

**Multi-drug therapies, their pharmacodynamic interactions and the role of microRNAs  
in pancreatic cancer models**

A thesis submitted for the award of Ph.D.



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## DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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## Acknowledgments

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## Research Outputs

### *Poster Presentations*

"Examination of miRNA dysregulation in pancreatic cancer PDX models, with parallel proteomic and transcriptomic investigations". Presented at American Association for Cancer Research conference, 2022

"Could pharmacodynamic modelling of FOLFIRINOX lead to more therapeutically advantageous treatment of pancreatic cancer?". Presented at American Association for Cancer Research – pancreatic special conference, Oct 2020

"SN-38, the driver of FOLFIRINOX toxicity in-vitro in Pancreatic Cancer". Presented at Irish Association for Cancer Research, Feb 2020

### *Public Engagement and Awards*

RTE brainstorm - The problem with pancreatic cancer – 13 Nov 2020.

LmFm radio interview for pancreatic cancer awareness month 2020.

Daily mirror article for pancreatic cancer awareness month 2020.

Finalist for the Irish Association of Cancer Research – Patrick J. Johnston award lay communication competition.

One of 6 students selected for the SFI-SSPC Day in the life of a scientist campaign 2021 which was shortlisted in the following category at the Pharma Industry Awards 2022 - Communications Award.

IRC-USI "why research matters" video competition finalist.

Silicon republic article "much is still not understood about pancreatic cancer", published 2022.

DCU Tell it straight winner 2021 in the video category for all years.

DCUs president's award for engagement 2022.

DCU Faculty of Science and Health Award for "Best Person Raising the Profile of the Faculty'.

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## **Abbreviations**

**2D:** Two-Dimensional  
**3D:** Three-Dimensional  
**5-FU:** 5-fluorouracil  
**ADEX:** Aberrantly Differentiated Endocrine Exocrine  
**AGO:** Argonaute protein  
**ANOVA:** One-Way Analysis of Variance  
**ATCC:** American Type Culture Collection  
**BMI:** Body Mass Indices  
**CA-125:** Cancer Antigen 125  
**CA19-9:** Carbohydrate Antigen 19-9  
**CEA:** Carcinoembryonic Antigen  
**CLL:** chronic lymphocytic leukaemia  
**CI:** Confidence Interval  
**CSC:** cancer stem cells  
**DAVID:** Database for Annotation, Visualization, and Integrated Discovery  
**DDIs:** Drug-Drug Interactions  
**DE:** differential expression  
**DMEM:** Dulbecco's Modified Eagle Medium  
**DNA:** Deoxyribonucleic Acid  
**DHA:** Docosahexaenoic acid  
**Dox:** doxycycline  
**ECOG:** Eastern Cooperative Oncology Group  
**ECOG-PS:** Eastern Cooperative Oncology Group performance status  
**ECACC:** European Collection of Cell Cultures  
**EMT:** epithelial-mesenchymal transition  
**EORTC:** European Organisation for Research and Treatment of Cancer  
**EVs:** extracellular vesicles  
**EXOs:** exosomes  
**FDA:** Food and Drug Administration  
**FBS:** Fetal Bovine Serum  
**GI:** Gastrointestinal  
**GO:** Gene Ontology  
**GFP:** Green Fluorescent protein  
**GSEA:** Gene Set Enrichment Analysis

**HT:** High-throughput

**IC50:** Half maximal inhibitory concentration

**IPMN:** Intraductal Papillary Mucinous Neoplasm

**KEGG:** Kyoto Encyclopedia of Genes and Genomes

**L2FC:** Log2 Fold Change

**lncRNA:** Long Non-Coding RNA

**LV:** Leucovorin

**MFE:** Minimum Free Energy

**MCN:** Mucinous Cystic Neoplasm

**MRE:** miRNA Response Element

**mRNA:** Messenger Ribonucleic Acid

**MVs:** microvesicles

**mtDNA:** Mitochondrial DNA

**ncRNAs:** Non-Coding RNAs

**OXPHOS:** Oxidative Phosphorylation

**OS:** Overall Survival

**PDX:** Patient-Derived Xenograft

**PBS:** Phosphate Buffered Saline

**PanIN:** Pancreatic Intraepithelial Neoplasia

**PFS:** Progression-Free Survival

**Pm:** plasmin

**PACT:** Protein activator of protein kinase R (PKR)

**PD:** Pharmacodynamic

**PD-L1:** Programmed Death-Ligand 1

**PD-1:** Programmed Cell Death Protein 1

**PDAC:** Pancreatic Ductal Adenocarcinoma

**PcG:** Polycomb Group

**PK:** Pharmacokinetic

**pre-miRNA:** precursor micro RNA

**pri-miRNA:** primary micro RNA

**PRODIGE4/ACCORD11:** A randomized phase III trial

**qPCR:** Quantitative Polymerase Chain Reaction

**RISC:** RNA-Induced Silencing Complex

**RNA:** Ribonucleic Acid

**RNApolIII:** RNA Polymerase II

**ROS:** Reactive Oxygen Species

**RQ:** Relative quantification

**SAS:** Statistical Analysis System  
**SCID:** Severely Combined Immunodeficiency  
**siRNAs:** Small Interfering RNAs  
**snoRNAs:** Small Nucleolar RNAs  
**SPNG:** Sponge  
**TAC:** Transcriptome Analysis Console  
**TAS:** Tumour Associated Stroma  
**TGA:** Transcriptional Gene Activation  
**TGS:** Transcriptional Gene Silencing  
**TME:** Tumour Microenvironment  
**TDMD:** Target-Directed miRNA Degradation  
**tetO:** the tet operon  
**TRBP:** TAR RNA-binding Protein  
**TRCs:** Tumour Repopulating Cells  
**UTR:** Untranslated Region

**Taylor-Jade Allen Coyle**

**Thesis Title: Multi-drug therapies, their pharmacodynamic interactions and the role of microRNAs in pancreatic cancer models**

## **Abstract**

This thesis investigates the pharmacodynamic interactions of the FOLFIRINOX regimen and the role of microRNAs (miRNAs) in pancreatic cancer models. FOLFIRINOX, a combination of 5-Fluorouracil, oxaliplatin, and SN-38, was evaluated for its pharmacodynamic interactions across various pancreatic cancer cell lines. Synergistic interactions were observed between oxaliplatin and SN-38 in PANC-1 and MIAPaCa-2 cells, while the triple combination showed strong synergy in MIAPaCa-2 and borderline additive effects in PANC-1. Antagonistic interactions were noted in primary PIN-127 and 3D PANC-1 models, underscoring the complexity of these drug interactions and the need for further optimisation in clinical settings. Parallel studies focused on the role of miRNAs in pancreatic cancer. A global comparison of miRNAs in pancreatic tumours, adjacent normal tissue, and patient-derived xenograft models identified several differentially expressed miRNAs which have not been previously linked to pancreatic cancer. Investigation into one miRNA, miR-6831-5p in particular showed that its overexpression in PANC-1 cells altered mitochondrial bioenergetics, purine nucleotide homeostasis, and apoptosis, potentially affecting gemcitabine sensitivity. These findings highlight the intricate interplay between miRNAs, metabolic pathways, and drug responses, offering new insights into pancreatic cancer. Further investigations revealed differential miRNA processing efficiencies of stably transfected pancreatic cancer cells. While vectors successfully increased miR-708 levels, they failed to elevate mature forms of miR-29b, -1290, -2467, and -6831 due to a block at the pre-miRNA stage. However, synthetic pre-miRNA mimics effectively generated mature miRNAs, indicating potential strategies to overcome miRNA processing barriers. These results demonstrated the distinct behaviour of different miRNAs with the same vector in the same cell line, the same miRNA between the two vector designs, and with the same miRNA across different cell lines. In addition, these findings on the structural and sequence differences between successful and non-successful vector designs could help inform future chimeric miRNA design strategies and act as a guide to other researchers on the intricate processing dynamics that can impact vector efficiency. By linking the investigation of drug interactions with insights into the role of miRNA, this thesis contributes to a more nuanced understanding of pancreatic cancer treatment and provides actionable strategies for enhancing therapeutic efficacy.

## **1. Introduction**

### ***1.1 Overview of Pancreatic Cancer***

Pancreatic cancer (PaC) is a particularly aggressive malignant tumour that originates in the cells of the pancreas, an organ situated behind the stomach (National Cancer Institute, 2024b). The pancreas produces exocrine secretions, including pancreatic juice, a mixture of digestive enzymes and bicarbonate from the acinar cells, which flow into the duodenum through the main and accessory pancreatic ducts. This coordinated release of digestive enzymes and bicarbonate into the duodenum is essential for the efficient breakdown and absorption of nutrients from food. The pancreas is also responsible for producing endocrine secretions, including glucagon and insulin from the islets of Langerhans, which are released directly into the bloodstream. The precise regulation of these hormones by the pancreas is essential for overall metabolic balance, and disruptions in this system can lead to conditions like diabetes (National Cancer Institute, 2024b).

PaC typically arises from the exocrine cells, with the most common form being pancreatic ductal adenocarcinoma (PDAC) (Halbrook *et al.*, 2023). PaC has often been described as having an asymptomatic progression, but in reality, there are common symptoms associated with the disease. However, the challenge lies in the fact that these symptoms—such as jaundice, weight loss, and abdominal pain—are nonspecific and can easily be mistaken for other gastrointestinal disorders. This frequent misattribution contributes to the difficulty in diagnosing pancreatic cancer at an early stage. PaC was the fourth leading cause of cancer related death in the European Union in 2022 (**European Commission**, 2022). Despite lower incidence, this cancer ranks among the deadliest cancers due to its low survival rates (Figure 1) (National Cancer Registry Ireland, 2023).

