 2022b; Uysal-Onganer et al., 2021). MiR-221, miR-155 and miR-126 are closely linked to PDAC progression at different stages (Greither et al., 2010; Komoto et al., 2009; Su et al., 2013; Uysal-Onganer et al., 2021). Following incubation of PDAC cells with $NiCl_2$, miR-221 expression was found to be significantly elevated both in Panc-1 and MiaPaCa-2 cells (385-fold, $p < 0.001$ and 5.8-fold, $p < 0.05$ respectively; **Figure 18A,D;** $n = 3$ for both). Similarly, significant upregulation of miR-155 was noted in both Panc-

1 (28.5-fold) and MiaPaCa-2 (151-fold) cells after $NiCl_2$ exposure (**Figure 18B,E;** n = 3, $p < 0.05$ for both), while $NiCl_2$ treatment significantly reduced miR-126 expression in Panc-1 (96.6-fold) and MiaPaCa-2 (100-fold) (**Figure 18C,F;** n = 3, $p < 0.0001$ for both). These results suggest that $NiCl_2$, increases oncogenic miR expressions such as miR-221 and miR-155 while reducing expression levels of tumour suppressor miR-126. To date, this is the first analysis to show that $NiCl_2$ could influence the expression levels of these miRs in PDAC.

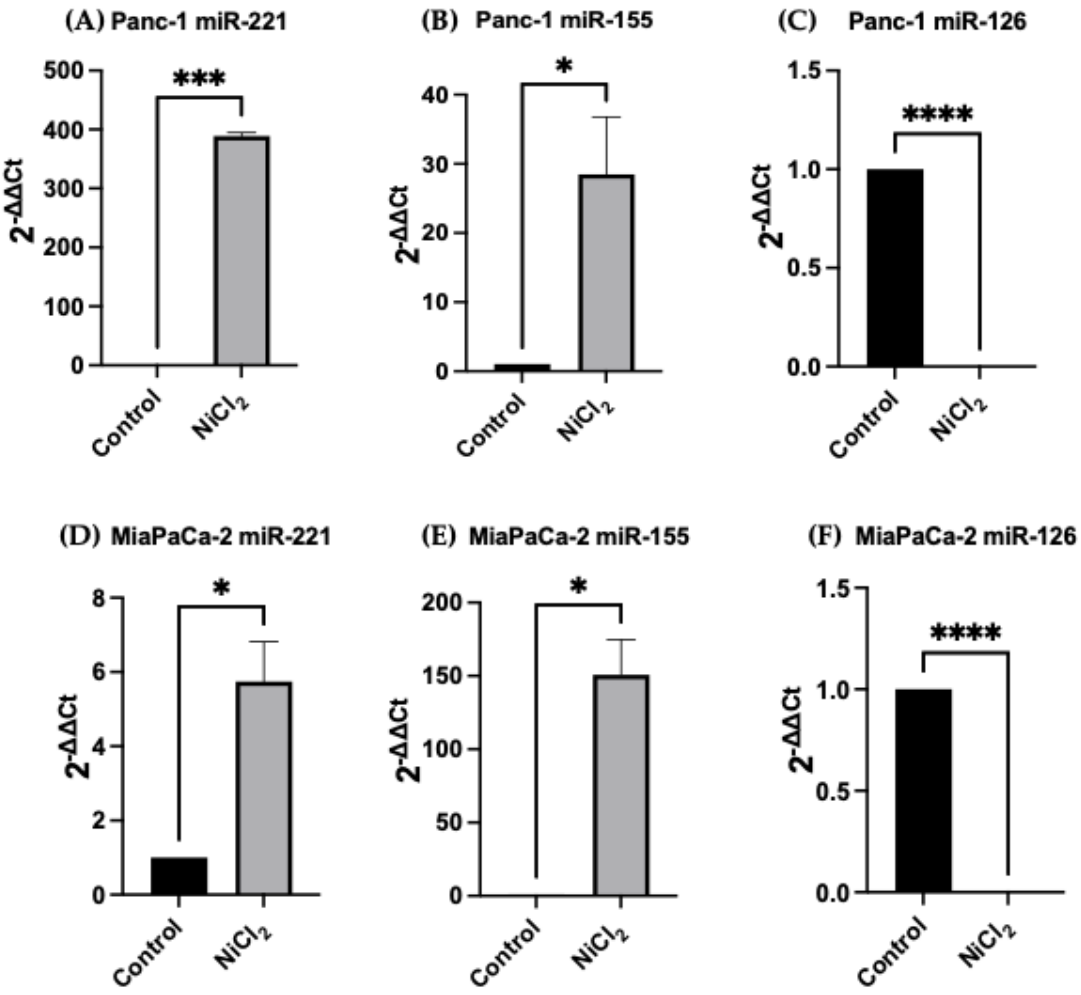


Figure 18: NiCl2 treatment mediated effects on miR expression levels in PDAC cells. (**A–C)**: Effects of $NiCl_2$ in the Panc-1 cell line: (**A**) miR-221 relative expression levels; (**B**) miR-155 relative expression levels; (**C**) miR-126 relative expression levels. (**D–F**): Effects of $NiCl_2$ in the MiaPaCa-2 cell line: (**D**) miR-221 relative expression levels; (**E**) miR-155 relative expression levels; (**F**) miR-126 relative expression levels. $NiCl_2$ treatment effects were evident in the expression levels of miR-221 ($p < 0.001$; Panc-1, $p < 0.05$; MiaPaCa-2), and miR-155 ($p < 0.05$ for all) compared to the control cells, while the expression levels of miR-126 are significantly downregulated ($p < 0.0001$ for all). The column graphic represents the average of three replicates of RNA isolated from the Panc-1 and MiaPaCa-2 cell lines. Data normalised according to RNU6 expression by fold analysis ($n = 3$, $p < 0.05$); Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2022c).

5.4. Discussion

Several studies have identified that Ni content in PDAC tissue is a reliable biomarker across different studies (Rahil-Khazen et al., 2002; Rezuke et al., 1987; Zhu et al., 2019). Studies examining the impact of exposure to various environmental factors on the development of PDAC are often limited by the relatively small number of cases of PCa and the ability to evaluate a small number of environmental factors in studies (Fryzek et al., 1997; Lo et al., 2007; Luckett et al., 2012). However, a clinical study and follow-up indicated the possibility of an association between PDAC risk and Ni exposure (Antwi et al., 2015). The incidence of PCa has been associated with occupational exposure to Ni in several studies (Alguacil et al., 2000; Andreotti and Silverman et al., 2012; Ojajarvi et al., 2007; Santibañez et al., 2010) and one meta-analysis (Ojajarvi et al., 2000).

Ni can stimulate global hypermethylation, which results in the suppression of key tumour suppressor genes, activation of proto-oncogenes and eliciting senescence (Yasaei et al., 2013). Oxidative stress is another important route for Ni-induced carcinogenesis (Valko et al., 2006), while the hypoxia-inducible signalling pathway is a key mechanical route for Ni-induced carcinogenesis, as the transcription factor hypoxia-inducible-factor-1 (HIF-1) is triggered (Salnikow et al., 2003).

Ni can interfere with miRs network to degrade mRNA or block protein synthesis (Sun et al., 2013). Specifically, a previous study indicated that $NiCl_2$ exposure could induce upregulation of miR-210 with stabilisation of the HIF-1α protein, which leads to a metabolism alteration (He et al., 2014b; Kulshreshtha et al., 2007). Furthermore, in a recent review, it has been mentioned that Ni exposure can cause aberrations in the expression levels of several miRs, which are implicated to play a crucial role in tumour growth, cell transformation and angiogenesis in malignancies such as colon and breast (Wang et al., 2021). Several studies have also suggested that Ni exposure can induce the expression levels of miR-152, miR-203, miR-4417, miR-222 and miR-210 (Li et al.,

2004). In this current study, the expression of miRs was found to be considerably dysregulated in PDAC cell lines, which were treated with $NiCl_2$ compared to corresponding control untreated cells. MiR-221 and miR-155 expression levels were significantly upregulated in Panc-1 and MiaPaca-2 PDAC cell lines treated with $NiCl_2$, while miR-126 was considerably downregulated.

Overexpression of miR-221 can have as an outcome the loss of expression of CDKNs, which is linked to liver fibrosis and unfavourable prognosis of PDAC (Almas et al., 2017). Importantly, when examining the relative expression levels of the selected miRs, miR-221 was significantly upregulated in PDAC cells that have been treated with $NiCl_2$ in comparison to the non-treated/control PDAC cells. In this current study, it was showed that the expression levels of miR-155 were significantly increased, when PDAC cells were exposed to $NiCl_2$ compared with the control Panc-1 and MiaPaCa-2 cells. MiR-155 is linked to clinical stage, lymph node metastasis and poor prognosis in PDAC patients (Han et al., 2012; Sasi et al., 2010; Zhang et al., 2012). MiR-126 downregulation has been associated with the clinical stage, lymph node metastasis, and the unfavourable overall survival of PDAC patients (Yuan et al., 2016). Several signalling pathways that miR-126 can moderate during PDAC development are RAS/ ERK, PI3K/Ak strain transforming (AKT)/mTOR, ADAM9/ EGFR/AKT, RAS homolog family member A (RhoA)/Rho-associated kinase (ROCK) and Wnt/β-catenin (Hu et al., 2019). Specifically, these pathways are responsible for angiogenesis, cell proliferation, vascular integrity, migration, and invasion in PDAC (Hu et al., 2019). The findings of the current study indicated that the expression levels of miR-126 were significantly downregulated in both Panc-1 and MiaPaCa-1 cells following $NiCl_2$ exposure related to the expression levels of control PDAC cells. Therefore, the recent study promotes some pioneer insights into the correlation between $NiCl_2$ exposure and expression levels of oncogenic and tumour suppressor miRs during PDAC pathogenesis.

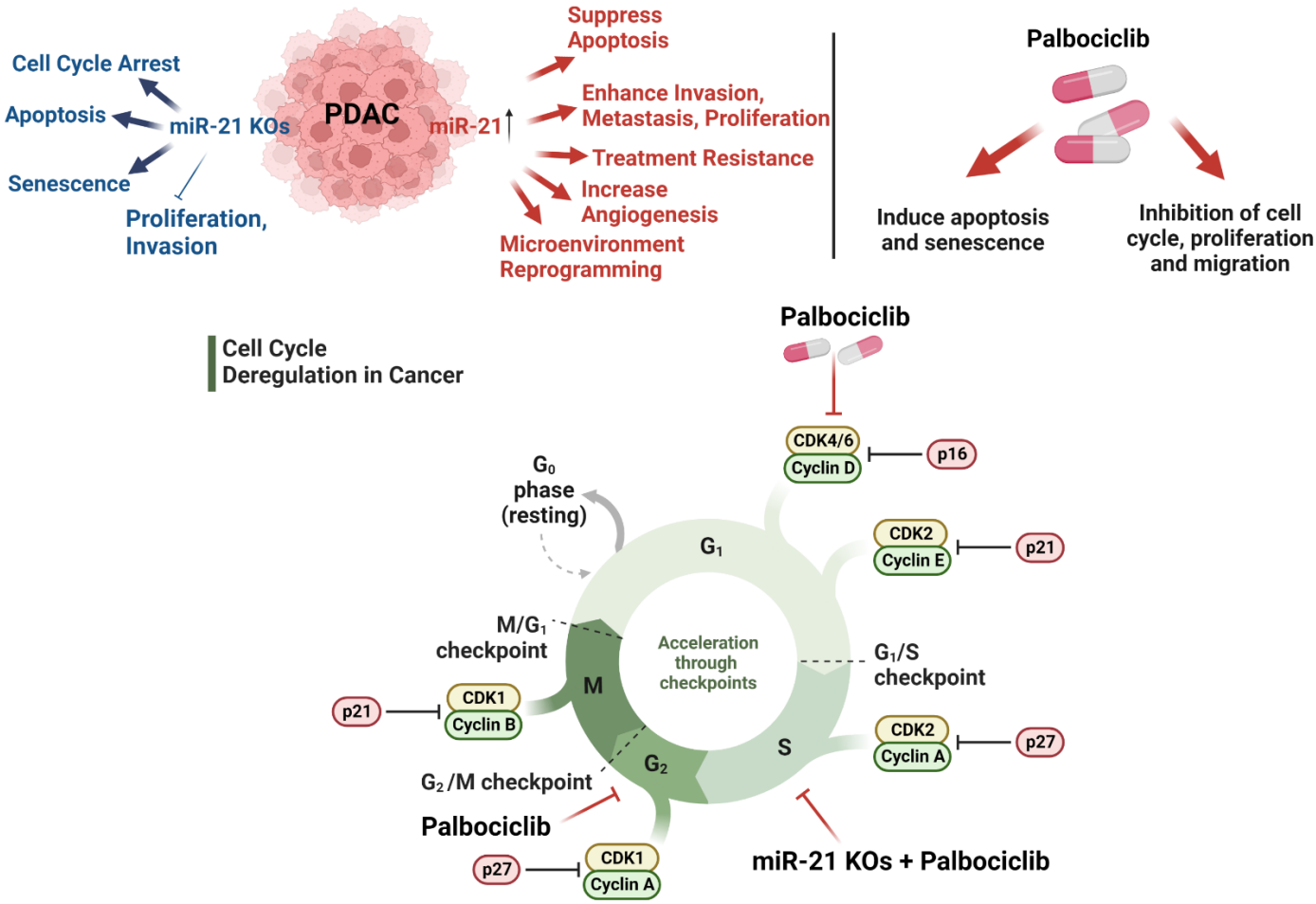
In summary, the findings of this study suggest novel roles of $NiCl_2$ on PDAC and specifically highlight that Ni plays a crucial role during PDAC development and underscores the role of miRs in response to $NiCl_2$ during PDAC pathogenesis.

5.5. Conclusions

PDAC cell lines, Panc-1 and MiaPaCa-2 were treated with $NiCl_2$, and the results showed increased pro-oncogenic properties via miR-221 and miR-155 and decreased anti-oncogenic role of miR-126. The results highlight new regulatory roles of $NiCl_2$ in PDAC pathogenesis via effects on miRs. With emerging research focus on miRs and the development of new approaches for understanding pathways relating to toxic effects of Ni, this part of the study provides new insights into the mechanisms of PDAC.

Chapter 6: Inhibiting CDK4/6 in Pancreatic Ductal Adenocarcinoma by targeting miR-21

Graphical Abstract



Adapted from Mortoglou et al., (2023). Created with BioRender.com

Highlights

- CDK4/6 inhibition decreases cellular proliferation, viability, and migration of Panc-1 and MiaPaCa-2 cells.
- CDK4/6 inhibition combined with miR-21 knockout result in cell cycle arrest of Panc-1 and MiaPaCa-2 cells.
- MiR-21 knockout could be used in PDAC treatment as apoptotic inducers.

6.1. Abstract

PDAC is characterised by alterations in several genes that drive carcinogenesis and limit therapeutic response. The two most common genetic aberrations in PDAC are the mutational activation of KRAS and loss *CDKN2A*, which culminate the activation of CDK4/6, that promote G1 cell cycle progression. Therapeutic strategies focusing on the CDK4/6 inhibitors such as Palbociclib (PD-0332991) may potentially improve outcomes in this malignancy. Primarily, miR-21 promotes cell proliferation and a higher proportion of PDAC cells in the S phase, while knockdown of miR-21 has been linked to cell cycle arrest at the G2/M phase and inhibition of cell proliferation. In this study, using a CRISPR/Cas9 loss-of-function screen, the expression of miR-21 was silenced in two PDAC cell lines (Panc-1 and MiaPaCa-2) and also in combination with PD-0332991 treatment, to examine the mechanisms of CDK4/6 inhibitors and miR-21 KOs on cell survival and death. This combination reduced cell proliferation, cell viability, increased apoptosis and G1 arrest *in vitro*. Furthermore, mitochondrial respiration and glycolysis of PDAC cells were analysed. Changes in protein content of treated cells, compared with controls were assessed by proteomic analysis, revealing KEGG pathways associated with PD-0332991 treatment and miR-21 knockout. The miRs expression signature profiles of the cells was analysed by small RNA-sequencing. The results demonstrated that combined targeting of CDK4/6 and silencing of miR-21 represents a novel therapeutic strategy in PDAC.

Keywords: pancreatic ductal adenocarcinoma; Palbociclib; microRNAs; CDK4/6 inhibition; apoptosis; cell cycle; senescence; proliferation

6.2. Introduction

Large-scale genomic studies have identified recurrent genetic alterations in PDAC, such as activation of oncogenes (mutant *KRAS* is found in >90% of PDAC) and inactivation of tumour suppressor genes *TP53*, *p16/CDKN2A*, *SMAD4*, and breast cancer gene 2 (*BRCA2)* in >50% of PDAC cases (Bailey et al., 2016a; Biankin et al., 2012; Cicenas et al., 2017; Jaffee et al., 2002; Maitra et al., 2008; Waddell et al., 2015; Witkiewicz et al., 2015a). Specifically, aberrations in the oncogenic *KRAS* (proto-oncogene, GTPase) gene result in the activation of the cyclin-dependent kinases (CDK) and mTOR signalling pathway, which are further linked to elevated cell survival (Bryant et al., 2014; Conway et al., 2019). The *CDKN2A* gene encodes p14/Arf and p16/Ink4a, which are potent inhibitors of CDK4 and CDK6 (Foulkes et al., 1997; Young et al., 2014). Mutated *CDKN2A* is the most common alteration in PDAC mouse models driven by oncogenic *KRAS* (Mueller et al., 2018), suggesting the relevance of activating CDK4/6 to promote cell-cycle entry. The loss of *CDK2NA* (p16) and tumour suppressor *TP53* in the cell cycle leads to uncontrolled cell proliferation, whereas mutations in *KRAS* and *TP53* play an important role in glucose metabolism, which disturbs cell maintenance (Bryant et al., 2014; Regel et al., 2012; Rivlin et al., 2011; Weissmueller et al., 2014). Hence, altered metabolic machinery in PDAC cells is a promising strategy for developing an effective therapy and disease management. Genetic alteration in PDAC results in the dysregulation of CDK4 and CDK6 through the inactivation of retinoblastoma (RB), which is a tumour suppressor protein (Cowan et al., 2014; Schutte et al., 1997; Witkiewicz et al., 2015a).

CDK4/6 inhibitors such as Palbociclib (PD-0332991), Ribociclib (LEE011), and Abemaciclib (LY2835219) are being examined as novel therapeutic agents in more than 300 preclinical and clinical trials for more than 50 different types of malignancies (Fassl et al., 2022). It has been shown that PD-0332991 displays antiproliferative activity in PDAC cell lines and patient-derived xenografts (PDX) (Chou et al., 2018;

Heilmann et al., 2014; Knudsen et al., 2019; Witkiewicz et al., 2015b). PD-0332991 has been also approved as a therapeutic agent for advanced metastatic hormone receptor-positive (HR+)/HER2-negative breast cancer (Eggersmann et al., 2019; Franco et al., 2014; Goel et al., 2018). Specifically, CDK4/6 inhibitors can induce apoptosis and are linked to canonical effects, including cell cycle inhibition and proliferation (Bonelli 2019; Dhir et al., 2019; Franco et al., 2014; Musgrove et al., 2011). Recent studies have revealed that PD-0332991 is also associated with non-canonical functions such as reversible senescence, metabolic rearrangement, and immunomodulation (Bonelli et al., 2019; Skowron et al., 2020), which could be pioneer approaches for cancer therapy (Nardella et al., 2011; Shao et al., 2019). During carcinogenesis, activating upstream mitogenic pathways, which elevate CDK4/6 activity leads to therapy resistance (Feng et al., 2019; Niu et al., 2019). Especially, mitogenic signals from receptor tyrosine kinases and downstream signalling events including RAS, PI3K, MAPK and mTOR progress the quiescent cells from G0 or G1 phase into S phase through CDK4 or CDK6 complex (Lim et al., 2013; Malumbres et al., 2001; Rodgers et al., 2014). Therefore, the continuous activation of cell cycle-related signalling pathways allows the utility of CDK inhibitors as therapeutic agents in cancer treatment (Jingwen et al., 2017) (**Figure 19**).

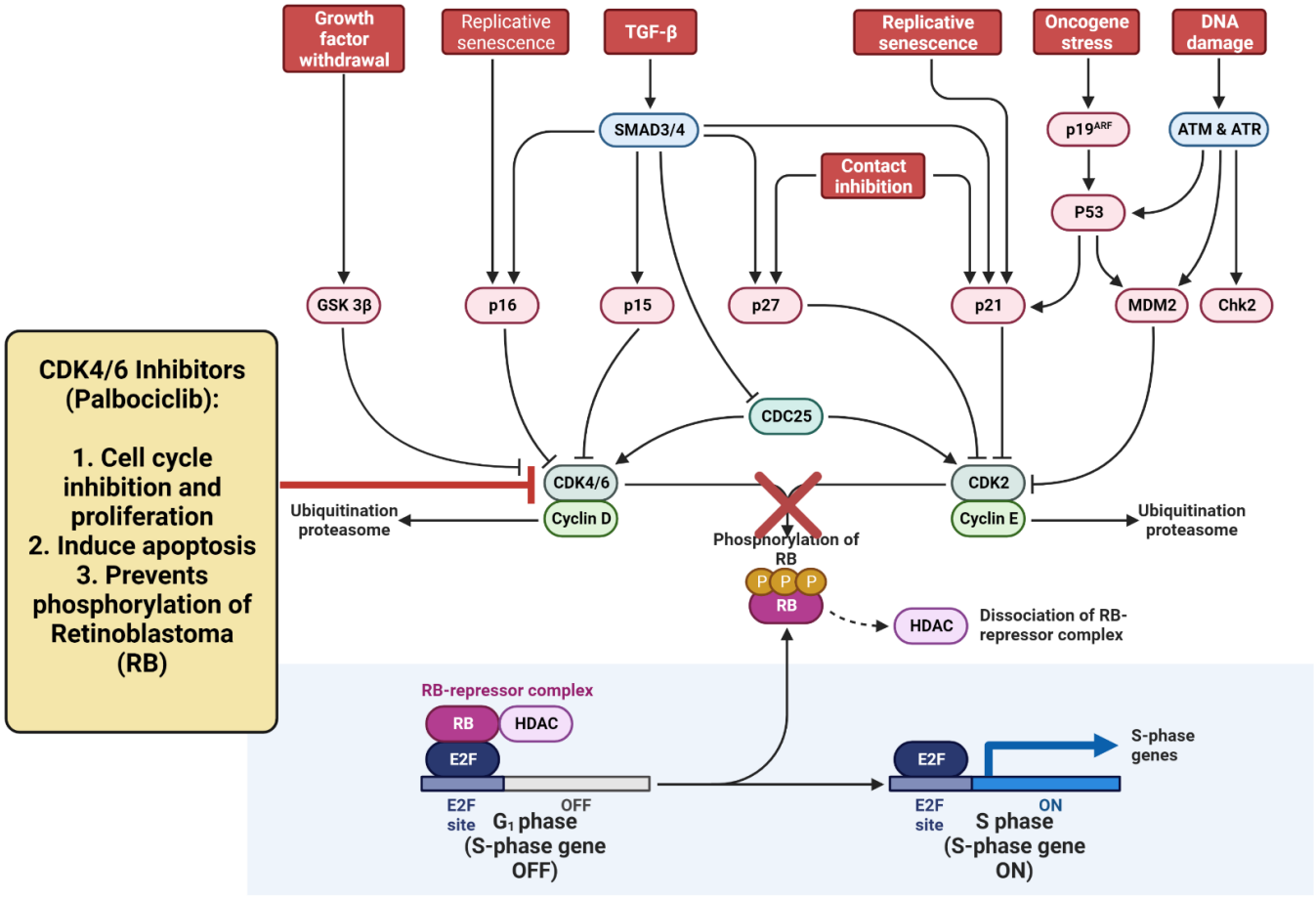


Figure 19: CDK4/6 inhibitors for PDAC therapy. D-cyclins (CycD) activate CDK4 and CKD6 in the G1 phase and stimulate cell cycle progression through the phosphorylation of the RB. In PDAC, CDK4/6 is overactivated, which leads to uncontrolled cell proliferation. CDK4/6 inhibitors have been shown to promote an effective therapeutic activity via the repression of CDK4/6 expression and the inhibition of cell proliferation. Using CDK4/6 inhibitors combined with targeting other signalling pathways may promote a pioneer strategy against therapy resistance. Adapted from Mortoglou et al., (2023). Created with BioRender.com.

Evidence demonstrates that miR-21 is an oncogenic miR presenting the highest specificity (0.80) and sensitivity (0.77) as an early PDAC diagnostic marker out of 7 key candidates (hsa-miR-31-5p, hsa-miR-210-3p and hsa-miR-155-5p, hsa-miR-217, hsa-miR-148a-3p and hsa-miR-375) (Qu et al., 2017; Zhou et al., 2014). MiR-21 confers resistance against CDK4/6 inhibitors and prevents their therapeutic efficiency. Previous reports indicated that thymic tumours of mTOR knockdown mice were characterised by upregulation of miR-21 and let-7a miRs and undetectable levels of the CDK6 protein. These tumour cells with reduced mTOR activity demonstrated greater resistance to treatment with PD-0332991 than tumour cells from mTOR wild-type mice. mTOR inhibition resulted in let-7 and miR-21 upregulation and consequent repression of CDK6 (Gary et al., 2020). Collectively, genetic, or pharmacological downregulation of mTOR resulted in let-7 and miR-21 increase and subsequently in downregulation of CDK6. Accordingly, simultaneous inhibition of the mTOR pathway and CDK6 activity by PD-0332991 exerted greater antitumour activity than either treatment alone, both *in vitro* in human T-cell acute lymphoblastic leukaemia/lymphoma and *in vivo* (Gary et al., 2020).

Since PDAC is a molecularly diverse disease showing several genetic alterations, understanding the mechanisms underlying PDAC carcinogenesis is crucial for early diagnosis, risk stratification, and targeted therapeutic strategies (Bailey et al., 2016b; Cancer Genome Atlas, 2017; Jones et al., 2008; Knudsen et al., 2016). This part of the study, aimed to evaluate the therapeutic potential of PD-0332991 in two PDAC cells lines (Panc-1 and MiaPaCa-2 cells) with different genetic features; and assess the synergistic effects of PD-0332991 treatment and miR-21 knock out not only in cellular proliferation, survival, cell cycle arrest, senescence, apoptosis, glycolytic and oxidative metabolism but also the protein content between the different samples and their miRs signature profiles (treated/non-treated wt control cells, treated/non-treated miR-21 KO2 cells).

6.3. Results

6.3.1. PD-0332991 Reduced Cell Viability and Proliferation in PDAC cell lines and their miR-21 KOs

In previous associated studies (**Chapter 3**), it was found that miR-21 was highly upregulated in both Panc-1 and MiaPaCa-2 cell lines compared to normal pancreatic cell line (HPDE) (Mortoglou et al., 2022a). Therefore, in this part of the current study, the effect of PD-0332991 treatment on relative cell viability and colony-forming ability in Panc-1 and MiaPaCa-2 PDAC wt cells and their miR-21 KOs was examined. For this purpose, MTT and colony formation assays were used. Panc-1 and MiaPaCa-2 wt cells and their miR-21 KOs were treated with PD-0332991 (2 μM, for 24 h) as has been previously optimised (Rencuzogulları et al., 2020; Sevgin et al., 2021). It was found that PD-0332991 treatment significantly decreased cellular viability of Panc-1 wt cells by 22.9% ($n = 3$; $p \leq 0.01$), miR-21 KO2 by 77.2% and miR-1 KO4 cells by 83% ($n = 3$; $p \leq 0.0001$, for all) (**Figure 20A**). In MiaPaCa-2 cells, PD-0332991 treatment significantly reduced cellular viability of MiaPaCa-2 wt cells by 31.7% ($n = 3$; $p \leq 0.001$), miR-21 KO2 by 49.6% and miR-21 KO4 by 75.16% ($n = 3$; $p \leq 0.0001$, for all). MiR-21 deletion alone significantly reduced the cellular viability of PDAC cells. Panc-1 miR-21 KO2 and KO4 decreased cellular viability by 52.45% and 75.6%, respectively ($n = 3$; $p \leq 0.0001$, for all). MiaPaCa-2 miR-21 KO2 and KO4 showed a similar trend by reducing cell viability by 15% and 38.76%, respectively ($n = 3$; $p \leq 0.01$, for KO2; $p \leq 0.001$, for KO4). Moreover, a combination of PD-0332991 treatment and miR-21 deletion resulted in a higher suppression of cellular viability in both Panc-1 and MiaPaCa-2 cells (**Figure 20B**).

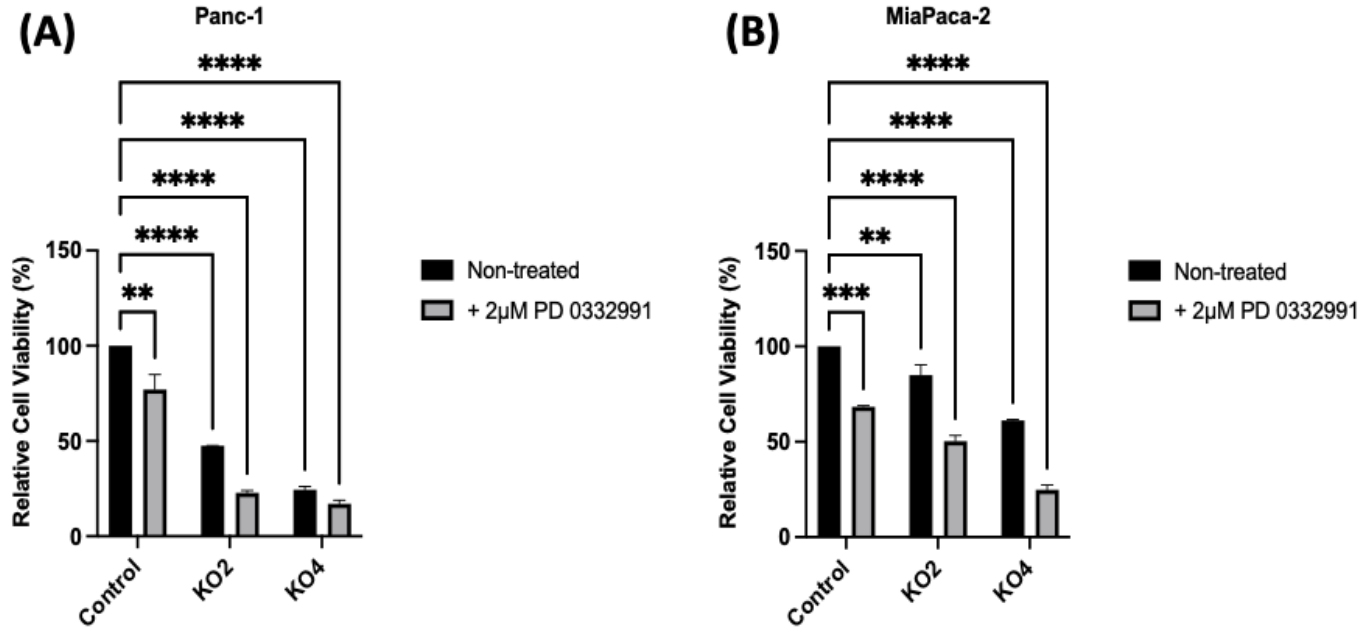
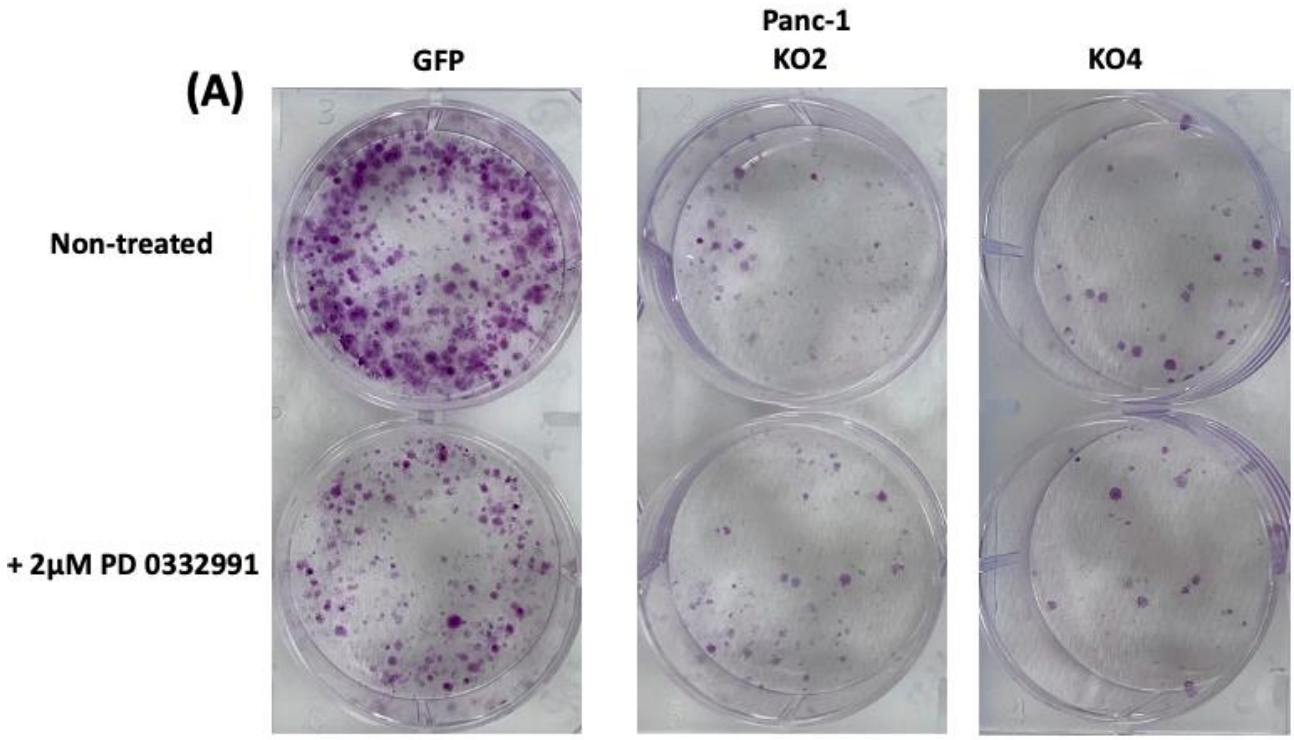
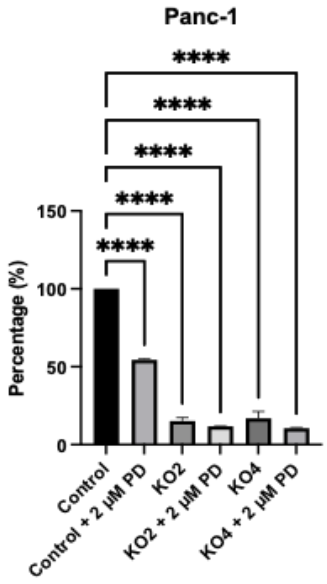


Figure 20: PD-0332991 decreased cell viability in Panc-1 and MiaPaCa-2 wt cells and their miR-21 KOs. (**A**) Panc-1 and (**B**) MiaPaCa-2 were treated with PD-0332991 (2 μM, for 24 h). Cells were incubated for 24 h and analysed by the MTT method. Cellular viability of treated Panc-1 miR-21 KO2 was also decreased by 50% compared to non-treated Panc-1 miR-21 KO2 ($n = 3$; $p \leq 0.01$), however miR-21 KO4 did not show the similar trend. Cellular viability of treated MiaPaCa-2 miR-21 KO2 and KO4 was also decreased by 40% and 59% compared to non-treated MiaPaCa-2 miR-21 KO2 and KO4 ($n = 3$; $p \leq 0.0001$, for all), respectively. The data are the mean ± SD of three technical repeats evaluated by One-way ANOVA and Bonferroni's multiple comparison test. Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2023).

Then, a colony-forming assay was conducted to examine the long-term effects of both PD-0332991 treatment (2 μM, for 24 h) and miR-21 KO. In line with the MTT assay results, PD-0332991 treatment significantly reduced the number of colonies in the Panc-1 wt cells, miR-21 KO2 and miR-21 KO4 by 46%, 89% and 90%, respectively ($n = 3$; $p \leq 0.0001$ for all) compared to untreated Panc-1 wt cells. Panc-1 miR-21 KO2 and KO4 alone also significantly reduced the number of colony formations by 84.5% and 83%, respectively ($n = 3$; $p \leq 0.0001$ for all) (**Figure 21A**). In the MiaPaCa-2 cell line, PD-0332991 treatment remarkably reduced the potential of colony formation of the treated MiaPaCa-2 wt cells, miR-21 KO2 and miR-21 KO4 cells by 90%, 92% and 66%, respectively ($n = 3$; $p \leq 0.0001$ for all). MiaPaCa-2 miR-21 KO2 and miR-21 KO4 alone also significantly reduced the colony numbers by 52% ($n = 3$; $p \leq 0.0001$) and 42.5 %, respectively ($n = 3$; $p \leq 0.001$ for all) (**Figure 21B**). The combination of PD-0332991 treatment and miR-21 KOs presented a more efficient therapeutic effect in the reduction of the colony formation in Panc-1 and MiaPaCa-2 cells.


(A)
Panc-1
GFP
KO2
KO4
Non-treated
+ 2µM PD 0332991


Panc-1
****
****
****
****
****
150
100
50
0
Percentage (%)
Control
Control + 2 µM PD
KO2
KO2 + 2 µM PD
KO4
KO4 + 2 µM PD

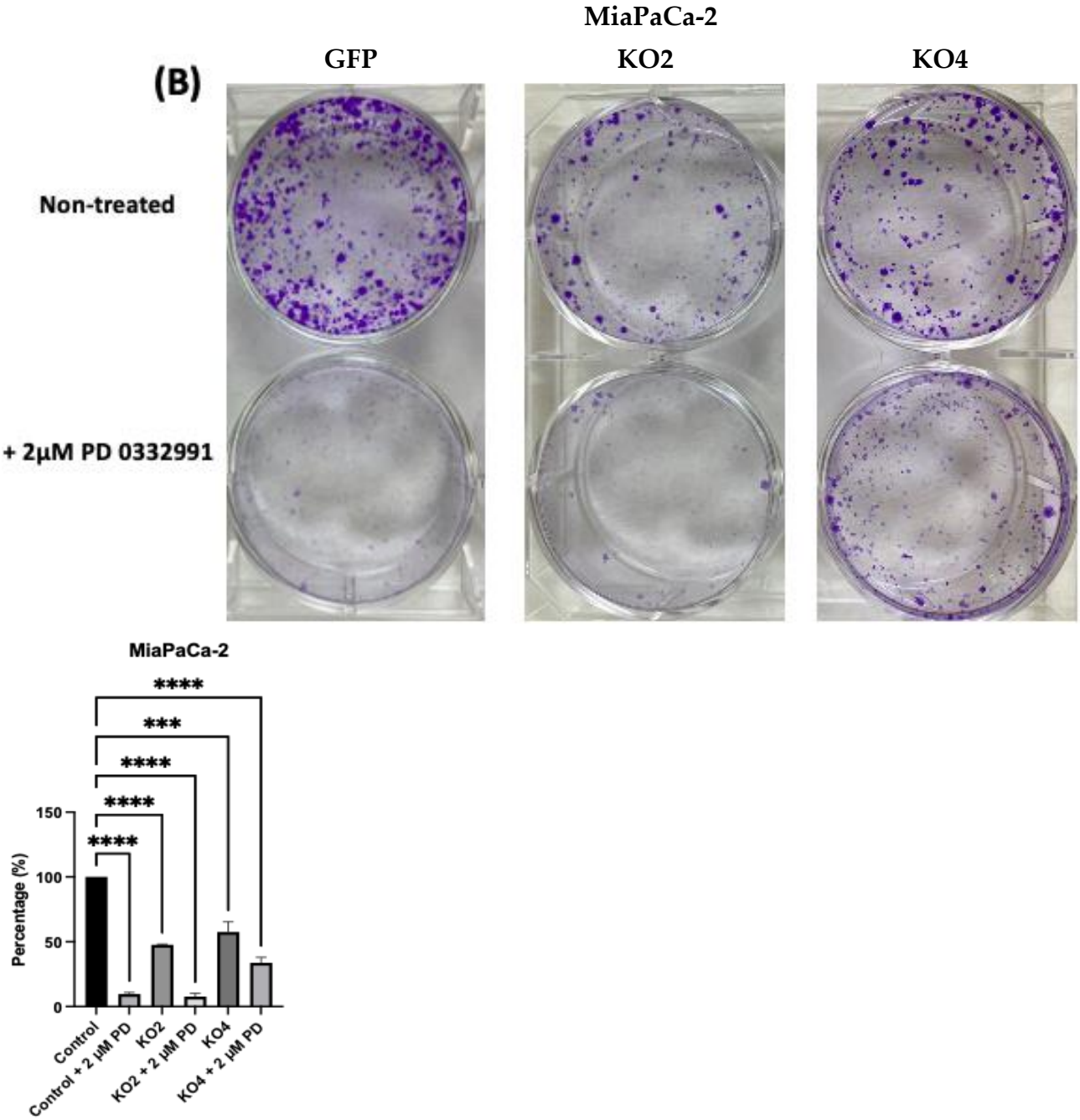


Figure 21: PD-0332991 decreased colony formation in Panc-1 and MiaPaCa-2 wt cells and their miR-21 KOs. The colony formation of (**A**) Panc-1 and (**B**) MiaPaCa-2 cells was counted after 10 days of treatment with media replenishment every 2 days. Graphs show the colony numbers quantification as percentages normalised to the control. The histograms represented the mean ± SD of three technical repeats; data evaluated by One-way ANOVA and Bonferroni's multiple comparison test. Panc-1 and MiaPaCa-2 cells were exposed to PD-0332991 (2 μM, for 24 h). Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2023).