### ***1.2 Epidemiological Landscape of Pancreatic Cancer: Regional Patterns and Demographics***

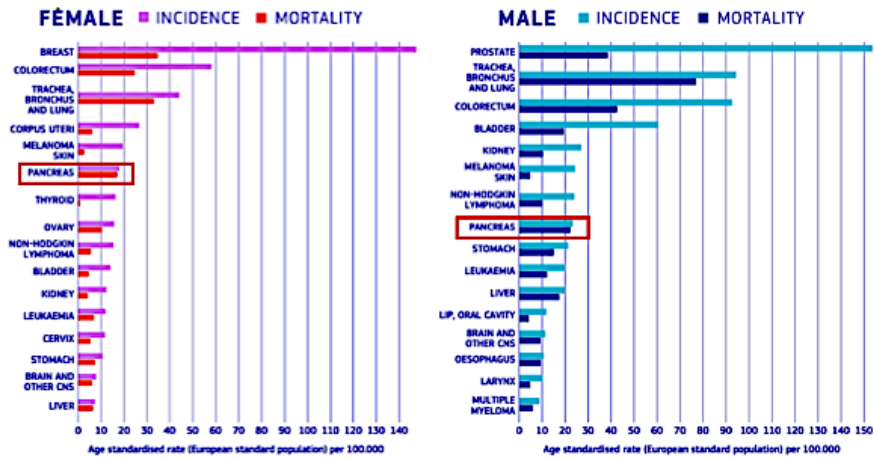
Worldwide, there were over 495,773 new cases of PaC in 2020 (Sung *et al.*, 2021) and accounts for 7.4% of all cancer cases in the European Union (European Cancer Information System, 2022). As the European population ages, a stable or increasing mortality trend is expected, leading to a rise in PaC deaths (**European Commission**, 2022). European projections indicate an increase in new cases by 2040, reaching 177,940 cases, with mortality rising to 169,389 deaths in 2040 (Partyka *et al.*, 2023). The substantial share of European cases may be attributed to both risk factors and advanced healthcare systems that facilitate detection and cause determination (T. Conroy, 2023). According to the National Cancer Registry Ireland (NCRI), there were 337 cases of PaC in 1994 versus 649 in 2020 (National

Cancer Registry Ireland, 2023). According to the NCRI, of the 624 cases diagnosed per year between 2018-2020, the number of deaths was 565 (National Cancer Registry Ireland, 2023).

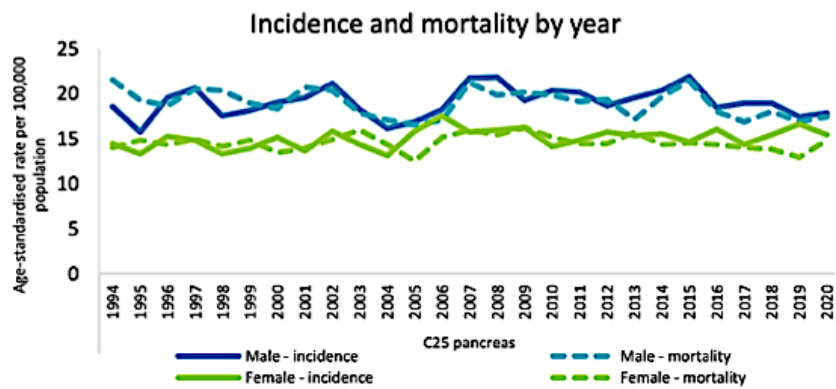
Although listed as one of the cancers with the poorest average prognosis, according to the American Cancer Society's Cancer Facts & Figures 2024 Report, the 5-year survival rate has now reached 13% (American Cancer Society, 2024). The 5-year survival rates for pancreatic cancer vary based on the type and stage of the disease. Based on people diagnosed with pancreatic cancer between 2013 and 2019, the 5-year survival rates for PDAC were about 40% if the cancer was localised, 16% if it had spread to nearby structures or lymph nodes (regional), and around 3% if it had metastasised to distant organs (distant) (American Cancer Society, 2024). In contrast, pancreatic neuroendocrine tumours generally have better survival rates, with approximately 95% for localised cases, 72% for regional cases, and 23% for distant cases (National Cancer Institute, 2024a). Rare pancreatic cancers, such as acinar cell carcinoma and intraductal papillary mucinous neoplasms, tend to have more variable survival rates but often show better outcomes if detected early (Ikezawa *et al.*, 2024).

There are slight differences in incidence and mortality rates between male and females. Males have a slightly higher incidence rate (cases per 100,000 per year) of 18 accompanied by a mortality rate (deaths per 100,000 per year) of 17.4 compared to 15.8 and 13.9 for women, respectively (National Cancer Registry Ireland, 2023). In addition, in 2018, Northern Ireland recorded 266 new cases of PaC, equating to an incidence rate of 16.5 per 100,000 individuals. During the same period, there were 241 deaths from PaC, resulting in a mortality rate of 15.1 per 100,000. Survival rates highlight the severity, with one-year survival at 25.7% (2012 – 2016) and a meagre five-year survival of 6.2% (2010 – 2014). This places Northern Ireland 32nd out of 36 countries in CONCORD-3, reflecting one of the poorest five-year survival rates globally (Allemani *et al.*, 2018).

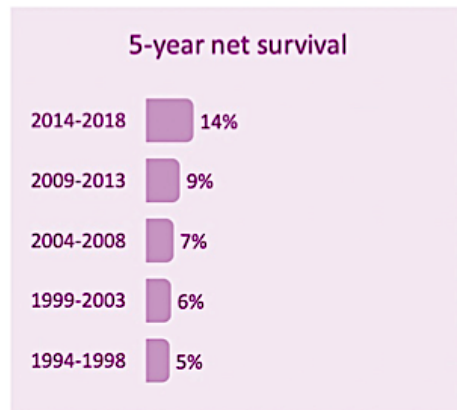
A



B.



C



D

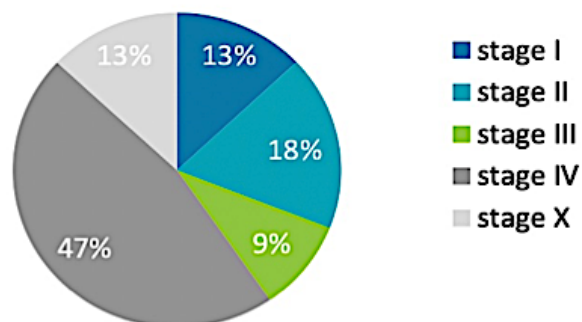


**Figure 1: Cancer statistics.** A. European age standardised incidence and mortality rates for each main cancer type. B. Age standardised incidence and mortality rates for pancreatic cancer per year from 1994-2020. C. Pancreatic cancer 5-year net survival rate. D. Percentage of stage of disease at diagnosis. Figure adapted from (National Cancer Registry Ireland, 2023) and (European Cancer Information System, 2022).

### **1.3 Pancreatic Cancer Risk Factors and Precursor Lesions**

Pancreatic cancer risk factors fall into two categories: modifiable (lifestyle, environmental, medical) and non-modifiable (age, gender, genetics, and ethnicity). Among the modifiable factors, smoking stands out as one of the most significant. A global meta-analysis revealed that smoking cigarettes raises the risk of PaC by a staggering 75%, even persisting for at least a decade after quitting (Iodice *et al.*, 2008). Diabetes type 2 also emerges as a noteworthy risk factor. It is associated with an increased risk of PaC, acting as both an early indicator and a contributing factor (Bosetti *et al.*, 2014). Surprisingly, this risk remains consistent across different regions, sexes, study designs, alcohol consumption levels, body mass indices (BMIs), and smoking statuses. The duration of diabetes plays a role, with the highest risk seen in patients diagnosed within a year (Ben *et al.*, 2011). A comprehensive study confirmed a two-fold excess risk of PaC in diabetics, persisting more than two decades after diagnosis, supporting diabetes as a causal factor (Klein, 2021). The relationship between diabetes and PaC remains uncertain, with questions lingering about whether hyperglycaemia, insulin resistance, and hyperinsulinemia play direct roles, or if diabetes serves as an indicator of underlying biological factors altering cancer risk. Moreover, the association between cancer and diabetes could be indirect, influenced by shared risk factors like obesity, supporting a chronic inflammatory hypothesis (Farias *et al.*, 2020). Obesity, defined as a body mass index (BMI) of  $\geq 30$  kg/m<sup>2</sup>, elevates the risk by 34-36%. However, it appears that obesity does not affect the prognosis of PaC patients (Téoule *et al.*, 2020). Another modifiable factor associated with PaC incidence is alcohol intake, with heavy alcohol consumption shown to increase the risk by 15% (Klein, 2021). Notably, alcohol consumption negatively impacts survival time after PaC diagnosis (Naudin *et al.*, 2018).

Pancreatic cancer has also been linked to chronic pancreatitis, cysts, and infections (Hu *et al.*, 2021). Chronic pancreatitis patients, for example, face a 16-fold higher pancreatic cancer risk (Hu *et al.*, 2021). While, infections like hepatitis B and *Helicobacter pylori* in the digestive tract can also increase the risk (Hu *et al.*, 2021). The three main precursor lesions to PaC are pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm (MCN), and intraductal papillary mucinous neoplasm (IPMN) (Distler *et al.*, 2014). Among these, PanIN is the most prevalent. PanIN, MCN, and IPMN are known to be early indicators of pancreatic

cancer (Paramythiotis *et al.*, 2023). IPMN, in particular, carries a heightened risk of advanced neoplasia, affecting approximately 62% of resected specimens. Individuals with IPMN also have a 1–8% greater likelihood of developing pancreatic ductal adenocarcinoma (PDAC) in other areas of the pancreas (Semaan *et al.*, 2023; Matthaei *et al.*, 2011). Studies on patients with a family history of pancreatic cancer or PDAC reveal the presence of microscopic pancreatic intraepithelial neoplasms (PanINs) in the vicinity of tumours and throughout the pancreas (Sipos *et al.*, 2009). PanINs are the most significant precursors to PDAC and typically manifest as small, flat or papillary lesions within the pancreatic ducts, often measuring less than 5 mm (Hruban, Maitra and Goggins, 2008). They are typically asymptomatic and can be categorised into three grades:

1. PanIN-1A and PanIN-1B: Low-grade lesions with minimal cytological and architectural abnormalities.
2. PanIN-2: Intermediate-grade lesions with mild to moderate cytological and architectural atypia, often exhibiting small fleshy projections known as papillae.
3. PanIN-3: High-grade lesions, also referred to as "carcinoma in situ," characterised by severe cytological and architectural abnormalities. PanIN-3 can display a papillary, flat, or a pattern of holes known as a cribriform pattern.

All PanINs are non-invasive and do not breach the basement membrane. PanINs serve as well-defined precursors to invasive PDAC. Molecular studies and genetically engineered mouse models have aided in understanding the progression from PanIN to invasive cancer (Hruban, Maitra and Goggins, 2008).

#### **1.4 Genetic, Epigenetic, and Metabolic Subtypes in Pancreatic Cancer Progression**

Pancreatic cancer's progression is driven by accumulating genetic and epigenetic changes, resulting in distinct molecular and metabolic subtypes that influence the disease's behaviour and treatment response. The *KRAS* oncogene mutation was identified as being a primary genetic alteration in PaC, in 1988 (Almoguera *et al.*, 1988). Today, our understanding of PaC genetics has deepened, enabling the creation of a coherent progression model (Hruban *et al.*, 2000). In genetic analyses of PanIN lesions, two key observations emerge. Firstly, while mutations don't follow a strict order, a pattern emerges: *KRAS* mutations and telomere shortening appear first (in PanIN-1), followed by *p16/CDKN2A* inactivation (typically in PanIN-2), and finally *TP53* and *SMAD4/DPC4* inactivation (typically in PanIN-3). Secondly, mutation prevalence increases with dysplasia degree, demonstrating a stepwise rise in *KRAS2* mutations with increasing PanIN dysplasia (Distler *et al.*, 2014). Most of the genetic alterations

found in invasive pancreatic adenocarcinomas are also detected in duct lesions. Notably, as cytological and architectural atypia in duct lesions intensify, the prevalence of these genetic alterations rises. Thus, PanIN lesions progress from seemingly harmless precursors to invasive cancers (see Figure 2) (Yakar, Bozkırlı and Ceyhan, 2022).

In addition to genetic mutations, gene expression can be influenced by epigenetic changes particularly promoter methylation (Distler *et al.*, 2014; Eyres *et al.*, 2021). For instance, the p16/CDKN2A gene is genetically inactivated in approximately 80% of invasive pancreatic cancers, and in another 15–20%, it is inactivated through promoter hypermethylation. The hypermethylation of several other genes (such as GATA6 and RB1) in pancreatic cancer patients, have been shown to start early in PanIN development and increasing during neoplastic progression (Hruban, Maitra and Goggins, 2008). A 2021 study by Eyres *et al.* demonstrated that epigenetic inactivation of loci, including GATA6, is linked to aggressive squamous-like PDAC subtypes. Remarkably, restoring this epigenetic control increased markers of classical pancreatic tumours, which are associated with better therapeutic responses and improved survival (Eyres *et al.*, 2021). These epigenetic alterations further contribute to the complex genetic landscape of pancreatic cancer and play a significant role in its progression.

Pancreatic cancer also exhibits distinct metabolic phenotypes that enable its aggressive growth and survival in nutrient-poor environments (Daemen *et al.*, 2015). These metabolic subtypes, including increased glycolysis (the Warburg effect), enhanced glutamine metabolism, and altered lipid metabolism, allow tumours to adapt to various stressors and sustain rapid proliferation (Espiau-Romera *et al.*, 2020). Research has identified specific metabolic subtypes within pancreatic ductal adenocarcinoma (PDAC), each with unique metabolic requirements and implications for disease progression. For instance, Daemen *et al.* identified three key metabolic subtypes in PDAC cell lines: slow-proliferating, glycolytic, and lipogenic (Daemen *et al.*, 2015). The slow-proliferating subtype is characterized by reduced cellular growth and low levels of amino acids and carbohydrates. In contrast, the glycolytic subtype exhibits high expression of glycolytic enzymes and related pathways, driving rapid growth and poor response to chemotherapy. The lipogenic subtype is defined by the overexpression of lipogenic genes and increased mitochondrial oxidative phosphorylation (OXPHOS), relying heavily on lipid metabolism (Daemen *et al.*, 2015).

These metabolic subtypes not only highlight the diversity within PDAC but also correlate with the tumour's molecular characteristics and clinical behaviour. The glycolytic subtype, for instance, aligns with more aggressive, quasi-mesenchymal tumours, while the lipogenic

subtype is associated with the classical, less aggressive form of PDAC. Understanding these subtypes is crucial, as they influence the tumour's response to treatment and offer potential targets for therapeutic intervention.

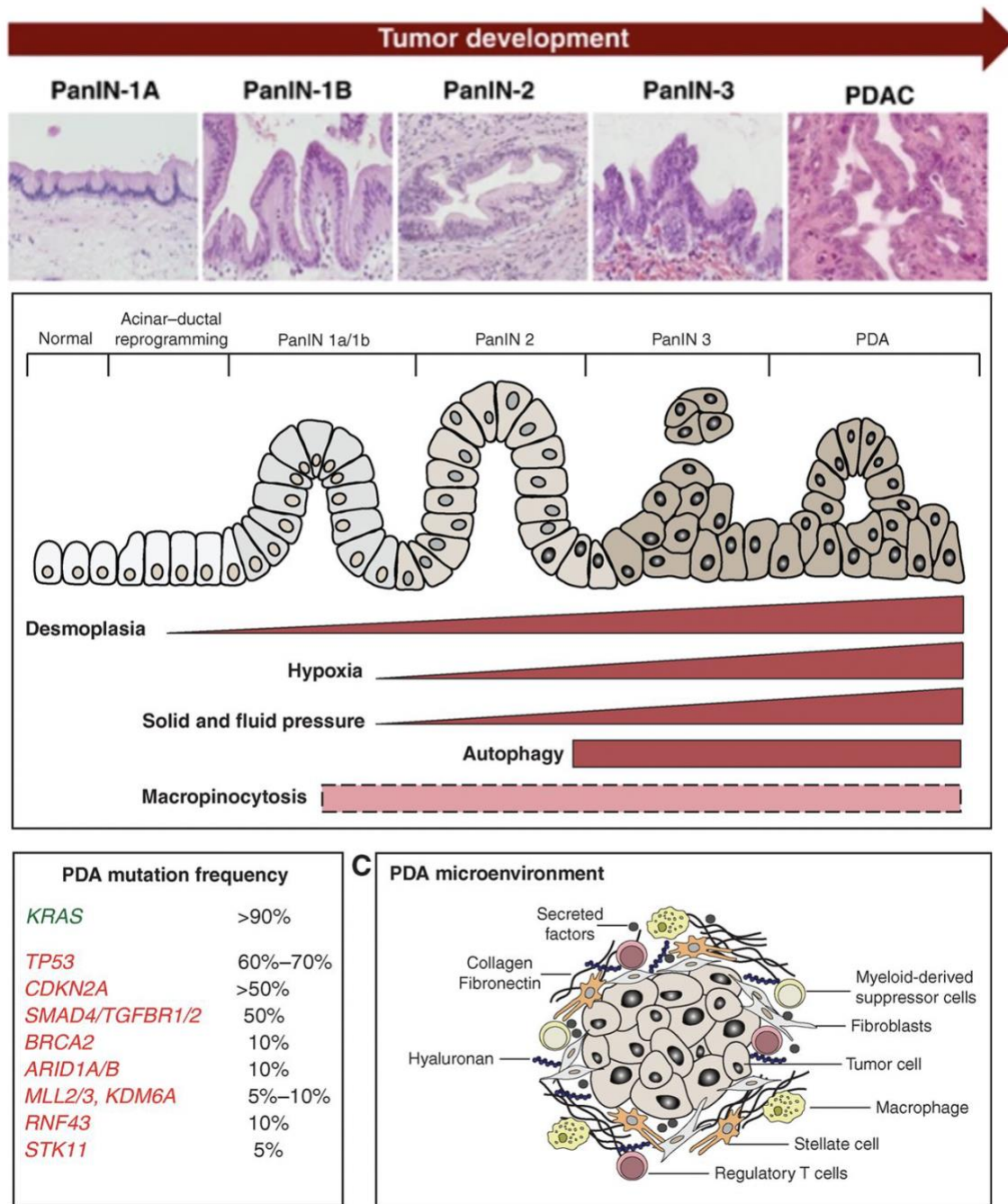


**Figure 2: Schematic of pancreatic cancer development and progression** – PaC arises from the multi-stage progression of precursor lesions known as pancreatic intraepithelial neoplasia (PanIN). *KRAS* are present in >90% of early stage PanIN lesions followed by a series of mutations in tumour suppressors at later stages. PDAC is also associated with evolving alterations in the tumour microenvironment including increasing hypoxia and desmoplasia. Adapted from Wörmann *et al* & Perera *et al.* (Wörmann *et al.*, 2014; Perera and Bardeesy, 2015)