6.3.2. PD-0332991 in Combination with miR-21 KOs Arrest the Cell Cycle and Induced Senescence

Flow cytometry was used to examine the effect of PD-0332991 on the cell cycle progression in Panc-1 and MiaPaCa-2 wt cells and their miR-21 KOs. To assess the effect of the CDK4/6 inhibitor on cell cycle distribution, Panc-1, MiaPaCa-2 wt cells and their miR-21 KOs were treated with PD-0332991 (2 μM, for 24 h). In the Panc-1 cell line, PD-0332991 treatment increased G1 cell cycle arrest by 34.45%, 37.3% and 35.9% in Panc-1 wt cells, miR-21 KO2 and miR-21 KO4, respectively ($n = 3$; $p \leq 0.0001$, for all), compared to non-treated Panc-1 wt cells. However, miR-21 deletion alone did not cause a significant G1 arrest in the Panc-1 cells. Additionally, both miR-21 deletion and PD-0332991 treatment suppressed the S phase. PD-0332991 treatment decreased the population of Panc-1 wt cells by 17.25% ($n = 3$; $p \leq 0.0001$), miR-21 KO2 by 18.45% ($n = 3$; $p \leq 0.0001$), and miR-21 KO4 by 17.83% ($n = 3$; $p \leq 0.0001$) in S phase. MiR-21 KO alone also reduced the population of Panc-1 miR-21 KO2 cells by 2.9% ($n = 3$; $p \leq 0.001$) and by 1.6% ($n = 3$; $p \leq 0.01$) in miR-21 KO4 cells in S phase, compared to Panc-1 wt cells. PD-0332991 treatment suppressed G2 phase by 16.55% ($n = 3$; $p \leq 0.001$) in Panc-1 wt cells, by 21.62% ($n = 3$; $p \leq 0.0001$) in Panc-1 miR-21 KO2 and by 17.9% ($n = 3$; $p \leq 0.001$) in Panc-1 miR-21 KO4 cells related to non-treated Panc-1 wt cells. Nonetheless, miR-21 knocking out alone did not show a significant reduction in the population of Panc-1 cells in the G2 phase of the cell cycle (**Figure 22A, Table 8**).

Table 8: Percentage of Panc-1 cell population in cell cycle.

Cell Cycle Phase	Control	Control + PD-0332991	KO2	KO2 + PD-0332991	KO4	KO4 + PD-0332991
G1	53.8%	87.4%	56%	91.95%	54.55%	87.25%
S	20.95%	3.7%	17.9%	2.4%	19.4%	2.6%
G2	25.25%	8.9%	26.1%	5.56%	26.05%	7.7%

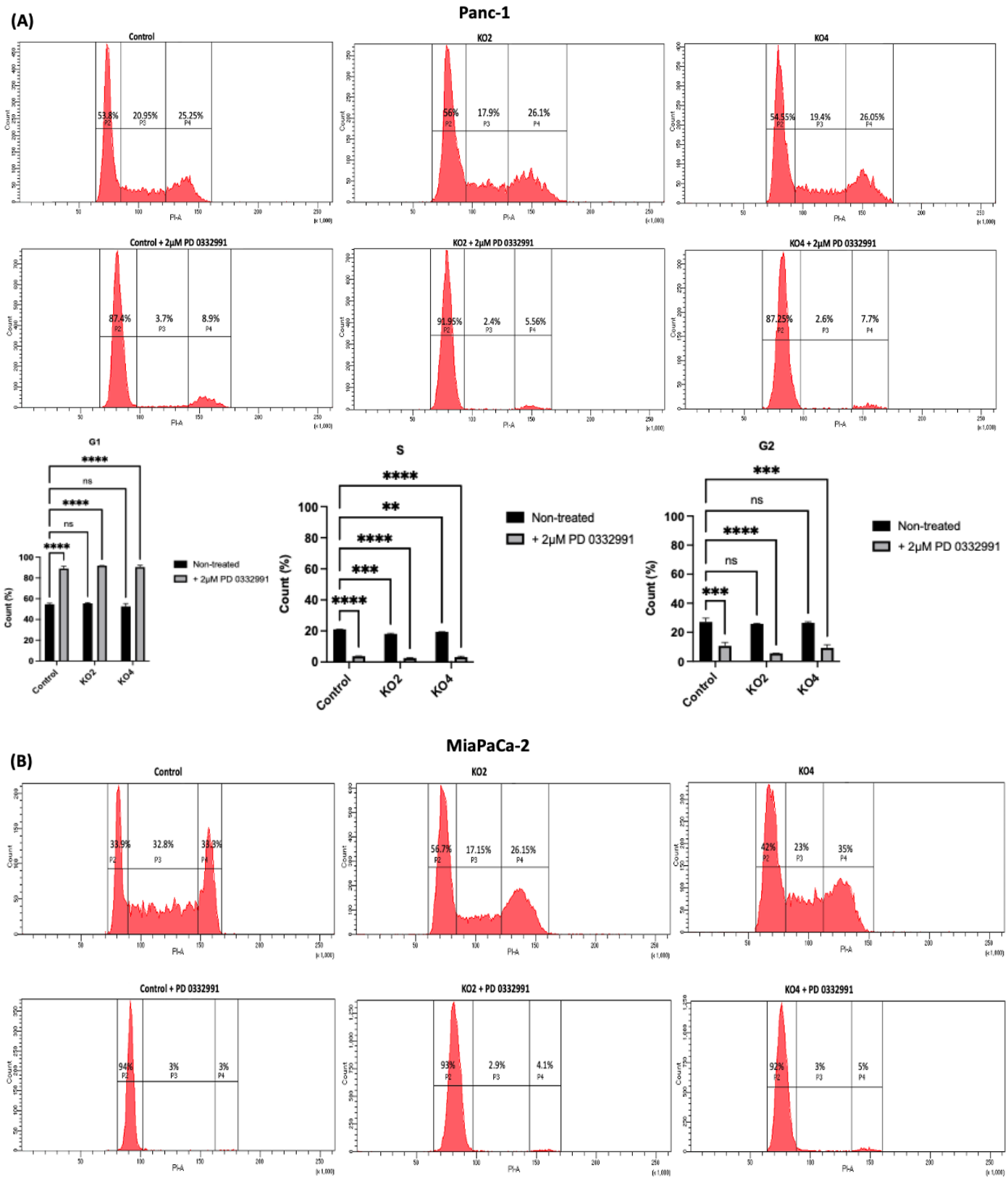
Adapted from Mortoglou et al., (2023).

In the MiaPaCa-2 cell line, it was showed that PD-0332991 treatment raised the G1 phase arrest rate by 60.5% in MiaPaCa-2 wt cells, by 61.9% in MiaPaCa-2 miR-21 KO2 and by 60.3% in MiaPaCa-2 miR-21 KO4 ($n = 3$; $p \leq 0.0001$ for all), compared to the non-treated MiaPaCa-2 wt cells. Additionally, miR-21 deletion alone presented a significant contribution in G1 phase arrest by increasing G1 cell cycle arrest by 25.25% ($n = 3$; $p \leq 0.0001$) in MiaPaCa-2 miR-21 KO2 cells and by 10.05% ($n = 3$; $p \leq 0.05$) in MiaPaCa-2 miR-21 KO4 cells compared to MiaPaCa-2 wt cells. It was also observed that miR-21 deletion alone and in combination with PD-0332991 treatment suppressed S cell cycle phase in MiaPaCa-2 cells. Specifically, PD-0332991 treatment suppressed S phase by 31.45%, 31.5% and 31.45% in MiaPaCa-2 wt cells, miR-21 KO2 and miR-21 KO4, respectively compared to non-treated MiaPaCa-2 wt cells ($n = 3$; $p \leq 0.0001$, for all). Similarly, in MiaPaCa-2 cells, miR-21 deletion alone, repressed S phase by 18.93% ($n = 3$; $p \leq 0.0001$) and 11.9% ($n = 3$; $p \leq 0.001$) in MiaPaCa-2 miR-21 KO2 and miR-21 KO4, respectively. A same trend was detected in the G2 phase of MiaPaCa-2 cells, where PD-0332991 treatment suppressed G2 phase by 29.85% ($n = 3$; $p \leq 0.0001$) in MiaPaCa-2 treated wt cells, by 30% ($n = 3$; $p \leq 0.0001$) in MiaPaCa-2 miR-21 KO2 cells and by 28.95% ($n = 3$; $p \leq 0.0001$) in MiaPaCa-2 miR-21 KO4 cells, compared to non-treated wt cells. Nevertheless, miR-21 deletion alone did not show significant suppression of G2 phase in MiaPaCa-2 cells (**Figure 22B, Table 9**).

Table 9: Percentage of MiaPaCa-2 cell population in cell cycle.

Cell Cycle Phase	Control	Control + PD-0332991	KO2	KO2 + PD-0332991	KO4	KO4 + PD-0332991
G1	33.9%	94%	56.7%	93%	42%	92%
S	32.8%	3%	17.15%	2.9%	23%	3%
G2	33.3%	3%	26.15%	4.1%	35%	5%

Adapted from Mortoglou et al., (2023).


(A)
Panc-1
Control
KO2
KO4
53.8%
20.95%
25.25%
56%
17.9%
26.1%
54.55%
19.4%
26.05%
Control + 2μM PD 0332991
KO2 + 2μM PD 0332991
KO4 + 2μM PD 0332991
87.4%
3.7%
8.9%
91.95%
2.4%
5.56%
87.25%
2.6%
7.7%
G1
S
G2
Count (%)
Non-treated
+ 2μM PD 0332991
Control
KO2
KO4
(B)
MiaPaCa-2
Control
KO2
KO4
33.9%
32.8%
33.3%
56.7%
17.15%
26.15%
42%
23%
35%
Control + PD 0332991
KO2 + PD 0332991
KO4 + PD 0332991
94%
3%
3%
93%
2.9%
4.1%
92%
3%
5%

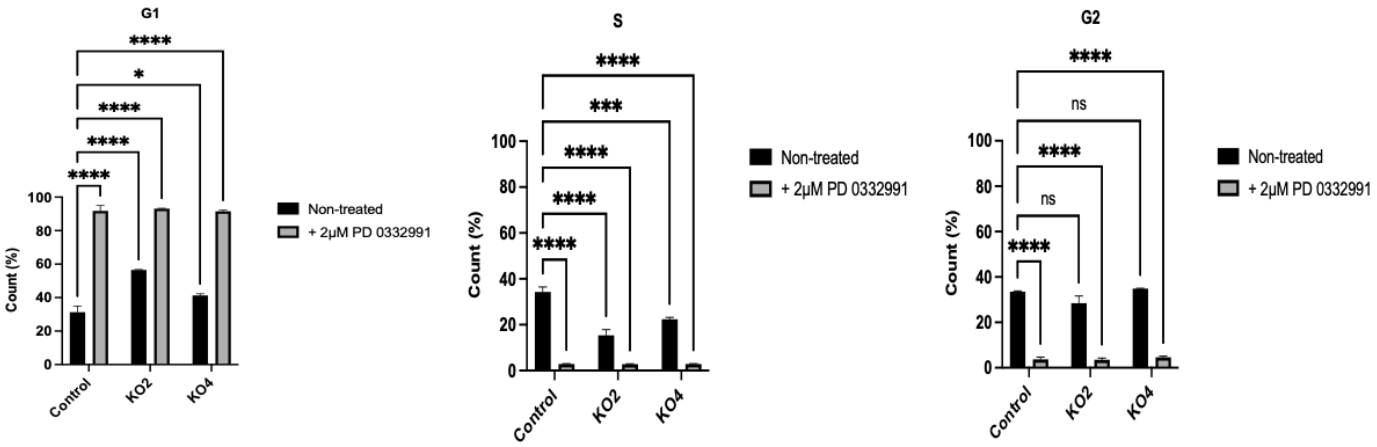
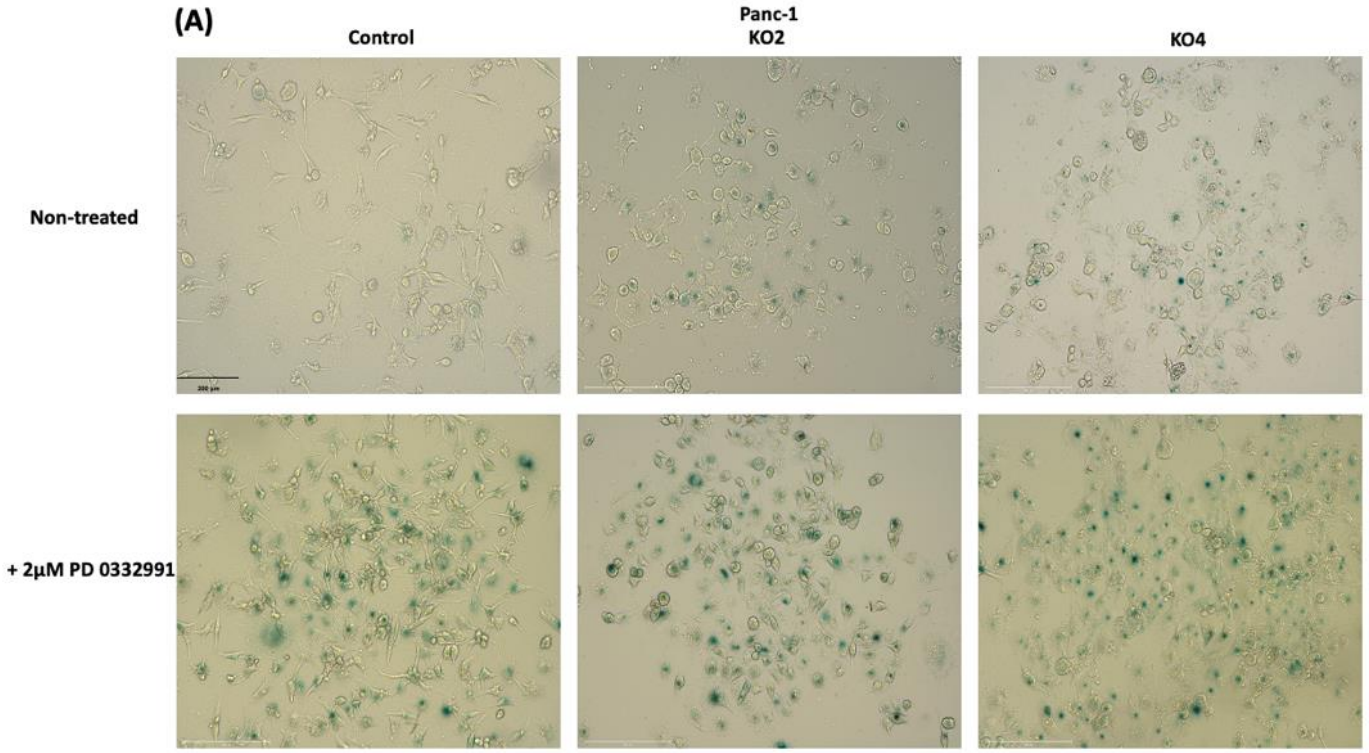
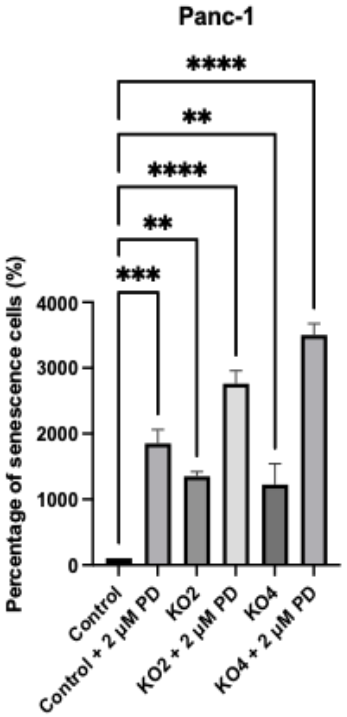


Figure 22: PD-0332991 induced cell cycle arrest in (A) Panc-1 and (B) MiaPaCa-2 wt cells and miR-21 KOs. Cells were treated with PD-0332991 (2 μM, for 24 h). After drug treatment, cells were fixed with 70% EtOH at +4°C for a week and stained with PI. The histograms represented the mean ± SD of three technical repeats; data evaluated by One-way ANOVA and Bonferroni's multiple comparison test. Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2023).

Next, the relationship between PD-0332991 treatment and the cellular senescence was further examined by performing a SA-β galactosidase test. After PD-0332991 treatment (2 μM, for 24 h) in Panc-1 and MiaPaCa-2 wt cells and their miR-21 KOs compared to non-treated wt cells. Panc-1 and MiaPaCa-2 treated wt cells and their treated miR-21 KOs stained positively with SA-β Gal compared to non-treated wt cells. Moreover, both Panc-1 and MiaPaCa-2 miR-21 KOs alone were stained positively with SA-β Gal (**Figure 23A,B**). Therefore, the results indicated that miR-21 KOs alone and in combination with PD-0332991 treatment triggered further senescence in both PDAC cell lines.


(A)
Control
Panc-1
KO2
KO4
Non-treated
+ 2μM PD 0332991


Panc-1
****
**
****
**
***
Percentage of senescence cells (%)
4000
3000
2000
1000
0
Control
Control + 2 μM PD
KO2
KO2 + 2 μM PD
KO4
KO4 + 2 μM PD

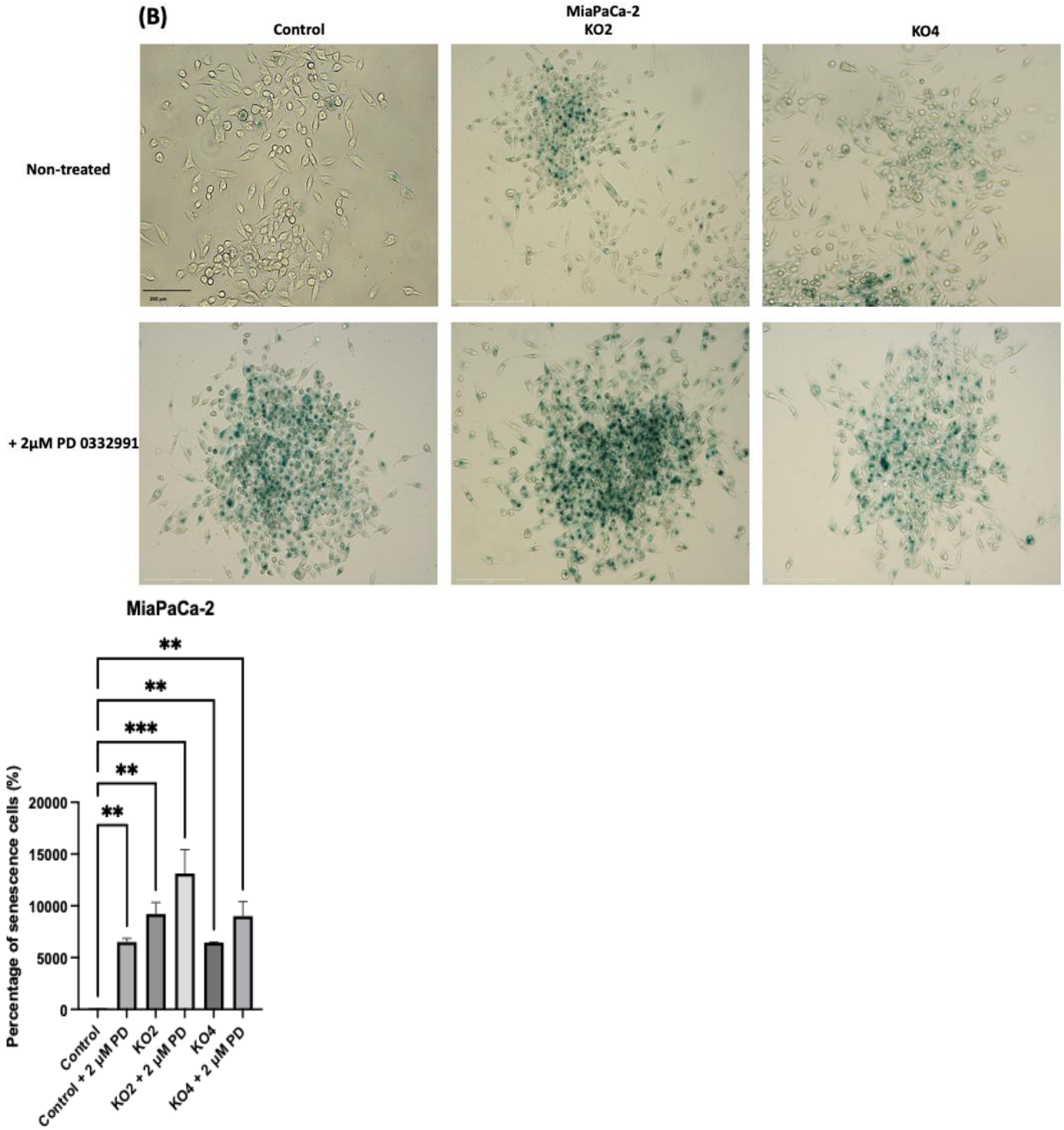


Figure 23: PD-0332991 treatment triggered cellular senescence in both (A) Panc-1 and (B) MiaPaCa-2 wt cells and their miR-21 KOs. Panc-1 and MiaPaCa-2 wt cells and their miR-21 KOs were exposed to Senescence β-Galactosidase Staining following treatment with the CDK4/6 inhibitor PD-0332991 (2 μM, for 24 h). After 24 h Senescence β-Galactosidase staining, cells were examined under the light microscope. The scale bar is 200 μm. Graphs show the cell numbers quantification normalised to

the control. The histograms represented the mean ± SD of three technical repeats; data evaluated by One-way ANOVA and Bonferroni's multiple comparison test. Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2023).

6.3.3. PD-0332991 Induced Apoptosis

The effects of PD-0332991 treatment (2 μM, for 24 h) and miR-21 KO in Panc-1 and MiaPaCa-2 PDAC cell lines were assessed on apoptosis induction through the Caspase 3/7 activation assay. No effects on apoptotic induction were observed in both either Panc-1 or MiaPaCa-2 wt cells treated with PD-0332991 compared to non-treated wt cells. However, PD-0332991 significantly induced apoptosis in Panc-1 miR-21 KO2 and KO4 by 350.4% and 412.2%, respectively ($n = 3$; $p \leq 0.01$, for all); and in MiaPaCa-2 miR-21 KO2 and KO4 by 291.8% and 342.33%, respectively ($n = 3$; $p \leq 0.01$, for KO2; $p \leq 0.001$, for KO4). Similarly, Panc-1 miR-21 KO2 and KO4 alone, increased Caspase 3/7 activity by 223.5% and 354.7%, respectively ($n = 3$; $p \leq 0.05$, for KO2; $p \leq 0.01$, for KO4) (**Figure 24A**), whereas MiaPaCa-2 miR-21 KO2 and KO4 alone significantly induced apoptosis by 164.1% and 250.1%, respectively ($n = 3$; $p \leq 0.05$, for KO2; $p \leq 0.01$, for KO4) (**Figure 24B**).

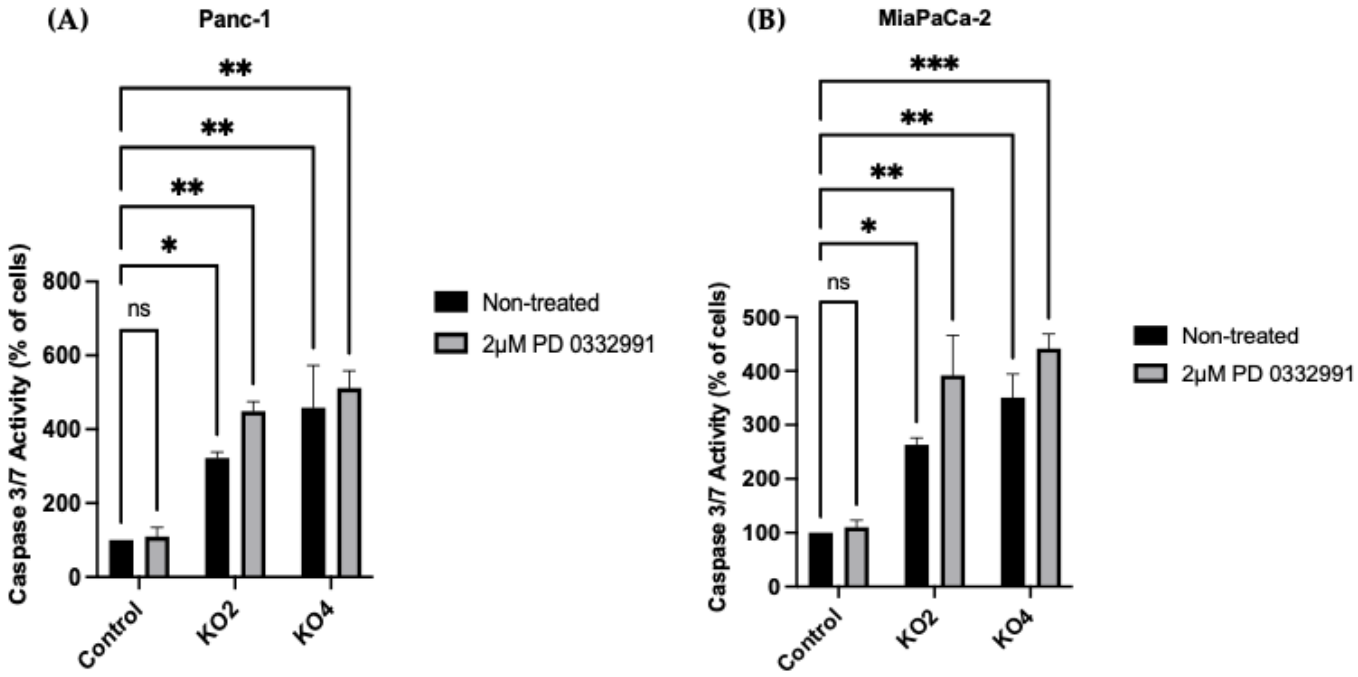
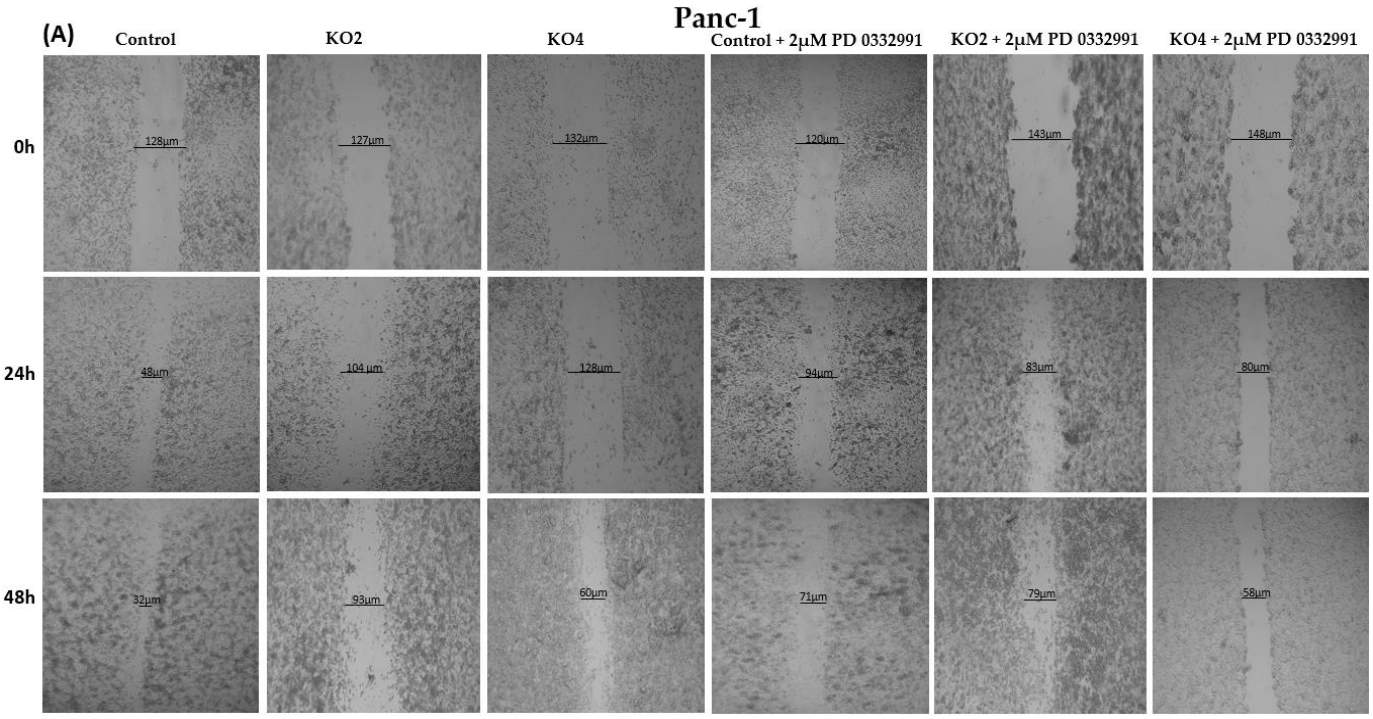
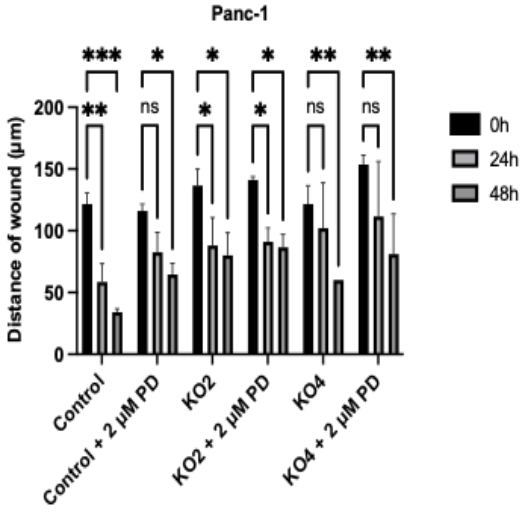


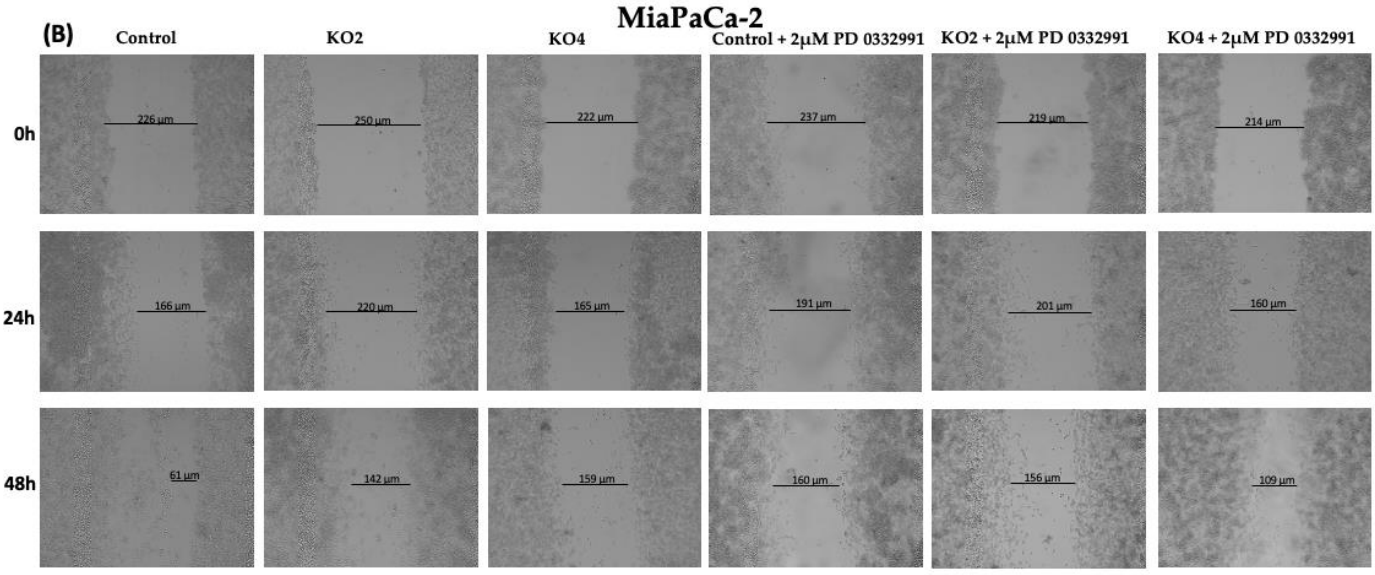
Figure 24: Effects of PD-0332991 on apoptotic cell death. (**A**) Panc-1 and (**B**) MiaPaCa-2 wt cells and their miR-21 KOs were treated with PD-0332991 (2 μM, for 24 h), and the caspase 3–7 activities were analysed. The histograms represented the mean ± SD from three technical repeats; data evaluated by One-way ANOVA and Bonferroni's multiple comparison test. Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2023).

6.3.4 PD-0332991 suppressed migration of Panc-1 and MiaPaCa-2 cells and their miR-21 KOs

Effects on cell motility was assessed for the PDAC cells treated with/out PD-0332991. For that purpose, a simple wound-healing assay was performed to confirm the potential effect of PD-0332991 on the migration of Panc-1 and MiaPaCa-2 wt cells and their miR-21 KOs. PD-0332991 treatment alone and in combination with miR-21 KO significantly reduced the wound healing capability of cells, while both Panc-1 and MiaPaCa-2 wt cells rapidly closed the wound site (**Figure 25A, B**) within 48 h.


Panc-1
(A)
Control
KO2
KO4
Control + 2μM PD 0332991
KO2 + 2μM PD 0332991
KO4 + 2μM PD 0332991
0h
24h
48h
128μm
127μm
132μm
120μm
143μm
148μm
48μm
104 μm
128μm
94μm
83μm
80μm
32μm
93μm
60μm
71μm
79μm
58μm


Panc-1
Distance of wound (μm)
200
150
100
50
0
***
**
*
ns
0h
24h
48h
Control
Control + 2 μM PD
KO2
KO2 + 2 μM PD
KO4
KO4 + 2 μM PD


MiaPaCa-2
(B)
Control
KO2
KO4
Control + 2μM PD 0332991
KO2 + 2μM PD 0332991
KO4 + 2μM PD 0332991
0h
24h
48h
226 μm
250 μm
222 μm
237 μm
219 μm
214 μm
166 μm
220 μm
165 μm
191 μm
201 μm
160 μm
61 μm
142 μm
159 μm
160 μm
156 μm
109 μm

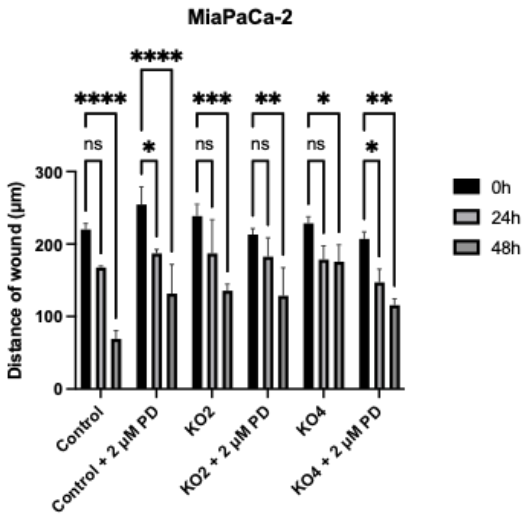
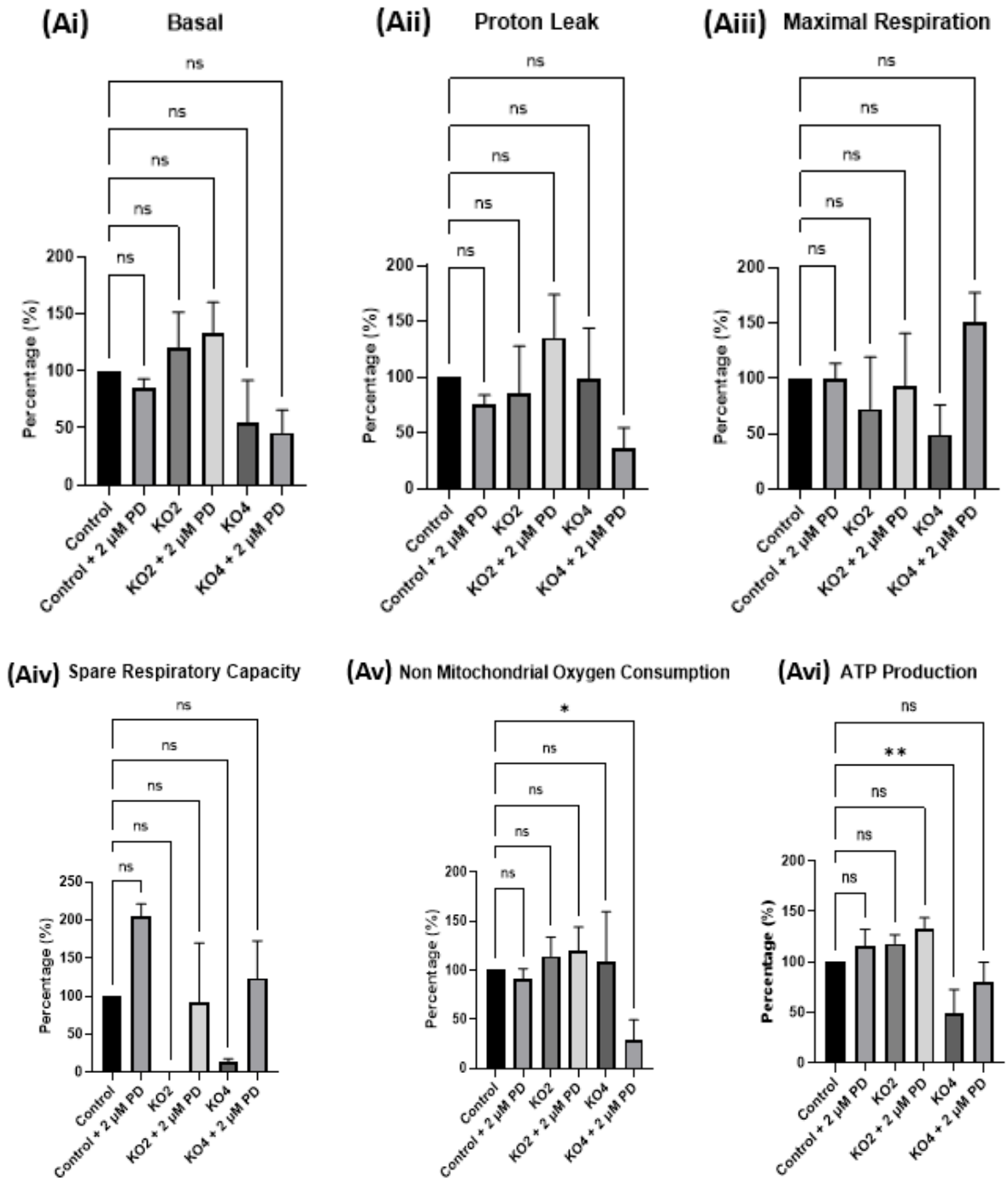


Figure 25: PD-0332991 decreased the motility of (A) Panc-1 and (B) MiaPaCa-2 wt cells and their miR-21 KOs. Wound healing assay was performed to compare the motility of Panc-1 and MiaPaCa-2 cells and their miR-21 KOs with/out PD-0332991. Images were obtained by using EVOS FL Auto 2 Imaging System (ThermoFisher, UK) with x10 magnification. The scale bar is 200 μm. The average distance of wound area was shown following measurement of at least 5 different areas for 0 h, 24 h, and 48 h incubation time. Each data point represents the mean ± SD of three technical repeats; data evaluated by One-way ANOVA and Bonferroni's multiple comparison test. Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2023).