Global gene expression analysis has been instrumental in identifying subtypes in various human tumour types. In 2011, Collisson and colleagues defined three molecular subtypes of PaC (Collisson *et al.*, 2011). To address the scarcity of available tumour specimens, this study integrated transcriptional profiles from primary PaC samples and cell lines, identifying three PaC subtypes: classical, quasi-mesenchymal, and exocrine-like. The classical subtype displayed high expression of adhesion-associated and epithelial genes, the quasi-mesenchymal subtype showed mesenchyme-associated gene expression, and the exocrine-like subtype had elevated expression of digestive enzyme genes, supported by immunohistochemical staining. GATA6 was highly expressed in the classical subtype, while KRAS mRNA levels were elevated in this subtype relative to others.

Building on this, Moffitt *et al.* in 2015 identified two major molecular subtypes, "Basal-like" and "classical," which they further divided into "normal" and "activated" stromal subtypes (Moffitt *et al.*, 2015). In 2016, Bailey *et al.* refined these classifications, renaming the quasi-mesenchymal subtype to "squamous," the classical subtype to "pancreatic progenitor," and the exocrine-like subtype to "Aberrantly Differentiated Endocrine Exocrine" (ADEX). Bailey *et al.* also identified an immunogenic class, characterized by significant immune infiltration and associated immune gene programs, including B cell signaling and T cell receptor pathways (Bailey *et al.*, 2016). Together, these genetic, epigenetic, and metabolic findings paint a comprehensive picture of pancreatic cancer's complexity, offering new avenues for personalised treatment approaches and highlighting the need for further research into these intricate processes.

### **1.5 Current Treatment Landscape and Emerging Therapies for Pancreatic Cancer**

Despite ongoing advancements in the treatment and diagnosis of pancreatic cancer, progress remains slow, with improvements often measured in months rather than years. Surgical resection, while potentially curative for some patients, is a clinical challenge; only about 20% of patients are eligible for surgery due to late-stage diagnoses (Strobel *et al.*, 2019). Moreover, resistance to chemotherapy and radiotherapy *are often* present from an early stage (Khan *et al.*, 2015). Although targeted strategies such as immunotherapy hold promise, they are not yet widely adopted and often yield inconsistent results in pancreatic cancer treatment. Diagnostic tools are advancing, but they still struggle with early detection, leading to diagnoses at more advanced, less treatable stages. This issue is compounded by the lack of effective screening methods and sensitive biomarkers (Yu *et al.*, 2016). Commonly used biomarkers like CA19-9, CA-125, and carcinoembryonic antigen (CEA) are not sufficiently robust or

specific for primary screening and diagnosis but can be useful for monitoring treatment response (Ren and Yu, 2018). *Hallmark* studies, while marking progress, are still yielding increased survival rates in the realm of months rather than years, coupled with substantial quality-of-life side effects. This underscores the imperative to persist in the pursuit of novel and more efficacious treatment alternatives.

The past four decades have seen fluoropyrimidines like 5-FU and antimetabolites such as gemcitabine as standard chemotherapies for pancreatic cancer (Chiorean and Covelev, 2015; Vreeland *et al.*, 2019; Riedl *et al.*, 2021). (Torphy, Fujiwara and Schulick, 2020)). Neoadjuvant radiation has been shown to improve surgical parameters for borderline resectable tumours by lowering nodal positivity and positive margin rates (Salamekh *et al.*, 2022). While the clinical benefit of chemoradiotherapy for resectable PaC is still debated, two randomised European studies, namely EORTC (Neoptolemos *et al.*, 2004) and ESPAC-1 (Neoptolemos *et al.*, 2009) concluded that radiation therapy offers no benefit after pancreatic cancer surgery. In 2023, a study conducted by Liu and Lefresne, evaluated palliative radiotherapy in PaC and found it was efficacious in improving pain and gastrointestinal bleeding and was generally well-tolerated in their patient cohort (Liu and Lefresne, 2023).

Pancreatic cancer (PaC) tumours are shaped by both the cancer cells' malignancy and the immunosuppressive tumour microenvironment (Chiorean and Covelev, 2015). This has intensified interest in immune therapies such as macrophage-targeted treatments (Nywening *et al.*, 2016), immune checkpoint inhibitors (Wainberg *et al.*, 2020), and stromal disruption (Jacobetz *et al.*, 2013). Specifically, the PD-1/PD-L1 interaction allows cancer cells to evade immune detection by suppressing immune responses (Zheng, 2017; Mucileanu, Chira and Mircea, 2021). Checkpoint inhibitors like pembrolizumab (Keytruda) (Zhao *et al.*, 2022), nivolumab (Opdivo) (Padrón *et al.*, 2022), and atezolizumab (Tecentriq) (Collienne *et al.*, 2022) have shown promise in clinical trials. However, their use in pancreatic cancer is still emerging, and more research is needed to refine these treatments and identify the patients who will benefit the most. While immune therapies show promise for pancreatic cancer, traditional chemotherapy, despite being less targeted, continues to be the primary approach in current clinical practice.

A 2013 study by Von Hoff *et al.* found the combination albumin-bound paclitaxel (nab-paclitaxel) and gemcitabine outperforms gemcitabine alone, although it was associated with increased myelosuppression and peripheral neuropathy noting these side effects seemed to be reversible (Von Hoff *et al.*, 2013). In 2011, Conroy *et al.* compared the efficacy of FOLFIRINOX (FFX) (5-fluorouracil (5-FU), oxaliplatin (Oxa) Irinotecan and leucovorin) to

gemcitabine as first-line therapy in patients with metastatic PaC and showed median overall survival was significantly increased by 4.3 months for patients receiving FOLFIRINOX compared with the gemcitabine group (Conroy *et al.*, 2011). In comparison to the FOLFIRINOX trial, the study by Von Hoff *et al.* included patients aged 75 and older (10%) and those with a poorer performance status (8% with an Eastern Cooperative Oncology Group (ECOG) performance status of 2). Despite these differences, FOLFIRINOX showed a clear survival benefit of 4.3 months over gemcitabine in treating pancreatic adenocarcinoma. In 2023 the FDA approved liposomal irinotecan (Onivyde), 5-fluorouracil (5-FU), leucovorin, and oxaliplatin also known as NALIRIFOX, as a first-line treatment for patients with metastatic PDAC after data from the NAPOLI 3 trial showed a statistically significant improvement in overall survival (OS) and progression-free survival (PFS) compared with patients treated with nab-paclitaxel in the study (Wainberg *et al.*, 2020). Hallmark clinical trials for advanced PaC are listed in Table 1 (Chiorean and Covelev, 2015) (Torphy, Fujiwara and Schulick, 2020)). A promising advance in understanding PaC has been the identification of expression-based subtypes, however, the current treatment landscape is lacking mainstream and effective treatment biomarkers. The COMPASS trial set out to perform whole genome sequencing and RNA sequencing of advanced PDAC to identify mutational and transcriptional features for better treatment selection (Aung *et al.*, 2018). They found that the best progression-free survival was observed in those treated with a modified FOLFIRINOX (m-FFX) who displayed a classical subtype. While this study marks significant progress, the main limitation of this and many other PDAC genomic studies is only a small proportion of patients are matched to targeted second-line treatment based on their potentially actionable genetic aberration as the field lacks biomarker directed clinical trials. This highlights a need for future biomarker driven clinical trials but also a need for other methods of optimising the treatment regimens that are currently available for patients.

Drug modeling explores the relationship between drug concentrations and their effects on biological systems (Gibiansky, 2008). Although still emerging in clinical practice, this field has gained importance with the increasing use of multi-drug combination therapies in treating aggressive cancers like pancreatic cancer (PaC). Drug-drug interactions (DDIs) define how one drug's effectiveness can be altered by another. These interactions are categorised into pharmacokinetic interactions—affecting the absorption, distribution, metabolism, or elimination of a drug—and pharmacodynamic interactions, which change a drug's effects due to the presence of another (Zhao *et al.*, 2023). By integrating pharmacokinetic and pharmacodynamic models, researchers can optimise dosage decisions and minimise side effects. Notably, Conroy *et al.* have made significant strides in advancing treatments for metastatic pancreatic cancer, with FFX emerging as the most effective therapy for this patient

group (Conroy *et al.*, 2011). Despite the extensive use of FFX in clinical trials, there remains a significant gap in understanding the pharmacological and toxicological interactions of its components. Molins and Jusko's introduction of psi ( $\Psi$ ) terms to interaction equations marked a pivotal development, enabling the evaluation of interactions among multiple drugs (Molins and Jusko, 2018). This foundational work set the stage for the research described in paper 2 of this thesis, which investigates the effects of individual and combined FFX drugs on pancreatic cancer cell survival, aiming to refine drug-specific parameters and clarify the nature of drug interactions.

**Table 1: Summary of landmark pancreatic cancer clinical trials and overall survival efficacy outcomes.** Table adapted from Chiorean *et al*, 2015. and Torphy *et al*, 2020.

| Study                         | Drug Therapy                                  | Overall survival outcomes |
|-------------------------------|-----------------------------------------------|---------------------------|
| Phase 3 PRODIGE 24/CCTG PA.6  | - mFOLFIRINOX vs gemcitabine                  | - 54.4 vs. 35 months      |
| Phase 2/3 PRODIGE 4/ACCORD 11 | FOLFIRINOX vs gemcitabine                     | - 11.1 vs. 6.8 months     |
| Phase 3 MPACT                 | - Nab-paclitaxel + gemcitabine vs gemcitabine | - 8.5 vs. 6.7 months      |
| Phase 3 NCIC CTG PA.3         | - Erlotinib + gemcitabine vs. gemcitabine     | - 6.24 vs. 5.91 months    |
| Phase 3 POLO                  | - Olaparib vs. placebo                        | - 18.9 vs. 18.2 months    |
| Phase 2 KEYNOTE-158           | - Pembrolizumab                               | - 4 months                |

## **1.6 RNA Therapy: Promise, Challenges, and Current Clinical Landscape**

RNA therapeutics represent a promising frontier in the treatment of various diseases, including cancer. These therapies operate by targeting specific genes or proteins involved in disease processes, with the goal of altering their activity to halt or reverse disease progression (Han *et al.*, 2023). RNA-based therapies offer a significant advantage over traditional treatments such as chemotherapy and radiation, as they have the potential to selectively target cancer cells while sparing healthy tissue. Increased specificity has the potential to enhance treatment efficacy but also minimise the debilitating side effects often associated with conventional therapies.

The success of RNA vaccines, particularly in the context of the COVID-19 pandemic, has underscored the potential of RNA therapeutics. This success has catalysed the exploration of RNA-based approaches in oncology, with a growing body of research investigating various RNA modalities, including messenger RNA (mRNA), antisense oligonucleotides (ASO), small interfering RNA (siRNA), and circular RNA (circRNA) (Han *et al.*, 2023). For example, a recent study provided preliminary evidence that adjuvant autogene cevumeran, an individualised mRNA neoantigen vaccine based on uridine mRNA–lipoplex nanoparticles, combined with atezolizumab and modified FOLFIRINOX, induces significant T cell activity in surgically resected PDAC patients, correlating with delayed recurrence (Rojas *et al.*, 2023). A forthcoming global randomised trial (IMCODE 003, BNT122) will hopefully reveal more insightful data (Rojas *et al.*, 2023).

However, the clinical application of RNA therapeutics is not without significant challenges. RNA molecules are inherently unstable and susceptible to degradation by RNases present in the bloodstream and tissues (Abdellatif *et al.*, 2023). This instability poses a major hurdle to the efficient delivery and uptake of therapeutic RNA by target cells, which is crucial for achieving the desired biological effect. Thus, the development of effective RNA delivery systems that can protect these molecules from degradation and enhance their cellular uptake is a critical area of ongoing research. Recent advancements in RNA delivery technologies have improved the target specificity and *in vivo* stability of these therapies, yet challenges remain, particularly in overcoming issues related to bioavailability, immunogenicity, and delivery specificity.

## **1.7 Non-coding RNAs in Cancer Therapy**

Non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), have emerged as important players in cancer biology, often being dysregulated in ways that contribute to disease progression. The ability of miRNA to influence specific mRNA translation and therefore expression of proteins that are involved in various diseases makes them interesting candidates to develop new therapeutic platforms. miRNAs can be manipulated either to suppress oncogenes or to restore the function of tumour suppressor genes. Despite their therapeutic promise, the clinical translation of ncRNA therapies has been hampered by delivery challenges and immune reactions. MRX34 is a lipid nanoparticle formulation of miR-34a that reached clinical trials stage for treatment of several malignancies including lung cancer. Although the study was withdrawn due to adverse immunogenic effects, the delivery system via encapsulation of miR-34 in a lipid formulation was key to promoting the therapeutic value of miR34.

A substantial proportion of the published research on ncRNA focusses on bioinformatics to elucidate the relationship between the differential expression of certain ncRNAs and tumour prognosis with further experimental characterisation needed to validate their efficacy. *In vitro* studies have been instrumental in elucidating the roles of miRNAs in disease and exploring their potential as therapeutic targets. Techniques such as the use of synthetic miRNA mimics, inhibitors, and CRISPR-Cas9 gene editing have allowed researchers to manipulate miRNA expression and assess their impact on cellular processes (Ijee *et al.*, 2023) (Javaid *et al.*, 2022). These studies provide critical insights into the mechanisms by which miRNAs contribute to cancer and offer a foundation for developing miRNA-based therapies. The primary objective of paper 3 of this thesis was to tackle challenges encountered in the design of miRNA expression vectors intended for use in cancer cells (Allen-Coyle *et al.*, 2024). Our approach involved a comprehensive evaluation of each main canonical biogenesis step to discern at which point dysfunctional processing of the miRNAs of interest occurs. Additionally, we investigated if these challenges persist when using non-cancerous cell lines. To the best of our knowledge, there was no existing study that has scrutinised the processing efficiency of these miRNAs in pancreatic cancer cells and assessed the levels of primary, precursor, and mature miRNA following transfection with stable vector designs.

## **1.8 miRNAs as Biomarkers for Diagnosis and Prognosis**

Extracellular miRNAs are found in biological fluids such as plasma, urine, and saliva marking them as stable biomarkers for various diseases, including cancer (Abreu, Liu and Tsongalis, 2017; Glinge *et al.*, 2017). These miRNAs are typically encapsulated within vesicles like exosomes, microvesicles, and apoptotic bodies, or bound to proteins such as AGO2, which protect them from degradation (Kosaka *et al.*, 2010). The presence of circulating miRNAs in tumour-derived exosomes has been correlated with distant cancer metastases, highlighting their potential prognostic significance. For instance, studies using miR-21, miR-210, miR-155, and miR-196a have shown promise in distinguishing pancreatic cancer patients, suggesting their utility in predicting disease progression (Wang *et al.*, 2009).

Despite the promising potential of miRNAs as prognostic tools, their clinical application faces significant challenges. Many existing studies suffer from small sample sizes, limiting broader clinical validation and raising concerns about the reliability of their findings (Slotwinski, Lech and Slotwinska, 2018). Additionally, variability in sensitivity and specificity across different studies further complicates their diagnostic utility. Notably, single miRNA profiling often results in lower diagnostic accuracy, thereby favoring the use of miRNA panels or sets as more reliable biomarkers (Bloomston *et al.*, 2007; Abreu, Liu and Tsongalis, 2017). A comprehensive meta-analysis by Sun *et al.* underscores this point, highlighting the enhanced diagnostic impact of miRNAs in pancreatic cancer when employed in sets exhibiting high sensitivity and specificity (Sun *et al.*, 2018). These observations emphasize the need for standardized miRNA profiling approaches to bolster their diagnostic and prognostic accuracy, paving the way for their integration into clinical protocols.

Paper 2 of this thesis undertakes a comprehensive comparison of miRNA profiles in PDAC tumours, adjacent normal tissue, and matched patient-derived xenograft (PDX) models (O'Neill *et al.*, 2023). By employing microarray screening, we identified key miRNAs that consistently appeared across multiple priority lists, focusing on those with limited existing literature documentation. This careful selection process has led to the focus of paper 4, where miR-6831-5p is investigated through *in vitro* studies using miRNA mimics. This exploration aims to unravel the functional implications of miR-6831-5p in cancer progression, further contributing to the understanding of miRNA roles in disease and their potential as therapeutic targets. The remainder of the introduction will provide a comprehensive background on miRNA biogenesis, their control of gene expression and elucidating how these small, yet powerful molecules can be harnessed to combat cancer at a molecular level.

## **1.10 microRNA biogenesis**

Non-coding RNAs (ncRNAs), are RNA molecules lacking an open reading frame and conserved codons in transcripts (Beermann *et al.*, 2016). These RNAs are categorised into subsets based on their length: small (20-25 nucleotides) and long (greater than 200 nucleotides) ncRNAs. Various types of ncRNAs contribute to cellular processes, such as micro RNAs (miRNAs), small nucleolar RNAs (snoRNAs), small interfering RNAs (siRNAs), transfer RNAs (tRNAs) and a well-conserved, highly stable backsplicing variant known as circular RNAs (Suzuki *et al.*, 2006; Bartel, 2004; O'Brien *et al.*, 2018; O'Carroll and Schaefer, 2013). Endogenously expressed, these ncRNAs regulate gene expression at the posttranscriptional level and are involved in diverse biological functions, ranging from developmental processes to their implication in diseases such as cancer and cardiovascular disorders (Bartel, 2004; O'Carroll and Schaefer, 2013; O'Brien *et al.*, 2018). In the following section, we will explore the biogenesis and functional significance of miRNAs in particular.

miRNAs constitute a class of non-coding RNAs approximately 22 nucleotides (nt) in length that regulate gene expression (O'Brien *et al.*, 2018). Discovered in the nematode *C. elegans* in 1993 by the Ambros and Ruvkun groups (Lee, Feinbaum and Ambros, 1993), these endogenous RNAs have garnered considerable attention for their roles in diverse biological functions, including the regulation of cell development, differentiation, and survival. Emphasising their significance, around one-third of all human genes are known to be regulated by miRNAs, with greater than 2000 miRNAs identified to date (Khan *et al.*, 2015). Furthermore, in recent years, the dysregulation of miRNAs has been implicated in the development of various human cancers (Beermann *et al.*, 2016).