6.3.5. Mitochondrial bioenergetics of PDAC cells following CDK4/6 inhibition and miR-21 deletion

The effect on key metabolic characteristics of PDAC cells, and their miR-21 KOs treated with PD-0332991 was assessed by using the Seahorse MitoStress Test. Parameters calculated in the form of bar chart included basal respiration, proton leak, maximal respiration, spare capacity, non-mitochondrial oxygen consumption and ATP-linked respiration (**Figure 26A,B**). In the Panc-1 cells, some alterations were observed in the basal respiration, proton leak, maximal respiration, and spare respiratory capacity of treated with PD-0332991 wt and miR-21 KOs compared to wt cells, however, these changes were not significant ($n = 3$; $p = 0.5066$, $p = 0.3019$, $p = 0.4615$, $p = 0.646$, for basal respiration, proton leak, maximal respiration, and spare respiratory capacity, respectively). There was a decrease by 71.6% ($n = 3$; $p \leq 0.05$) in the non-mitochondrial oxygen consumption of treated Panc-1 miR-21 KO4 cells (**Figure 26Av**) and a reduction by 50.4% ($n = 3$; $p \leq 0.01$) in the ATP production of Panc-1 miR-21 KO4 in relation to Panc-1 wt cells (**Figure 26Avi**). In the MiaPaCa-2 cells, changes were detected in the basal respiration, proton leak and ATP production, however, they were non-significant ($n = 3$; $p = 0.3342$, $p = 4202$, $p = 0.5027$, for basal respiration, proton leak and ATP production, respectively). Compared MiaPaCa-2 miR-21 KO2 and KO4 cells with MiaPaCa-2 wt cells, it was detected that miR-21 knockout alone, resulted in the increase of maximal respiratory capacity by 62.53% and 60.25%, respectively ($n = 3$; $p \leq 0.05$, for all) (**Figure 26Biii**). Furthermore, the non-mitochondrial oxygen consumption of MiaPaCa-2 miR-21 KO2 cells was increased by 102.5% in comparison to MiaPaCa-2 wt cells ($n = 3$; $p \leq 0.05$) (**Figure 26Biv**). Moreover, in the MiaPaCa-2 cell line, there was not an activity in the spare respiratory capacity.


(Ai) Basal
(Aii) Proton Leak
(Aiii) Maximal Respiration
(Aiv) Spare Respiratory Capacity
(Av) Non Mitochondrial Oxygen Consumption
(Avi) ATP Production
Percentage (%)
ns
*
**
Control
Control + 2 µM PD
KO2
KO2 + 2 µM PD
KO4
KO4 + 2 µM PD

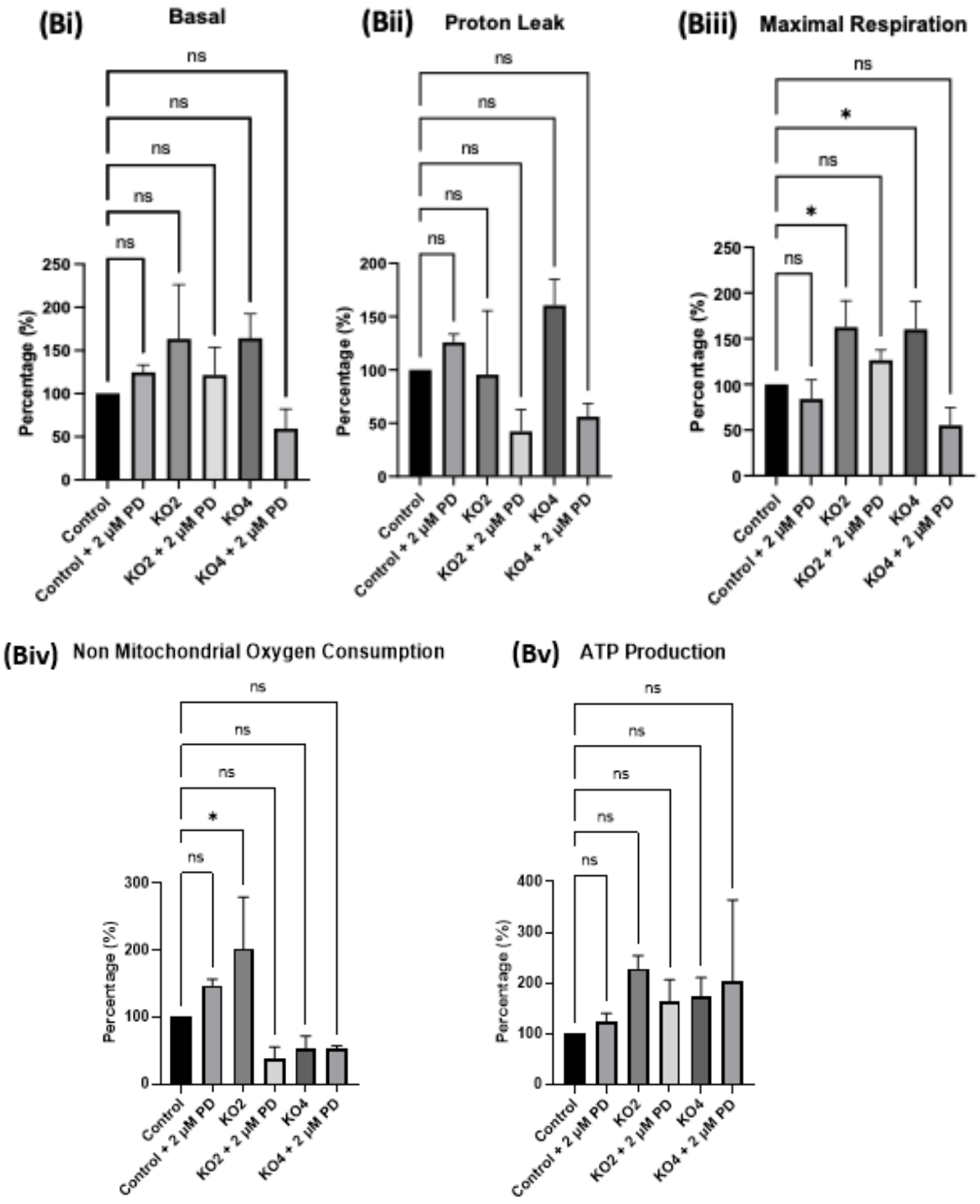


Figure 26: Mitochondrial respiration function parameters of (A) Panc-1 cells and (B) MiaPaCa-2 cells and their miR-21 KOs (KO2, KO4) treated with PD-0332991 (2 µM, for 24 h) using the Seahorse XFe24 extracellular flux analyser. Changes of mitochondrial respiration were analysed with basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial oxygen consumption and ATP production. The data are the mean ± SD of three technical repeats and three

biological repeats evaluated by One-way ANOVA and Bonferroni's multiple comparison test. Exact p-values are indicated (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$); error bars indicate SD. Adapted from Mortoglou et al., (2023).

6.3.6. Proteomic Profiles of PDAC cells and their miR-1 KO2s with/out PD-0332991 treatment

Total proteomic content of each group of cells for both Panc-1 and MiaPaCa-2 cell lines were compared using LC-MS/MS analysis. Protein hits (shared and unique - from the analysis using the R-script) are shown in **Figure 27** and are further listed and summarised in **Supplementary Tables 2-5**.

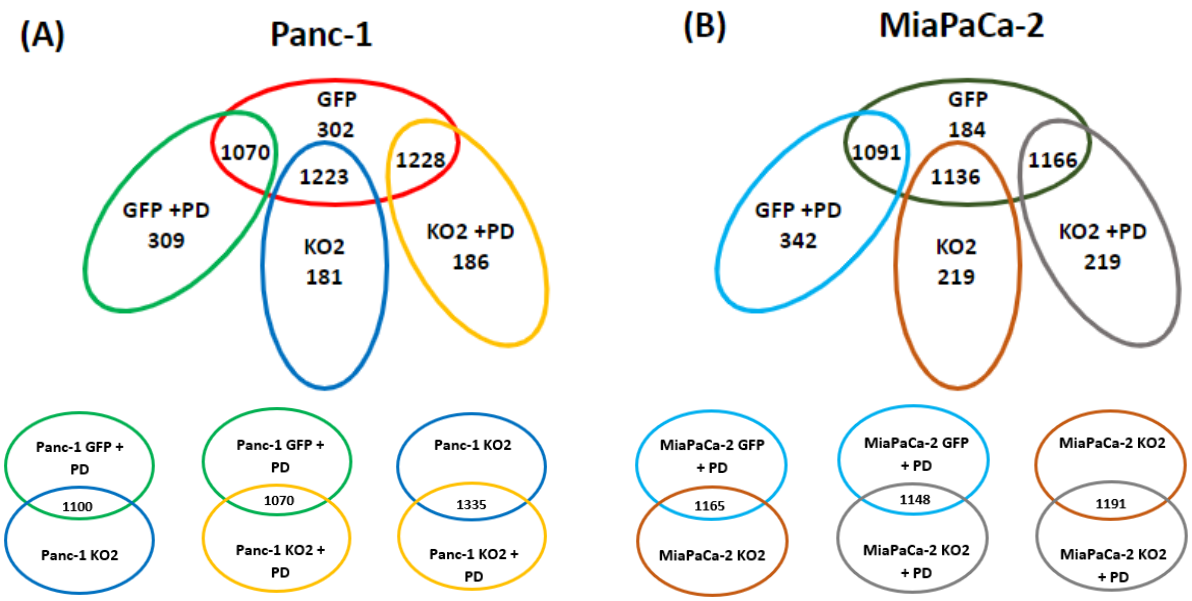
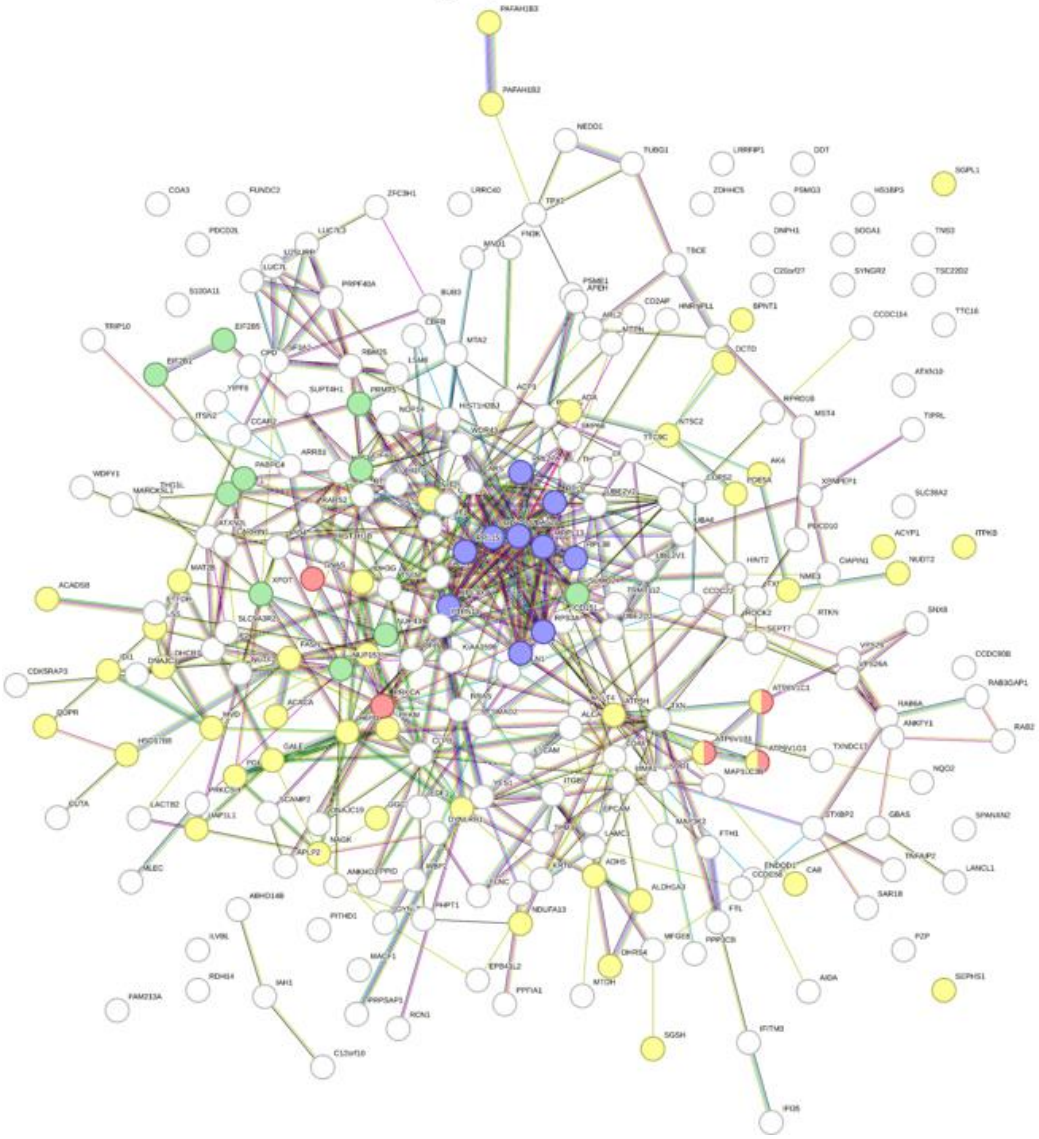


Figure 27: Venn diagram comparing the accession numbers of proteins (unique and shared protein hits) identified in four different conditions (GFP, GFP + PD-0332991, miR-21 KO2, miR-21 KO2 +PD-0332991) in both (A) Panc-1 and (B) MiaPaCa-2 PDAC cell lines. Adapted from Mortoglou et al., (2023).

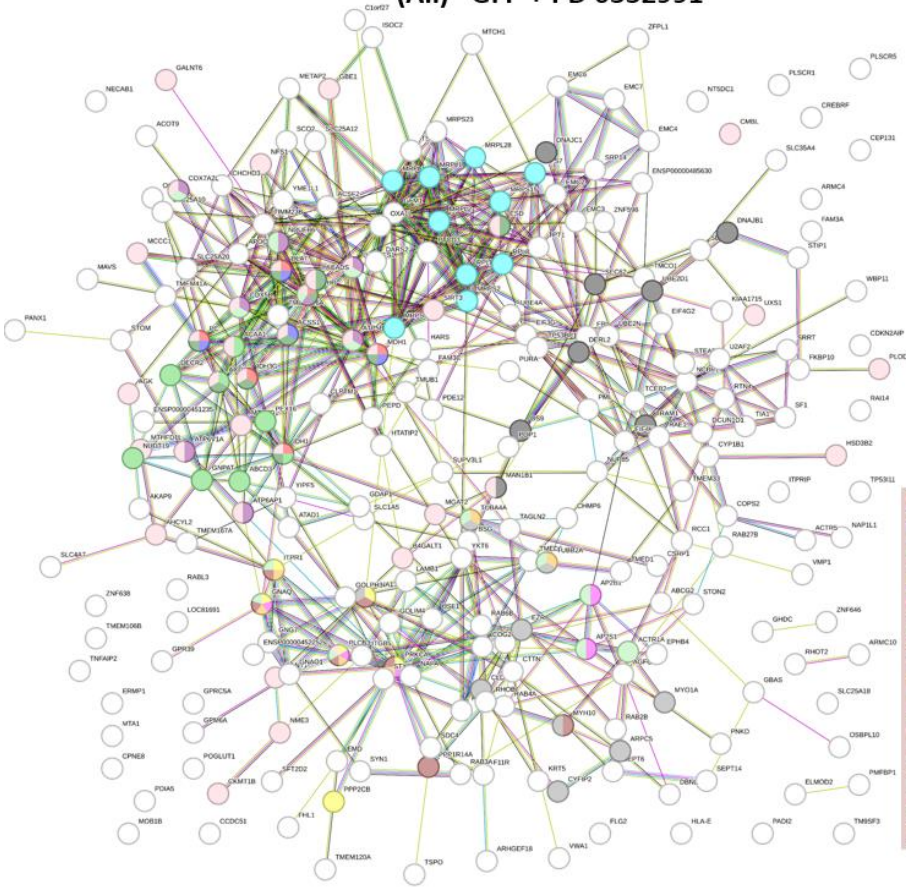
Protein lists for each group were then analysed to STRING (Searching Tool of Retrieval of Interacting Genes/Proteins; **https://string-db.org/**, accessed on October 2022) to illustrate protein-interaction networks and pathway analysis (**Figure 28A, B**). KEGG and Reactome specific pathways for each group are presented in **Table 10**. Additionally, common KEGG pathways between non-treated Panc-1 wt and treated wt include ribosome and metabolic pathways. In MiaPaCa-2 cells, the common KEGG pathway between all subtypes was ribosome; spliceosome was common between MiaPaCa-2 wt and treated wt, whereas KEGG pathway specific to MiaPaCa-2 treated wt and MiaPaCa-2 miR-21 KO2 was mRNA surveillance. Another example of a common KEGG pathway between MiaPaCa-2 treated wt, miR-21 KO2 and treated miR-21 KO2 was RNA transport.

Panc-1

(Ai) GFP


KEGG
Vibrio cholerae infection
Ribosome
RNA transport
Metabolic pathways

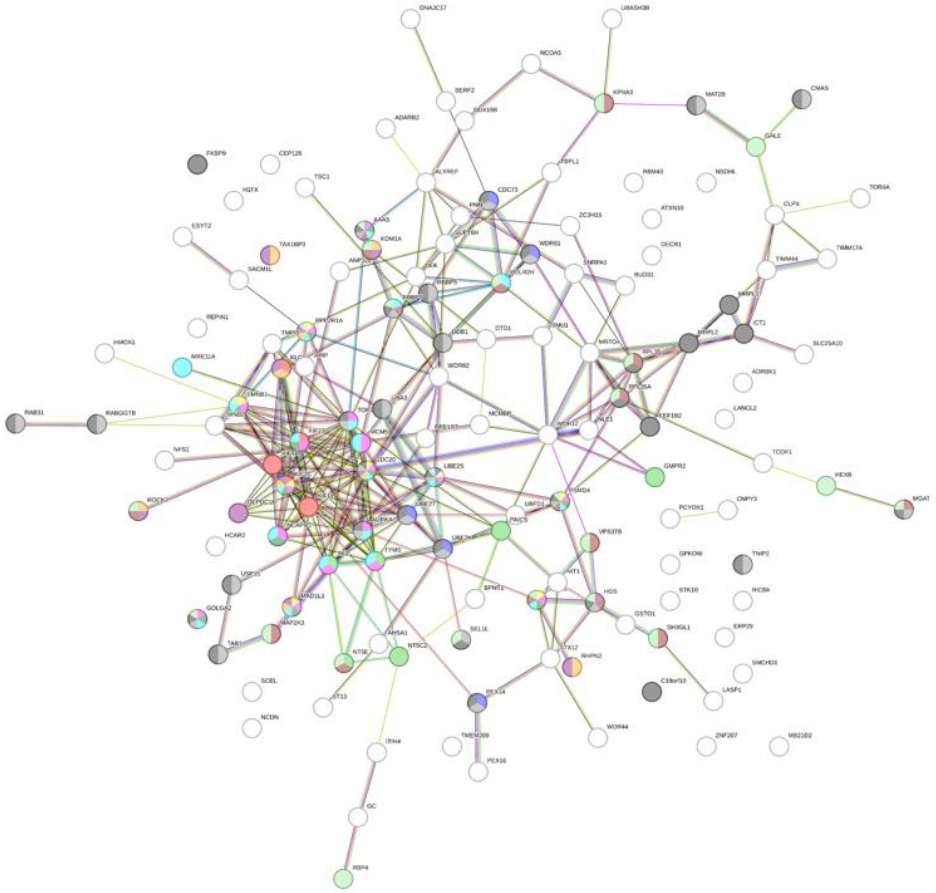
(Aii) GFP + PD 0332991



KEGG

- Citrate cycle (TCA cycle)
- Pyruvate metabolism
- Peroxisome
- Long-term depression
- Endocrine and factor-regulated calcium reabsorption
- Carbon metabolism
- Ribosome
- Gap junction
- Oxidative phosphorylation
- Vascular smooth muscle contraction
- Pathogenic *E.coli* infection
- Protein processing in endoplasmic reticulum
- Huntington disease
- Metabolic pathways

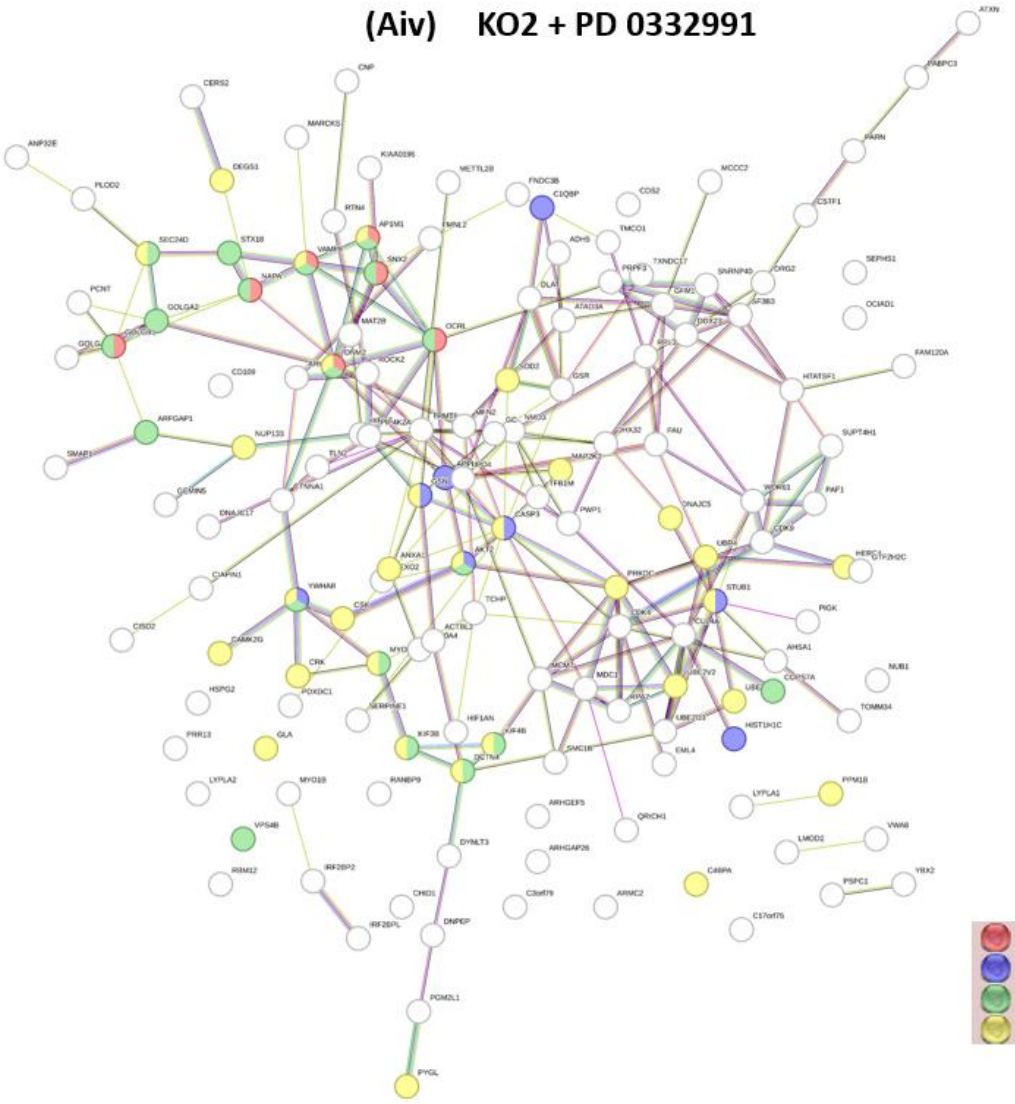
(Aiii) KO2



Reactome

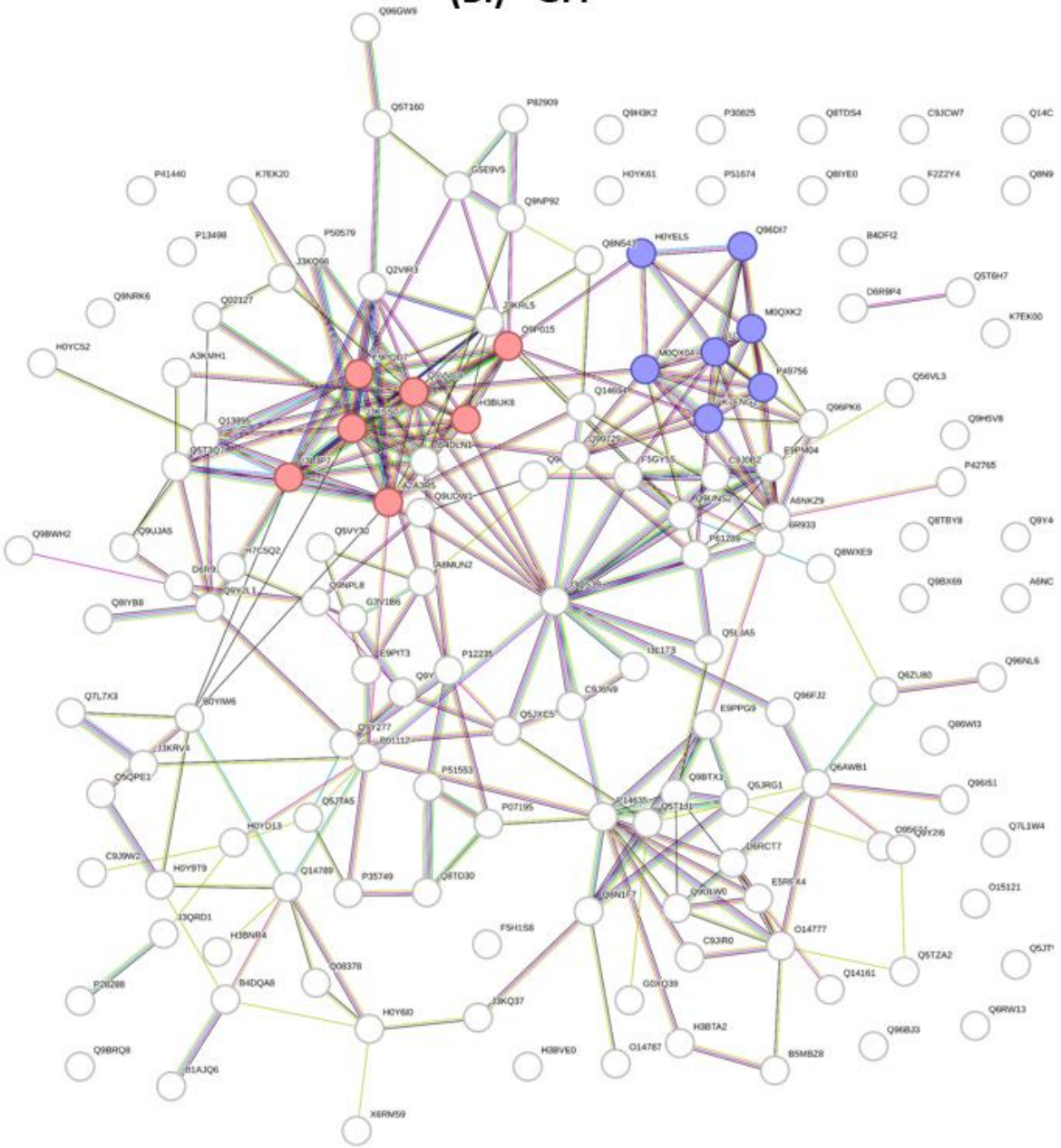
- Kinesins
- Protein ubiquitination
- Metabolism of nucleotides
- Mitotic anaphase
- Cell cycle, mitotic
- M phase
- Cell cycle
- RHO GTPase effectors
- Signaling by Rho GTPases
- Infectious disease
- Post-translational protein modification
- Metabolism of proteins
- Disease

(Aiv) KO2 + PD 0332991

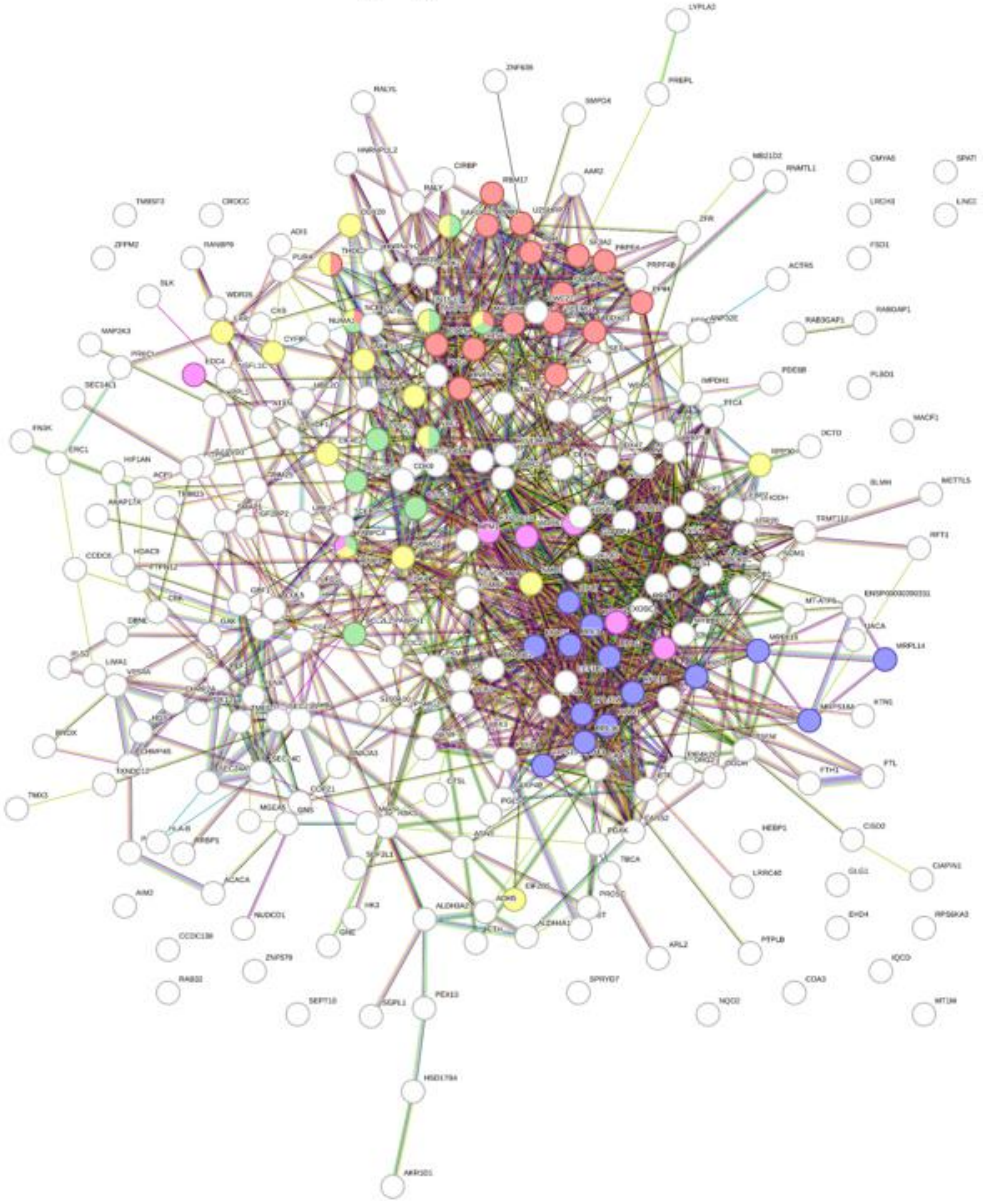


MiaPaCa-2

(Bi) GFP



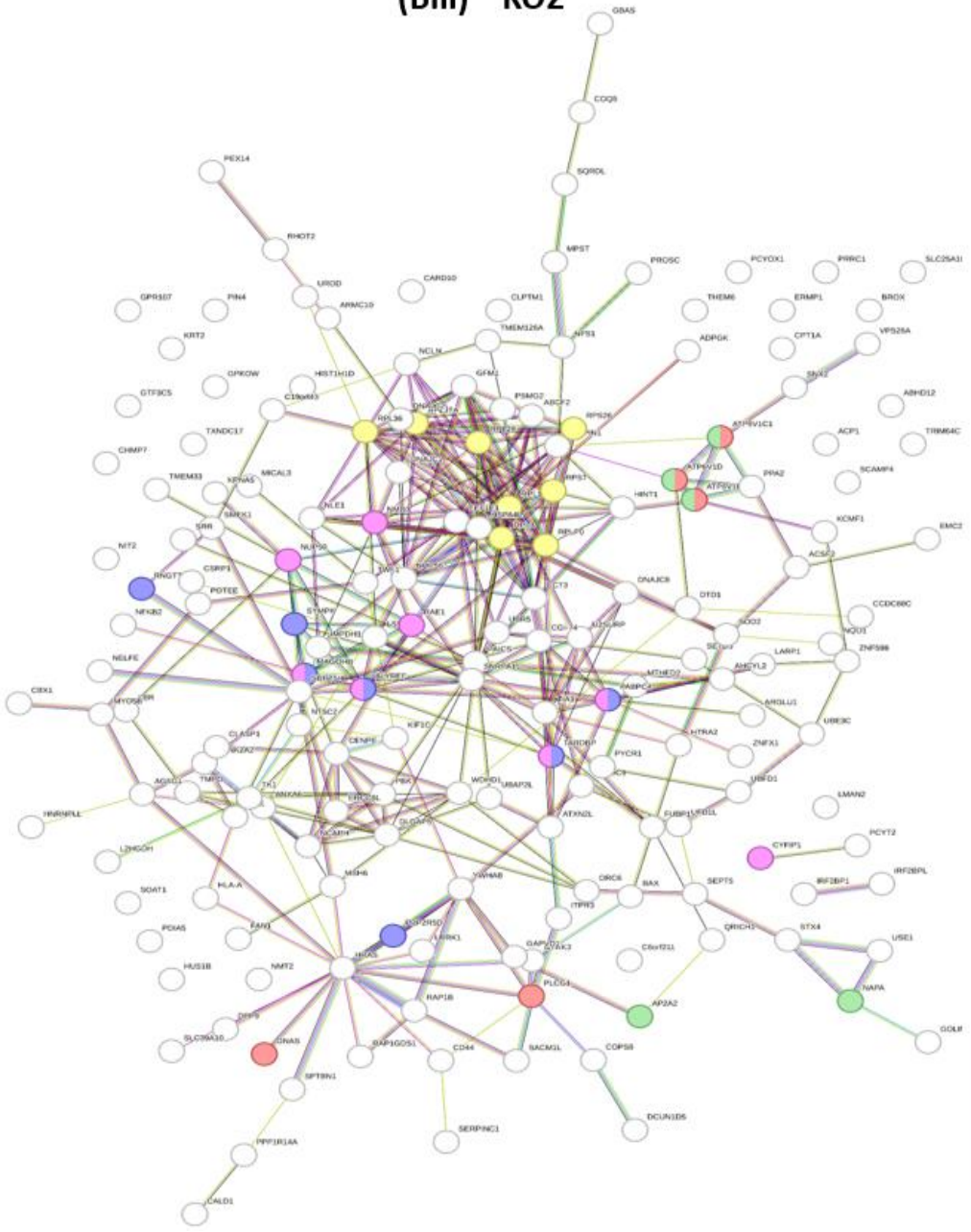
(Bii) GFP + PD 0332991



KEGG

- Spliceosome
- Ribosome
- mRNA surveillance pathway
- RNA transport
- RNA degradation

(Biii) KO2


KEGG
Vibrio cholerae infection
mRNA surveillance pathway
Synaptic vesicle cycle
Ribosome
RNA transport

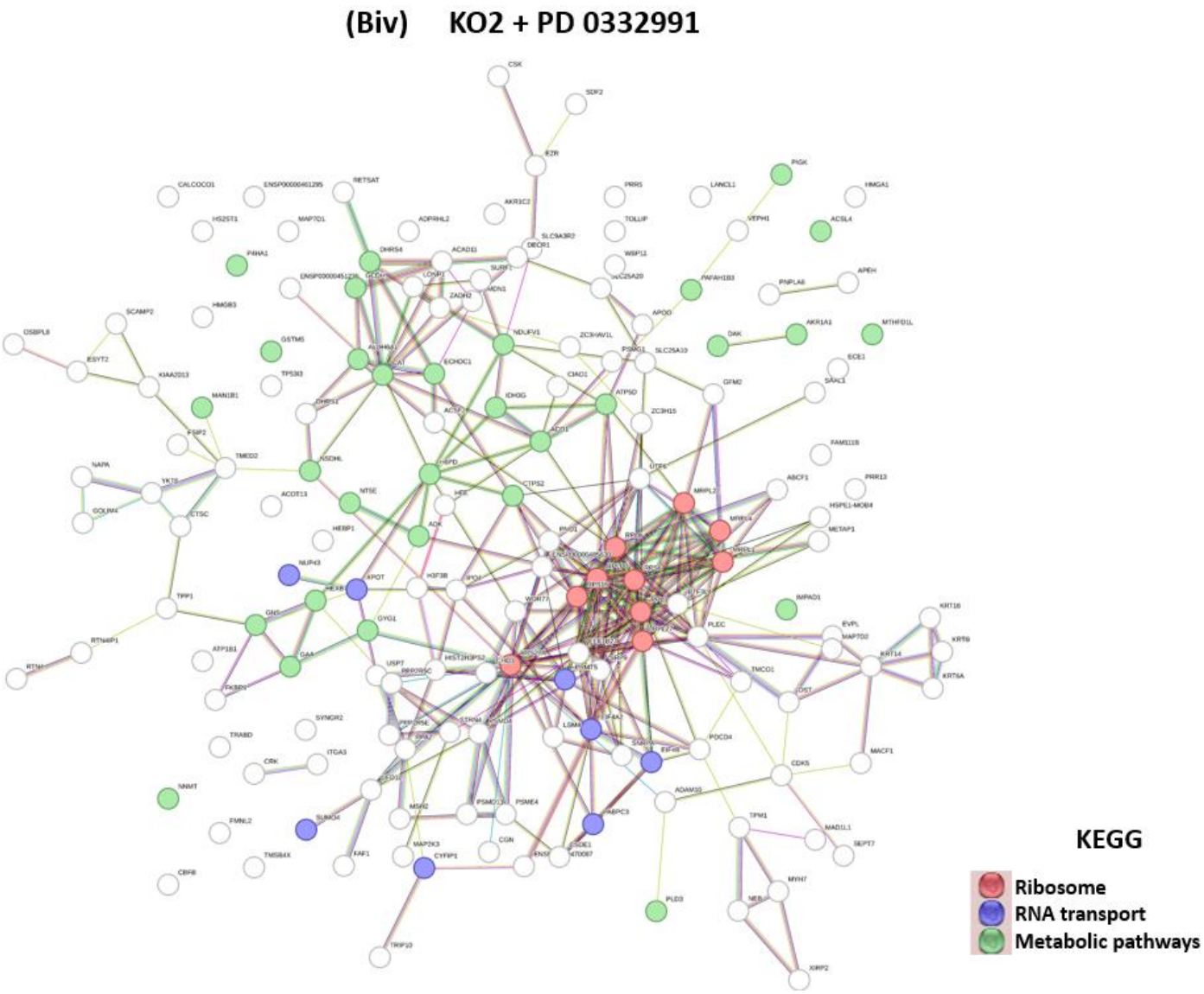


Figure 28: KEGG and Reactome pathway analysis for protein network analysis for each examined group, presenting predicted protein networks annotating associated KEGG and Reactome pathways for total protein of (A) Panc-1 (Ai) GFP (Aii) GFP + PD-0332991 (Aiii) miR-21 KO2 (Aiv) miR-21 KO2 + PD-0332991 and (B) MiaPaCa-2 (Bi) GFP (Bii) GFP + PD-0332991 (Biii) miR-21 KO2 (Biv) miR-21 KO2 + PD-0332991. Adapted from Mortoglou et al., (2023).

Table 10: KEGG and Reactome pathways for total proteomic content of treated and non-treated with PD-0332991 Panc-1 and MiaPaca-2 wt and miR-21 KO2s, as examined by LC-MS/MS analysis.

Panc-1 GFP	Panc-1 GFP+PD	Panc-1 KO2	Panc-1 KO2+PD	MiaPaCa-2 GFP	MiaPaCa-2 GFP+PD	MiaPaCa-2 KO2	MiaPaCa-2 KO2+PD
Metabolic pathways (45)	Citrate cycle (TCA cycle) (6)	Cell Cycle (20)	Membrane Trafficking (19)	Ribosome (7)	Spliceosome (18)	Ribosome (8)	Ribosome (10)
Ribosome (10)	Carbon metabolism (10)	Cell Cycle, Mitotic (17)	Golgi Associated Vesicle Biogenesis (7)	Spliceosome (7)	RNA transport (17)	mRNA surveillance pathway (7)	RNA transport (8)
RNA transport (10)	Ribosome (10)	Metabolism of proteins (34)	Immune System (31)		Ribosome (14)	RNA transport (8)	Metabolic pathways (30)
Vibrio cholerae infection (5)	Peroxisome (8)	Post-translational protein modification (26)	Programmed Cell Death (8)		mRNA surveillance pathway (10)	Vibrio cholerae infection (5)	

Huntington disease (14)	M Phase (12)	RNA degradation (7)	Synaptic vesicle cycle (5)
Long-term depression (6)	Protein ubiquitination (6)		
Metabolic pathways (37)	Metabolism of nucleotides (6)		
Oxidative phosphorylation (8)	Kinesins (5)		
Endocrine and other factor-regulated calcium reabsorption (5)	Disease (24)		
Pathogenic Escherichia coli infection (9)	Signalling by Rho GTPases (11)		
Gap junction (6)	RHO GTPase Effectors (9)		

	Protein processing in endoplasmic reticulum (8)	Infectious disease (16)
	Pyruvate metabolism (4)	Mitotic Anaphase (8)
	Vascular smooth muscle contraction (7)	

() Indicates Observed Gene Count. Adapted from Mortoglou et al., (2023).