Approximately 10% of known miRNA genes are located within exons, and when encoded in the sense direction with the coding gene, they follow the transcription patterns of their host genes (Rodriguez *et al.*, 2004). It is plausible that miRNAs within introns or exons of cell lineage- or function-specific genes contribute to controlling genetic networks based on the expected function of the host gene product. Supporting this model, miR-338, originating from introns of genes like apoptosis-associated tyrosine kinase, suppresses mRNAs that act as negative regulators of neuronal differentiation (Barik, 2008). Regardless of genomic location, miRNA biogenesis involves a highly conserved process, with nuclear processing of primary miRNA transcripts by the Microprocessor complex (Drosha and DGCR8), cytoplasmic export by Exportin-5, and further processing by Dicer and the Argonaute family (O'Carroll and Schaefer, 2013). The processing of miRNAs involves one of two pathways known as the

canonical or non-canonical biogenesis pathways, both of which begin with the processing of RNA polymerase II/III transcripts, either post- or co-transcriptionally as shown in Figure 3 (O'Brien *et al.*, 2018). As mentioned above, the dominant canonical pathway consists of three main steps centred around Drosha/DGCR8 and Dicer. The non-canonical pathway utilises different combinations of proteins involved in the canonical pathway and can be broadly classified as either Drosha/DGCR8- or Dicer-independent pathways (Stavast and Erkeland, 2019).

A defining characteristic of miRNAs is their integration into the RNA-Induced Silencing Complex (RISC) (Stavast and Erkeland, 2019; Jo *et al.*, 2015). Several non-canonical pathways have been identified based on this principle. The first such pathway, known as the mirtron pathway, was initially recognised in *Drosophila* (Wong and Rasko, 2021). In this pathway, splicing and debranching of very short hairpin introns of mRNA coding genes generate pre-miRNAs independently of Drosha/DGCR8 processing (Ruby, Jan and Bartel, 2007). These pre-miRNAs are directly exported and processed by Dicer. In mammals, only a limited number of mirtrons, including miR-877, miR-702, and miR-1124–miR-1141, have been described so far. Additionally, tRNA and snoRNA-derived fragments have been shown to enter the RISC complex independently of Drosha/DGCR8 but depend on Dicer (Havens *et al.*, 2012). Deletion of DGCR8 specifically eliminates the canonical miRNA biogenesis pathway, providing insights into its contributions in various cellular processes such as skin development, cardiovascular development, mature cardiomyocyte function and survival, as well as excitatory neuron function and survival (Abdelfattah, Park and Choi, 2014).

The evolutionary conservation of miRNAs within genes suggests an adaptive response to environmental pressures, coordinating specific cell functions. Perturbations in miRNA biogenesis and processing have been increasingly linked to various diseases, particularly cancer (Peng and Croce, 2016). Dysregulation of miRNA expression or aberrant processing can lead to either an overexpression or depletion of specific miRNAs, influencing downstream gene targets and contributing to the development and progression of diseases (Beermann *et al.*, 2016). Understanding the nuances of miRNA biogenesis, including both canonical and non-canonical pathways, is crucial for unravelling the molecular mechanisms underlying diseases and may offer potential therapeutic avenues for intervention.

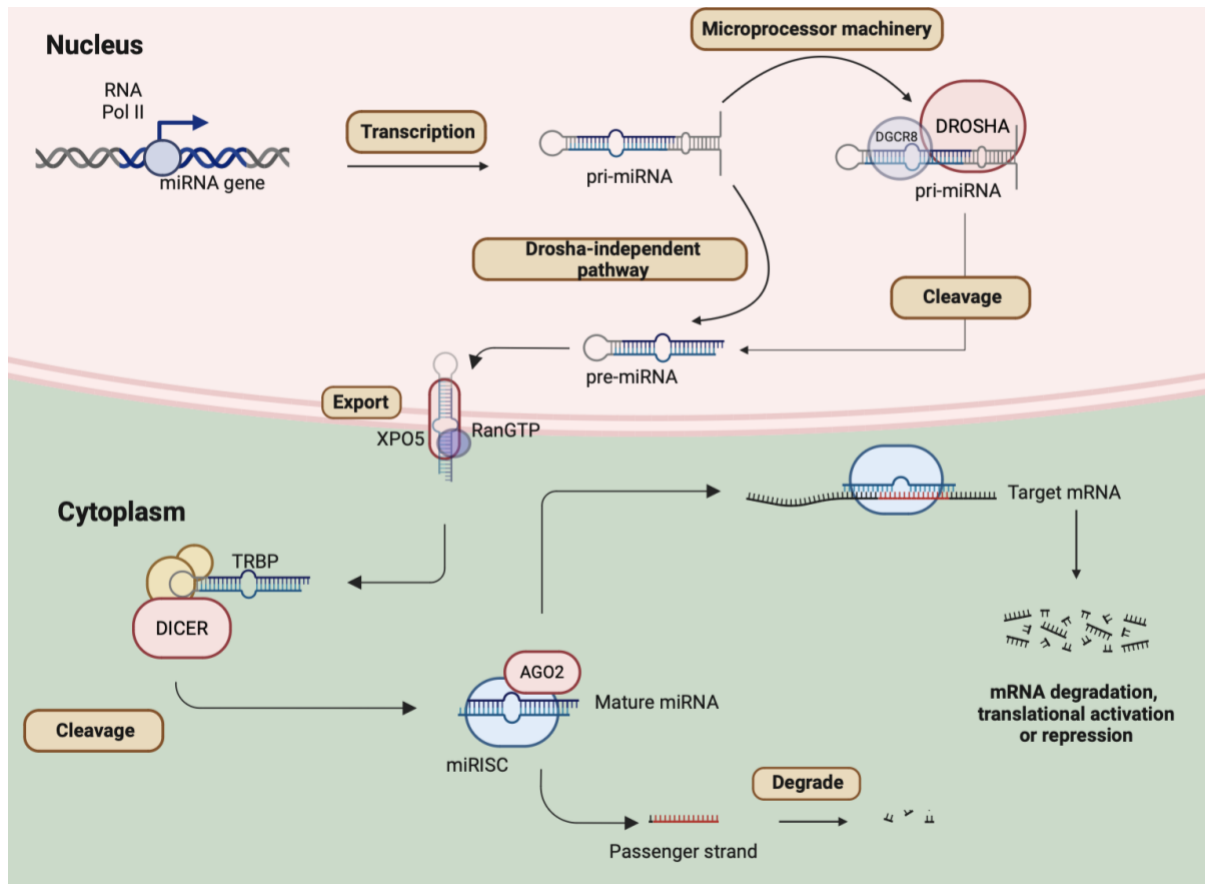


**Figure 3: MicroRNA Biogenesis** - Canonical microRNA (miRNA) biogenesis initiates with the generation of the primary miRNA transcript (pri-miRNA). The microprocessor complex, consisting of Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8), cleaves the pri-miRNA, generating the precursor miRNA (pre-miRNA). Subsequently, Exportin5/RanGTP facilitates the transport of pre-miRNA to the cytoplasm, where it undergoes processing to form the mature miRNA duplex. Loading either the 5p or 3p strand into the Argonaute (AGO) family of proteins results in the formation of the miRNA-induced silencing complex (miRISC). In non-canonical pathways, small hairpin RNAs (shRNA) can undergo DROSHA and/or Dicer-independent cleavage. Regardless of the pathway, all lead to the formation of a functional miRISC complex. In most instances, miRISC binds to target mRNAs, inducing degradation or translational activation/repression. Figure generated using BioRender.com.

### **1.11 miRNA in control of gene expression**

miRNAs exert their function through precise binding of their seed region (position 2-8 at the 5' end) to the miRNA response element (MRE) on target mRNAs, influencing the fate of the latter (Huntzinger and Izaurralde, 2011). Traditionally viewed as essential cytoplasmic regulators governing post-transcriptional gene expression via mRNA degradation or translational repression, miRNAs have recently revealed distinctive nuclear functions, significantly broadening their functional spectrum in gene expression guidance (Forman and Collier, 2010). Core to the functionality of miRNAs is the formation of the minimal RISC (AGO2 and miRNA), which acts as a platform to recruit additional proteins for various biological functions such as proteins required for RISC assembly (Dicer, TRBP and Hsp90), translational regulation, mRNA binding proteins and proteins involved in protein metabolism are all required for the mechanism of RISC mediated gene expression (O'Brien *et al.*, 2018). The efficacy of this binary complex is illustrated by the fact that miRNAs can selectively identify mRNA targets through Watson and crick base-pairing, while allowing for detach-and-reattach dynamics across various targets, enabling sequential control over a broad range of molecules (Catalanotto, Cogoni and Zardo, 2016).