6.3.7. MiRs signature profiles of PDAC 3D culture cells and their miR-21 KOs with/out PD-0332991

For the examination of miRs signature profiles of PDAC cells, PDAC 3D culture cells have been generated. Specifically, it has been shown previously that organoids can reproduce more effectively the characteristics of tumour microenvironment and are more suitable for basic and translational research on PDAC (Below et al., 2022). MiRs signature profiles for each group of cells for both Panc-1 and MiaPaCa-2 PDAC 3D cell culture were compared by using small RNA-sequencing analysis. PDAC 3D culture cells were used for this study, as it has been suggested previously to be not only more physiologically relevant compared to monolayer cell culture models (two-dimensional cultures) but also more effective for genome editing (Dossena et al., 2020; Fatehullah et al., 2016). MiRs signature profiles (shared and unique - from the analysis using Galaxy software) are shown in **Figure 29, Figure 30 and Table 11** and are further listed and summarised in **Supplementary Table 6,7**.

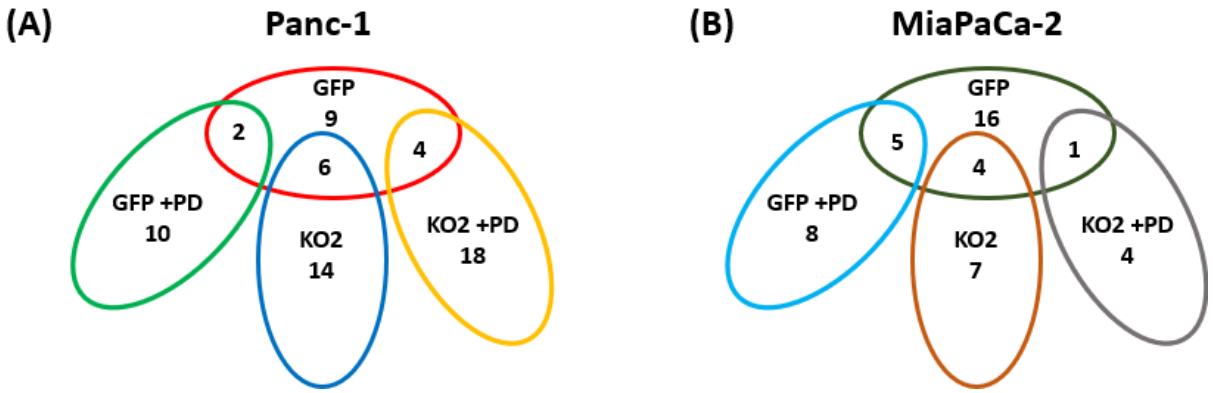


Figure 29: Venn diagram comparing the aligned reads of miRs (unique and shared) identified in four different conditions (GFP, GFP + PD-0332991, miR-21 KO2, miR-21 KO2 +PD-0332991) in both (A) Panc-1 and (B) MiaPaCa-2 PDAC cell lines.

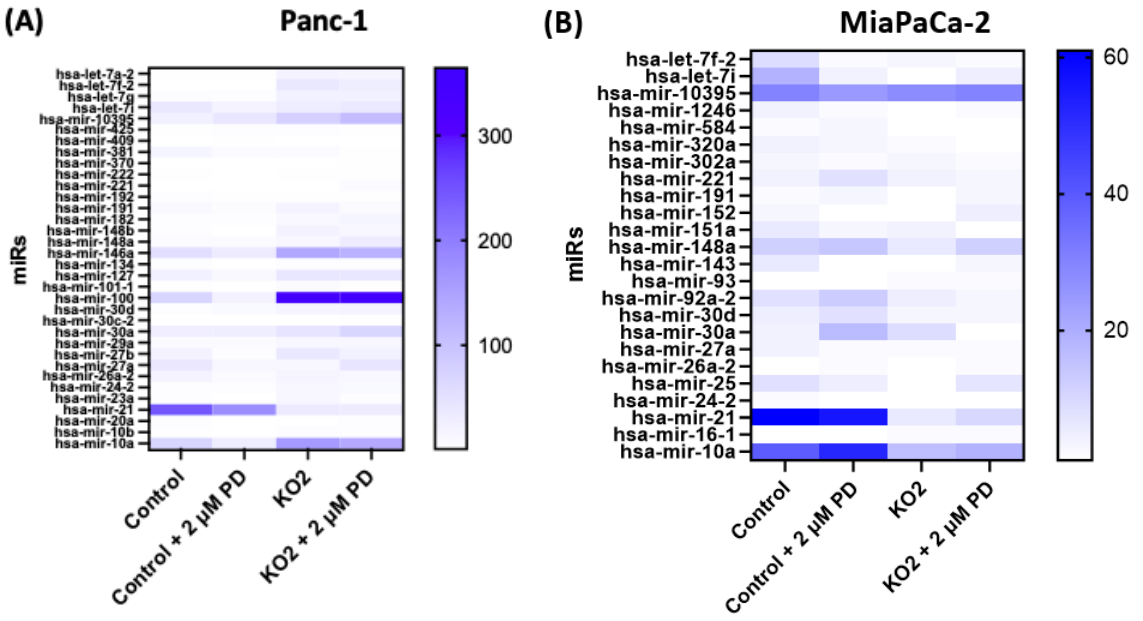


Figure 30: Heatmaps presenting the common miRs in four different conditions (GFP, GFP + PD-0332991, miR-21 KO2, miR-21 KO2 +PD-0332991) comparing the aligned reads of miRs between (A) Panc-1 and (B) MiaPaCa-2 PDAC 3D culture cells.

Table 11: Uniquely expressed miRs between PDAC 3D culture cells.

Panc-1 GFP	Panc-1 GFP + PD	Panc-1 KO2	Panc-1 KO2 + PD	MiaPaCa-2 GFP	MiaPaCa-2 GFP + PD	MiaPaCa-2 KO2	MiaPaCa-2 KO2 + PD
hsa-miR-4521 (1)	hsa-miR-10397 (1)	hsa-miR-671 (1)	hsa-let-7e (1)	hsa-miR-3615 (1)	hsa-let-7b (1)	hsa-miR-5587 (1)	hsa-miR-1301 (1)
hsa-miR-663a (1)	hsa-miR-3652 (3)	hsa-miR-656 (1)	hsa-miR-4427 (1)	hsa-miR-1251 (1)	hsa-miR-4657 (1)	hsa-miR-381 (1)	hsa-miR-361 (1)
hsa-miR-497 (1)	hsa-miR-3609 (2)	hsa-miR-641 (1)	hsa-miR-3613 (1)	hsa-miR-671 (1)	hsa-miR-574 (1)	hsa-miR-302b (1)	hsa-miR-216b (1)
hsa-miR-342 (1)	hsa-miR-1538 (2)	hsa-miR-589 (1)	hsa-miR-1181 (3)	hsa-miR-615 (1)	hsa-miR-328 (1)	hsa-miR-192 (1)	hsa-miR-99a (1)
hsa-miR-205 (1)	hsa-miR-1182 (1)	hsa-miR-505 (1)	hsa-miR-889 (1)	hsa-miR-424 (1)	hsa-miR-146a (2)	hsa-miR-186 (1)	
hsa-miR-200a (1)	hsa-miR-431 (1)	hsa-miR-495 (1)	hsa-miR-654 (1)	hsa-miR-365a (1)	hsa-miR-140 (1)	hsa-miR-125b-1 (1)	

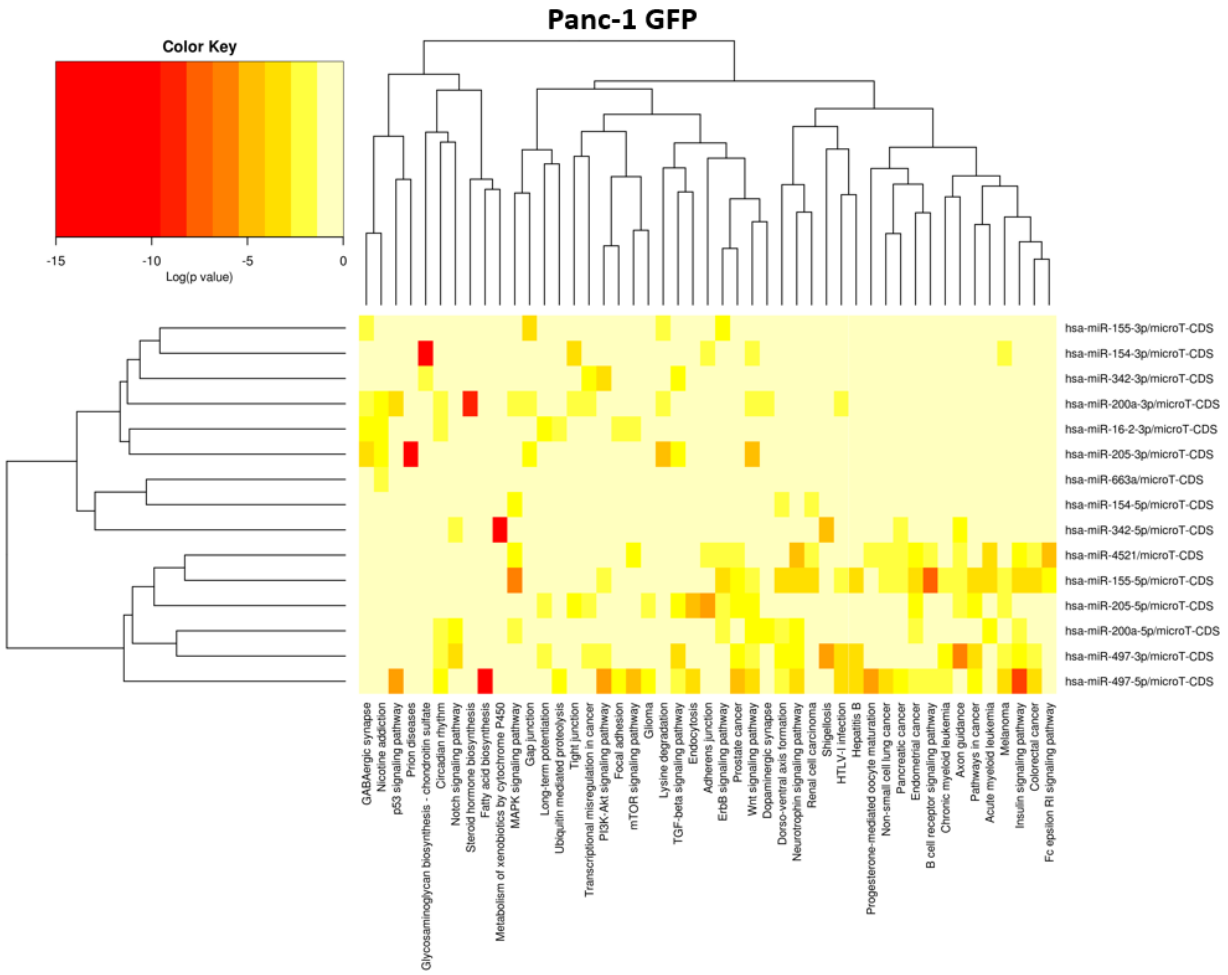
	(1)					
hsa-miR-155 (1)	hsa-miR-429 (1)	hsa-miR-494 (2)	hsa-miR-543 (2)	hsa-miR-320d-2 (1)	hsa-miR-100 (1)	hsa-miR-125a (1)
hsa-miR-154 (1)	hsa-miR-194-2 (1)	hsa-miR-485 (1)	hsa-miR-539 (1)	hsa-miR-320b-2 (2)	hsa-miR-10b (1)	
hsa-miR-16-2 (1)	hsa-miR-99a (3)	hsa-miR-454 (1)	hsa-miR-328 (1)	hsa-miR-197 (1)		
	hsa-miR-34a (1)	hsa-miR-320a (1)	hsa-miR-324 (1)	hsa-miR-181a-2 (1)		
		hsa-miR-196a-2 (2)	hsa-miR-302c (1)	hsa-miR-106b (2)		
		hsa-miR-193a (2)	hsa-miR-302b (1)	hsa-miR-101- (1)		
		hsa-miR-101-2 (1)	hsa-miR-195 (1)	hsa-miR-98 (1)		
		hsa-miR-98	hsa-miR-129-2	hsa-miR-30e		

(1)	(1)	(1)
	hsa-miR-125b-1 (4)	hsa-miR-30b (1)
	hsa-miR-96 (1)	hsa-miR-18a (1)
	hsa-miR-93 (2)	
	hsa-miR-29b-1 (1)	

(Aligned Reads)

Then the signalling pathways, which are associated with specific miRs in each of the four different conditions (GFP, GFP + PD-0332991, miR-21 KO2, miR-21 KO2 +PD-0332991) in each of the PDAC cell lines (Panc-1, MiaPaCa-2) were accessed by using DIANA tools and specifically mirPath (version 3). Results are shown in **Figure 31.**

(A)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-5
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Log(p value)
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hsa-miR-154-3p/microT-CDS
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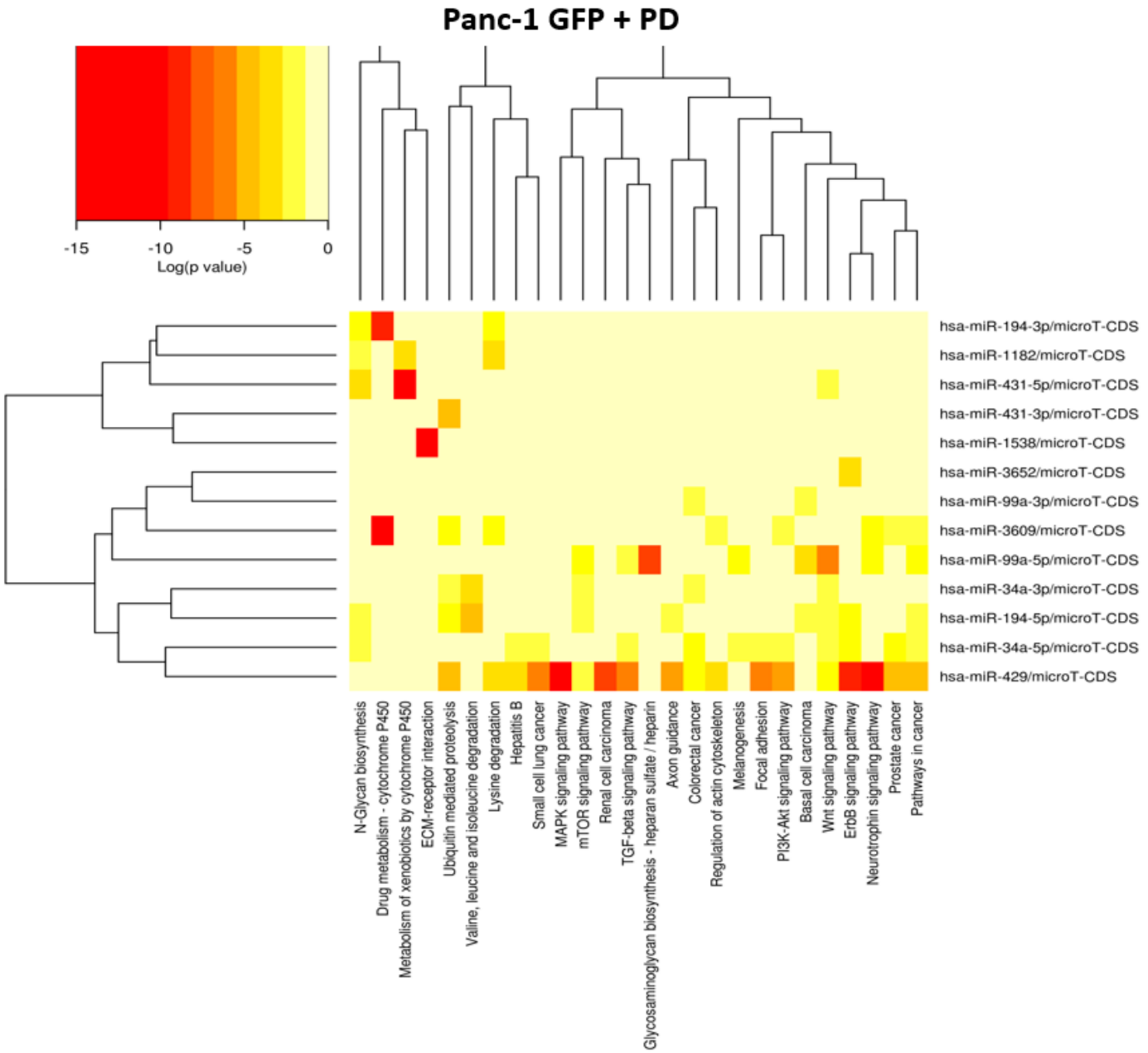
hsa-miR-200a-3p/microT-CDS
hsa-miR-16-2-3p/microT-CDS
hsa-miR-205-3p/microT-CDS
hsa-miR-663a/microT-CDS
hsa-miR-154-5p/microT-CDS
hsa-miR-342-5p/microT-CDS
hsa-miR-4521/microT-CDS
hsa-miR-155-5p/microT-CDS
hsa-miR-205-5p/microT-CDS
hsa-miR-200a-5p/microT-CDS
hsa-miR-497-3p/microT-CDS
hsa-miR-497-5p/microT-CDS
GABAergic synapse
Nicotine addiction
p53 signaling pathway
Prion diseases
Glycosaminoglycan biosynthesis - chondroitin sulfate
Circadian rhythm
Notch signaling pathway
Steroid hormone biosynthesis
Fatty acid biosynthesis
Metabolism of xenobiotics by cytochrome P450
MAPK signaling pathway
Gap junction
Long-term potentiation
Ubiquitin mediated proteolysis
Tight junction
Transcriptional misregulation in cancer
PI3K-Akt signaling pathway
Focal adhesion
mTOR signaling pathway
Glioma
Lysine degradation
TGF-beta signaling pathway
Endocytosis
Adherens junction
ErbB signaling pathway
Prostate cancer
Wnt signaling pathway
Dopaminergic synapse
Dorso-ventral axis formation
Neurotrophin signaling pathway
Renal cell carcinoma
Shigellosis
HTLV-I infection
Hepatitis B
Progesterone-mediated oocyte maturation
Non-small cell lung cancer
Pancreatic cancer
Endometrial cancer
B cell receptor signaling pathway
Chronic myeloid leukemia
Axon guidance
Pathways in cancer
Acute myeloid leukemia
Melanoma
Insulin signaling pathway
Colorectal cancer
Fc epsilon RI signaling pathway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-10
-5
0
Log(p value)
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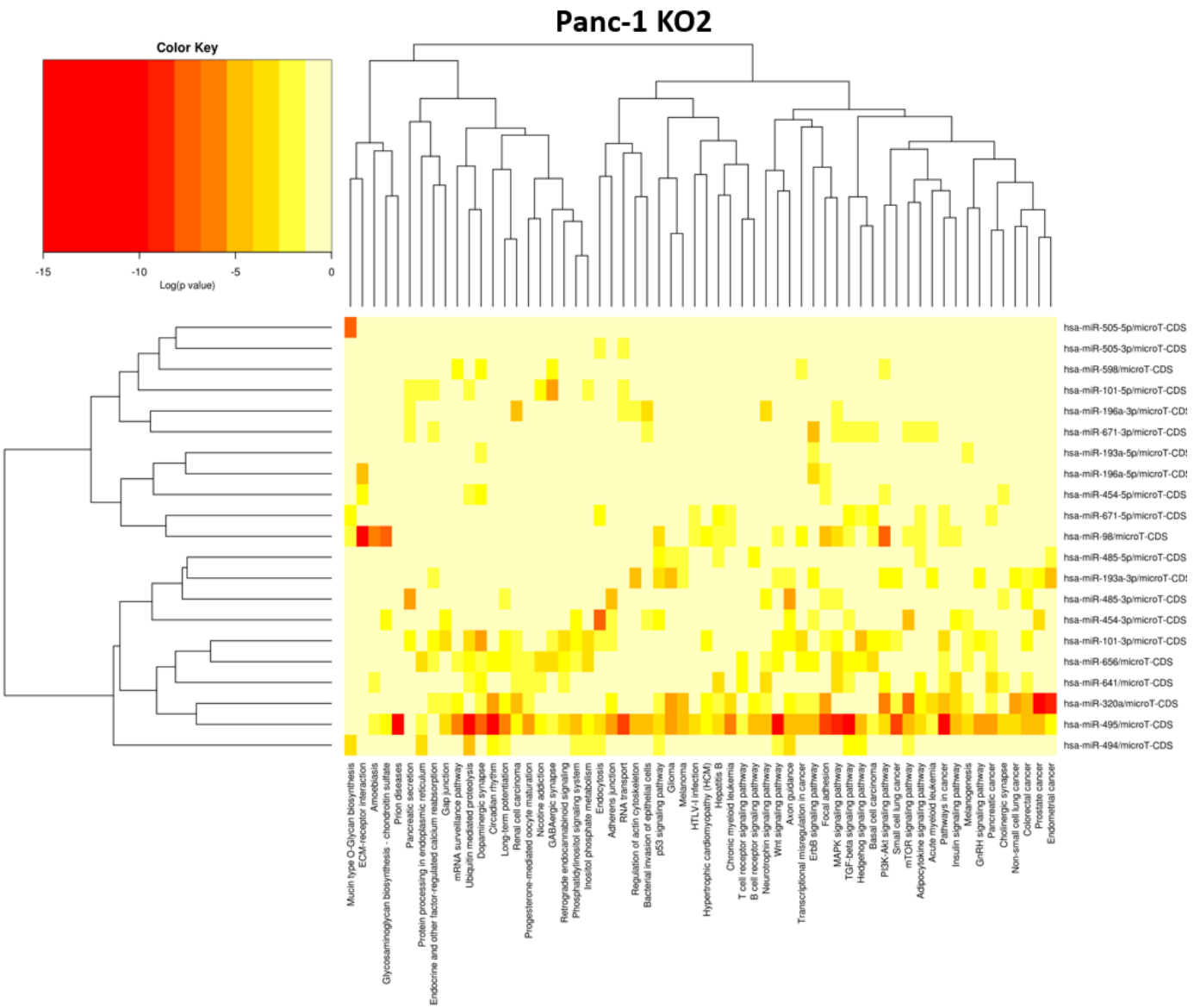
hsa-miR-1182/microT-CDS
hsa-miR-431-5p/microT-CDS
hsa-miR-431-3p/microT-CDS
hsa-miR-1538/microT-CDS
hsa-miR-3652/microT-CDS
hsa-miR-99a-3p/microT-CDS
hsa-miR-3609/microT-CDS
hsa-miR-99a-5p/microT-CDS
hsa-miR-34a-3p/microT-CDS
hsa-miR-194-5p/microT-CDS
hsa-miR-34a-5p/microT-CDS
hsa-miR-429/microT-CDS
N-Glycan biosynthesis
Drug metabolism - cytochrome P450
Metabolism of xenobiotics by cytochrome P450
ECM-receptor interaction
Ubiquitin mediated proteolysis
Valine, leucine and isoleucine degradation
Lysine degradation
Hepatitis B
Small cell lung cancer
MAPK signaling pathway
mTOR signaling pathway
Renal cell carcinoma
TGF-beta signaling pathway
Glycosaminoglycan biosynthesis - heparan sulfate / heparin
Axon guidance
Colorectal cancer
Regulation of actin cytoskeleton
Melanogenesis
Focal adhesion
PI3K-Akt signaling pathway
Basal cell carcinoma
Wnt signaling pathway
ErbB signaling pathway
Neurotrophin signaling pathway
Prostate cancer
Pathways in cancer


Panc-1 KO2
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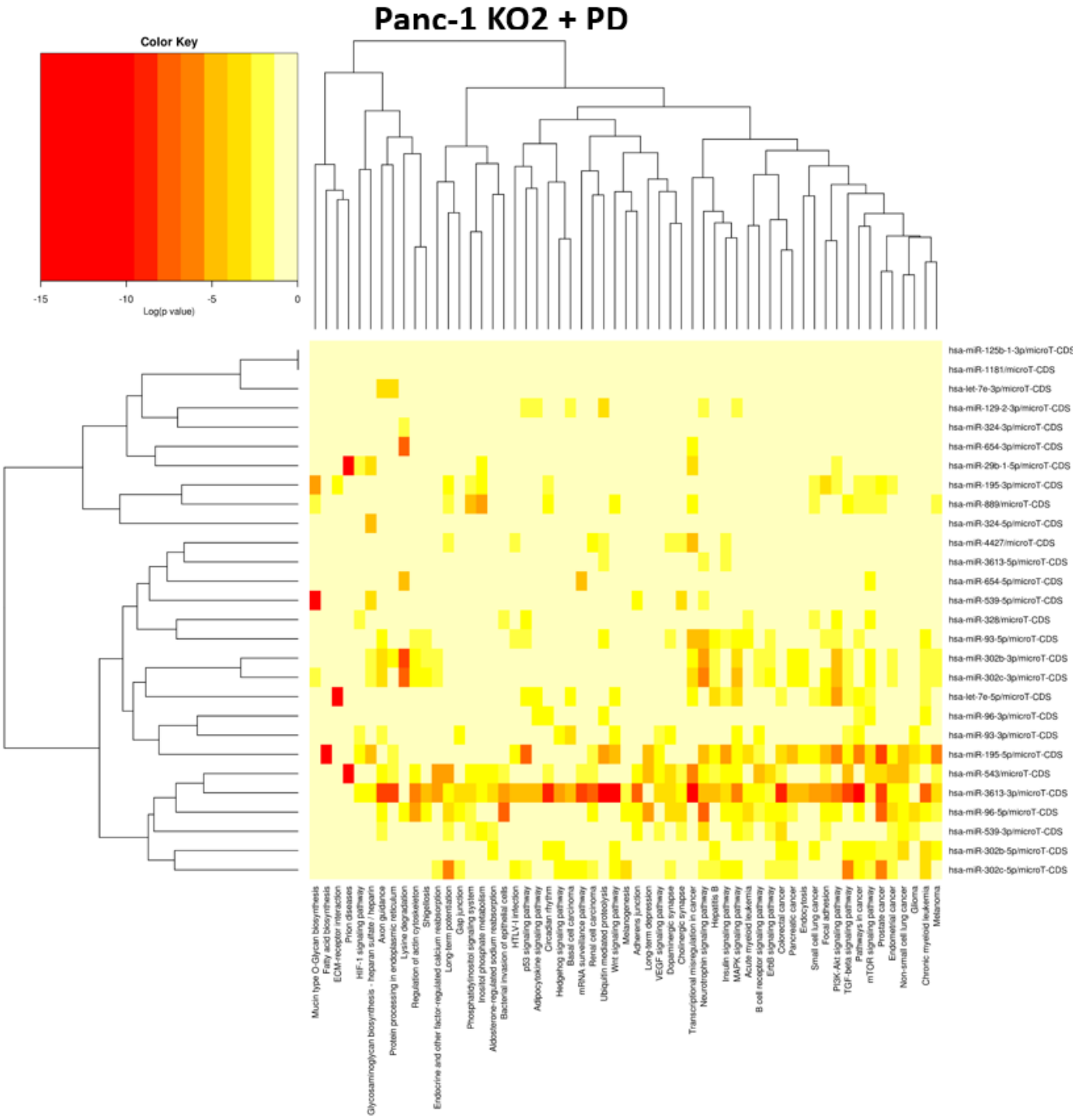
hsa-miR-494/microT-CDS
Mucin type O-Glycan biosynthesis
ECM-receptor interaction
Amoebiasis
Glycosaminoglycan biosynthesis - chondroitin sulfate
Prion diseases
Pancreatic secretion
Protein processing in endoplasmic reticulum
Endocrine and other factor-regulated calcium reabsorption
Gap junction
mRNA surveillance pathway
Ubiquitin mediated proteolysis
Dopaminergic synapse
Circadian rhythm
Long-term potentiation
Renal cell carcinoma
Progesterone-mediated oocyte maturation
Nicotine addiction
GABAergic synapse
Retrograde endocannabinoid signaling
Phosphatidylinositol signaling system
Inositol phosphate metabolism
Endocytosis
Adherens junction
RNA transport
Regulation of actin cytoskeleton
Bacterial invasion of epithelial cells
p53 signaling pathway
Glioma
Melanoma
HTLV-I infection
Hypertrophic cardiomyopathy (HCM)
Hepatitis B
Chronic myeloid leukemia
T cell receptor signaling pathway
B cell receptor signaling pathway
Neurotrophin signaling pathway
Wnt signaling pathway
Axon guidance
Transcriptional misregulation in cancer
ErbB signaling pathway
Focal adhesion
MAPK signaling pathway
TGF-beta signaling pathway
Hedgehog signaling pathway
Basal cell carcinoma
PI3K-Akt signaling pathway
Small cell lung cancer
mTOR signaling pathway
Adipocytokine signaling pathway
Acute myeloid leukemia
Pathways in cancer
Insulin signaling pathway
Melanogenesis
GnRH signaling pathway
Pancreatic cancer
Cholinergic synapse
Non-small cell lung cancer
Colorectal cancer
Prostate cancer
Endometrial cancer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Log(p value)
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hsa-miR-324-3p/microT-CDS
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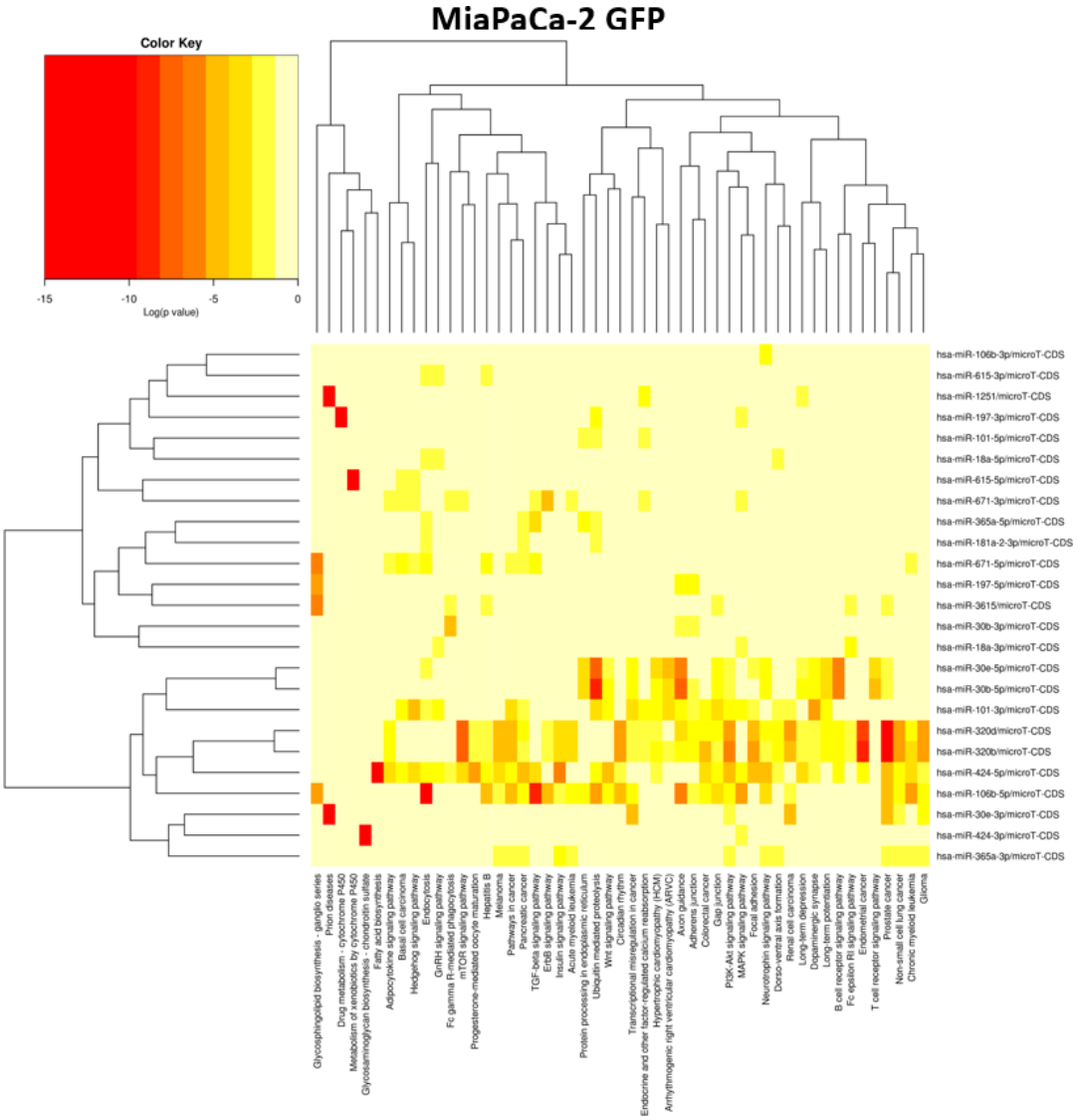
hsa-miR-96-5p/microT-CDS
hsa-miR-539-3p/microT-CDS
hsa-miR-302b-5p/microT-CDS
hsa-miR-302c-5p/microT-CDS
Mucin type O-Glycan biosynthesis
Fatty acid biosynthesis
ECM-receptor interaction
Prion diseases
HIF-1 signaling pathway
Glycosaminoglycan biosynthesis - heparan sulfate / heparin
Axon guidance
Protein processing in endoplasmic reticulum
Lysine degradation
Regulation of actin cytoskeleton
Shigellosis
Endocrine and other factor-regulated calcium reabsorption
Long term potentiation
Gap junction
Phosphatidylinositol signaling system
Inositol phosphate metabolism
Aldosterone-regulated sodium reabsorption
Bacterial invasion of epithelial cells
HTLV-I infection
p53 signaling pathway
Adipocytokine signaling pathway
Circadian rhythm
Hedgehog signaling pathway
Basal cell carcinoma
mRNA surveillance pathway
Renal cell carcinoma
Ubiquitin mediated proteolysis
Wnt signaling pathway
Melanogenesis
Adherens junction
Long term depression
VEGF signaling pathway
Dopaminergic synapse
Cholinergic synapse
Transcriptional misregulation in cancer
Neurotrophin signaling pathway
Hepatitis B
Insulin signaling pathway
MAPK signaling pathway
Acute myeloid leukemia
B cell receptor signaling pathway
ErbB signaling pathway
Colorectal cancer
Pancreatic cancer
Endocytosis
Small cell lung cancer
Focal adhesion
PI3K-Akt signaling pathway
TGF-beta signaling pathway
Pathways in cancer
mTOR signaling pathway
Prostate cancer
Endometrial cancer
Non-small cell lung cancer
Glioma
Chronic myeloid leukemia
Melanoma

(B)


MiaPaCa-2 GFP
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Log(p value)
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hsa-miR-18a-5p/microT-CDS
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hsa-miR-424-3p/microT-CDS
hsa-miR-365a-3p/microT-CDS
Glycosphingolipid biosynthesis - ganglio series
Prion diseases
Drug metabolism - cytochrome P450
Metabolism of xenobiotics by cytochrome P450
Glycosaminoglycan biosynthesis - chondroitin sulfate
Fatty acid biosynthesis
Adipocytokine signaling pathway
Basal cell carcinoma
Hedgehog signaling pathway
Endocytosis
GnRH signaling pathway
Fc gamma R-mediated phagocytosis
mTOR signaling pathway
Progesterone-mediated oocyte maturation
Hepatitis B
Melanoma
Pathways in cancer
Pancreatic cancer
TGF-beta signaling pathway
ErbB signaling pathway
Insulin signaling pathway
Acute myeloid leukemia
Protein processing in endoplasmic reticulum
Ubiquitin mediated proteolysis
Wnt signaling pathway
Circadian rhythm
Transcriptional misregulation in cancer
Endocrine and other factor-regulated calcium reabsorption
Hypertrophic cardiomyopathy (HCM)
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
Axon guidance
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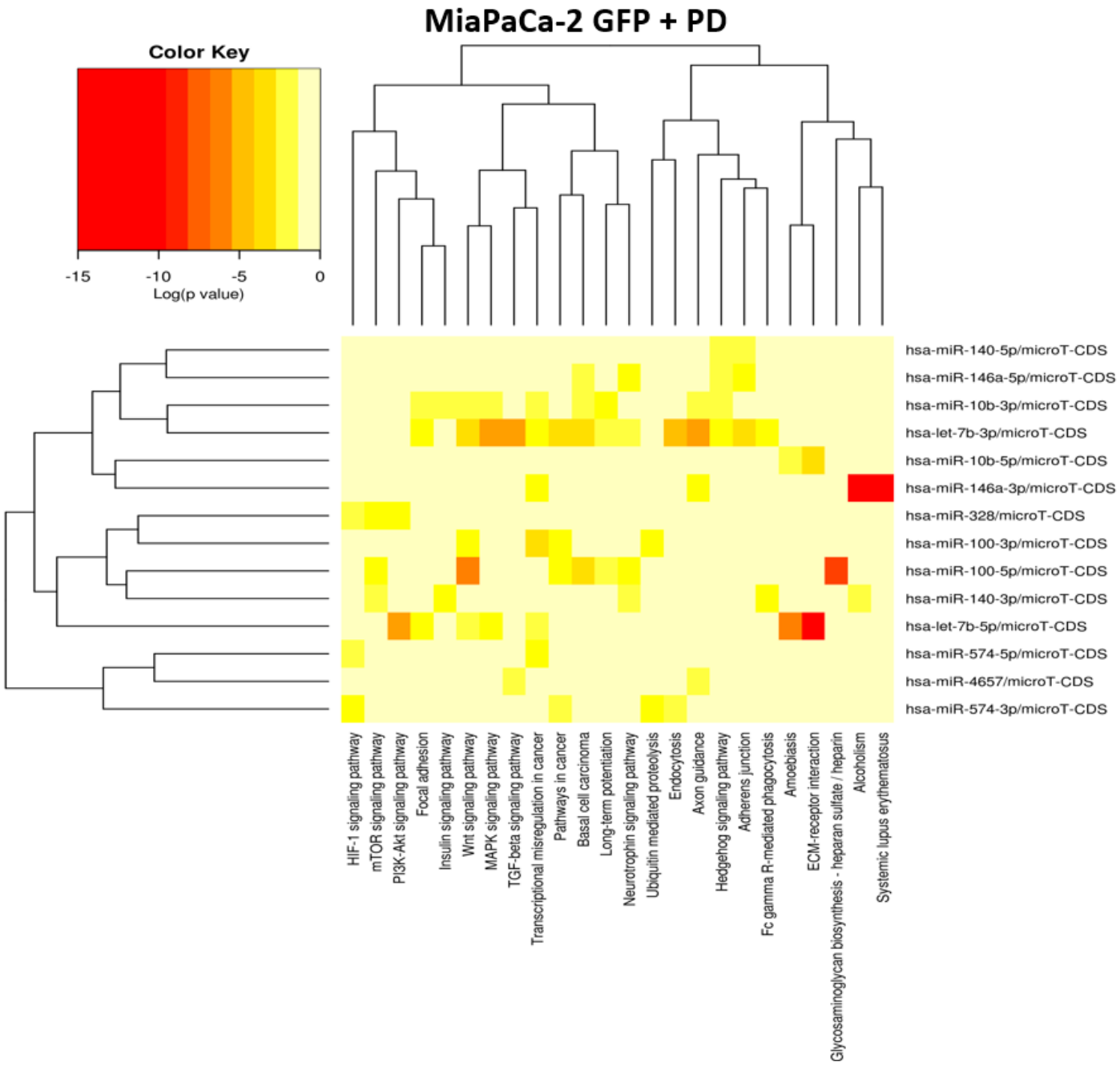
Gap junction
PI3K-Akt signaling pathway
MAPK signaling pathway
Focal adhesion
Neurotrophin signaling pathway
Dorso-ventral axis formation
Renal cell carcinoma
Long-term depression
Dopaminergic synapse
Long-term potentiation
B cell receptor signaling pathway
Fc epsilon RI signaling pathway
Endometrial cancer
T cell receptor signaling pathway
Prostate cancer
Non-small cell lung cancer
Chronic myeloid leukemia
Glioma


MiaPaCa-2 GFP + PD
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Log(p value)
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hsa-miR-10b-5p/microT-CDS
hsa-miR-146a-3p/microT-CDS
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hsa-miR-140-3p/microT-CDS
hsa-let-7b-5p/microT-CDS
hsa-miR-574-5p/microT-CDS
hsa-miR-4657/microT-CDS
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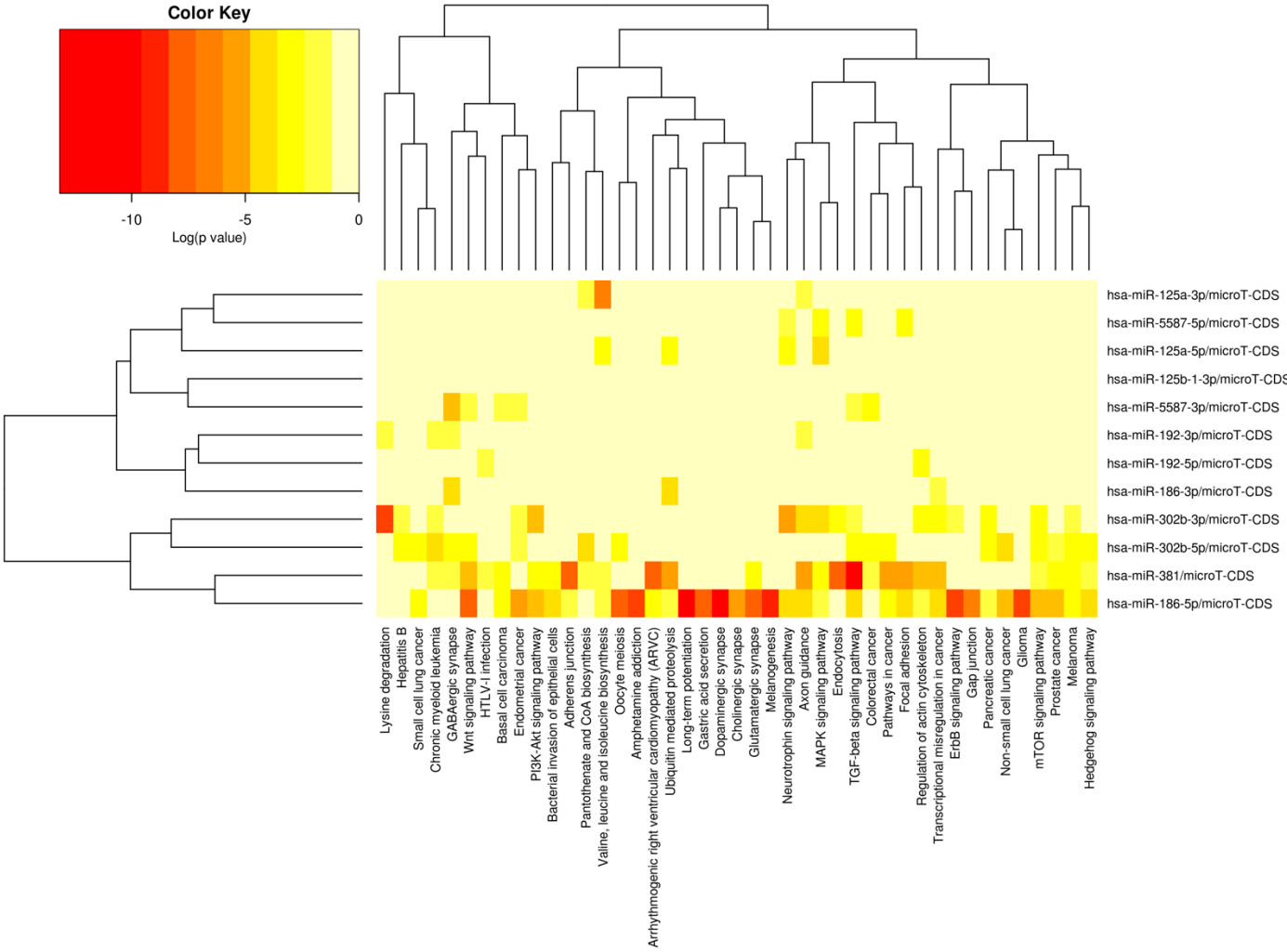
HIF-1 signaling pathway
mTOR signaling pathway
PI3K-Akt signaling pathway
Focal adhesion
Insulin signaling pathway
Wnt signaling pathway
MAPK signaling pathway
TGF-beta signaling pathway
Transcriptional misregulation in cancer
Pathways in cancer
Basal cell carcinoma
Long-term potentiation
Neurotrophin signaling pathway
Ubiquitin mediated proteolysis
Endocytosis
Axon guidance
Hedgehog signaling pathway
Adherens junction
Fc gamma R-mediated phagocytosis
Amoebiasis
ECM-receptor interaction
Glycosaminoglycan biosynthesis - heparan sulfate / heparin
Alcoholism
Systemic lupus erythematosus

MiaPaCa-2 KO2



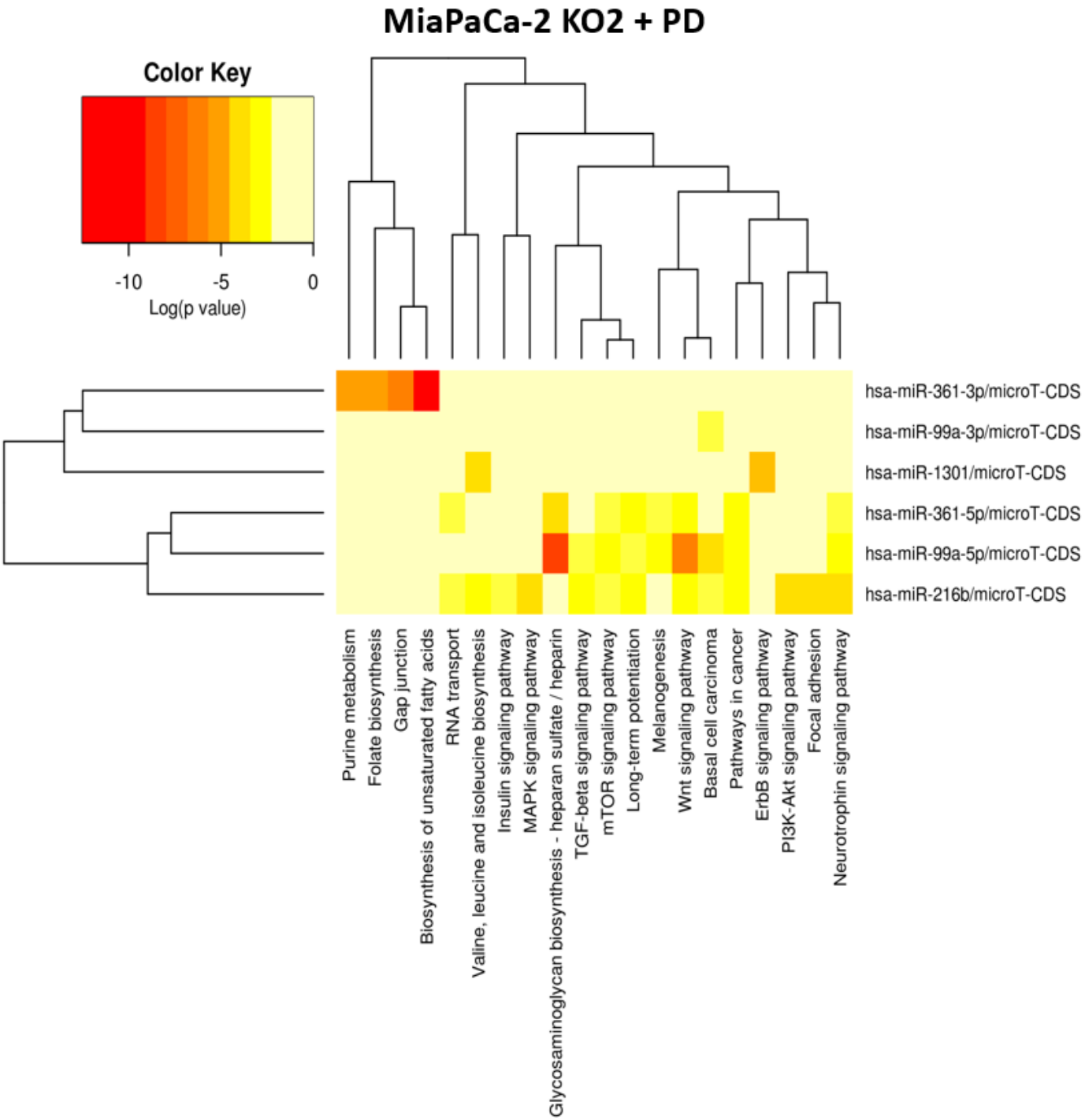


Figure 31: MirPath signalling pathways related to each of the four different conditions (GFP, GFP + PD-0332991, miR-21 KO2, miR-21 KO2 +PD-0332991) in (A) Panc-1 and (B) MiaPaCa-2 PDAC 3D culture cells. DIANA tools mirPath was used for the designing of heatmaps.

6.4. Discussion

In this part of the study the effect of the CDK4/6 inhibitor, PD-0332991 was analysed in combination with miR-21 KOs. It was found that PD-0332991 could be an effective therapeutic strategy for PDAC, affecting not only cell proliferation, viability but also apoptosis.

Results showed that PD-0332991 reduced cell viability through regulation of cell survival in both Panc-1 and MiaPaCa-2 cells in line with previous study by Rencuzogulları et al. (2020). It was also found that miR-21 KOs alone have decreased cell viability in two different PDAC cell lines (Panc-1 and MiaPaCa-2), while PD-0332991 treatment in combination with miR-21 deletion resulted in a higher reduction of cell viability in PDAC cells. Previously, other studies reported that *in vitro* inhibition of miR-21 could lead to a significant reduction in cell viability in several cancer types including PDAC (Chan et al., 2005; Meng et al., 2006; Meng et al., 2007; Si et al., 2007**)**. To the best of my knowledge, this is the first time that the combination of PD-0332991 treatment and miR-21 knocking out in cell viability of PDAC cells, has been studied. Furthermore, the findings provide strong evidence that CDK4/6 inhibition alone and in combination with miR-21 deletion can have subtle effects on the suppression of cellular proliferation in both Panc-1 and MiaPaCa-2 cells. It has been reported that miR-21 plays a key role in cell proliferation of PDAC cells by targeting the MAPK/ERK and PI3K/AKT signalling pathways (Zhao et al., 2018). Another study indicated that PD-0332991 treatment reduced the colony numbers of Panc-1 and MiaPaCa-2 by 25% and 15%, respectively (Sevgin et al., 2021). This current study showed for the first time the combinatory effects of miR-21 deletion with CDK4/6 inhibition that led to reduction of colony formation in PDAC, *in vitro.* CDK4/6 play a central role in cell-cycle entry and G1 progression and therefore it has been considered as attractive therapeutic targets in numerous tumour types combined with available drugs, which target other cellular pathways (Asghar et al., 2015; Malumbres, 2019; Sherr et al., 2016;

Turner et al., 2017). Several studies have noted that the genetic inhibition of CDK4/6 activity can minimise tumour progression in select models (Landis et al., 2006; Malumbres et al., 2006; Santamaria et al., 2007; Yu et al., 2001). Therefore, the effects on cell cycle arrest following PD-0332991 treatment observed may be of some interest. Results showed that PD-0332991 treatment induced cell cycle arrest at the G1 phase in both Panc-1 and MiaPaCa-2 cells, while combination of PD-0332991 treatment and miR-21 deletion resulted in a more efficient trigger of G1 arrest *in vitro*. These results are in line with the findings of previous studies, where PD-0332991 promoted cell cycle arrest at the G1 phase and further led to the accumulation of cyclin D1 (Rencuzogulları et al., 2020; Sevgin et al., 2021; Zhang et al., 2017b). Previously, it was shown that CDK4/6 inhibition stimulates cytostasis via cell-cycle arrest in the G1 phase, which has as a further consequence cell growth inhibition (George et al., 2021). Furthermore, in this current study it was observed that the effective therapeutic potential of PD-0332991 was achieved by reducing the number of PDAC cells in the S phase of cell cycle progression. MiR-21 KO alone resulted in suppression of S phase in PDAC cells, however, in combination with PD-0332991 a higher decrease in PDAC cells number was observed in S phase. It has been demonstrated that upregulation of miR-21 prompted the transition from G0/G1 phase to S phase under EGF stimulation in PDAC, therefore, miR-21 may play a regulatory role in the inducement of G1/S transition in PDAC (Zhao et al., 2018). Moreover, based on this current study, the combination of PD-0332991 treatment and miR-21 deletion arrests PDAC cells in the G2-phase, which has been demonstrated in a similar trend with previous studies (Rencuzogulları et al., 2020; Sevgin et al., 2021). Cyclin-CDK complexes moderate cell cycle through the phosphorylation of RB and has as a result the suppression of E2F transcription factor family that allows cells to divide (O'Leary et al., 2016). The CDK4/6 inhibitors act at the G_1-to-S cell cycle checkpoint and this checkpoint is moderated through D-type cyclins, CDK4 and CDK6 (O'Leary et al., 2016), which prevent the phosphorylation of RB protein (George et al., 2021). Further non-cell cycle related effects of CDK4/6 include direct activation of vascular endothelial growth

factor (VEGF) transcription, promotion of angiogenesis and NF-κB activation via the p65 transcription factor (Abedin et al., 2010; Handschick et al., 2014; Kollmann et al., 2013). The need for developing effective therapeutic strategies based on CDK4/6 inhibitors in combination with other agents, which are clinically active, is vital (**Figure 32**).

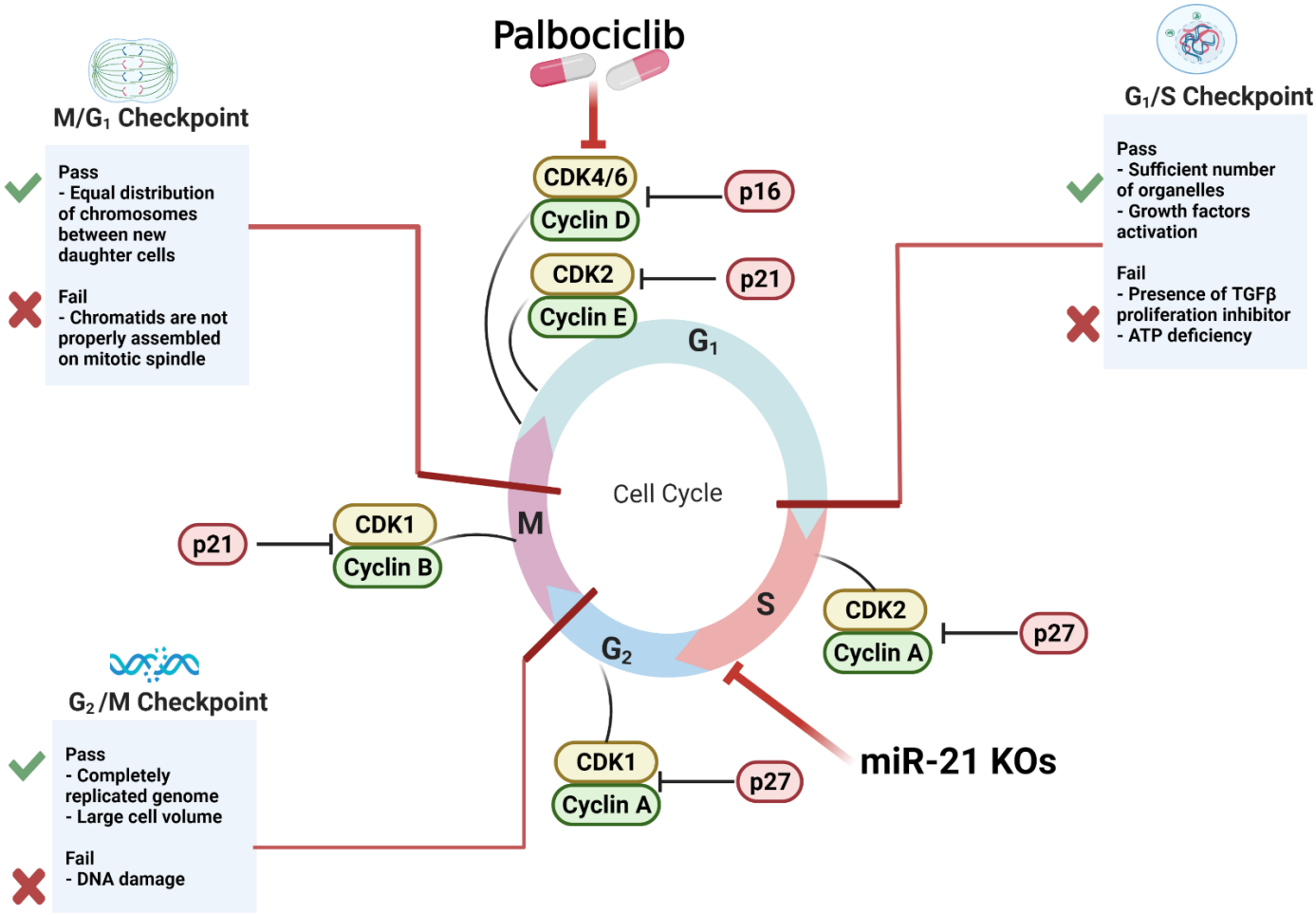


Figure 32: PD-0332991 and miR-21 KOs as coregulatory partners in cell cycle arrest. Cyclins are synthesised in specific phases of the cell cycle and results in the activation of CDKs, and the cyclin-CDK complexes, which regulate progression of the cell throughout the cycle. There are two major classes of CDK inhibitors including the CDK4 (INK4) family ($p16^{INK4A}$), which blocks the formation of cyclin D-CDK4/6 complexes and the CDK-interacting protein/kinase inhibitory protein family ($p21^{Cip1}$ and $p27^{Kip1}$), which act on all cyclin-CDK complexes. Initiation of the cell cycle occurs in G1 phase, which is mainly regulated by the activation of CDK4 and CDK6 kinases that are downstream of mitogenic signals (Baker et al., 2012; Duronio et al., 2013; Morrison et al., 2012). CDK4 and CDK6 activation are positively moderated through the binding of several D-type cyclins such as cyclin D2, which are highly expressed during G1/S phase (Pagano et al., 1994). Inhibition of CDK4/6 by using PD-0332991 in combination with miR-21 knockout, can be an effective mechanism of cell cycle arrest in PDAC cells. PD-0332991 blocks cell cycle progression from G1 to S phase by

preventing the CDK4/6-cyclin D1-mediated phosphorylation of RB and is also closely associated with G2 phase, while PD-0332991 treatment together with miR-21 KOs are linked to S and G2 phases of cell cycle in PDAC. Adapted from Mortoglou et al., (2023). Created with BioRender.com.

Importantly, a previous *in vitro* study reported that Panc-1 and MiaPaCa-2 cells treated with PD-0332991 stained positively with SA-β Gal, which indicates the inducer role of PD-0332991 in terms of senescence (Sevgin et al., 2021). This finding is consistent with this current study, which revealed that PD-0332991 alone and in combination with miR-21 deletion triggered cellular senescence in two different PDAC cell lines (Panc-1 and MiaPaCa-2). Several studies have shown that several miRs including miR-21 are differentially expressed in senescent cells and play a regulatory role in cellular senescence (Creemers et al., 2012; Jung et al., 2014; Williams et al., 2017). Particularly, miR-21 level is upregulated during the ageing process and linked to various malignancies including PDAC (Lee et al., 2007; Nakata et al., 2011; Olivieri et al., 2016). Popov et al. (2022) reported that miR-21 is a senescence-associated miRs (SA-miRs), which controls cell transition during cell cycle (G1/S or G2/M checkpoints) through targeting CDKs and CDK inhibitors (Bueno et al., 2011). Moreover, a previous study has indicated that cyclin D–CDK4/6 complexes lead to the phosphorylation of FOXM1, which protects cancer cells from senescence (Anders et al., 2011). Furthermore, it has been described previously that inhibition of CDK4/6 in preclinical models leads to the activation of RB, which can further induce senescent-like arrest (Dean et al., 2012a; Dean et al., 2012b; Finn et al., 2009; Fry et al., 2004; Konecny et al., 2011; McClendon et al., 2012; Toogood et al., 2005).

According to a study by Franco et al., (2014), almost all CDK4/6 inhibitors, including PD-0332991 can induce apoptosis in numerous malignancies. Moreover, cyclin D–CDK4/6 complexes also contribute not only in apoptosis but also in glucose metabolism and cell differentiation (Hydbring et al., 2016; Lee et al., 2014). Here, results showed that PD-0332991 treatment induced higher apoptotic rate in both Panc-1 and MiaPaCa-2 miR-21 KOs but not in their treated wt cells, whereas miR-21 KOs alone triggered apoptosis in PDAC cells. Similarly, a previous study showed that miR-21 overexpression results in the prevention of apoptosis (Park et al., 2009), and Buscaglia et al. (2011) reviewed that miR-21 is closely associated with several stages

of oncogenic life including stimulation of cell proliferation, invasion and metastasis, genome instability inflammation, immune destruction, abnormal metabolism, angiogenesis, and suppression of apoptosis. Further studies have also revealed that CDK4/6 inhibitors contribute to tumour cell apoptosis (Helsten et al., 2016; Minton et al., 2017), while a similar study determined that CDK4/6 inhibitors can stimulate not only caspase-dependent apoptosis through the regulation of Bax and Bak proteins in MiaPaCa-2 cells but also caspase-independent apoptosis in Panc-1 cells through raised cleavage of PARP (Sevgin et al., 2021).

Overexpression of miR-21 promotes cell invasion in PDAC, while inhibition of miR-21 reduces cell proliferation, invasion, and chemoresistance for gemcitabine (Moriyama et al., 2009). The current study found that the combination of PD-0332991 and miR-21 KO cells decreased the cell motility significantly when used in combination. Other studies have investigated that miR-21 expression is related to invasion-related genes such as *VEGF* (Wey et al., 2005). Previously, it has been shown that PD-0332991 suppressed migration and invasion in oesophageal squamous cell carcinoma (ESCC) cells (Chen et al., 2017).

Mitochondria are key regulators of cellular energy metabolism, with mitochondrial respiration generating most of the intracellular ATP. Therefore, the current study assessed the key parameters of mitochondrial respiration in PDAC cells following PD-0332991 treatment and miR-21 knocking out. Specifically, basal respiration represents the energetic demand of cells under basal conditions, the oxygen consumption of basal respiration used to meet ATP synthesis and leads to mitochondrial proton leak. ATP-linked respiration is associated with the reduction in OCR after the injection of the oligomycin, which is the portion of basal respiration. Proton leak can be described as the remaining basal respiration, which has not coupled to ATP synthesis after oligomycin injection and can be a sign of mitochondrial damage. Additionally, maximal respiration represents the maximum capacity that the electron respiratory chain can achieve and is measured by the injection of the uncoupler FCCP. In this

study, it was revealed that MiaPaCa-2 miR-21 KO2 and KO4 increased maximal respiration compared to MiaPaCa-2 wt cells, whereas a previous study showed that anti-miR-21-5p-transfected H9C2 cells presented a high increase in maximal respiration (Nasci et al., 2019). Spare respiration is the difference between maximal and basal respiration, which indicates the ability of the PDAC cells to respond to alterations in energetic demand and demonstrates the fitness of the PDAC cells. Besides, non-mitochondrial respiration is the oxygen consumption caused by cellular enzymes and not mitochondria after the injection of rotenone and antimycin A (Gu et al., 2021). In the current study, slight changes were found in the basal respiration, proton leak and spare respiratory capacity rates (albeit all non-significant). This might be explained by the high variability between the different biological repeats and wells in each experiment and by interventions that were introduced to the cells such as miR-21 deletion and compound treatments (PD-0332991), which can have as a further result changes in the cell numbers during the culture. One further explanation could be the unequal distribution of cells within the well, and particularly, in the centre of the well, where the Seahorse microchamber measures oxygen concentration (Little et al., 2020).

In this current study, the proteomic profiles of two different PDAC cell lines (Panc-1 and MiaPaCa-2) were assessed following PD-0332991 treatment and miR-21 knockout, compared with Panc-1 and MiaPaCa-2 wt cells. Large-scale proteomic signatures between wt and treated cells were identified. To further understand changes in the pathobiological and physiological pathways associated with Panc-1 and MiaPaCa-2 wt cells versus cells treated with PD-0332991 and miR-21 KO2s, protein–protein interaction network analysis was carried out by using String analysis. Particularly, String analysis revealed several KEGG and Reactome signalling pathways, which are involved in PDAC development. In this study, large-scale changes were identified in the proteome signatures between Panc-1 and MiaPaCa-2 PDAC cell lines and their miR-21 KOs, which could be used to further understand the molecular mechanisms

of PDAC progression. Several KEGG pathways were identified as specific to the total proteomic profile of Panc-1 wt, including vibrio cholerae infection and RNA transport, whereas pathways related to metabolism and ribosome were also linked to Panc-1 PDAC cells. Moreover, KEGG and Reactome pathways relating to the PD-0332991 treatment are linked to a range of physiological and pathobiological mechanisms in Panc-1 cells. This included citrate cycle, pyruvate metabolism, peroxisome, long-term depression, endocrine and factor regulated calcium reabsorption, carbon metabolism, gap junction, oxidative phosphorylation, vascular smooth muscle contraction, pathogenic *E.coli* infection, protein processing in endoplasmic reticulum and Huntington disease. In this study, proteomics analysis revealed that oxidative phosphorylation is linked to Panc-1 cells treated with PD-0332991. It has been shown previously that increased oxidative phosphorylation promotes stem and immune-evasive properties of pancreatic cancer stem cells. Therefore, this pathway could facilitate the discovery of new CSC-specific hallmarks for the development of PDAC target-based therapies (Valle et al., 2020). A recent study has revealed that PD-0332991 can result in the dysregulation of metabolites in several metabolic pathways in central carbon metabolism in PDAC cells (Qin et al., 2020), whereas PD-0332991 can also affect the regulation of the isoform M2 of pyruvate kinase (PKM2) (Amelio et al., 2014). In addition, Reactome pathways relating specific to Panc-1 miR-21 KO2 included kinesins, protein ubiquitination, metabolism of nucleotides, mitotic anaphase, cell cycle (mitotic), M phase, cell cycle, RHO GTPase effectors, signalling by Rho GTPases, infectious disease, post-translational protein modification, metabolism of proteins and disease. Particularly, miR-21 can moderate cellular proliferation, migration, invasion, metastasis, and survival through the inhibition of tumour suppressor RhoB not only in colorectal and breast cancer but also in PDAC (Connolly et al., 2010; Liu et al., 2011; Vennin et al., 2020). Hence, Rho GTPases could be used as potential targets in PDAC therapy by reducing the metastatic predisposition of PDAC cells (Vennin et al., 2020). PD-0332991 can promote G0/G1 cell cycle arrest through the inducement of senescence in fibroblasts, breast cancer, melanoma and PDAC (Jost et al., 2021).

Furthermore, Reactome pathways particular for the Panc-1 treated miR- 21 KO2 were Golgi associated-vesicle biogenesis, programmed cell death, membrane trafficking and immune system. This indicates that critical immune-related pathways are influenced by combination of miR-21 deletion and PD-0332991 treatment in Panc-1 cells as well as apoptosis and membrane trafficking pathways in Panc-1 PDAC cells. Specific KEGG pathway for MiaPaCa-2 treated wt cells was RNA degradation and spliceosome, as well as ribosome pathway in MiaPaCa-2 wt cells. For MiaPaCa-2 miR-21 KO2 were Vibrio cholerae infection and synaptic vesicle cycle. A previous study showed that numerous miRs can regulate synaptic mRNA expression (Paschou et al., 2012). Additionally, metabolic and energy pathways were influenced by miR-21 deletion and PD-0332991 treatment in MiaPaCa-2 PDAC cells, whereas no KEGG pathways were specific to the MiaPaCa-2 wt cells proteome.

In vitro organoid models have been shown to be an accurate way for the demonstration of several *in vivo* biological processes such as tissue renewal, stem cell functions, and responses to drugs, mutations or damage (Fatehullah et al., 2016). Moreover, it has been proposed that organoids can provide effective model systems for expression profiling research studies and analysis of rare cell lineages, which are difficult to be accessed *in vivo* (Grun et al., 2015). Additionally, it has been suggested that human organoids can be used to assess the effectiveness of novel bioactive chemical compounds, which could be used for the development of personalised treatment regimens (Meng, 2010). A further study by Li et al., (2019a) has showed that 3D cell culture models such as organoids, present similarities with human's organs in structure and functions as well as in cellular proliferation, differentiation, self-renewal, self-assembly and genetic stability. Thus, for this study, PDAC 3D culture cells have been chosen to assess the effects of miR-21 deletion and PD-0332991 in PDAC cell lines (Panc-1 and MiaPaCa-2).

Small RNA-sequencing data has revealed that in Panc-1 wt cells, miR-154, miR-155, miR-16-2, miR-200a, miR-205, miR-342, miR-4521, miR-497 and miR-663a were uniquely expressed, while in Panc-1 wt cells treated with PD-0332991, miR-10397, miR-1182, miR-1538, miR-194-2, miR-34a, miR-3609, miR-3652, miR-429, miR-431 and miR-99a. Further miRs, which are uniquely expressed in Panc-1 miR-21 KO2 cells were miR-101-2, miR-193a, miR-196a, miR-320a, miR-454, miR-485, miR-494, miR-495, miR-505, miR-589, miR-641, miR-656, miR-671 and miR-98, whereas in Panc-1 miR-21 KO2 treated with PD-0332991 cells, let-7e, miR-1181, miR-125b-1, miR-129-2, miR-195, miR-29b-1, miR-302b, miR-302c, miR-324, miR-328, miR-3613, miR-4427, miR-539, miR-543, miR-654, miR-889, miR-93 and miR-96 were uniquely expressed. It was also noted that miR-21 alone and in combination with PD-0332991 treatment reduced the expression levels of miR-21 in Panc-1 cells. Most of the miRs, which have been expressed in Panc-1 wt cells show oncogenic properties and involved in several biological processes including cellular proliferation, migration, invasion and apoptosis (Dooley et al., 2017; Ferrari et al., 2020; Li et al., 2014d; Zhou et al., 2018), whereas miR-205, miR-4521 and miR-497 can act both as tumour suppressor and oncogenic in different types of malignancies (Ayesha et al., 2021; Kuthethur et al., 2023; Luo et al., 2021). Additionally, PD-0332991 treatment in Panc-1 wt cells promoted the expression of miR-1538, miR-34a and miR-3609. Specifically, a previous study has showed that miR-1538 inhibits colorectal cancer growth and metastasis through the regulation of DNA methyltransferases 3A **(**DNMT3A) (Zhang et al., 2022). It has been also revealed that restoration of miR-3609 expression not only can sensitise breast cancer to Adriamycin by blocking PD-L1 expression (Li et al., 2019b) but also increase the antitumour effect of Sorafenib in hepatocellular carcinoma cells (Shao et al., 2020). Based on the findings of the current study, these miRs could be also promising targets for treatment of PDAC. MiR-21 deletion alone in Panc-1 cells, resulted in the expression of miR-485, miR-505, miR-641 and miR-98. Particularly, a recent study observed that expression of miR-485 inhibits cell proliferation, colony formation and cell migration in osteosarcoma (Du et al., 2018); miR-505 prevents cell growth and EMT

through targeting of MAP3K3 via the AKT-NFκB pathway in non-small cell lung cancer cells (Tang et al., 2019). MiR-641 could also reduce cellular proliferation of gastric cancer cells and glioblastoma development through the suppression of the phosphorylation of AKT (Hinske et al., 2017; Wang et al., 2019b), whereas miR-98-5p could lead to the inhibition of gastric cancer cell stemness and chemoresistance through the of targeting branched-chain aminotransferases 1 (Zhan et al., 2021). Combination of miR-21 deletion and PD-0332991 treatment in Panc-1 cells resulted in the expression of let-7. Previous studies have suggested that the restoration of the expression level of let-7 family members can be used as effective therapeutic tool for aggressive malignancies (Barh et al., 2010; Yu et al., 2007). Thus, the current small RNA-sequencing results may provide novel insights for potential clinical of applications of the expressed miRs.

In MiaPaCa-2 3D culture cells, which were developed in the current study, small RNA-sequencing results showed that miR-101, miR-106b, miR-1251, miR-181a-2, miR-18a, miR-197, mR-30b, miR-30e, miR-320b-2, miR-320d-2, miR-3615, miR-365a, miR-424, miR-615, miR-671 and miR-98 were expressed only in MiaPaCa-2 wt cells, where let-7b, miR-100, miR-10b, miR-140, miR-146a, miR-328, miR-4657 and miR-574 were only expressed in MiaPaCa-2 wt cells treated with PD-0332991. MiR-125b-1, miR-186, miR-192, miR-302b, miR-381 and miR-5587 were uniquely expressed in MiaPaCa-2 miR-21 KO2 cells, whereas miR-1301, miR-361, miR-216b, miR-99a were only expressed in MiaPaCa-2 miR-21 KO2 treated with PD-0332991 cells. It was also noted that miR-21 alone and combined with PD-0332991 treatment decreased the expression levels of miR-21 in MiaPaCa-2 3D culture cells, which could further lead to the inhibition of the oncogenic properties of this miR. In MiaPaCa-2 wt cells, most of the miRs, which were expressed present oncogenic roles through the promotion of cellular proliferation, migration, invasion and inhibition of apoptosis (Dastmalchi et al., 2021; Gao et al., 2015; Liu et al., 2022; Wang et al., 2018c; Yuan et al., 2021), whereas miR-320d-2 and miR-98 play a tumour suppressor role in bone tumour and

endometrial cancer, respectively (Huang et al., 2021; Zhang et al., 2021a). Furthermore, small RNA sequencing data exhibited that PD-0332991 treatment alone is related with the expression of miR-140 in MiaPaCa-2 cells; a previous study suggested that upregulation of miR-140-5p could result in the inhibition of cellular proliferation and enhancement of apoptosis of ovarian cancer cells through the suppression of Wnt-related genes, and blocking nuclear transport of â-catenin (Wu et al., 2020). Besides, the expression of miR-192 was also promoted via the deletion of miR-21 in MiaPaCa-2 cells. It has been previously observed that miR-192 is downregulated in renal and ovarian cancer and associated with poor clinical outcome (Kondetimmanahalli et al., 2018). Therefore, miR-21 deletion could promote a better clinical outcome of PDAC cases via the expression of miR-192, however further *in vitro* studies are needed to discover the specific mechanisms of its action. In the current study, it was also found that miR-21 deletion alone prompted the expression of miR-302b in MiaPaCa-2 cells. Liu et al., (2016) have pointed that upregulation of miR-302b inhibited cell proliferation and G1/S phase conversion rate in gastric cancer. Hence, the results of the current study could provide not only new perspectives for research of cellular regulation and proliferation in PDAC but also new targets for PDAC diagnosis and treatment. A previous study has found that miR-216b is downregulated in malignancies such as pancreatic, colorectal and gastric cancer (Wu et al., 2018). In this study, it was found that the combination of miR-21 deletion and PD-0332991 treatment in MiaPaCa-2 PDAC 3D culture cells promoted the expression of miR-216b, which could further lead to the inhibition of cellular proliferation, migration, invasion and promotion of apoptosis (Wu et al., 2018). Moreover, it has been revealed that miR-361 was expressed in MiaPaCa-2 miR-21 KO2 cells treated with PD-0332991, whereas previous studies have noted that miR-361 downregulation promoted shorter survival rates and in lung and colon cancer (Bottani et al., 2019; Ma et al., 2015c). **Figure 33** summarises all the miRs, which are related not only with miR-21 deletion alone and PD-0332991 treatment alone but also with the combination of these on miRs signature profiles in Panc-1 and MiaPaCa-2 PDAC 3D cell models.

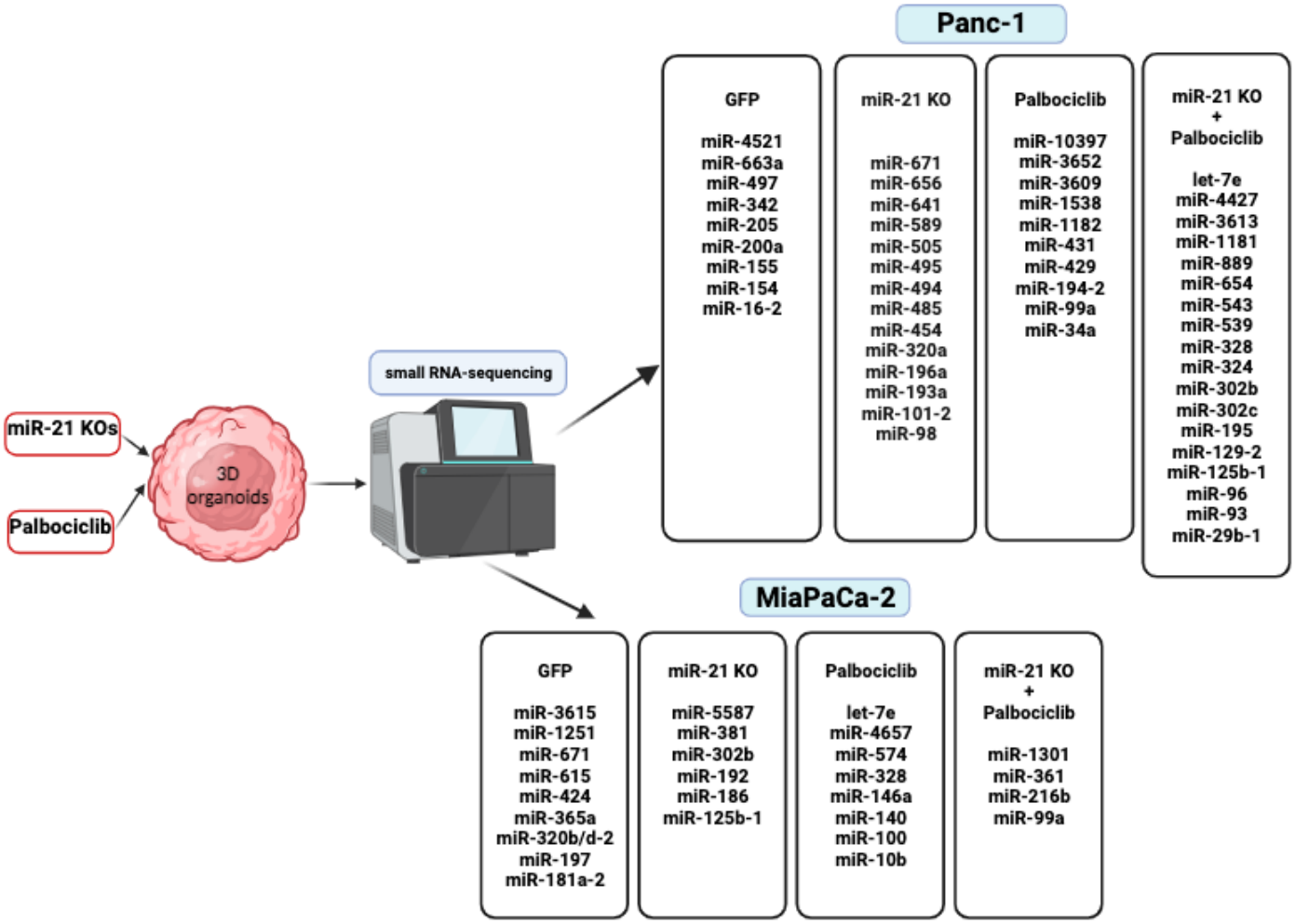


Figure 33: MiRs signature profiles of both miR-21 deletion and PD-0332991 treatment. MiRs profiles for each of the PDAC cell lines are described based on the small RNA-sequencing data results of the 3D PDAC models. Created with Biorender.com.

6.5. Conclusions

In conclusion, this part of the study found that PD-0332991 could be used as a promising candidate for combined drug therapy in PDAC. Moreover, a novel function of miR-21 KOs in the regulation of cell cycle progression, cell proliferation, apoptosis, and senescence was identified, which may provide a therapeutic potential for PDAC in combination with PD-0332991 treatment. Therefore, the results provide preliminary mechanistic insights of PD-0332991, which can be used further for a clinical evaluation for PDAC therapy, as the administration of PD-0332991 as a single agent is not sufficient enough to decrease poor progression of PDAC cells. Further studies are needed to understand how specific tissue and serum and plasma biomarkers could predict the effectiveness of this CDK4/6 inhibitor to provide optimal treatment combinations.