Until recently the primary function of the miRNA-RISC (miRISC) was believed to be post-transcriptional mRNA regulation in the cytoplasm either through hindering of translation or enhancing mRNA degradation (Jo *et al.*, 2015). In recent years, multiple reports have indicated the presence of miRISC complexes in the nuclei of mammalian cells. Through techniques such as Western blots, cell fractionation, and immunofluorescence, the nuclear fractions have been shown to contain AGO proteins, Dicer, TRBP, and Gw182/TNRC6, forming multi-protein complexes (Gagnon *et al.*, 2014). Studies on HeLa nuclear and cytoplasmic extracts have suggested differences in the composition and binding stability of miRISC complexes between the nucleus and cytoplasm. Gagnon and colleagues observed that nuclear Dicer, programmed with exogenous 21-nt siRNAs, retains catalytic activity along with AGO2 and can target nuclear RNAs, inducing their degradation (Gagnon *et al.*, 2014). Notably, the loading process seems to occur in the cytoplasm, despite the concurrent presence of Dicer and TRBP in the nucleus. The current hypothesis proposes that the assembly of miRNA-AGO2 takes place outside the nucleus, where crucial loading factors are available. Once formed, the minimal RISC may then be imported into the nucleus, aligning with earlier analyses suggesting a smaller size for the nuclear RISC compared to its cytoplasmic counterpart (Weinmann *et al.*, 2009). These findings indicate that the minimal RISC could serve as a central element within a broader machinery dedicated to gene

expression modulation in the nucleus. The specific mechanism governing the shuttling of miRISC between the cytoplasm and nucleus remains unanswered. It is probable that the RISC can employ multiple routes to access the nuclear compartment. Karyopherins, a protein group, aid RISC entry into the nucleus through nuclear pores by recognising specific sequences (Weinmann *et al.*, 2009). Importin 8 (IPO8) is crucial for AGO-miRNA complex import, and silencing IPO8 reduces nuclear AGO2 levels. AGO2, lacking a nuclear localisation signal, partners with TNRC6A, which has an NLS, enabling the minimal miRISC complex to shuttle between the nucleus and cytoplasm. This complex, involving IPO8, TNRC6A, and AGO2, might form in cytoplasmic P-bodies and bind to the nuclear pore complex for nuclear entry (Gagnon *et al.*, 2014). Several studies propose the presence of a motif-dependent mechanism that selectively transports miRNAs into the nucleus. One study found a hexanucleotide element (AGUGUU) at the 3' end of miR-29b responsible for its selective nuclear localisation in HeLa cells, and transferring this motif to other small RNAs induces their nuclear accumulation (Hwang, Wentzel and Mendell, 2007). MiRNAs with a 3' ASUS element or a 3'-terminal guanine nucleotide are also suggested to preferentially localise within the nucleus (Khudayberdiev *et al.*, 2013). The system for selectively translocating miRNAs into the nucleus may vary among different cell types.

The similar size of miRNAs to siRNAs and the presence of miRISC in the nucleus hint at a novel mechanism for miRNA-guided gene expression regulation at the transcriptional level, involving transcriptional gene activation (TGA) or transcriptional gene silencing (TGS) as displayed in Figure 4 (Castel and Martienssen, 2013). Human miR-373 was the first identified miRNA activating gene transcription (TGA) by inducing E-cadherin (CDH1) and cold-shock domain-containing protein 2 (CSDC2) transcription (Place *et al.*, 2008). TGA requires a gene promoter DNA sequence complementary to the miRNA's 5'-seed region. Recruitment of AGO proteins, especially AGO2, and RNAPIII enrichment, along with chromatin mark modifications, accompany miRNA TGA. Long non-coding RNA (lncRNA) or promoter-associated RNA (pRNA) are capable of inducing gene silencing through repressive complex recruitment. Potential mechanisms involve miRNA targeting of complementary sequences within these lncRNAs or pRNAs, promoting exclusion of repressive complexes (Younger, Pertsemliadis and Corey, 2009; Younger and Corey, 2011). The miRNA may also bind to the 5' UTR promoter region of a nascent RNA or a non-coding promoter transcript, recruiting a protein complex to shift chromatin structure and increase gene transcription levels (Younger, Pertsemliadis and Corey, 2009).

As mentioned earlier, miRNAs can induce TGS (Kim *et al.*, 2008). Possible mechanisms involve miRNA-targeted pRNA serving as a platform for an inhibitory protein complex, including RISC elements, polycomb group (PcG) components like EZH2, and chromatin modulators like tri-methyl histone H3 lysine 27 (H3K27me3) (Miao *et al.*, 2016; Adilakshmi, Sudol and Tapinos, 2012; Benhamed *et al.*, 2012; Gonzalez, Pisano and Serrano, 2008). This interaction positions the inhibitory complex near the promoter region, leading to modifications in chromatin structure that establish a non-permissive transcriptional state. Alternatively, miRNAs may interact with promoter regions through direct binding between RNA and single-stranded DNA complementary sequences. Ciolfi and colleagues found that miR-223 induced TGS of *NFI-A* by interacting with two DNA sequences in the *NFI-A* promoter region that are complementary to the miR-223 seed sequence (Zardo *et al.*, 2012). Their investigation confirmed the nuclear entry of miR-223 where the miRNA was observed to localise at its complementary sequences on the *NFI-A* promoter. Since, RNA synthesis is blocked during metaphase, this finding may support a view whereby miR-223 directly binds to DNA on specific sequences (Zardo *et al.*, 2012). The studies described here demonstrate the tremendous versatility in miRNA function with clear roles in both the cytoplasm and nucleus with direct involvement in gene regulation at both the transcriptional and post-transcriptional level.

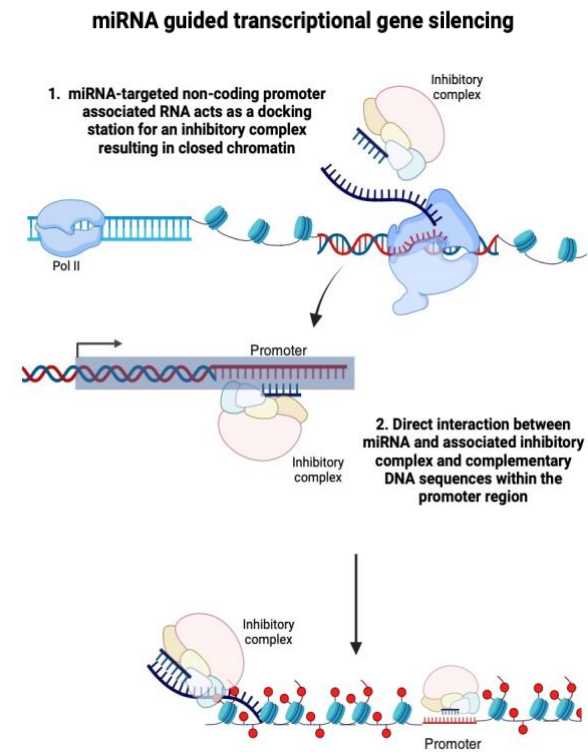
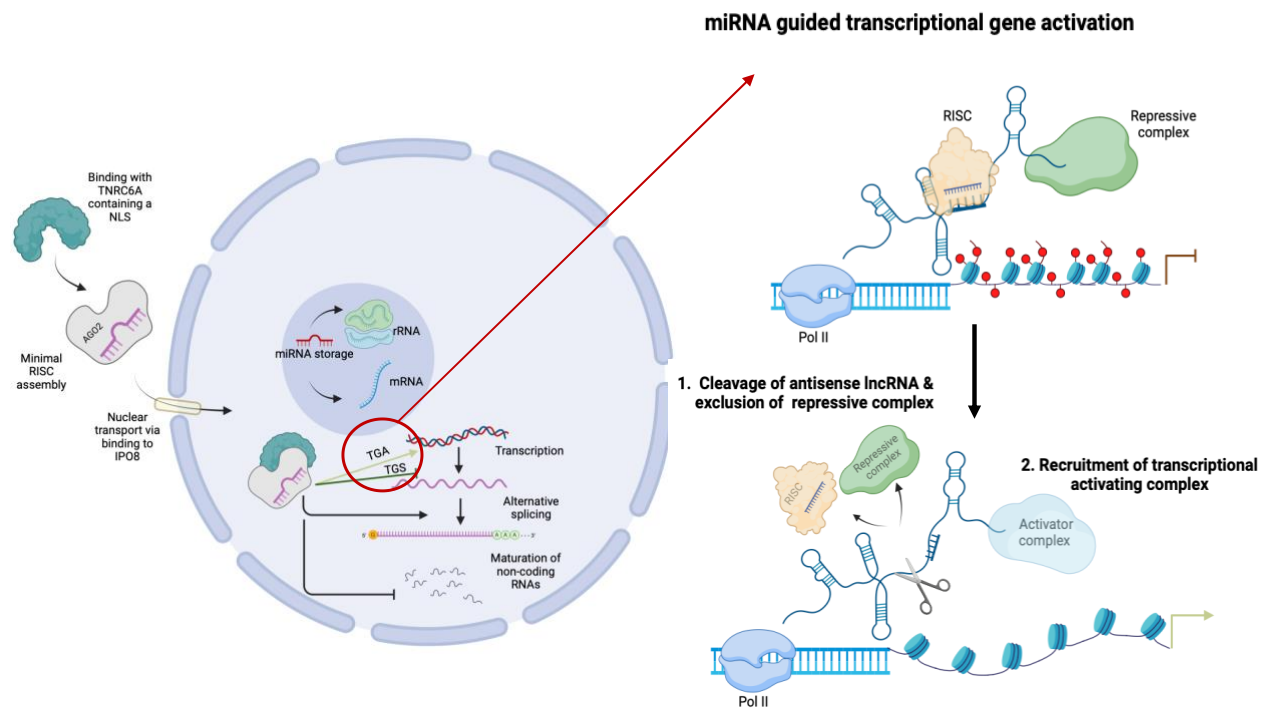