General Discussion

PDAC is the most lethal malignancy worldwide. The high mortality rate is due to asymptomatic progression of clinical features in metastatic stages of the disease, which renders standard therapeutic options futile. Hence, the molecular mechanisms, which are linked to the aggressive features of this tumour, should be further examined to develop better diagnostic, prognostic and therapeutic agents. MiRs can control cell growth, proliferation, apoptosis, differentiation, metastasis and angiogenesis. Throughout this thesis, I have managed to **1**) Review miR-mediated pathways and therapeutic resistance mechanisms of PDAC by summarising the involvement of miR-mediated metabolic mediators and their affected regulatory pathways through modulating different genes, which are responsible for PDAC progression (Mortoglou et al., 2021b); **2**) Underline the roles and functions of miRs in PDAC diagnosis and therapy by explaining the roles of miRs in PDAC prognosis and metastasis and evaluated the recent developments in miR-based therapies in PDAC, which have shown significant promise in controlling tumour progression and metastasis (Mortoglou et al., 2021a); **3**) Examine the expression levels of selected miRs *in vitro*. This study identified that several miRs such as miR-21, miR-221, miR-155 miR-126 are significantly dysregulated in PDAC cell lines (Panc-1, MiaPaCa-2 and BxPC3) compared to normal pancreatic ductal epithelial cell line (HPDE). MiR-21 was found to be the most oncogenic miR between them and therefore, it was selected for further examination (Mortoglou et al., 2022a); **4**) Generate miR-21 knockouts by using two different PDAC cell lines (Panc-1 and MiaPaCa-2) via CRISPR-mediated approaches to evaluate the role of miRs in PDAC stemness. Following miR-21 KOs from both Panc-1 and MiaPaCa-2 cell lines, reversed expressions of EMT and CSCs markers were observed. The expression patterns of key CSC markers, including CD44, CD133, CD24, CXCR4, and ALDH1, were changed depending on miR-21 status. MiR-21 suppressed cellular invasion of Panc-1 and MiaPaCa-2 cells, as well as the cellular proliferation of MiaPaCa-2 cells. Results suggested that miR-21 is involved in the

stemness of PDAC cells, may play roles in mesenchymal transition, and that miR-21 poses as a novel, functional biomarker for PDAC aggressiveness (Mortoglou et al., 2022a); **5**) Determine the effects of the environmental toxins cadmium chloride and nickel chloride in the expression levels of both oncogenic (miR-221, miR-155) and tumour suppressor miR (miR-126) in Panc-1 and MiaPaCa-2 PDAC cell lines. Recent reviews have showed that metals such as nickel and cadmium can cause dysregulation of miRs that are implicated to play an important role in cell transformation, tumour growth and angiogenesis, while they can also promote tumourigenesis through epigenetic mechanisms such as DNA methylation, histone post-transcriptional modifications and non-coding RNA regulation or other pathways including inducing of HIF-1α to moderate miR-210. This study is the first to date that nickel and cadmium exposure effects miR-155 and miR-221 and miR-126 expression levels in PDAC *in vitro* (Mortoglou et al., 2022b/c); **6**) Evaluate the effects of miR-21 knockouts and PD-0332991 treatment alone, and the combination of these two factors in cellular proliferation, migration, apoptosis, cell cycle arrest, senescence, mitochondrial bioenergetics, proteins contents and miRs expression profiles. In particular, this study revealed that CDK4/6 inhibition decreases cellular proliferation, viability, and migration; CDK4/6 inhibition combined with miR-21 knockout result in cell cycle arrest and that miR-21 knockouts could be used as apoptotic inducers. Pancreatic 3D models from Panc-1 and MiaPaCa-2 cells have been also used to examine the miRs expression profiles in two different conditions (miR-21 deletion and PD-0332991 treatment) by using small RNA-sequencing analysis. Results indicated that miR-21 deletion could alter the expression levels of several miRs by inhibiting the oncogenic properties of oncomiRs and enhance the expression levels of tumour suppressors in both Panc-1 and MiaPaCa-2 PDAC cell lines (Mortoglou et al., 2023).

In this study, CRISPR/Cas9 was used for the knockout of the expression of the oncogenic miR-21. It has been described that CRISPR/Cas9 is an effective and stable strategy to activate or silence miRs in cancerous cells compared to the standard

methods, as miR knockdown through CRISPR/Cas9 approaches can achieve not only a 75-99% success rate with low off-target activity (Chang et al., 2016; Friedland et al., 2013; Narayanan et al., 2016) but also can target numerous pre-miR structures in one application (Cao, Xiao and Yan, 2018). Previous studies have suggested the use of CRISPR/Cas as a promising therapeutic approach for miRs targeting both *in vivo* and *in vitro* in human malignancies (El Fatimy et al., 2017; Godden et al., 2022; Huo et al., 2017; Narayanan et al., 2016). The results of these studies have shown that miRs gene knockdown by using CRISPR/Cas technology can reduce survival of cancerous cells. Furthermore, Cas9 is an example of class 2 putative enzyme, which has been noted as a programmable miR gene-targeting module for biotechnological applications because of its various uses in genome engineering in several species (Zhang et al., 2021b). In addition, CRISPR/Cas gene-targeted cancer therapy is also entering preclinical trials with promising applications in cancer immunotherapy based on miR therapeutic strategies. CRISPR/Cas system is being used widely as a genetic engineering tools by taking advantage of the endogenous DNA repair machinery of cells, however, the potential use of CRISPR/Cas as a miR targeting platform in tumour therapy needs to be further explored (Cyranoski, 2016; Hussen et al., 2023).

To date, there is no definitive conclusion for the clinical applications of miRs in PDAC due to the heterogeneity in methodologies between studies. Through this current study, specific miR signature profiles were identified based on *in vitro* data, which could prove useful tools for the early PDAC diagnosis and prognosis. Moreover, according to small RNA-sequencing data, this research suggested that miR-21 can modulate expression of other miRs and/or genes, which are interrelated with metastasis in PDAC and hence the connectivity and complexity of the regulatory network of the miRs during PDAC development is increased. By targeting specific regulatory network of miRs, researchers will be able to facilitate not only the development and advancement of miR-based clinical applications, but also illuminate the gap between genotypic and phenotypic features of PDAC.

Conclusively, miRs research is one of the most widely discussed area in science the last decades with miRs applications in translational research to have been increased with much room for growth. Besides, there are a number of commercial efforts to arise these developments to the clinical practice, whereas Phase I clinical trials, which use miR-based therapeutics in human cancers, have showed encouraging insights (Grimaldi, Salvatore and Incoronato, 2021). Bioinformatics tools have also presented promising discoveries for the identification of novel miRs, their mechanisms of actions, and their target genes, which could improve researcher's knowledge for the roles of miRs in malignancies progression, prognosis, diagnosis and treatment. Therefore, the findings of this research could be the cornerstone of a pioneer precision medicine era of research, which could minimise the poor outcome of this malignancy and promote therapeutic strategies based on miRs signature expression profiles.

List of Associated Publications

Paper 1: Mortoglou, M., Miralles, F., Mould, R.R., Sengupta, D., Uysal-Onganer, P. (2023). Inhibiting CDK4/6 in Pancreatic Ductal Adenocarcinoma via microRNA-21. *European Journal of Cell Biology*, *102*(2), 151318. https://doi.org/10.1016/j.ejcb.2023.151318

Paper 2: Mortoglou, M., Miralles, F., Arisan, E.D., Dart, A., Jurcevic, S., Lange, S., and Uysal-Onganer, P. (2022a). microRNA-21 Regulates Stemness in Pancreatic Ductal Adenocarcinoma Cells. *International Journal of Molecular Sciences*, *23*(3), 1275. https://doi.org/10.3390/ijms23031275

Paper 3: Mortoglou, M., Manić, L., Buha Djordjevic, A., Bulat, Z., Đorđević, V., Manis, K., Valle, E., York, L., Wallace, D., and Uysal-Onganer, P. (2022c). Nickel's Role in Pancreatic Ductal Adenocarcinoma: Potential Involvement of microRNAs. *Toxics*, *10*, 148. https://doi.org/10.3390/toxics10030148

Paper 4: Mortoglou, M., Buha Djordjevic, A., Djordjevic, V., Collins, H., York, L., Mani, K., Valle, E., Wallace, D., and Uysal-Onganer, P. (2022b). Role of microRNAs in response to cadmium chloride in pancreatic ductal adenocarcinoma. *Archives of Toxicology*, *96*(2), 467–485. https://doi.org/10.1007/s00204-021-03196-9

Paper 5: Mortoglou, M., Wallace, D., Buha Djordjevic, A., Djordjevic, V., Arisan, E.D., and Uysal-Onganer, P. (2021b). MicroRNA-Regulated Signaling Pathways: Potential Biomarkers for Pancreatic Ductal Adenocarcinoma. *Stresses*, *1*(1), 30–47. https://doi.org/10.3390/stresses1010004

Paper 6: Mortoglou, M., Tabin, Z.K., Arisan, E.D., Kocher, H.M., and Uysal-Onganer, P. (2021a). Non-coding RNAs in pancreatic ductal adenocarcinoma: New approaches for better diagnosis and therapy. *Translational Oncology*, *14*(7), 101090. https://doi.org/10.1016/j.tranon.2021.101090

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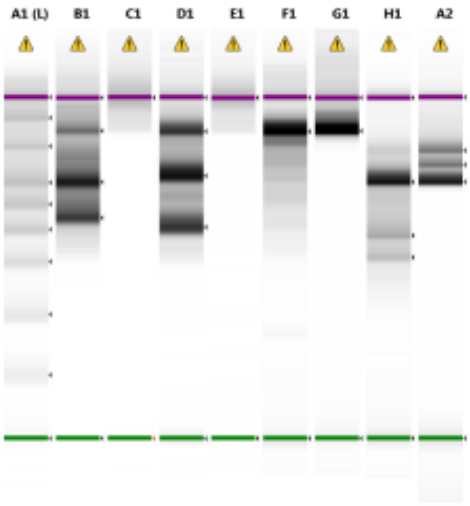
Appendices

Appendix I: Supplementary Figure 1 (S1)

Gel Image



Default image (Contrast 100%), Image is Scaled to view larger Molecular Weight range

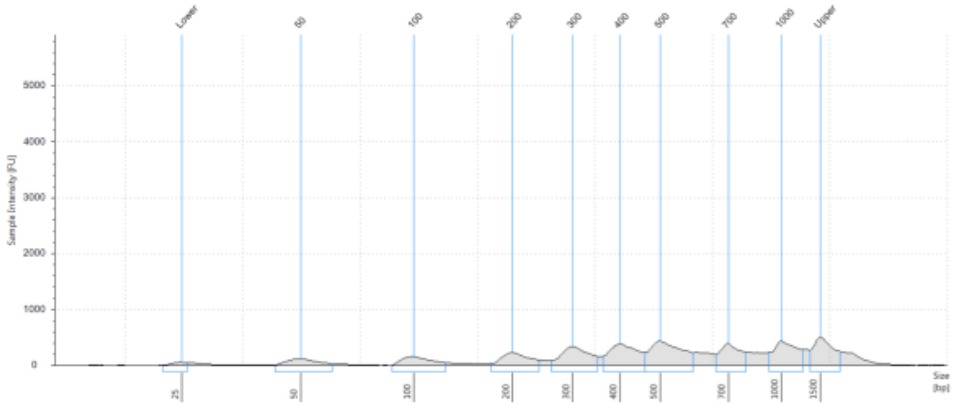
Sample Info

Well	Conc. [pg/µl]	Sample Description
A1	1640	Ladder
B1	5630	KO 2+
C1		KO 2-
D1	4970	KO 4+
E1		KO 4-
F1	2520	GFP+
G1	2490	GFP -
H1	3360	C+
A2	3480	C-

Supplementary Figure 1: Agarose gel results checking for amplification.

Appendix II: Supplementary Figure 2 (S2)

A1: Ladder



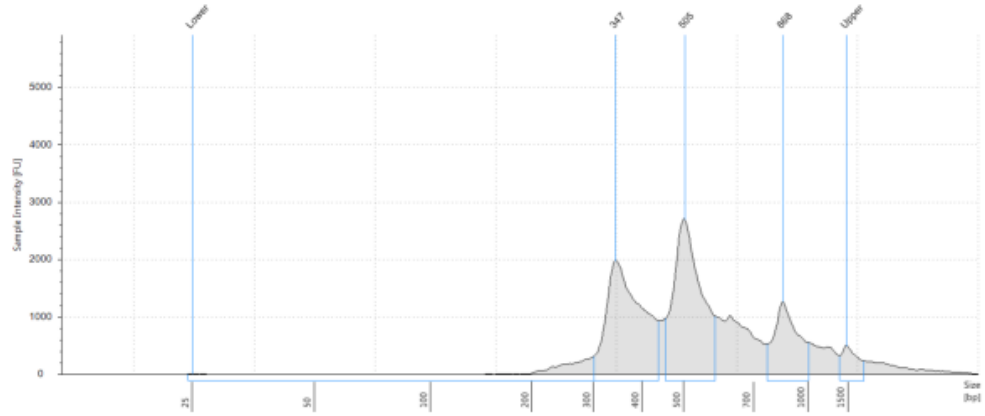
Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
A1	1640	Ladder	⚠	Caution! Expired ScreenTape device; Ladder

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	16.2	-	998	-		Lower Marker
50	82.7	-	2540	5.03		
100	109	-	1680	6.62		
200	150	-	1150	9.12		
300	231	-	1180	14.03		
400	274	-	1050	16.65		
500	344	-	1060	20.92		
700	197	-	434	12.00		
1000	257	-	395	15.62		
1500	250	250	256	-		Upper Marker

B1: KO 2+



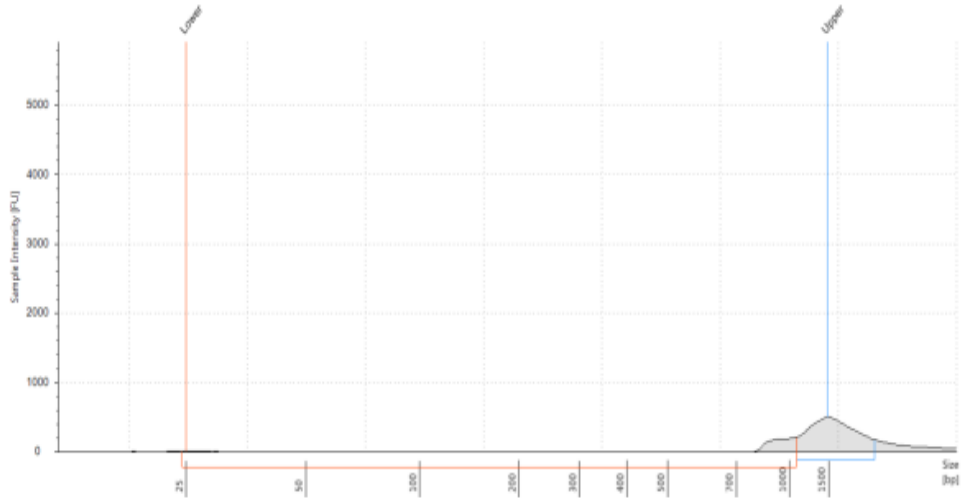
Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
B1	5630	KO 2+	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	262	-	16100	-		edited Lower Marker
347	2250	-	9970	39.94		
505	2430	-	7410	43.17		
868	951	-	1690	16.89		
1500	250	250	256	-		Upper Marker

C1: KO 2-



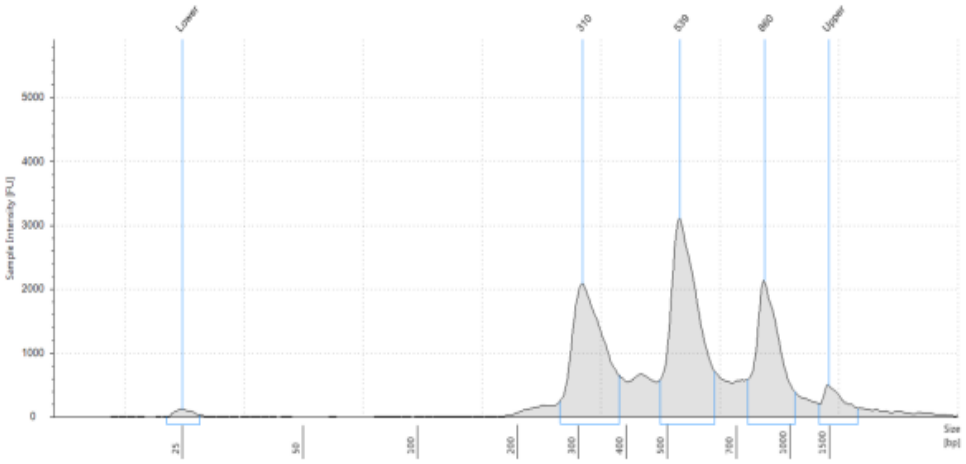
Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
C1		KO 2-	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	54.3	-	3340	-		edited Lower Marker
1500	250	250	256	-		Upper Marker

D1: KO 4+



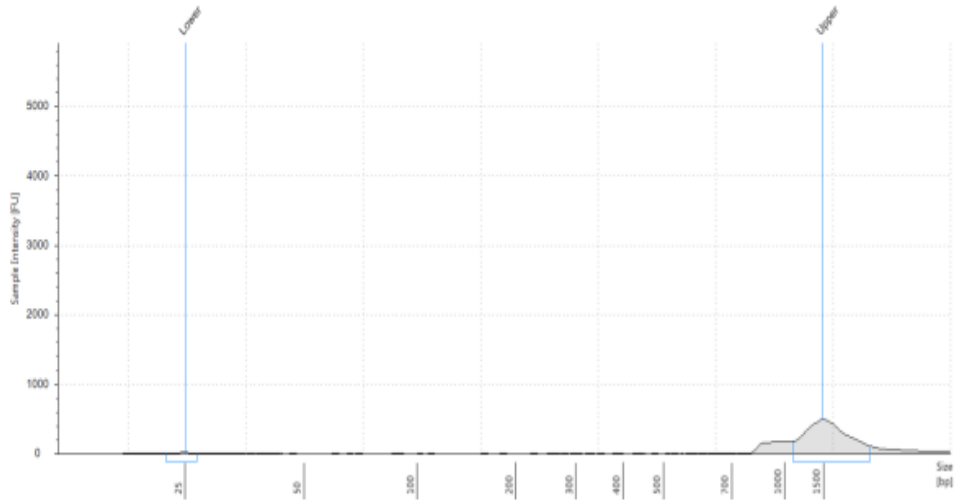
Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
D1	4970	KO 4+	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	48.7	-	3000	-		Lower Marker
310	1620	-	8060	32.67		
539	2120	-	6060	42.70		
860	1220	-	2190	24.63		
1500	250	250	256	-		Upper Marker

E1: KO 4-



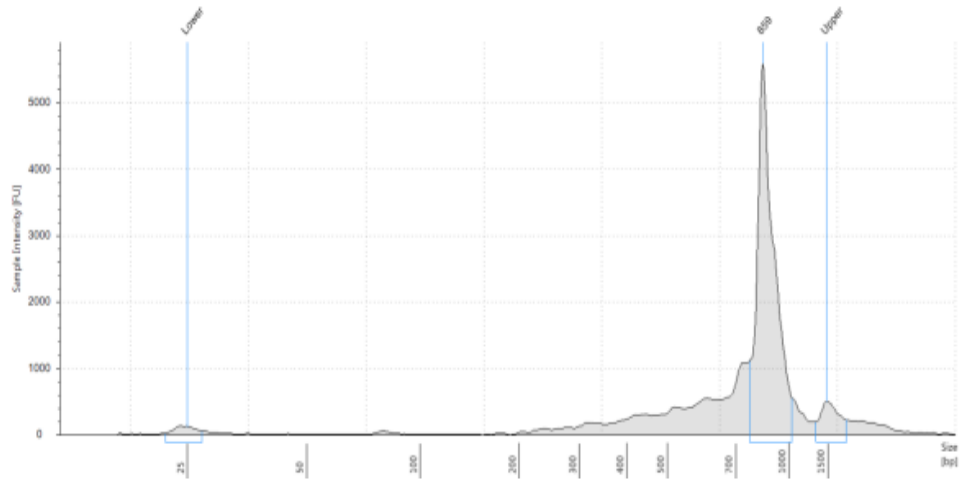
Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
E1		KO 4-	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	2.75	-	170	-		Lower Marker
1500	250	250	256	-		Upper Marker

F1: GFP+



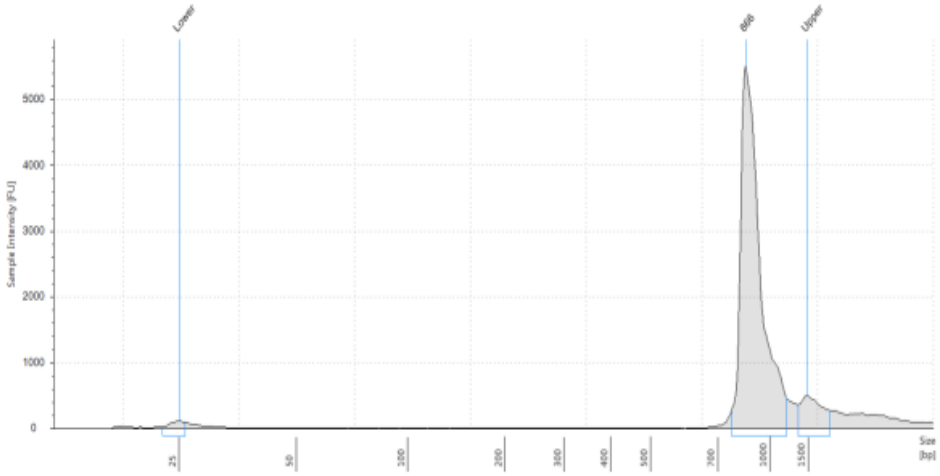
Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
F1	2520	GFP+	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	72.7	-	4480	-		Lower Marker
859	2520	-	4510	100.00		
1500	250	250	256	-		Upper Marker

G1: GFP -



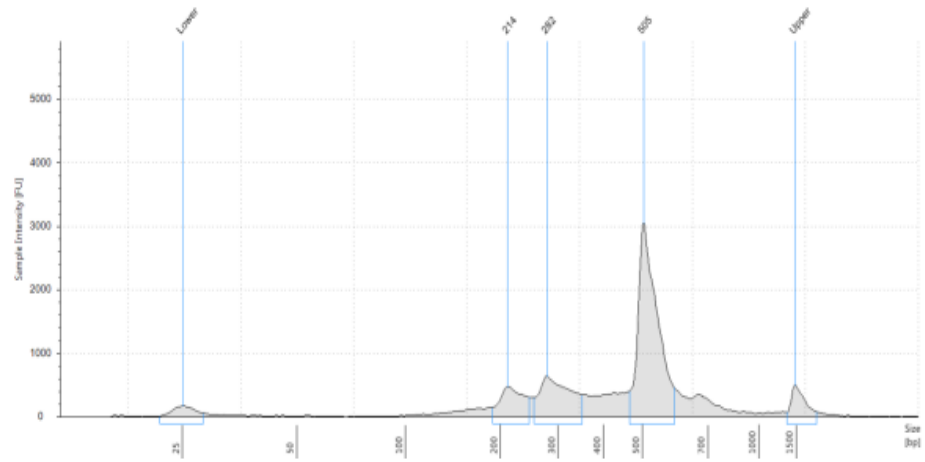
Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
G1	2490	GFP -	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	34.2	-	2110	-		Lower Marker
866	2490	-	4430	100.00		
1500	250	250	256	-		Upper Marker

H1: C+



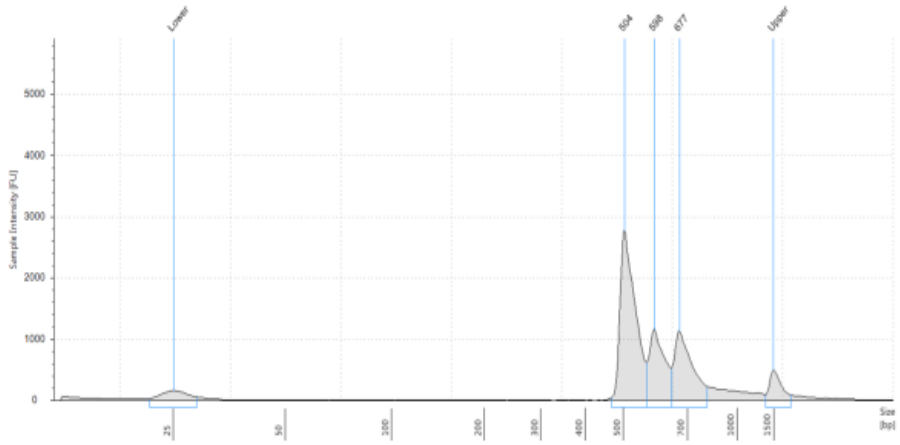
Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
H1	3360	C+	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	139	-	8560	-		Lower Marker
214	413	-	2970	12.29		
282	729	-	3980	21.68		
505	2220	-	6760	66.02		
1500	250	250	256	-		Upper Marker

A2: C-



Sample Table

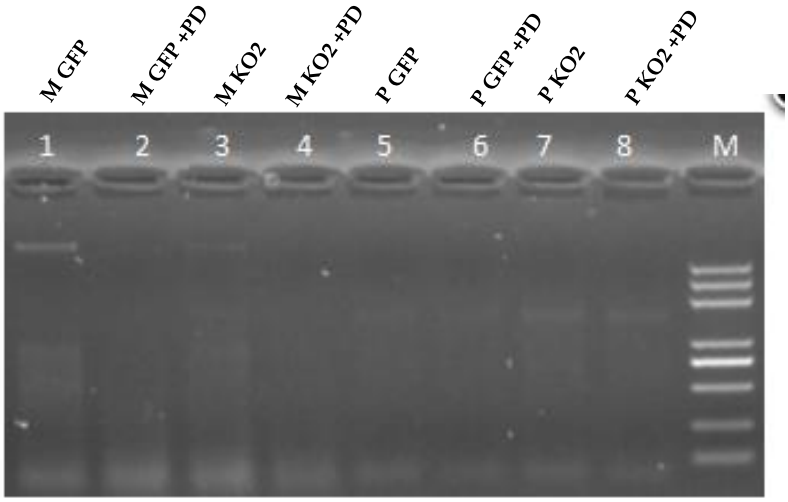
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
A2	3480	C-	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	178	-	11000	-		Lower Marker
504	1840	-	5630	52.97		
598	759	-	1950	21.81		
677	878	-	1990	25.22		
1500	250	250	256	-		Upper Marker

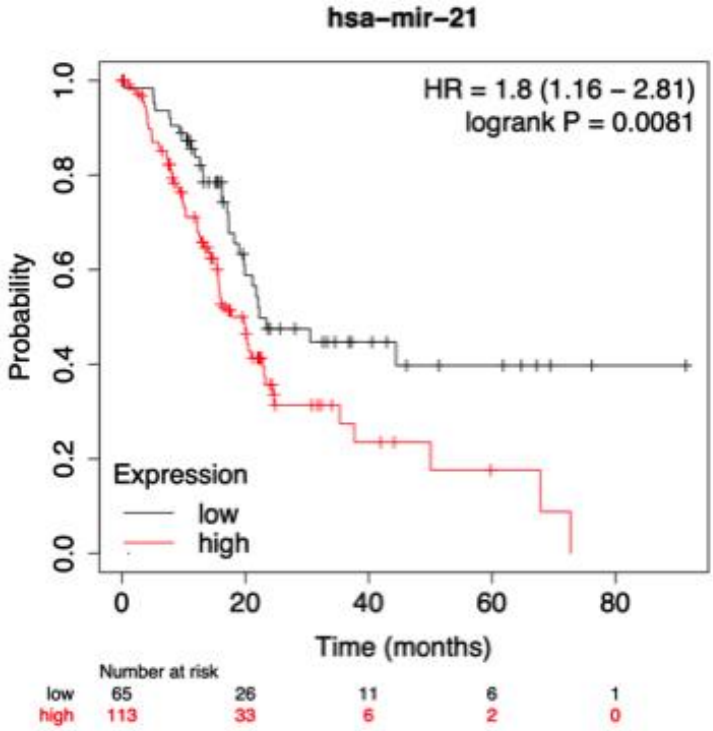
Supplementary Figure 2: Cas9-gRNA editing efficiency was estimated from Sanger electropherograms of short polymerase chain reaction products around the gRNA regions in Cas9-gRNA transduced cells.

Appendix III: Supplementary Figure 3 (S3)



Supplementary Figure 3: Gel electrophoresis results of MiaPaCa-2 and Panc-1 PDAC 3D culture cells and miR-21 KO2s treated with Palbociclib for Small RNA-sequencing checking RNA degradation. Gel Concentration: 1%; Voltage: 180v; Run Time: 16 minutes.

Appendix IV: Supplementary Figure 4 (S4)



Supplementary Figure 4: MiR-21 is one of the most oncogenic miRs related to PDAC prognosis; overexpression of miR-21 was detected in PDAC patients and correlated with poor prognosis and overall survival according to the TCGA dataset. Kaplan–Meier plots were used for the overall survival ratio for miR-21 low and high expression profile patients (178 patients in total). X-axis survival time cut-off, y-axis survival probability. According to the TCGA database, the survival ratio of the low expression cohort (median 22.2 months) was longer than in the high expression cohort (median 19.77 months), based on data from 178 patients. The high expression profile of miR-21 (113 people with high expression of miR-21, compared with 65 people with low expression of miR-21) was significantly correlated with overall survival. Adapted from Mortoglou et al., 2022a.

Appendix V: Supplementary Table 1

Supplementary Table 1: Quality Control Results from small RNA-sequencing.

Sample	total_reads	N% > 10%	low quality	5_adapter_contamine	3_adapter_null or insert_null	with ployA/T/G/C	clean reads
M GF	13018201 (100.00%)	1 (0.00%)	0 (0.00%)	2221 (0.02%)	166051 (1.28%)	90272 (0.69%)	12759656 (98.01%)
M GFP+PD	10609534 (100.00%)	1 (0.00%)	0 (0.00%)	1892 (0.02%)	164816 (1.55%)	75086 (0.71%)	10367739 (97.72%)
M KO2	9884808 (100.00%)	2 (0.00%)	0 (0.00%)	1096 (0.01%)	143666 (1.45%)	74420 (0.75%)	9665624 (97.78%)
M KO2+PD	9915996 (100.00%)	0 (0.00%)	0 (0.00%)	1565 (0.02%)	174503 (1.76%)	61111 (0.62%)	9678817 (97.61%)
P GFP	12541430 (100.00%)	59 (0.00%)	0 (0.00%)	10417 (0.08%)	193513 (1.54%)	43612 (0.35%)	12293829 (98.03%)
P GFP+PD	11449711 (100.00%)	5 (0.00%)	0 (0.00%)	11188 (0.10%)	182472 (1.59%)	33513 (0.29%)	11222483 (98.02%)
P KO2	10836736 (100.00%)	3 (0.00%)	0 (0.00%)	1484 (0.01%)	115827 (1.07%)	34015 (0.31%)	10685407 (98.60%)
P KO2+PD	13050851 (100.00%)	2 (0.00%)	0 (0.00%)	2582 (0.02%)	185668 (1.42%)	39051 (0.30%)	12823548 (98.26%)

M = MiaPaCa-2 PDAC cell line, P = Panc-1 PDAC cell line.

Appendix VI: Supplementary Table 2

Supplementary Table 2: Unique proteins with their Protein ID for Panc-1 cells.

Panc-1 GFP	Panc-1 GFP + PD	Panc-1 KO2	Panc-1 KO2 + PD
P49327	A0A087WWY3	P22234	P78527-2
P05787-2	Q13885	C9JDR0	P06396-2
P49589-3	P15311	P20700	P31946-2
E7EPK1	P68366	B3KQV6	O00159-3
E9PHY5	Q14247-2	Q58FF6	Q5STU3
D6RG13	P16070	Q16576	O00469
A0A0B4J2A4	A0A140T962	A0A087WUL9	M0R0P9
Q9UHB6	O00469-2	H7BXI1	Q562R1
P09104	A0A0A0MT26	Q5TA02	Q9NVI7-2
A0A286YF22	P13647	M0QXU7	Q9Y4G6
P32004	Q15067-2	P55036	Q9NQC3-5
H0YD13	A0A087WUZ3	Q16531	H0YDD4
H0YKS8	F8W0P7	H0Y8E6	Q9H361
Q14315	P63010-3	A0A286YFD6	E9PK47
Q9NQR4	P10515	Q9UBB4	Q5T4S7
F8VW21	H0Y4R1	P33992	Q9HC35-2
A0A0C4DGI3	B9A041	F8W8D4	2A0A087WTV6
Q9UHB9-4	F5H2F4	C9J9W2	Q15393
Q14376	P35613	Q14DU5	O75116
P20340	P31948	P46734	Q9NZB2
A0A1B0GVU7	R4GMT0	Q8IUC4	F5GXZ9
J3QT28	M0QXL5	F2Z388	H0YBL1
P49902	Q8WUD1	P49959	B4DGJ5
P48449	O75746	Q9BTT0	A0A1B0GV93
E7EQV9	C9JW69	Q5URX0	O60749-2
Q13310-2	P11498	Q03252	Q15819
P62987	Q9NUI1	E5RFV2	Q96G23
Q15428	Q9NQC3-3	A0A0A0MSW4	P33993-3
K7ELL7	P21912	P35659	A0A1B0GWA2
P06753-2	Q9H1K4	F8WDN7	O95433
P06899	P17252	Q969H8	O95372
P63092	P35580	H0YNJ6	O15066
P30046	K7ENG2	Q16763	Q9Y2T7
P63173	A0A0A0MS99	M0R1T5	H0Y711
P16401	P50402	H0YN81	V9GYA7
A1L0T0	F8VU65	D6RF35	Q14789-2
Q53H82	Q9NRW1	P30040	Q6P996

H7C393	Q53H12	P42166	M0QZR4
Q15813	Q9NX40	H0YMA0	E7ENJ6
B4E0Y9	H0YIV4	H0YL53	E5RHT6
G3V113	Q15758	G3V438	A0A087WV29
E5RI99	E7EQB8	Q3KNR6	H7BXS3
A0AVT1	E9PKZ0	O14907	P29966
G3V2F7	P09110	Q15021	O43592
D6RD69	A0A087WUX8	A6NF51	D6RD67
K7EK07	H0Y8C6	P52732	B4DP64
O95861	P20336	H7BXC7	M0R027
O75947	Q6DU44	P04183	H0YB71
D6RAN4	B3KWE1	C9JLU1	H0YL19
Q9UN86	P17600	H3BRL3	Q9NZL9
Q9UPN3-5	H0Y449	Q5JUV6	P15927
P61201	Q9UM00-2	A0A087WXD9	P63272
E9PJ81	Q15363	Q9NPQ8	A0A0U1RR07
P00813	Q5SRD1	F8WB72	D6RFE4
P51553	Q6UB35	H0Y630	Q9UJW0
P18084	A0A096LPI6	A0A087WW40	Q08378
P02792	M0R080	P04818	Q08379
F8VR84	Q92520	Q6UXN9	F5GYG5
O75436	Q14643	H0YGT2	P42574
O15127	H0YLA2	E5RJR3	F8WAU4
Q9UL26	A0A096LPK7	Q9NVM6	P16403
H7C286	Q5D862	F6RGN5	Q8IYB5
O95470	J3KTJ8	C9JIR0	P05121-2
Q8WWM7	P43897	Q8TEA8	Q2VIQ3
H0Y2X5	X6RJP6	I3L3E4	J3KS45
E7EPB3	Q96AY3	F2Z2G2	P35221
P02794	H0Y5J4	Q86X76	O43395
C9IZ01	B1AMS2	C9J177	J3KRV4
E9PF63	B4DDD6	Q9BT09	P18077
Q8N163	K7EL20	Q9NS86	D6RA11
H7C492	A8MXH2	Q92522	A0A087WVC1
Q3KQV9	M0R058	A0A0J9YWY6	Q13619
sp|cRAP106|P10599|THIO_HUMAN	Q9UBX3	Q6ZU80	Q9NTZ6
P36404	Q9HD45	A0A0A0MT60	P09543-2
Q9BRA2	B5MCP9	Q96GX9	D6RBJ7
E9PS44	F8WAR4	E7EVL6	Q5GLZ8
P11766	Q14344	A8MZ87	P50750
Q96DA6	A0A087X106	A0A0J9YVR1	H3BSK9

Q96JB5	Q658P3	H7BYN4	J3KP97
Q86UE4	Q9BUP3	Q13636	A0A0U1RRM6
O60869	F8WF48	Q7KZ85	P11802
Q16134	Q5JXH7	A0A087WW00	E9PPA0
H3BPR2	P62714	Q9UKD2	Q9UNE7
B1AHE3	Q8NFJ5	A0A087WYR3	I3L3B0
F5GYW6	D3DQV9	P21589	E9PDF6
A0A096LNJ1	G3V1U3	H0Y9X2	I3L0K2
H0Y368	P09471	O95239	Q6FI81
J3KN01	Q9NPA0	Q5VY30	P49903
Q16822	P28288	O00505	O75351
O94919	X6RA14	M0QYE0	C9JA08
E7ESE2	E9PND2	B7ZKJ8	F5H1Y4
P48509	O43819	Q5T653	O95613
K7EP07	Q13232	B7ZC06	Q5SWX3
Q9UHV9	P20338	G3V3B3	A6NGJ0
Q9BRX8	Q9NXV6	Q5QPP1	Q15386
B1AKR6	I3L0M9	Q92917	A0A1W2PQD2
A0A087WY82	P31431	H0Y5L2	Q12774
X6R9L0	F8W785	Q2TAY7	Q01968
J3QRU1	Q15006	Q12834	Q53EP0
M0R261	Q96FZ7	H0Y8R1	H0YK99
Q32MZ4	A0A0A0MRF4	Q9BTE3	Q9NWT6
A8MYV2	C9J0B2	B1AHA8	Q9NSS8
F5GX99	Q86YS6	O94804	Q14676
C9JHU5	Q96AB3	H0Y3V8	Q9BT92
B4DP31	C9JA17	H0Y3M2	B8ZZF0
Q9P0J0	O00194	A0A087WZ13	E9PR30
A6NG10	H7C2U6	H0YJH7	X6RDA4
P61956	Q9Y5Y5	Q9NVX2	Q8NEN0
A0A087WY85	P62745	A0A286YF56	I3L297
E7EX17	O95139	P53611	D6R918
P08237	A0A087WTU3	Q8IYB1	A0A0A0MRX9
F8VUY8	P29590	A0A0G2JR38	Q9BUQ8
sp|cRAP100|P00441|SODC_HUMAN	Q12888	F8VZG8	H7C477
P11047	C9IY40	Q6P1J9	H0YI31
E9PIX6	H7BZK2	A0A0A0MS70	Q96PY5
P09417	Q9Y624	Q15750	H0YN14
H0YE57	Q13445	Q9NXH8	E9PC44
P49790	H7BXQ8	A0A0G2JQK5	E9PPH5
H0Y3Z4	C9JZ87	A0A087WTW5	Q6YHK3

A2ACR1	Q96HN2-2	Q8TF42	Q9H1B7
C9JL85	Q96ER9	Q9GZL7	B1AK81
M0R0M7	Q8IXI1	H3BU82	P98160
H0YFI1	B8ZZY2	F2Z2E7	F5H248
H0YJX6	A0A0G2JIC6	H7C2N1	E7EQI7
P31949	J3QL54	H3BP78	Q2TAL8
Q06124	H0YK61	P11388	H3BM14
Q8TEX9	A0A0A0MS41	Q9NPD8	A3KMH1
Q9BX68	H0YBD2	H0Y704	J3QKV7
P21283	E9PHM6	A0A087X271	Q8WUM0
A0A0U1RR22	Q14139	A0A0A0MRG9	A0A1B0GUB2
X6R3R3	X5D2R7	S4R369	H0YD29
Q9NQG5	H3BQQ2	A0A1W2PNP0	P41240
O75157	Q96DG6	Q96SK2	Q9UKG1
H3BTL1	Q96RP9	D6RGJ2	Q9H3Z4
A0A0A0MSZ4	O95159	A0A1C7CYX0	P0CE67
Q2TAA2	G3V3G9	J3KTL8	Q05048
Q9NQW7	E7ER77	Q9HCD5	B9ZVU2
F8WDS9	Q03169	C9J4P8	A0A087WTD7
Q9HBH5	Q9Y2J8	Q15291	H7C3S1
C9J5E3	G3XAN4	D6RDM7	Q6PCE3
Q8NFH3	P26439-2	J3KS15	Q6P5Q4
Q9H479	A0A087WTV2	J3KQ96	J9JIC5
D6W592	Q8N490-2	A0A0A0MSW3	A0A0G2JQ41
E7EST3	H0YAF1	A0A075B712	A0A087WXL3
F8VXU5	A6QRJ1	A0A0A0MRT6	H0YI90
Q5VTU3	P27105	F2Z2X8	O95453
O75348	Q8NBU5	Q9H9H4	A0A0A0MQV9
Q92506	C9JRP1	Q6ZSC3	B9DI82
D3DTZ5	Q8TBQ9	F6Y5H0	Q15785
Q96BJ3	Q6PCB0	F8W8W4	Q96S59
Q9Y5X2	P47985	Q92574	Q5T3N0
A0MZ66	Q5T2S8	O60341	O95674
H3BRV9	Q5QPD0	P25098	Q8TEQ6
C9JKM8	Q96L35	Q8NFW8	F8W8N5
E9PJD9	S4R3U9	Q9UBB6	A0A024RCG2
Q5T5U6	H3BPK7	H3BMQ5	C9JXZ5
B4DQI4	F8WBI5	Q8WU90	Q9UJA5
Q9NRX4	H7C0B3	B8ZZY7	K7EMQ3
P45954	H0YAX3	Q99661	P04003
O75323	Q13190	E9PMM3	E5RI06

F2Z2Q9	H7C1U8	A3KFJ0	I3L1L3
A0A087WWM0	I3L2J8	A0A0U1RR30	Q7Z5L9
B7Z5N5	O60262	Q5JSH3	Q6P1K8
E9PM35	E9PJF7	Q8TDS4	Q5JXC5
C9JT21	Q6ZU15	Q9NS39	B4DJV5
J3KNE3	B1ALC0	A0A087X0K9	Q6P1Q9
P50583	H0YBD9	D6R9U2	A0A087WVD7
H0Y7N8	A0A1B0GUK8	Q7Z6U0	H0YFG1
H0YJ63	H0YC04	C9JCD9	H0YHV1
F5GWZ1	H0YC35	I3L165	Q8WVM0
H3BN50	M0R1E3	Q9H307	A0A0A0MR06
E9PQU5	H0YKU5	A0A087X0R8	F8VP70
Q4VC31	A0A0U1RQT9	B1AJQ6	Q8NDV3
H0YF06	Q5HYI8	O75381	H0YIV7
H0YEY4	Q9NUB1	E9PB61	Q7L7V1
A0A0A0MT32	F8VSD4	Q99595	P26447
F5GX14	H0YGU3	J3QSG3	H7C487
F5GX77	O15228	Q59GN2	A0A0J9YXA3
J3KPP4	D6RAA6	Q8WUY9	O43719
A0A087WUI8	Q86YQ8	H0YK07	H0YDW2
D6R9S0	O96005	A0A0D9SEI3	H7C4F9
A0A1W2PQY5	Q96HV5		O15121
A0A0A0MRL6	H7BZL2		A0A075B7D2
P20742-2	E9PGM4		P06280
O75976	A0A087WZG4		D6RC71
A0A087WT48	D6RF87		I3L1N9
P35219	F8WCN3		
Q13136	K7ELS8		
A0A0C4DFT3	A0A0G2JJA7		
A0A0G2JMS7	Q96EY7		
M0R0F9	Q9BXB5		
Q15061	A0A087WTS4		
H3BPG7	H0YG82		
F2Z2Y6	Q96RD7		
P27144	A0A087WUK2		
B0QZ43	D3DWX8		
Q9GZT9	Q9H9F9		
C9IZG4	Q8WXE9		
M0QXS6	K7EK01		
Q59FY4	Q7Z434		
H0Y8D9	Q14746		

Q9GZN8	Q9Y2W2
Q9Y2W1	B1AR60
H0Y746	Q8TB36
K7EQ23	A0A087WYM3
H0Y579	J3KNA0
A0A1B0GUE3	Q96I63
Q5TD07	J3KSI8
O75663	D6RBS5
R4GMU1	A0A0D9SES7
Q9BWH2	V9GYN3
A0A087WX59	Q96IC2
M0QYC1	G3V342
F5GXJ9	K7EJB8
Q9NZM3-3	E7EVJ5
D6RHC4	M0QYT5
F8W8C3	A0A024R4K9
Q13144	H7C3K7
A0A087X0Z7	A0A087WUM0
A0A0C4DGG8	H7C5Q2
Q8NHV4	F6T1Q0
G3V2U7	K7EN15
A0A087WWG1	Q8TBY8
H0YAT2	Q8TB61
P0C7P4	V9GYE4
H3BP35	Q8IWB1
A0A0C4DG91	I3L1T3
A0A087X1H5	Q969M3
Q9BST9	Q8N2G8
P07305	Q8NCL4
F6TR53	P51668
K7ELD9	O15015
O94776-2	A0A075B6F3
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J3KRN5	Q9BV81
M0QZK8	Q9Y697
Q9ULW0	H0YK03
P51688	A2IDC6
H3BLS7	A0A0C4DFL7
F5H0D0	Q09161
Q9H9A6	A0A096LNH0
A0A0D9SFB0	C9J4N6

2X6RLN4	Q99575
H7C5E8	Q8IUR6
Q9BYD1	Q5TIH2
D6R9E3	H7BYL6
H0YKK6	O43194
R4GMX2	H0Y5W8
P10301	Q8NBZ7
Q9BRP1	P82914
H0YEF4	Q00577
P27987	H7C561
B5MCA4	H7C033
Q8NEE8	Q8N987
O60293	Q9P0K7
O60826	A0A0C4DH82
Q5MJ10	H0YAZ0
H7C0X7	Q9Y399
Q96M63	A8MXV4
G5E9C5	H0Y4T7
Q9Y5K6	A0A1B0GVI3
O15355	A0A140T947
Q9BT73	Q01970
P49006	E9PK80
A0A0D9SFK7	Q86XA6
A0A087X2C3	P51674
R4GNG3	Q6PI48
A0A286YEX5	B1AH87
O75400	B0QZ36
A0A087X114	G3V599
H0YKI8	J3QLR8
Q15293	H3BR35
H7C5Q3	F5GYT8
A0A0G2JHC2	H0Y886
Q9Y2U5	L0R6Q1
A0A0A0MRN5	H0Y6Y8
K7EKE5	Q15056
Q15042	H7C3A1
Q96EC8	H7C213
A0A0A0MTN0	E9PI90
Q9P2R3	G3XAI2
J3KS23	Q96KC8
Q5F2F8	P10606

G3XAJ4	Q9BYD6
G3V153	A8MT19
C9J363	Q5SWX8
A0A087WXX9	P82932
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Q92541	B4DVA9
A0A087WZX7	H0YG20
H0Y4S9	H7C2U8
Q08752	O14493
K7EPV0	Q9H4A6
A0A087X0D5	Q14554
E5RIQ8	U3KQ32
Q9GZP4	B4E321
Q8IWB7	E5RG67
H0YA47	D6RCK3
A0A075B788	V9GYZ1
Q5T160	Q9C0E8
P80217	H0Y8C3
Q06481	A0A0C4DFQ1
	M0QYZ2
	A0PG75
	Q16678
	Q10469
	P50148
	C9JCW7
	Q14966
	C9J0H3

Appendix VII: Supplementary Table 3

Supplementary Table 3: Unique proteins with their Protein ID for MiaPaCa-2 cells.

MiaPaCa-2 GFP	MiaPaCa-2 GFP + PD	MiaPaCa-2 KO2	MiaPaCa-2 KO2+PD
A0A087WWY3	P21333	P35908	P15311
P42765	P14618-2	B4DUR8	K7EKE6
P12235	Q15149-2	E5RK69	Q15149-8
H0YD13	P07237	Q01082	P52895
A0A0A0MRV0	P39023	Q6S8J3	P02538
Q8N1F7	Q14669-4	P31946	Q16181
P35749	A0A0A0MSX9	P22234	E7EQG2
P61289	Q03001-8	H0Y2P0	P04259
Q5JTV8-3	Q5T6W2	P42167	Q9NQC3
A0A087X253	Q9H2G2-2	G5E9G0	P13674
Q99729-3	E9PCR7	Q13148	P08779
B0YIW6	P63241	F5H7Y6	
K7ENG2	P13797	P54920	F6WQW2
Q9Y277	Q9P2E9	A0A087WU27	P43246
O14787-2	E7EWE5	P46779	Q9UNM6
Q9Y2L1	D6RG13	K7EMW4	sp|cRAP099|P62979|RS27A_
J3QS39	P25789	H3BNV2	Q15738
A6NCQ0	H3BPE1	Q9Y383	sp|cRAP017|P04040|CATA_
E9PQD7	P27635	C9J381	A0A096LPI6
J3KQ37	B1ANR0	J3QKT4	M0R210
C9J9W2	P18124	E7EQV9	F8W9J4
Q96PK6-5	Q9BQG0	A0A0A0MSW4	P26006
H7C5Q2	E9PRC8	E9PDE8	Q16698
P07195	P53992	F5GYZ5	M0R0Y2
F5H1S8	K7EIG1	H0YM31	W4VSQ9
sp|cRAP096|P01112|RASH_HUMAN	P06748-2	E7EMB6	H0Y6Y4
F5GY55	A0A0G2JS82	Q13310-2	Q15363
Q2VIR3	J3QQ67	D6RBV2	A0A140T912
A0A087WVN4	Q86UP2-3	O94973	Q5VWC4
A0A286YFA2	K7ER96	O75323	A0A0D9SF54
P51553	Q14980	B5MCP9	B4DKB2
A0A0C4DG40	Q9UJU6	E7ER77	K7EK07
H7C2U6	O43172	P62854	F8VW21
A0A0C4DGP4	P30475	Q96KB5	P02533

Q9UNS2	E7ENU7	O60749-2	Q8TEX9
D6R9P4	H3BR35	P52701	Q9H361
C9J6N9	P08243	F8W8W4	A0FGR8-2
Q6AWB1	P19784	Q92797	S4R3N1
Q02127	Q14498	P25325	G5E9Q7
Q5TZA2	K7EJM5	P01112	H7BY10
Q96FJ2	Q14258	P57088	P15927
H3BVE0	F8W9S7	Q02224-3	Q9BTZ2
H0YBP9	E5RHG8	F8VPE8	E9PKZ0
Q08378	P84103	E9PB61	A8MXH2
P50579	P55795	E9PP21	Q6UB35
A0A0A0MRJ7	Q5JTH9	Q7L576	E9PF16
Q5T1J1	P14678	A0A0A0MTS7	Q6NUM9
A0A087X0P0	P78406	P50416	A2A3R7
P14635	P46778	K7ERV3	Q5TEC6
J3QRD1	C9K0R9	Q9NQR4	Q8WU90
Q6RW13	B5ME97	Q7Z460	P07686
Q9NRK6	O00764	D6RGV9	Q8IY17
Q5LJA5	H3BM42	Q5JWF2	Q9BQA1
Q9Y2I6	C9JQD4	O15131	A0A286YER3
Q8N543	Q7L576-2	Q07812	A0A0A0MRA3
Q5T3Q7	H7C3I1	A0A1W2PQ47	C9JNP9
D6R918	Q01105	O96005	P10253
Q9BTX1	Q99543-2	B0QZ65	Q9NRL3
Q14694	Q13618	Q9NVX2	Q02252
J3KQ96	A0A1B0GVU0	H0YMA0	J3KRT0
Q5VVC8	P46087	C4P0D6	Q9NTX5
P49756	A0A0C4DGG9	C9J4Z3	H0YG07
E9PPG9	P16989	Q96HN2-2	H7C1U8
O95674	Q92890	Q5HYK3	P46108
Q9H5V8	P20290	K7ERP6	Q709F0
A0A0C4DG98	P46734-2	F8VS81	P53634
G3V1B6	A0A087WVC1	B4DFG0	P14550
Q6ZU80	P20645	F8W726	Q9NRF8
A0A0A0MSL3	Q9Y3U8	P16402	Q96LJ7
Q5QPE1	Q5VVC9	A0A087X211	H7C393
H3BPC4	Q15437	F2Z3H0	J3KS45
J3KSS0	Q16695	J3KMX1	B1ANM7
Q9NPL8	D6RDY6	Q6PKG0	Q00535
Q96GW9	A0A1W2PQ43	C9JVN9	R4GMU1
O15121	Q15428	Q8TEA8	Q9BYD6

Q9UJA5	A0A087WUS0	O00267	K7ES61
A0A0G2JH50	O60502	Q8WWM7	E9PLT0
A6NKZ9	H0Y2W2	Q14554	Q9UPN3-5
H7C170	A0A1W2PQS6	E9PGZ4	J3KTJ8
P82909	Q9Y6M1-3	Q15006	G3V3G9
I3L1L3	Q9H223	Q92917	A0A0J9YVR1
B4DLN1	A0A087WXU3	F8WAU4	Q5VY93
Q96T76	D6RBJ9	Q9Y6N5	A0A087WY67
Q8IYE0	Q9BV57	Q2NKX8	K7ELD9
C9JIR0	Q6P2E9	P16190	F8WBJ6
A3KMH1	H3BP71	S4R3Q6	Q9UBX3
H0YBE1	Q9H7D7	F6RGN5	H0YE29
Q7L7X3-3	A0A0A0MRP6	H7BXQ8	A0A1W2PQB6
A0A088AWN3	E5RG77	C9JHK9	Q8NE71
A0A1W2PP20	Q5T4L4	E9PFR3	H0Y827
H0Y390	P35659	A0A0G2JM12	H0YCY6
H0YK61	Q9NRV9	Q96KC8	Q92947
H3BNR4	Q9Y223	E7EMW7	Q12873
Q8IYB8	Q9BUQ8	H7BYV1	B4E1S2
P51674	F2Z2Q9	A0A0U1RRM6	P20929
A0A1B0GW15	Q05209	C9J6D1	O95456
Q9Y4C2	H0YNH8	J3QSB5	J3KQY1
E9PIT3	H7BZ48	M0R317	P62328
P30825	H3BUR4	K7EM13	A4UGR9-8
B4DFI2	O15160	J3KS05	A0A087X2H1
P13498	P50750	B8ZZU9	A0A087WXC5
H0YC52	B4DXZ6	O43896	E9PE20
P28288	M0R261	P36543	H0YDT8
K7EK00	K7ENR6	E5RG67	Q9P2M7
X6RM59	A8MZF9	G3V559	Q93009
A0A0G2JQT1	Q7RTV0	A0A1B0GX65	O43592
Q9P015	B3KT61	H0YIZ9	H0YKP3
B4DQA8	J3KPP6	H7BZL2	Q9Y4Z0
U3KQK1	A0A087WV29	C9IZG3	P40261
A0A087WWQ2	Q5TZA2-2	O75717	F6S8M0
I3L3P7	P11802	Q86TI2	A0A0J9YWR7
Q9BWH2	A0A0A0MSZ9	D6RD60	P21589
Q9UDW1	P00533	I3L102	B5MCW2
C9JCW7	Q13868	I3L0K2	P12883
Q9H3K2	C9JA08	D6W592	K7EKI0
Q7L1W4	Q7KZ85	Q7RTP6-5	O95302

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Q14C86	G3V4T2	A0A0A0MR85	H7BZH6
A8MUN2	Q9BYJ9	Q9P219	O14773
H3BTA2	G3V192	Q8WUX9	Q53EL6
I3L1T3	H3BPB3	A0A0C4DFM1	H0YDP7
Q5JXC5	P49589-3	C9JFM5	H0Y3V8
Q9BRQ8	G3V5E1	O43464	Q8N4Q0
Q86WI3	Q9BY32	G3V4E5	F8W7W8
Q5JRG1	X6RLX0	A0A1W2PQD2	Q15526
Q56VL3	P0CG29	A0A0A0MS29	F5GWX2
A0A1W2PNX8	Q12996	Q6IN85	P09012
Q8N987	Q9BTV5	B1APM4	C4P0D4
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D6RCT7	2D6W592	H3BQQ2	P17096
Q8TD30	B1AMU3	O75381	Q9Y2W2
Q14789	Q15424	Q9ULV0	C9JP81
G0XQ39	H3BT65	O00567	H0YFY6
M0QXK2	P61956	Q38SD2	P05026
D6R933	Q9NX58	Q8IU81	H0YB03
Q96I51	Q96EY1	Q05682	F8VQX7
Q8TBY8	A0A087WVD7	P06132	Q9NPJ3
J3KRL5	X6RAL5	A0A1W2PPK1	G3V2L6
F2Z2Y4	P52790	Q9P2E3	Q3KQU3
B1AJQ6	G5E9W3	B0QZ36	Q14D04
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Q96DI7	Q5QPQ0	M0QYT5	Q16537
Q5T160	F5GXQ0	H0Y4T6	P46734
A0A087WZC3	M0R0A1	P52306	H3BN93
J3KRV4	D6RFE4	Q5VUC6	Q96PY5
A0A087WW43	A0A1W2PPT5	P01008	A0A087WX58
Q96NL6	Q8N339	A0A0A0MRN5	K7EPI1
Q9BX69	Q9Y3P9	C9JXK0	F2Z2G2
2E5RFX7	B3KXD6	G3XAJ4	P55263
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Q5VY30	Q03701	A0A087WTS4	A0A0C4DGV3
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Q9ULW0	Q9Y3C6	A0A087WVV7	H0Y430
C9J0B2	Q59FY4	Q969U7-2	A0A0D9SFL3
K7EK20	Q5QPL9	Q15386	H0YA63
H0YFA9	Q9H0S4	K7EJB8	A0A087X2B1
Q5T6H7	F8VVA7	A0A087WY61	P41240
H3BUK8	E9PLA7	Q8NHY5	H3BRS6
H0YEL5	D6RGZ2	I3L294	A0A096LNV3
Q5JTA5	Q5QPA5	H0YBG2	Q8IV08
G5E9V5	Q96P11-3	Q9H993	A0A087X102
E9PM04	Q96DY2	Q969E2	J3KND1
Q96BJ3	P36404	A0A087WZK0	Q13362-4
A0A0G2JLL4	F6T1Q0	A0A087X0K1	A0A1W2PPU6
Q8WXE9	Q9UJX2	E9PEB5	P21399
B5MBZ8	Q8IYB5	H0Y8D9	Q6SJ93
H0YK18	P36406	B8ZZY2	H7C2N1
P41440	B9ZVN9	Q14573	H0YK87
H0Y9T9	Q8WUM0	K7ELS0	E7EX17
H0Y2Q1	O14530	A6NLI5	K7ERY7
A0A0A0MRF4	P07711	Q9BRR6	A0A0C4DGV0
M0QX04	B7WP74	A0A087WW31	H0Y3T6
Q9NP92	I3L165	Q9Y2M0	Q5T8Q9
O14777	O60306	Q96M27	H7C3K7
Q13895	Q15042	H0YJV2	Q9NYH9
Q8TDS4	H0Y6N5	C9J470	H0YG82
	Q5TF55	D6RF87	Q8IYS2
	C9J6C5	C9IY70	Q96H79
	D6W5Y5	O00461	G3V0I5
	E9PPH5	A0A087WWV1	J3QRM1
	Q5W111	B7Z7U4	F8VP70
	P00374	A0A087WUY5	Q96T17
	Q9HC36	A0A0A0MRE5	Q99470
	A0A286YFF7	Q9ULF5	G3V1R2
	Q6P4A8	Q5JUV6	B1AHG4
	C9J679	A0A0J9YXA3	Q969S9-5
	A0A087WUB9	Q5T7U1	A0A1B0GTB0
	Q9H9F9	Q9NWB6	I3L4J1
	A0A0A0MTN3	F5H6P7	A0A0A0MT56
	F5H4C6	E7EV59	M0QYV0

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	P51857-2	P18615	H0YL50
	I3L297	G5E994	H7BZ75
	G3V220	H3BRL3	H0YDU8
	C9JJP5	A0A087WTT2	P53582
	D6R905	H0YB56	H0YB34
	Q9Y221	G3XAH0	F2Z2Y8
	A0A075B6E5	I3L2C6	Q9P1Z2
	Q8WXF1	V9GY71	Q6EEV6
	I3L2W1	H7BZK4	G5E9W8
	Q96KR1	F2Z2E7	Q7LGA3
	Q9H477	Q8WUY1	F8WDS9
	O95104	D6RBD7	E9PL10
	A0A087WVZ3	Q9H1B7	C9JPE1
	A0A087WVZ9	E9PI90	O60488
	Q9NSS8	H3BT22	H0YG20
	E5RHG6	Q9UKX7	A0A0J9YVR0
	P49406	I3L3N0	M0R389
	F5GX77	O75937	A0A1B0GTN0
	Q16204	Q5VW32	O76071
	H0YAS9	M0QXB0	Q9NX46
	A0A0C4DG91		A0A087WWW0
	Q5TD07		
	H3BLV9		
	Q13637		
	P24928		
	F5GZM3		
	I3L2X8		
	H0Y9Z9		
	Q99459		
	Q92968		
	M0QXX8		
	Q9NP77		
	H0YAT2		
	A0A087WWP4		
	Q9UBV8		
	A2A2Q9		
	A0A0U1RQT9		
	Q14690		
	I3NI32		
	X6RAJ1		

	Q9UKV0-5
	Q9UN37
	B8ZZZ7
	F8WFB5
	Q1KMD3
	Q96S59
	Q9H9A6
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	P60903
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	A0A0A0MRN4
	O95470
	O75343
	Q8TBX8
	E9PMD4
	Q93034
	Q9H444
	A0A0A0MTN0
	Q6P1L8
	H7C213
	Q5C9Z4
	Q9UKG1
	A0A1B0GUE9
	H0YMF9
	Q9H479
	H0YDJ3
	O00507
	A6PVI3
	A0A087WWM4
	E9PL41
	Q8NAF0
	P51812
	Q14684
	H7C3S1
	C9JT21

	D6RE78
	Q8N3K9
	Q96M89
	Q8WTT2
	O95486
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	A0A1B0GVN9
	Q8NCU8
	A0A087WT38
	Q68E01-2
	J3KSL7
	O00178
	F8VQE1
	H7BXF4
	F5H3U9
	Q92538
	Q92503
	P00846
	H0YBL1
	D6RDM7
	Q9UHI6
	Q4J6C6
	H7C579
	Q9C0C9
	Q59GN2
	A0A0D9SEI3
	A0A075B7D2
	O95801
	Q96I25
	O75691
	Q92615
	F5H4E8
	H3BTB7
	Q01780
	P41743
	Q6Y1H2
	H0YKJ4
	Q5VU10

	B4E1E4
	S4R3E9
	G3V5V3
	G3V4N7
	P02792
	I3L0E3
	A0A0D9SGE8
	A0A1W2PRV5
	B5MDE0
	K7EPV0
	P32929
	A0A087WWK8
	K7ESE8
	O95881
	C9JKM8
	P35913
	H0YCY8
	Q9BZE4
	A0A1B0GUB2
	I3L1N9
	Q13131
	Q13131

Appendix VIII: Supplementary Table 4

Supplementary Table 4: Common proteins with their Protein ID between Panc-1 cells.

Panc-1 GFP/GFP + PD	Panc-1 GFP/KO2	Panc-1 GFP/KO2 + PD
Q09666	Q09666	Q09666
P35579	P35579	P35579
Q15149-4	Q15149-4	Q15149-4
O75369	O75369	O75369
P21333	Q60FE5	Q60FE5
Q9Y490	P21333	P21333
Q9NZM1	Q9Y490	Q9Y490
O43707	Q9NZM1	Q9NZM1
P08670	O43707	O43707
P08238	P08670	P08670
P11021	P08238	P08238
P18206	P11021	P11021
P10809	P18206	P18206
P42704	P10809	P10809
P07900	P42704	P42704
P46940	P07900	P07900
P78527	P46940	P46940
P14618	P78527	P14618
Q86UP2	P14618	Q86UP2
P12814	Q86UP2	P12814
P38646	P12814	P38646
P11142	P38646	P11142
P06733	P11142	P06733
A0A087WVQ6	P06733	A0A087WVQ6
P07814	A0A087WVQ6	P07814
P14625	P07814	P14625
P26038	P14625	P26038
Q14204	P26038	Q14204
P13639	Q14204	P13639
P11216	P13639	P11216
Q14697	P11216	Q14697
P13667	Q14697	P13667
P29401	P13667	P29401
Q14697-2	P29401	Q14697-2
P07437	Q14697-2	P07437

P55072	P07437	P55072
A0A0G2JIW1	P55072	A0A0G2JIW1
P78371	A0A0G2JIW1	P78371
P05783	P78371	P05783
P11717	P05783	P11717
P07355	P11717	P07355
A0A087X054	P07355	A0A087X054
P68371	A0A087X054	P68371
P34932	P68371	P34932
P08727	P34932	P08727
P60709	P08727	P60709
P11413	P60709	P11413
P50990	P11413	P50990
P04075	P50990	P04075
P30101	P04075	P30101
P04406	P30101	P04406
Q92616	P04406	Q92616
P00558	Q92616	P00558
Q14152	P00558	Q14152
P02545	Q14152	P02545
Q01813	P02545	E7EQR4
P40939	E7EQR4	Q01813
P22314	Q01813	P40939
P68104	P40939	P22314
P31939	P22314	P68104
P02545-2	P68104	P31939
O60701	P31939	P02545-2
Q9BVA1	P02545-2	O60701
P49368	O60701	Q9BVA1
Q99832	Q9BVA1	P49368
P25705	P49368	Q99832
P07237	Q99832	Q01082
P48643	Q01082	P25705
Q86VP6	P25705	P07237
P13489	P07237	P48643
P06576	P48643	Q86VP6
P33176	Q86VP6	P13489
P50991	P13489	P06576
Q13509	P06576	P33176
P22626	P33176	P50991
P27824	P50991	Q13509

P31150	Q13509	P22626
P35241	P22626	P27824
Q71U36	P27824	P11586
P00338	P11586	P31150
P26640	P31150	P35241
Q92598	P35241	P31948-2
P04083	P31948-2	Q71U36
P60842	Q71U36	P00338
P15144	P00338	P26640
Q9Y6N5	P26640	Q92598
P41250	Q92598	P60842
E9PLK3	P60842	P15144
P18669	P15144	Q9Y6N5
P00367	Q9Y6N5	P41250
P68363	P41250	E9PLK3
P12956	E9PLK3	P18669
Q01518	P18669	P00367
O95373	P00367	P68363
P17987	P68363	P12956
Q9BQE3	P12956	Q01518
Q9P2E9	Q01518	O95373
P13010	O95373	P17987
P02786	P17987	Q9BQE3
Q9BSJ8	Q9BQE3	Q9P2E9
P49411	Q9P2E9	P13010
Q13200	P13010	P02786
Q14764	P02786	Q9BSJ8
P27348	Q9BSJ8	P49411
E7EUI8	P49411	Q13200
P40926	Q13200	Q14764
Q13813	Q14764	P27348
Q05639	P27348	E7EUI8
P60174	E7EUI8	P40926
P22102	P40926	Q13813
P55060	Q13813	Q05639
Q00839	Q05639	P12268
P50395	P12268	P60174
sp|cRAP095|Q06830|PRDX1_HUMAN	P60174	P22102
P27797	P22102	P55060
Q96KP4	P55060	Q00839
P05556	Q00839	P50395

A0A0A0MS51	P50395	sp|cRAP095|Q06830|PRDX1_HUMAN
A0A1B0GVD5	sp|cRAP095|Q06830|PRDX1_HUMAN	P27797
P53618	P27797	Q96KP4
P54136	Q96KP4	P05556
P26639	P05556	A0A1B0GVD5
A0A087WSW9	A0A0A0MS51	P53618
P19367	A0A1B0GVD5	P54136
P19338	P53618	P26639
P04843	P54136	A0A087WSW9
sp|cRAP006|P08758|ANXA5_HUMAN	P26639	P19367
P07195	A0A087WSW9	P19338
P17655	P19367	P04843
F8W6I7	P19338	Q14247
Q16658	P04843	P49588
P51659	Q14247	sp|cRAP006|P08758|ANXA5_HUMAN
Q9Y230	P49588	P07195
P00533	sp|cRAP006|P08758|ANXA5_HUMAN	P17655
A0A140T913	P07195	F8W6I7
P08133	P17655	Q16658
sp|cRAP017|P04040|CATA_HUMAN	F8W6I7	P51659
P55084	Q16658	Q9Y230
P17301	P51659	P00533
P24752	Q9Y230	A0A140T913
Q8IVF2	P00533	sp|cRAP017|P04040|CATA_HUMAN
O60664	A0A140T913	P55084
P13797-3	sp|cRAP017|P04040|CATA_HUMAN	P17301
A0A024R571	P55084	P24752
J3KPF3	P17301	Q8IVF2
P36578	P24752	O60664
Q14974	Q8IVF2	P13797-3
P53396	O60664	A0A024R571
B5ME19	P13797-3	J3KPF3
A0A0A0MTS2	A0A024R571	P36578
B1AK87	J3KPF3	Q14974
P26641	P36578	P53396

P34897	Q14974	B5ME19
P63104	P53396	A0A0A0MTS2
P56192	B5ME19	O00410
P09525	A0A0A0MTS2	B1AK87
P61978	O00410	P26641
P10316	B1AK87	P34897
G3V1A4	P26641	P63104
P13746	P34897	P56192
P62258	P63104	P09525
Q7KZF4	P56192	P61978
Q99714	P09525	P10316
Q13011	P61978	G3V1A4
P48735	P10316	P13746
P54577	G3V1A4	P62258
Q9Y678	P13746	Q7KZF4
O75083	P62258	Q99714
P68032	Q7KZF4	Q13011
Q99460	Q99714	P48735
P62195-2	Q13011	P54577
P00505	P48735	Q9Y678
P23526	P54577	O75083
P17174	Q9Y678	P68032
P53621	O75083	Q99460
Q9UQ80	P68032	P62195-2
E7EUU4	Q99460	P00505
Q07065	P62195-2	P23526
P63244	P00505	A2A274
P32322	P23526	P17174
A0A1C7CYX9	P17174	P53621
P07737	P53621	Q9UQ80
P52209	Q9UQ80	E7EUU4
Q9Y265	E7EUU4	Q07065
E9PCR7	Q07065	P63244
A6NLN1	P63244	P32322
P17812	P32322	A0A1C7CYX9
Q9UHD8	A0A1C7CYX9	P07737
sp|cRAP034|P09211|GSTP1_HUMAN	P07737	P52209
P48444	P52209	Q9Y265
P04181	Q9Y265	A6NLN1
P28838	A6NLN1	P17812
Q9NSE4	P17812	Q9UHD8

P07954	Q9UHD8	sp|cRAP034|P09211|GSTP1_HUMAN
P30041	sp|cRAP034|P09211|GSTP1_HUMAN	P48444
Q15019	P48444	P04181
Q15084	P04181	P28838
P09622	P28838	Q9NSE4
P12270	Q9NSE4	P07954
P31946	P07954	P30041
Q12931	P30041	Q15019
P40227	Q15019	Q15084
M0QWZ7	Q15084	P09622
P61158	P09622	P12270
P04792	P12270	Q12931
O00299	P31946	P40227
O43776	Q12931	M0QWZ7
B0QY89	P62826	P61158
P16615	P40227	P04792
P61981	M0QWZ7	O00299
P20073	P61158	O43776
O95573	P04792	B0QY89
Q13838	O00299	P16615
O60763	O43776	P61981
sp|cRAP094|P62937|PPIA_HUMAN	B0QY89	P20073
Q9NYU2	P16615	O95573
P36871	P61981	O60763
Q32Q12	P20073	sp|cRAP094|P62937|PPIA_HUMAN
Q02790	O95573	P41252
P40763	Q13838	Q9NYU2
Q15738	O60763	P36871
P25786	J3KSZ0	Q32Q12
Q14980	sp|cRAP094|P62937|PPIA_HUMAN	P40925
P51572	Q9NYU2	Q02790
P47897	P36871	P40763
P37837	Q32Q12	Q15738
A0A087X2I1	P40925	P25786
P23141	Q02790	Q14980
P23284	P40763	P51572
P62191	P25786	P47897

P13804	Q14980	P37837
O14980	P51572	A0A087X2I1
Q96AG4	P47897	P23141
P35998	P37837	P23284
A0A1W2PQ51	A0A087X2I1	P62191
P61224	P23141	P13804
Q9BUF5	P23284	O14980
O95831	P62191	Q96AG4
P43686	P13804	P35998
Q5T4U5	O14980	A0A1W2PQ51
P26006	Q96AG4	P61224
Q14240	P35998	Q9BUF5
P49748	A0A1W2PQ51	O95831
Q15365	P61224	P43686
P51149	Q9BUF5	Q5T4U5
O43242	O95831	H3BMS6
P05362	P43686	P26006
P61160	Q5T4U5	Q14240
A0A087X1X7	H3BMS6	P49748
A6NKB8	P26006	Q15365
P12004	Q14240	P51149
H0YN18	P49748	O43242
P14868	Q15365	P05362
Q9UJZ1	P51149	P61160
P27695	O43242	A0A087X1X7
P50416	P05362	A6NKB8
O14818	P61160	P12004
E7EVA0	A0A087X1X7	H0YN18
K7EJE8	A6NKB8	P14868
A0A087X2D8	P12004	Q9UJZ1
P55884	H0YN18	P27695
P52907	P14868	P50416
Q92973	Q9UJZ1	O14818
P29317	P27695	E7EVA0
Q6DD88	P50416	K7EJE8
Q8WUM4	O14818	A0A087X2D8
P09960	E7EVA0	P55884
P31040	K7EJE8	P52907
P49915	A0A087X2D8	Q92973
P50454	P55884	P29317
P23396	P52907	Q6DD88

P84095	Q92973	Q8WUM4
P30048	P29317	P09960
P30086	Q6DD88	P31040
Q5T5C7	Q8WUM4	P49915
P22695	P09960	P50454
P43490	P31040	P23396
Q9H0U4	P49915	P84095
Q99497	P50454	P30048
P07099	P23396	P30086
P30508	P84095	Q5T5C7
Q13162	P30048	P22695
P54886	P30086	P43490
Q15181	Q5T5C7	Q9H0U4
P62820	P22695	Q9NY33-4
P13929	P43490	P10768
P62701	Q9H0U4	Q99497
Q12904	P10768	P07099
Q13263	Q99497	P14550
Q06210	P07099	P30508
P05023	P14550	Q13162
P30084	P30508	P54886
Q13177	Q13162	Q15181
P00387	P54886	P62820
P46777	Q15181	P62701
P52788	P62820	Q12904
Q9ULV4	P13929	O94760
A0A0C4DG17	P62701	Q13263
P33991	Q12904	A0A0G2JH68
Q9NSD9	O94760	Q06210
Q96QK1	Q13263	P05023
Q9HDC9	A0A0G2JH68	P30084
Q9P2J5	Q06210	Q13177
P61106	P05023	P00387
P30044	P30084	P46777
Q9NQC3-2	Q13177	P52788
Q96I99	P00387	Q9ULV4
E7EX90	P46777	A0A0C4DG17
P62136	P52788	P33991
P15531	Q9ULV4	Q9NSD9
A2AEA2	A0A0C4DG17	Q96QK1
A0A024QZX5	P33991	Q9HDC9

P13674-2	Q9NSD9	Q9P2J5
Q13501	Q96QK1	P61106
Q8NBS9	Q9HDC9	P30044
O00267	Q9P2J5	P29692-3
A0A024R4E5	P61106	O15067
Q53GQ0	P30044	E7EX90
P60981	P29692-3	P62136
Q04917	O15067	P15531
Q16222	E7EX90	A2AEA2
Q7Z6Z7	P62136	A0A024QZX5
O15371	P15531	Q13501
P61586	A2AEA2	Q8NBS9
Q5JR08	A0A024QZX5	O00267
P00492	P13674-2	A0A024R4E5
D6RER5	Q13501	P60981
P60900	Q8NBS9	P63010
Q13347	O00267	Q04917
P09429	A0A024R4E5	Q16222
P05141	Q53GQ0	Q7Z6Z7
Q12907	P60981	O15371
E9PBS1	P63010	P61586
Q9UNM6	Q04917	Q5JR08
Q15046	Q16222	P00492
A0A087X117	Q7Z6Z7	D6RER5
P78417	O15371	P60900
Q9Y3I0	P61586	Q13347
P39023	Q5JR08	P09429
P28066	P00492	P05141
Q9UBQ7	D6RER5	Q12907
P61221	P60900	E9PBS1
P05455	Q13347	Q9UNM6
A0A0A0MTJ9	P09429	Q15046
Q15907	B3KS98	A0A087X117
P07384	P05141	P78417
G8JLD5	Q12907	Q9Y3I0
P51665	Q15046	P39023
Q9HB71	A0A087X117	P28066
P31751	Q9Y3I0	P18465
Q9BR76	P39023	Q9UBQ7
P62424	P28066	P61221
P31947	Q9UBQ7	Q9Y281

O00148	P61221	P05455
P61019	Q9Y281	A0A0A0MTJ9
Q9HAV7	P05455	Q15907
O00116	A0A0A0MTJ9	P07384
Q9UBT2	Q15907	G8JLD5
O76003	P07384	P51665
O95202	G8JLD5	Q9HB71
P46060	P51665	Q9BR76
A0A087WTT1	Q9HB71	P62424
Q12906	P31751	P31947
O00232	Q9BR76	O00148
Q29836	P62424	E7EMB1
P09601	P31947	P61019
Q96HE7	O00148	Q9HAV7
Q14008	E7EMB1	Q9UBT2
P04179	P61019	O76003
Q15691	Q9HAV7	O95202
P62269	O00116	P46060
J3KTA4	Q9UBT2	A0A087WTT1
P21964	O76003	Q12906
P14866	O95202	O00232
H7BZJ3	P46060	H0Y3P2
Q99536	A0A087WTT1	P09601
M0R0F0	Q12906	Q96HE7
A0A087X0X3	O00232	Q14008
P15880	H0Y3P2	P04179
P61604	Q96HE7	Q15691
P29373	Q14008	H0YL72
Q9NUQ9	P04179	P62269
A0A087WUT6	Q15691	J3KTA4
P08559	P62269	P21964
P42224	J3KTA4	P14866
A0A0C4DGS1	P21964	H7BZJ3
R4GNH3	P14866	Q99536
A0A087WT44	H7BZJ3	M0R0F0
P36957	Q99536	Q9UJU6
Q9Y4W6	M0R0F0	P15880
sp|cRAP054|P04264|K2C1_HUMAN	P63241	P61604
Q3ZCQ8	Q9UJU6	P29373
P51991	P15880	Q9NUQ9
P50552	P61604	A0A087WUT6

P23381	P29373	P42224
P06748	Q9NUQ9	A0A0C4DGS1
J3KQ32	A0A087WUT6	R4GNH3
Q96CS3	P08559	A0A087WT44
A0A024RA52	E7ETU9	P36957
O60506	P42224	Q9Y4W6
F8VYE8	A0A0C4DGS1	sp|cRAP054|P04264|K2C1_HUMAN
P62879	R4GNH3	Q3ZCQ8
A0A1W2PPZ5	A0A087WT44	P51991
Q92878	P54578	P50552
P41091	P36957	P23381
O14828	Q9Y4W6	P06748
J3QQ67	sp|cRAP054|P04264|K2C1_HUMAN	J3KQ32
P60228	Q3ZCQ8	Q96CS3
G5EA31	P51991	A0A024RA52
Q9Y266	P50552	O60506
Q15366-3	P23381	Q9UJU6-2
P26583	P06748	F8VYE8
P45974	J3KQ32	P62879
P19623	Q96CS3	Q9BWD1
Q7L2H7	A0A024RA52	A0A1W2PPZ5
P48047	O60506	Q92878
P61204	Q9UJU6-2	P41091
P12236	F8VYE8	O14828
Q08J23	P62879	J3QQ67
F8WCF6	Q9BWD1	P60228
A6NEM2	A0A1W2PPZ5	G5EA31
P09972	Q92878	sp|cRAP104|P12081|SYHC_HUMAN
P60891	P41091	Q9Y266
Q12765	O14828	Q15366-3
Q9H488	J3QQ67	P26583
P20618	P60228	P45974
P50995	G5EA31	P19623
P39019	sp|cRAP104|P12081|SYHC_HUMAN	Q7L2H7
X1WI28	Q9Y266	P48047
P62834	Q15366-3	P46926
Q14103	P26583	P61204

F6S8M0	P45974	P12236
P62873	P19623	Q08J23
P11177	Q7L2H7	E5RHG8
G8JLB6	P48047	F8WCF6
Q14847	P46926	A6NEM2
P30153	P61204	P09972
O76094	P12236	P60891
P31930	Q08J23	Q12765
Q9Y5M8	E5RHG8	Q9H488
P62263	F8WCF6	P20618
Q13283	A6NEM2	P50995
O43615	P60891	P30740
P62241	Q12765	P39019
D6REX3	Q9H488	X1WI28
Q9UBS4	P20618	F8VV59
Q12797	P50995	Q14103
Q13409	P30740	F6S8M0
A0A087WYT3	P39019	P62873
P16152	X1WI28	P11177
E5KLJ9	F8VV59	G8JLB6
C9JFR7	P62834	Q14847
P08240	Q14103	P30153
O75396	F6S8M0	O75874
O60749	P62873	O76094
P38117	P11177	P31930
Q8TCS8	G8JLB6	Q9Y5M8
O00425	P30153	P62263
F8W1A4	O76094	Q13283
Q969V3	P31930	O43615
P62140	Q9Y5M8	M0R210
Q02878	P62263	P62241
O14672	Q13283	D6REX3
sp|cRAP099|P62979|RS27A_HUMAN	P62241	Q9UBS4
A0A0J9YXF2	D6REX3	Q12797
B7Z7P8	Q9UBS4	Q13409
Q969X5	Q12797	A0A087WYT3
P46821	Q13409	P16152
P06756	A0A087WYT3	Q9BXP5
O00303	P16152	E5KLJ9
Q04837	Q9BXP5	P15170-2
M0QX65	E5KLJ9	I3L4X2

A0A0A0MTN3	P15170-2	C9JFR7
P52597	I3L4X2	P08240
O75533	C9JFR7	O75396
Q9Y3F4	P08240	P38117
Q9H223	O75396	Q8TCS8
P36542	O60749	O00425
Q9P0L0	P38117	F8W1A4
Q16698	Q8TCS8	Q969V3
P25205	O00425	P62140
J3KQ48	F8W1A4	Q02878
P16435	A0A1W2PNV4	P37802
P63000	Q969V3	sp|cRAP099|P62979|RS27A_HUMAN
Q8TAT6	P62140	A0A0J9YXF2
P47755	Q02878	B7Z7P8
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Q9Y3U8	B0QYN7	A0A0A0MRM9
O75306	Q9BTV4	P01023
P48960	J3KQL8	Q9Y5P6
C9JA28	P53007	Q96JJ7
P08579	Q6NVY1	sp|cRAP096|P01112|

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C9JJ19	O60502-3	Q9HAB8
Q9NZ08	P35270	P13073
Q9Y3T9	Q7L9L4	P46939
A6NP52	Q07666	J3KMX3
O15460	H0Y2W2	P68036
Q8NFH5	F8VVL1	B0QYN7
P35914	Q9P035	Q9BTV4
O14949	P60602	J3KQL8
Q9UBV2	A0A0A0MTH3	O60502-3
Q14677	P10644	Q7L9L4
G3V325	Q13057	Q07666
I3L0E3	E7EQU1	F8VVL1
E9PK85	P32119	Q9P035
R4GMR5	Q9Y295	P60602
P05121	B3KQ25	A0A0A0MTH3
G5E9V5	Q9H0V9	P10644
A0A087X163	P46778	Q13057
A0A087WUD3	O60488	E7EQU1
I3L3B4	Q06203	P32119
F8W7Q4	E9PPG9	Q9Y295
Q9H9J2	A0A0B4J1Z1	B3KQ25
A0A0B4J239	Q7Z2W4	O15230
Q8IVL5	Q92900	O60488
Q5RI15	P62273	A0A087WXC5
Q15125	Q9H2G2	Q9Y5X3
P0DN76	O96019	F5GY55
Q8N111	B4DZG7	Q06203
H0Y5N2	A0A087WUS0	E9PPG9
A0A0R4J2E8	Q8TCT9	Q7Z2W4
Q8N357	P52948	Q92900
A0A1B0GW05	P13473	P62273
Q10713	Q8WY22	Q9H2G2
K7EJT5	P46108	O96019
P47914	A0A087WY71	B4DZG7
A0A0A0MR02	Q6P2Q9	A0A087WUS0
F8WBG6	Q14914	Q8TCT9
E9PF19	O15270	P52948
O76031	P51858	P13473
P52306	P10155	A0A087WY71
Q53EU6	Q08209-2	Q6P2Q9

A0A0J9YXN7	P00403	Q14914
Q5SRE5	I3L2M4	O15270
Q9Y3D6	B5MC98	P51858
Q6UW02	Q5QPQ0	P10155
Q92504	P62330	Q08209-2
G0XQ39	A0AVL1	P00403
G5EA06	H0Y5B4	I3L2M4
I3L0N3	P36507	B5MC98
O14656	Q9UJS0	K7ER96
Q9UJG1	C9JIG9	Q8IY17
O75915	sp|cRAP041|P35527|K1C9_HUMAN	P62330
Q96S66	Q7L5N7	A0AVL1
A0A0A0MSA9	A0A0A6YYA0	Q9UJS0
Q29RF7	H0YDT8	C9JIG9
O15173	E9PD53	sp|cRAP041|P35527|K1C9_HUMAN
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J3QL14	P62304	A0A0A6YYA0
Q8NE62	A0A087X211	H0YDT8
A0A0C4DH52	O95816	Q15717
R4GN98	H3BPE7	H0YLR3
C9JU35	Q7L7X3	E9PD53
H0Y8C4	F8W930	Q04941
A0A1B0GTB8	P49770	E9PJF0
Q8N766	O43809	P62304
J3QL56	P48147	O95816
P82933	A0A087WVM4	H3BPE7
Q9BW60	Q5VTU8	Q7L7X3
J3QLI9	H0Y827	P61457
E9PI70	Q96N66	F8W930
O95302	D6R9W4	P49770
O60783	P33897	O43809
P36551	E5RHC5	P48147
A0A0C4DGX7	P50570	A0A087WVM4
Q9NRN7	G3V126	Q5VTU8
C9JP16	A0A0A0MSJ0	Q96N66
A0A087X1X9	Q5VZU9	D6R9W4
Q3KQU3	A8MXL6	P33897
C9IZQ1	Q96RS6	A0A0A0MT83
O00115	P30419	A0A0A0MSJ0

Q96FQ6	P56385	Q5VZU9
O43402	P05026	A8MXL6
D6RCD0	Q8N5M9	Q96RS6
P00846	Q7Z4H8	P30419
C9JKI3	Q8NFV4	P56385
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Q9Y679	P46063	A0A1W2PP11
O00592	P14174	P05026
P08648	E7EMW7	Q8N5M9
Q9NQW6	Q8NF37	Q7Z4H8
Q8NFQ8	Q9Y3B4	Q8NFV4
O00330	Q6NUK1	Q9NPJ3
K7EJ34	A0A087WT27	P46063
A0A0G2JSE9	P35250	P14174
O95182	G5EA30	E7EMW7
F8VNT9	Q13618	Q8NF37
A0A087WYD6	J3QLE5	M0QXF7
Q9NX14	F8W6G1	Q9Y3B4
Q13641	Q9NZ45	O14908
Q9BXT2	Q9BVC6	A0A087WT27
O43172	P63218	P35250
Q8NBN7	Q99470	B1AHB1
Q96HS1	A0A087WU53	G5EA30
Q9UQ35	Q5SRQ3	Q13618
A6NEM5	Q9UG63	J3QLE5
A0A087WTI3	Q9NR30	B4DFG0
O43815	Q9NXG2	O94906
H3BUY0	P36405	F8W6G1
Q96DZ1	A0A087X1E4	Q9NZ45
A0A0D9SF63	P09543	P63218
P51809	Q9HCN4	Q99470
P53701	O95347	A0A087WU53
	Q9BV40	Q9UG63
	H0YCY6	Q9NR30
	Q9Y3U8	Q9NXG2
	O75306	Q9HCN4
	A0A087X027	O95347
	P48960	H0YCY6
	A0A087WZT0	Q9Y3U8
	C9JA28	O75306
	P08579	A0A087X027

	C9JJ19	P48960
	A6NP52	A0A087WZT0
	B4DQA8	C9JA28
	Q9P0J7	P08579
	P36639	Q9NZ08
	O15460	Q9Y3T9
	O14949	A6NP52
	Q9Y237	B4DQA8
	Q14677	Q9P0J7
	G3V325	P36639
	I3L0E3	F8VVA7
	E9PK85	O15460
	O00743	A0A0U1RQF8
	R4GMR5	Q9Y237
	X6RAL5	G3V325
	P05121	A0A096LNY6
	A0A087WUD3	E9PK85
	I3L3B4	O00743
	A8MVM1	R4GMR5
	F8W7Q4	H0YEN0
	E9PL10	A0A087WUD3
	U3KQK1	I3L3B4
	Q9H9J2	F8W7Q4
	A0A0B4J239	E9PL10
	A0A0U1RQV4	U3KQK1
	Q14258	A0A024RCR6
	F2Z3N7	A0A0B4J239
	G3V1V0	A0A0U1RQV4
	P20929	A0A0J9YX13
	Q5TEJ0	Q14258
	Q5RI15	H3BQK0
	Q9NR50	F2Z3N7
	A0A087X1P5	G3V1V0
	Q99442	O00507
	Q15125	P20929
	P0DN76	Q5RI15
	Q9BRX2	Q9NR50
	A0A0R4J2E8	A0A087X1P5
	B4DXZ6	Q99442
	Q8N357	Q15125
	Q96P70	P0DN76

	A0A1B0GW05	J3QKS7
	Q10713	H0Y2Y8
	K7EJT5	Q9BRX2
	P47914	A0A0R4J2E8
	A0A0A0MR02	B4DXZ6
	F8WBG6	Q8N357
	A0A087WWT1	Q96P70
	A0A087X1K9	A0A1B0GW05
	Q5HY57	Q5T9C2
	A0A140T998	Q10713
	E9PF19	K7EJT5
	P52306	P47914
	O14617	A0A0A0MR02
	Q53EU6	F8WBG6
	C9J2P0	A0A087WWT1
	Q9Y6K5	Q5HY57
	O43252	H0YE29
	Q5SRE5	A0A140T998
	Q9Y3D6	E9PF19
	C9JJP5	P52306
	Q92504	O14617
	G5EA06	Q53EU6
	I3L0N3	C9J2P0
	Q3ZAQ7	O43252
	Q9NZL4	P53582
	P62877	Q5SRE5
	Q9UJG1	Q9Y3D6
	O75915	C9JJP5
	Q5JPT2	Q92504
	G5E9W8	G0XQ39
	Q96S66	G5EA06
	Q6PKG0	I3L0N3
	A0A0G2JPA8	Q3ZAQ7
	Q29RF7	Q9NZL4
	A0A075B6E5	A0A0X1KG71
	Q96DV4	Q8NAF0
	P35244	P42785
	O00483	Q5JPT2
	C4P0D4	M0R1L7
	A0A0C4DH52	Q6PKG0
	H0Y8C4	A0A0G2JPA8

	Q8N766	Q29RF7
	H0YAS9	A0A087WWM4
	Q5TB53	Q96DV4
	H0Y8L3	P35244
	Q9Y2V2	Q5T181
	A0A0C4DGX4	C4P0D4
	P82933	J3QL14
	P53794	Q8NE62
	Q9BW60	R4GN98
	Q8WW12	H0Y8C4
	J3QLI9	Q8N766
	E9PI70	Q5TB53
	H0YEL5	M0QXK2
	O60783	H0Y8L3
	Q92600	Q9Y2V2
	A0A0C4DGX7	A0A0C4DGX4
	Q9NRN7	P53794
	O75368	Q9BW60
	C9JP16	Q8WW12
	Q9Y3C6	J3QLI9
	H0YG54	H0YEL5
	A0A087X1X9	O95302
	Q3KQU3	O60783
	J3KSN1	P36551
	Q15404	A0A0C4DGX7
	C9IZQ1	Q9Y3C6
	A8MTH1	Q92538
	F8VPD4	B8ZZZ7
	Q96FQ6	Q3KQU3
	E1P5H9	Q15404
	A0A0C4DGY8	C9IZQ1
	J3KS79	F8VPD4
	C9JKI3	Q96FQ6
	H3BND8	B1ANM7
	O95881	A0A096LNV8
	P50238	J3KS79
	O00592	P00846
	G5E9Z2	C9JKI3
	P08648	H3BND8
	Q9NQW6	A0A075B720
	Q8NFQ8	P42285

	A0A0G2JSE9	Q9Y679
	A0A0A0MRV8	O00592
	F5H442	H3BPK3
	G3V1N2	A0A0A0MRF6
	H0Y895	G5E9Z2
	Q9NX14	A0A075B6G3
	Q9Y6G9	Q8NFQ8
	Q9BXT2	A0A0G2JSE9
	O43172	A0A0A0MRV8
	Q96HS1	F5H442
	A0A024R7W5	G3V1N2
	B1AL79	F8VNT9
	H3BUY0	Q9Y6G9
	Q9NP77	Q9BXT2
	O00203	O43172
	Q13451	Q96HS1
	P51809	A0A024R7W5
	O00170	B1AL79
	Q15645	Q96SU4
	Q14116	Q13033
	H3BRE0	O00203
		E9PHA6
		H0YHF8
		O00170
		Q15645
		H3BRE0

Appendix IX: Supplementary Table 5

Supplementary Table 5: Common proteins with their Protein ID between MiaPaCa-2 cells.

MiaPaCa-2 GFP/GFP + PD	MiaPaCa-2 GFP/KO2	MiaPaCa-2 GFP/KO2 + PD
P35579	P35579	P35579
O75369	O75369	O75369
P07900	P07900	P07900
P08238	P08238	P08238
P42704	P42704	P42704
P10809	P10809	P10809
P08670	P08670	P08670
P11021	P11021	P11021
P38646	P38646	P38646
P13639	P13639	P13639
A0A087WVQ6	A0A087WVQ6	A0A087WVQ6
P11216	P11216	P11216
P05787	P05787	P05787
P14618	P14618	P14618
O43707	O43707	O43707
P06733	P06733	P06733
P11142	P11142	P11142
P12814	P02545	P02545
P02545-2	P12814	P12814
P34932	P02545-2	P02545-2
P78527	P34932	P34932
P04406	P78527	P78527
P35580	P04406	P04406
P26038	P35580	P35580
P05783	P26038	P26038
Q14204	P05783	P05783
P04083	Q14204	Q14204
P50990	P04083	P04083
E7EQR4	P50990	P50990
P13667	E7EQR4	P13667
P60709	P13667	P60709
P04075	P60709	P04075
P14625	P04075	P14625
P78371	P14625	P78371

P30101	P78371	P30101
P55072	P30101	P55072
Q02790	P55072	Q02790
Q92598	Q02790	Q92598
P06576	Q92598	P06576
P00558	P06576	A0A0U1RQF0
P07437	P00558	P00558
P68371	P07437	P07437
P46940	P68371	P68371
P22314	P46940	P46940
P19338	P22314	P22314
P49411	P19338	P19338
P29401	P49411	sp|cRAP108|P02787|TRFE_HUMAN
P07814	P29401	P49411
P00352	P07814	P29401
P50991	P00352	P07814
A0A0G2JIW1	P50991	P00352
P68104	A0A0G2JIW1	P50991
P48643	Q13885	A0A0G2JIW1
P25705	P68104	P68104
P02786	P48643	P48643
Q99832	P25705	P25705
P18206	P02786	P02786
P17987	Q99832	Q99832
P49368	P18206	P18206
P04350	P17987	P17987
P50395	P04350	P49368
P22102	P50395	P04350
Q14697	P22102	P50395
B9A067	Q14697	P22102
P11586	B9A067	Q14697
P60842	P11586	B9A067
P00505	P60842	P11586
P31939	P00505	P60842
P40939	P31939	P00505
Q14152	P40939	P31939
P00367	Q14152	P40939
P34897	P00367	Q14152
P61978	P34897	P00367
Q13200	P61978	P34897
P04843	Q13200	P61978

P60174	P04843	Q13200
P22626	P60174	P04843
Q16222	P22626	P60174
P54886	Q16222	P22626
P04181	P54886	Q16222
P40926	P04181	P54886
P18669	P40926	P04181
P12268	P18669	P40926
A0A087X054	P12268	P18669
Q12931	A0A087X054	P12268
P21796	Q12931	A0A087X054
P27824	P21796	Q12931
P54136	P27824	P21796
E9PLK3	P54136	P27824
Q13509	E9PLK3	P54136
P55060	Q13509	E9PLK3
P27348	P55060	Q13509
O75083	P27348	P55060
P53396	O75083	P27348
Q01518	P53396	O75083
Q09666	Q01518	P53396
P68363	Q09666	sp|cRAP095|Q06830|PRDX1_HUMAN
P55084	P68363	Q01518
P49419-2	Q71U36	Q09666
P31040	P55084	P68363
P12236	P49419-2	Q71U36
P33991	P31040	P55084
P41250	P12236	P49419-2
P05141	P33991	P31040
P52209	P41250	P12236
P04792	P05141	P33991
Q9BQE3	P52209	P41250
Q00839	P04792	P05141
P49748	Q9BQE3	P52209
P35241	Q00839	P04792
P52789	P49748	Q9BQE3
P07355	P35241	Q00839
P54652	P07355	P49748
P61158	P54652	P35241
P19367	P61158	P07355
P23526	P19367	P61158

P35998	P23526	P19367
P26641	P35998	P23526
Q05639	P26641	P35998
P40227	Q05639	P26641
P63244	P40227	Q05639
P49588	P63244	P40227
P62191	P49588	P63244
O14980	P62191	P49588
P68032	O14980	P62191
Q15365	P68032	O14980
P12956	Q15365	P68032
Q9UQ80	P12956	Q15365
O95373	Q9UQ80	P12956
P63104	O95373	Q9UQ80
Q9Y230	P63104	O95373
P27797	Q9Y230	P63104
P00491	P27797	Q9Y230
P33176	P00491	P27797
P62195-2	P33176	P00491
P07737	P62195-2	P33176
P13010	P07737	P62195-2
Q13838	P13010	P07737
P62258	Q13838	P13010
P50454	P62258	Q13838
Q9NZM1	P50454	P62258
Q96AG4	A0A0D9SFS3	P50454
A6NLN1	E9PK54	A0A0D9SFS3
P28838	Q9NVI7-2	Q9NVI7-2
G3V1A4	Q9NZM1	Q9NZM1
A0A087X2I1	Q96AG4	Q96AG4
O95831	A6NLN1	A6NLN1
Q9Y4W6	P28838	P28838
P61106	G3V1A4	G3V1A4
P33993	A0A087X2I1	A0A087X2I1
P21980	O95831	O95831
O00410	Q9Y4W6	Q9Y4W6
P20700	P61106	P61106
P31930	P33993	P33993
Q8WUM4	P21980	P21980
A0A0A0MR02	O00410	O00410
B5ME19	P20700	P20700

O94925-3	P31930	P31930
P40925	Q8WUM4	Q8WUM4
Q9HB71	A0A0A0MR02	A0A0A0MR02
B1AK87	B5ME19	B5ME19
O60506	O94925-3	O94925-3
Q14974	P40925	P40925
F8W6I7	Q9HB71	Q9HB71
P00338	B1AK87	B1AK87
J3KPX7	O60506	O60506
K7EJE8	Q14974	Q14974
P35232	F8W6I7	F8W6I7
J3KSZ0	P00338	P00338
Q99714	J3KPX7	J3KPX7
Q7KZF4	K7EJE8	P35232
Q13263	P35232	J3KSZ0
Q9ULV4	Q5T9A4	Q99714
Q15046	Q99714	Q7KZF4
P61981	Q7KZF4	Q13263
Q92973	Q13263	Q9ULV4
J3KPF3	Q9ULV4	Q15046
Q15084	Q15046	P61981
P07954	P61981	Q92973
P22695	Q92973	J3KPF3
P18754	J3KPF3	Q15084
Q9BUF5	Q15084	P07954
P24752	P07954	sp|cRAP094|P62937|PPIA_HUMAN
Q9UJZ1	P22695	P22695
Q14103	P18754	P18754
P49915	Q9BUF5	Q9BUF5
P55884	P24752	P24752
Q03252	Q9UJZ1	Q9UJZ1
P14866	Q14103	Q14103
A0A0A0MTS2	P49915	P49915
B5MDF5	P55884	P55884
Q86VP6	Q03252	Q03252
Q04828	P14866	P14866
P09622	A0A0A0MTS2	A0A0A0MTS2
P12004	B5MDF5	B5MDF5
Q9NSE4	Q86VP6	Q86VP6
A0A087WSW9	H7BZ94	H7BZ94
Q99460	Q04828	Q04828

B0QY89	P09622	P09622
P62879	P12004	P12004
P30153	Q9NSE4	sp|cRAP104|P12081|SYHC_HUMAN
Q8NBS9	A0A087WSW9	Q9NSE4
P16615	Q99460	A0A087WSW9
A2A274	B0QY89	Q99460
P23381	P62879	B0QY89
P31946-2	P30153	P62879
G8JLD5	Q8NBS9	P30153
P62873	P16615	Q8NBS9
P16152	A2A274	P16615
O14818	P23381	A2A274
P26640	G8JLD5	P23381
P14868	P62873	P31946-2
P46777	P16152	G8JLD5
P51572	O14818	P62873
E7EUU4	P26640	P16152
P52788	Q14669	O14818
P25786	P14868	P26640
P23284	P46777	Q14669
Q9Y2Z4	P51572	P14868
O00148	E7EUU4	P46777
P26639	P52788	P51572
P43686	P25786	E7EUU4
P23396	P23284	P52788
P29317	Q9Y2Z4	P25786
Q9Y265	O00148	P23284
O60664	P26639	Q9Y2Z4
Q12907	P43686	O00148
Q96CS3	P23396	P26639
A0A0C4DG17	P29317	P43686
Q32Q12	Q9Y265	P23396
P31948-2	O60664	P29317
P36957	Q96CS3	Q9Y265
P62820	A0A0C4DG17	O60664
P05023	Q32Q12	Q12907
Q13283	P31948-2	Q96CS3
P27695	P36957	A0A0C4DG17
Q13347	P62820	Q32Q12
P30041	P05023	P31948-2
Q9Y3F4	Q13283	P36957

P46060	P27695	P62820
Q15366-3	Q13347	P05023
A0A1C7CYX9	P30041	Q13283
Q15233	Q9Y3F4	P27695
O43242	P46060	Q13347
R4GNH3	Q15366-3	P30041
O00299	A0A1C7CYX9	Q9Y3F4
P51149	Q15233	P46060
P61019	O43242	Q15366-3
P25205	R4GNH3	A0A1C7CYX9
Q15907	O00299	Q15233
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Q9UHD8	Q8NF37	O15144
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Q99536	Q15061	Q12792
Q13564	P42677	Q14318
Q99541	P49903	Q96A33
Q8WVC2	E9PGT1	Q8NF37
Q8TDB8	E9PD53	P01023
Q9Y295	J3KT73	Q15061
Q9UMS4	A0A0C4DG89	P42677
Q00688	P62280	Q96IX5
O43819	P17600	P49903
Q8N766	E9PHY5	E9PGT1
P49773	Q5T0D2	E9PD53
Q9BSH4	Q14683	P61201
Q15067	P40121	P34896
P53701	K7EMT0	J3KT73
E9PF19	Q9ULC4	A0A0C4DG89
P51858	A0A0G2JH68	P62280
A0A087X2G1	Q9NQW6	Q02750
A0A087WY55	Q3LIE7	E9PHY5
P08240	A8MXQ1	Q5T0D2
Q9Y3T9	H3BLU7	Q14683
Q99808	Q9Y679	P40121
Q9UBR2	H3BPE7	D6RJ96
P30043	Q9UHD8	K7EMT0
P61088	R4GMR5	Q9ULC4
A0A087WZ13	Q99536	A0A0G2JH68
Q12904	P01024	A8MXQ1
P62857	Q99541	H3BLU7
Q9NYU2	Q8WVC2	Q9Y679
A0A096LNY6	A0A087X2D0	H3BPE7
P07099	Q8TDB8	Q9UHD8
Q6IBS0	H0YHA7	R4GMR5
A0A087WVM4	Q9Y295	Q99536

K7EM18	Q9UMS4	P01024
Q16186	Q12797	Q13564
P35606	Q00688	Q99541
Q9H845	O43819	Q8WVC2
Q07666	Q8N766	A0A087X2D0
H0YDJ9	A0A087WTG3	Q8TDB8
D6RCD0	Q9BSH4	H0YHA7
A0A0D9SF53	P53701	Q9UMS4
U3KQ56	B5MC82	Q12797
X6R5C5	E9PF19	Q00688
Q13547	P51858	O43819
P00403	A0A087X2G1	Q8N766
Q15717	P12277	P49773
Q16658	Q9BTE3	A0A087WTG3
Q09028	A0A087WY55	Q9BSH4
Q6NVY1	Q9Y3T9	A0A0D9SG72
P62318	Q99808	Q15067
O43809	Q9UBR2	P53701
J3QRU1	P30043	E9PF19
Q9Y570	P61088	P51858
J3QQY2	J3QLE5	Q6NUQ4
I3L0N3	A0A087WZ13	A0A087X2G1
K7ERF1	I3L2B0	P12277
Q15404	Q12904	A0A087WY55
Q92769	P62857	P08240
Q5SRQ3	Q9NYU2	Q9Y3T9
F5H6E2	A0A1W2PPS8	Q99808
P0DN76	E7EQB2	Q9UBR2
F8VUY8	A0A096LNY6	P30043
P09874	P07099	P61088
P36639	Q6IBS0	J3QLE5
O60684	A0A087WVM4	A0A087WZ13
P37108	Q16186	I3L2B0
Q9P035	P35606	Q12904
O15145	Q9H845	P62857
P45954	Q07666	Q9NYU2
E5RFV2	H0YDJ9	E7EQB2
H7C2I8	D6RCD0	A0A096LNY6
O15511	A0A0D9SF53	P07099
P62829	H0YGT2	Q6IBS0
Q7L1Q6	Q13547	Q16186

B4DJ81	P00403	P35606
O60749	Q15717	E9PF63
M0R027	Q16658	Q9H845
C9IZ01	Q09028	Q07666
A6NKB8	Q6NVY1	H0YDJ9
O14744	P62318	D6RCD0
A0A087WWJ2	O43809	H3BV90
C9JLU1	O14672	A0A0D9SF53
P30040	J3QRU1	H0YGT2
Q9BRA2	A0A1W2PNR9	X6R5C5
Q02809	Q9Y570	Q13547
P30740	Q6NUK1	P00403
Q5T123	J3QQY2	Q15717
E9PND2	I3L0N3	Q16658
A0A024R4M0	K7ERF1	Q09028
O15269	Q15404	Q6NVY1
P27694	Q92769	P62318
P08237	F5H6E2	O43809
J3KTE4	P0DN76	J3QRU1
Q9P258	F8VUY8	Q9Y570
Q5SRE5	P09874	Q6NUK1
Q8TCT9	E9PJH7	I3L0N3
P30050	P36639	H0YHI8
P60602	O60684	K7ERF1
Q96TA1	A0A087WUK2	Q92769
G3V1C3	P37108	Q5SRQ3
O75844	Q9P035	F5H6E2
H7C1E4	P29992	P0DN76
A0A0U1RR32	O15145	P09874
M0QYE0	M0R181	E9PJH7
Q9NZL9	B4DDK6	P36639
P49207	P45954	O60684
P62750	I3L1P8	P37108
B4DP72	E5RFV2	Q9P035
Q9P287	H7C2I8	P29992
Q92900	O15511	O15145
P35270	P62829	M0R181
Q8N183	A0A087X027	D6RAN4
O96019	Q7L1Q6	B4DDK6
P23919	B4DJ81	P45954
M0QXL5	Q96I63	I3L1P8

Q04941	O14744	H7C2I8
Q13045	C9JLU1	O15511
A0A075B7C0	P30040	P62829
O94906	Q02809	A0A087X027
A0A0C4DGQ8	P30740	Q7L1Q6
A0A024R571	Q5T123	O60749
P53999	A0A024R4M0	Q5T948
Q8N5M9	O15269	C9IZ01
A0A087WYS1	P27694	Q96I63
O14907	P08237	A6NKB8
A0A087WYR0	J3KTE4	C9JLU1
Q99442	Q9P258	P30040
Q8IVL6	Q5TB53	F5H6N1
J3KSR8	Q5SRE5	Q9BRA2
Q14914	Q8TCT9	Q02809
P52732	P30050	P30740
Q9NQ88	P60602	Q5T123
A0A087WU14	Q8NC51	E9PND2
Q08211	Q96TA1	A0A024R4M0
H0Y6E7	H0Y2Y8	O15269
Q9BRX2	C9JY26	P27694
G5E9Q6	G3V1C3	P08237
P36871	O75844	J3KTE4
Q96N66	H0Y5J4	Q9P258
H0YLR3	Q3KNR6	Q5TB53
Q9UG63	A0A0U1RR32	Q5SRE5
A0A0A0MT83	Q9NZL9	Q8TCT9
Q16762	P49207	P30050
G3V2E7	P62750	P60602
Q5VTR2	Q9H2G2	Q8NC51
C9JIG9	A0A087X163	Q96TA1
Q0VDF9	Q92900	H0Y2Y8
B8ZZU8	P35270	C9JY26
H0YKH6	Q8N183	G3V1C3
J3KQJ1	O96019	O75844
P30419	P23919	Q3KNR6
P61009	M0QXL5	A0A0U1RR32
P10412	Q04941	Q9NZL9
O95197	Q13045	P49207
P30044	A0A075B7C0	Q9H2G2
O96011	A0A0C4DGQ8	B4DP72

Q9UN86	A0A024R571	Q9P287
A0A0A6YYA0	Q9UBI6	Q92900
A0A0A0MSV9	Q5RI15	P35270
A0A0A0MRI2	P53999	Q8N183
M0R0F9	Q8N5M9	O96019
P61457	A0A087WYS1	P23919
E7EUH7	O14907	M0QXL5
P62304	A0A087WYR0	Q04941
Q5VTU3	Q8IVL6	Q13045
Q9Y3B4	Q14914	A0A075B7C0
Q9H832	M0QXF9	O94906
M0QXF7	P52732	H0YK42
Q9Y697	Q08211	sp|cRAP005|P01008|ANT3_HUMAN
Q53EU6	H0Y6E7	A0A0C4DGQ8
O95297	G5E9Q6	A0A024R571
H3BPJ9	P36871	Q9UBI6
H0Y5B4	H3BUY0	Q5RI15
Q86U90	Q96N66	P53999
Q96ER9	Q9NZ45	Q8N5M9
P07858	A0A0A0MT83	A0A087WYS1
F5GWZ1	Q16762	O14907
E7EN38	E9PJT1	A0A087WYR0
E7ETB3	G3V2E7	Q8IVL6
Q70UQ0	A0AVL1	J3KSR8
Q9BV86	C9JIG9	Q14914
P52948	Q0VDF9	M0QXF9
G3V126	B8ZZU8	P52732
P12532	J3KQJ1	A0A087WU14
F8VVL1	P30419	H0Y6E7
F8VPD4	P61009	Q9BRX2
Q03135	O95197	G5E9Q6
A0A0A0MRE3	P30044	P36871
P09382	O96011	H3BUY0
Q5JNZ5	A0A1B0GUA3	Q96N66
Q5T1J5	Q08379	H0YLR3
Q9BR76	A0A0A6YYA0	Q9NZ45
P09417	A0A0A0MSV9	sp|cRAP041|P35527|K1C9_HUMAN
A8MXV4	A0A0A0MRI2	Q9UG63
E5RGI7	M0R0F9	A0A0A0MT83
Q9NR50	P61457	Q8N5K1
Q5HY57	E7EUH7	Q16762

P22307	P62304	P35250
Q7Z2W4	Q5VTU3	G3V2E7
Q13151	Q9Y3B4	A0AVL1
A0A087X1K9	Q9H832	Q0VDF9
H0YGJ7	M0QXF7	B8ZZU8
E9PK85	P46013	J3KQJ1
P08579	Q53EU6	P10412
Q5RKV6	H0Y5L2	O95197
P62424	H3BPJ9	P30044
O00186	H0Y5B4	O96011
G3V180	A0A1W2PRR9	A8MWP7
Q9HCN4	Q96ER9	Q08379
A0A087WUC6	Q2NKQ5	A0A0A6YYA0
H7C5E2	H0Y2V6	A0A0A0MSV9
P11279	P07858	A0A0A0MRI2
O94874	A0A1W2PP11	P61457
B4DWR3	Q70UQ0	P62304
Q5TFE4	P0C0S5	Q5VTU3
D6RAX2	Q9BV86	Q9Y3B4
C9JA28	Q8N490-2	B1AH49
Q9GZN8	G3V126	Q9H832
Q08752	P12532	M0QXF7
O75400	F8VVL1	Q6UW02
A0A0B4J1Z1	F8VPD4	Q9Y697
J3QL86	Q9H9J2	Q53EU6
A0A087WUD3	Q03135	O95297
Q96DV4	H3BNT2	H3BPJ9
K7EJT5	Q14232	H0Y5B4
Q9H0D6	A0A0A0MRE3	A0A1W2PRR9
A0A0A0MT32	P09382	Q01970
G3V325	A0A0A0MRX2	Q96ER9
F8W7Q4	Q9BR76	Q2NKQ5
F5H1S9	P09417	H0Y993
P11388	A8MXV4	P07858
P26885	Q9NR50	E7ETB3
E9PMR4	Q5HY57	P0C0S5
H3BN98	O15091	Q96P11-2
H0Y6Y8	P22307	Q9BV86
G5EA06	Q7Z2W4	I3L1U4
O95816	Q13151	Q8N490-2
Q9Y696	Q5VTU8	G3V126

Q8WUA2	P30038	Q9NTZ6
Q9Y3D6	A0A087X1K9	P12532
A6NEM2	E9PK85	F8VVL1
A0A0B4J239	P04920	F8VPD4
P47914	P08579	Q9H9J2
Q6UW68	A0A087WU53	Q03135
I3L245	Q5RKV6	H3BNT2
P50570	Q6PI48	A0A0A0MRE3
X6RAY8	H0YAG8	K7EQQ1
I3L3B4	Q15056	P09382
P04080	P62424	A0A0A0MRX2
Q8N357	O00186	Q5JNZ5
P13473	Q14344	Q5T1J5
G5E9Z2	G3V180	Q9BR76
G5EA09	Q9HCN4	P09417
O95376	A0A087WUC6	A8MXV4
A0A0S2Z5D0	P11279	Q9NR50
A0A0C4DFQ1	O94874	Q5HY57
Q96G03	A0A286YFD6	O15091
D6RBW1	B4DWR3	P22307
Q9BVC6	B7ZKJ8	Q13151
K4DI93	Q5TFE4	Q5VTU8
H0YIZ1	D6RAX2	O14949
Q8WWC4	C9JA28	P30038
Q9H4E7	Q5JR89	A0A087X1K9
H0YDX7	Q9GZN8	E9PK85
Q06124	Q08752	P04920
O75663	O75400	P08579
P48147	A0A087X1X9	A0A087WU53
H0Y8R1	H0Y579	Q6PI48
Q9NX47	Q96JJ7	H0YAG8
I3L3Q4	A0A0B4J1Z1	Q15056
E7ES00	A0A087WUD3	P62424
Q9NRN7	Q96DV4	O00186
P42126	K7EJT5	G3V180
Q6UXN9	Q9H0D6	Q9HCN4
Q10471	G3V325	A0A087WUC6
P42285	F8W7Q4	P11279
Q15125	A0A087WUX8	O94874
Q9NXH8	E9PMR4	B4DWR3
Q5R3B4	A0A096LNH5	B7ZKJ8

G5EA54	Q7Z5L9	Q5TFE4
J3KN29	Q5BJD5	C9JA28
Q8NFH4	H0YFA4	Q9GZN8
A0A0A0MSA9	H3BN98	P31937
X6R2S6	H0Y6Y8	A0A087X1X9
A0A087WT27	B4DEB1	A0A0A0MR13
O14556	A0A0J9YXF2	Q96JJ7
F2Z3N7	Q29RF7	A0A0B4J1Z1
H3BTL1	Q9Y696	O00330
A0A0X1KG71	Q9Y3D6	A0A087WUD3
C9IZQ1	A6NEM2	Q96DV4
O95239	A0A0B4J239	K7EJT5
F8VQQ3	P47914	Q9H0D6
A0A0A0MS31	O60502-3	A0A0A0MT32
Q15018	Q6UW68	G3V325
Q9BW60	Q5TIH2	F8W7Q4
P14174	P50570	F6VRR5
H0Y630	X6RAY8	P26885
P13984	I3L3B4	A0A087WUX8
M0QXB4	Q8N357	E9PMR4
A0AVT1	P13473	Q7Z5L9
K7EJL1	G5E9Z2	Q5BJD5
P61970	Q5H9A7	H0YFA4
H0YGW7	A0A0G2JPA8	Q07812-4
Q9P2X0	Q8N4V1	H3BN98
A0A0U1RRM8	Q9H0V9	G5EA06
A0A0A0MSJ0	Q5T4U8	A0A0J9YXF2
A0A024RCR6	Q8WYA6-2	Q9Y696
Q9NXG2	A0A0C4DFQ1	Q9Y3D6
Q6ZSC3	Q96G03	A6NEM2
Q14696	D6RBW1	A0A0B4J239
P62273	A6NF51	P47914
Q15291	Q9BVC6	O60502-3
Q27J81	J3KS15	Q6UW68
A0A024R4K9	K4DI93	Q96E11
Q9H4L5	O75947	Q5TIH2
E7ETY7	Q7Z434	P50570
Q2NL82	Q3ZAQ7	H7C492
M0QZK8	Q8WWC4	I3L3B4
Q5URX0	P21266	P04080
Q96FQ6	B4DP31	Q8N357

H0Y8B3	Q9H4E7	P13473
P49662	H0YDX7	G5E9Z2
H7C1N3	K7EK42	J3QLI9
Q9BXT2	Q06124	H0YD21
O75874	O75663	A6NJH9
Q9BZL1	P48147	Q5H9A7
H0Y7L5	Q9HBH5	A0A0G2JPA8
Q13426	Q9NX47	Q8N4V1
A0A087X0M4	P42126	Q9H0V9
F5GX99	Q6UXN9	G5EA09
Q13451	Q96RW7	O95376
P62906	A0A087X1N7	Q8WYA6-2
O43324	H0Y449	A0A0C4DFQ1
H7BYH4	Q8WW12	Q96G03
Q8TEQ6	Q15125	D6RBW1
E3W990	Q5R3B4	Q9BVC6
P09914	Q8NFH4	J3KS15
P10155	A0A0A0MSA9	Q9H9P8
O15270	X6R2S6	K4DI93
E5RFK2	Q9HBH1	O75947
Q96DA6	A0A087WT27	Q7Z434
O60701	D6RBL0	Q3ZAQ7
Q9NX14	Q8TB61	H0YIZ1
O14908	F2Z3N7	Q8WWC4
P22059	Q13033	P21266
C9J8H1	C9IZQ1	B4DP31
H7C4F9	O95239	K7EK42
J3KR44	F8VQQ3	O75663
Q8NFW8	Q15018	P48147
A0A0C4DFT3	P53794	A0A087WWH0
Q9NUQ3	Q9BW60	P42126
A0A0B4J2B4	V9GYM8	Q6UXN9
	P14174	Q96RW7
	P13984	Q10471
	M0QXB4	Q8WW12
	A0AVT1	Q15125
	K7EJL1	Q8NFH4
	P61970	A0A0A0MSA9
	Q9P2X0	X6R2S6
	A0A0A0MSJ0	Q9HBH1
	Q8NBU5	A0A087WT27

	A0A024RCR6	O14556
	Q92504	Q9Y223-2
	C9JJ19	F2Z3N7
	Q6ZSC3	Q13033
	C9J8M3	A0A0X1KG71
	Q14696	C9IZQ1
	P62273	F8VQQ3
	Q27J81	A0A0A0MS31
	A0A024R4K9	Q15018
	Q2NL82	A0A0G2JQ92
	M0QZK8	A0A087WSW7
	P49662	P49006
	Q99805	Q9BW60
	H7C1N3	P14174
	A0A087WYR3	H0Y630
	Q9P0K7	E5RJI7
	Q9BXT2	P13984
	O75874	M0QXB4
	H0Y7L5	K7EJL1
	Q13426	P61970
	A0A0A0MTQ5	A0A024RCR6
	F5GX99	Q92504
	Q13451	A0A087X0K9
	P62906	Q14696
	M0R072	P62273
	P10155	A0A286YFE3
	P31949	Q27J81
	Q96DA6	A0A024R4K9
	O60701	Q9H4L5
	P22059	E7ETY7
	Q9H3P7	M0QZK8
	J3KR44	G3V1N2
	A0A0C4DFT3	P49662
	Q53HL2	H7C1N3
	A0A0B4J2B4	A0A087WYR3
	Q96IC2	H0YHU8
		Q9BXT2
		O75874
		H0Y7L5
		Q13426
		A0A0A0MTQ5

	Q9UHG3
	F5GX99
	Q13451
	P62906
	O43324
	H7BYH4
	P09914
	J3KS17
	P10155
	O15270
	A8MT70-2
	Q96DA6
	O60701
	O14908
	P22059
	C9J8H1
	H7C4F9
	Q9H3P7
	J3KR44
	B5MCN0
	Q969X5
	A0A0C4DFT3
	H0YFF0
	A0A0B4J2B4
	Q96IC2

Appendix X: Supplementary Table 6

Supplementary Table 6: Common miRs between Panc-1 3D culture cells.

Panc-1 GFP/GFP + PD	Panc-1 GFP/KO2	Panc-1 GFP/KO2 + PD
hsa-miR-339	hsa-miR-652	hsa-miR-374a
hsa-miR-126	hsa-miR-503	hsa-miR-181b-1
	hsa-miR-493	hsa-miR-146b
	hsa-miR-330	hsa-miR-28
	hsa-miR-181a-1	
	hsa-miR-106b	

Results are obtained from small RNA-sequencing.

Appendix XI: Supplementary Table 7

Supplementary Table 7: Common miRs between MiaPaCa-2 3D culture cells.

MiaPaCa-2 GFP/GFP + PD	MiaPaCa-2 GFP/KO2	MiaPaCa-2 GFP/KO2 + PD
hsa-let-7a-2	hsa-miR-629	hsa-miR-532
hsa-miR-1307	hsa-miR-589	
hsa-miR-302c	hsa-miR-204	
hsa-miR-183	hsa-miR-20a	
hsa-miR-30c-2		

Results are obtained from small RNA-sequencing.