**Figure 4: Panel A: Schematic Representation of Nuclear miRNA Functions** - In the nucleus, miRNAs exhibit diverse functions. They can activate (TGA) or inhibit (TGS) gene transcription, impede the maturation of non-coding RNAs (LncRNA, pri-miRNA, pre-miRNA), and influence alternative splicing. In the nucleolus, miRNAs may impact the maturation of ribosomal RNAs (rRNAs) and messenger RNA (mRNAs). **Panel B: Mechanisms of miRNA guided TGA** - lncRNAs or promoter-associated RNAs (pRNAs) can silence gene expression by recruiting a transcriptional repressive complex. miRNAs targeting a complementary sequence within the lncRNA or pRNA induce cleavage, promoting exclusion of the repressive complex and derepression of sense transcription. Alternatively, miRNAs induce gene activation by recruiting a protein complex with transcriptional activators. **Panel C: Mechanisms of miRNA-Guided TGS** - miRNAs can inhibit gene expression at the transcriptional level by targeting non-coding promoter-associated RNA, creating a docking platform for a protein inhibitory complex. This interaction modifies the chromatin structure, establishing a non-permissive transcriptional status. Alternatively, miRNA-guided recognition and interaction with promoter regions may occur through direct RNA-DNA interaction. Figure Created with BioRender.com. microRNA dysregulation in cancer

### **1.12 microRNA dysregulation in cancer**

There are numerous mechanisms by which miRNAs can become dysregulated. The main categories include; gene sequence alterations, abnormality of transcription factors, epigenetic modifications, competing endogenous RNAs (ceRNAs) and dysfunction of miRNA biogenesis/processing (Lee *et al.*, 2008). Genomic variations of miRNA genomic loci can give rise to alterations of miRNA expression. As many miRNA genes are located at cancer associated sites they are more prone to deletion, translocation, or amplification. For example, the miR-15a/miR-16-1 cluster has been shown to locate within 13q14 and is deleted at high frequency in B-cell chronic lymphocytic leukaemia (CLL) (Lin and Gregory, 2015). miRNAs can also become dysregulated due to alterations of transcriptional activators or repressors. The most well-defined transcription factor-miRNA relationship is that of tumour suppressors p53 and the miR-34 family. miR-34a is a direct target of p53 and can be significantly upregulated by this molecule (Melo *et al.*, 2010). Epigenetic modifications including DNA methylation and/or histone modifications alter gene expression without altering the DNA sequence. Loss of miR-34a expression was associated with the frequent hypermethylation of its promoter on CpG island in many cancer types including pancreatic (29.1%) and colon (13.0%) (Ali Syeda *et al.*, 2020). ceRNAs are a relatively new concept, but they play a significant role in disease including cancer by acting as molecular sponges for miRNAs, influencing gene expression. Dysregulation of ceRNA networks is implicated in various aspects of oncogenesis, impacting critical pathways related to cell proliferation, apoptosis, and metastasis (Si *et al.*, 2019; Ling *et al.*, 2020). The biogenesis and maturation of miRNAs is a

complex pathway comprised of numerous key enzymes and proteins including Drosha and Dicer1, DGCR8, XPO5, TRBP2 and AGO. The dysregulation of these components at any step of the process may lead to abnormal expression of miRNAs and are often deregulated in cancer (Lee *et al.*, 2008; Lin and Gregory, 2015; Melo *et al.*, 2010; Ali Syeda *et al.*, 2020).

The observation that certain components of the biogenesis pathway can be dysfunctional in cancer cells served as the basis of experimental procedures detailed in paper 3. In this chapter, the goal was to generate inducible stable miRNA overexpressing pancreatic cancer cell lines. During the generation of these cell lines we made an intriguing discovery that despite successfully increasing certain miRNA levels such as miR-708, with two different vector designs, mature forms of miR-29b, -1290, -2467, and -6831 remained unaffected. Our detailed investigation ruled out alternative strand selection by the RISC complex and transcriptional interference at the pri-miRNA level. Drosha processing and pri-miRNA export from the nucleus also appeared normal. The accumulation of pre-miRNA was observed only in cell lines where mature miRNA expression was not achieved, indicating a block at the pre-miRNA stage. Others have shown defects in miRNA biogenesis as a mechanism by which miRNAs can lead to cancer progression as both the canonical and noncanonical pathways involve several intermediate factors that are tightly regulated. For example, low levels of dicer expression and functionality are linked to the development and poor survival prognosis of patients with the following cancer types: breast, lung, liver, bladder, and colorectal (Iliou *et al.*, 2014; Allegra *et al.*, 2014; He *et al.*, 2007). Exportin-5 was found to play an oncogenic role in CRC, where high expression levels associated with the worst clinicopathology and poor CRC patient survival (Shigeyasu *et al.*, 2017). Overexpression of Ago1 and Ago2, Dicer, and Drosha were observed in ovarian cancer (Vaksman *et al.*, 2012).

### **1.13 miRNAs in Cancer initiation, Progression and Treatment Response**

Numerous miRNAs exhibit aberrant expression and are likely implicated in the development of PDAC (Baradaran, Shahbazi and Khordadmehr, 2019). This was demonstrated by Yu *et al.* who identified 107 aberrantly expressed miRNAs by comparing different grades of PanIN lesions with control pancreatic duct samples (Yu *et al.*, 2012). They identified miR-196b as the most selectively differentially expressed miRNA, with its downregulation limited to PanIN-3 lesions suggesting it could be utilised for pancreatic screening (Yu *et al.*, 2012). Chen *et al.* used *in-vitro* and *in-vivo* assays to reveal that miR-96 decreased the invasive and metastatic nature of cancer cells and confirmed a direct relationship between miR-96 and KRAS by demonstrating that there was perfect base pairing between the 'seed sequence' of miR-96 and its target KRAS (Yu *et al.*, 2010).

miR-21 can contribute to the inhibition of cell cycle arrest, apoptosis and gemcitabine sensitivity. miR-21 exerts its tumour promoting functions through upregulation of Bcl-2, RECK, FasL, p85 $\alpha$  and PDCD4 (Baradaran, Shahbazi and Khordadmehr, 2019) and is frequently reported to be upregulated in pancreatic cancer where a meta-analysis demonstrated that its overexpression is associated with poor prognosis (Khan *et al.*, 2015). A 2019 study by Zhang *et al.* revealed that overexpression of the long non-coding RNA (lncRNA) Cancer Susceptibility Candidate 2 (CASC2) inhibited the migration and invasion of PANC-1 cells by significantly inhibiting the expression of miR-21 and PTEN (Zhang *et al.*, 2019). Levels of Cancer Susceptibility Candidate 2 (CASC2) expression are lower in pancreatic cancer tissue and cell lines. LncRNAs are a class of RNA molecules with a length of greater than 200 nt which as their name suggests do not encode for proteins. LncRNAs that are functionally active in the nucleus can regulate gene expression both transcriptionally and epigenetically (Beermann *et al.*, 2016). Increasing evidence suggests that lncRNAs including CASC2 act as competing endogenous RNAs (ceRNA) via sponging miRNAs.

### **1.14 miRNAs Role in Metastasis and Interaction with the Tumour Microenvironment**

miRNAs have also been implicated in the highly metastatic nature of pancreatic cancer with these miRNAs referred to as metastamiRs (Srivastava *et al.*, 2014). miRNA expression profile data generated by Kadera *et al.* indicated that miR-100 was expressed at significantly higher levels in metastatic pancreatic cell lines in contrast to non-metastatic cell lines (Kadera *et al.*, 2013). A common feature of many cancers is the loss of the tumour suppressor miR-143,

overexpression of this miRNA in PaC cells has been shown to downregulate a number of genes associated with metastasis. Furthermore, *in-vivo* miR-143 restoration assays carried out by Hu *et al.* inhibited liver metastasis and tumour growth (Khan *et al.*, 2015). It is clear from the studies reviewed that miRNAs are key players in pancreatic cancer initiation, progression and metastasis (Hu *et al.*, 2012). A 2015 study conducted by Keklikoglou *et al.* (Keklikoglou *et al.*, 2015) found miR-206 was significantly downregulated in PDAC tumours and PaC cell lines. They revealed that miR-206 is a novel negative regulator of NF- $\kappa$ B signalling which is constitutively activated in pancreatic cancer and functions as a tumour suppressor through combinatorial targeting of the oncogenes KRAS (affecting tumour vascularisation) and ANXA2 (effecting invasiveness through ECM degradation). Overexpression of miR-206 altered cell cycle kinetics in PaC cells resulting in suppressed cell proliferation, migration and invasion of PaC cells.

Studies reveal that miRNAs regulate genes influencing cellular responses to chemotherapy drugs (Si *et al.*, 2019). For example, miR-21 was shown to be involved in the drug resistance of gastric, pancreas and colorectal cancer to Trastuzumab, gemcitabine and 5-FU, by targeting *PTEN* (Eto *et al.*, 2014), *FasL* (Wang *et al.*, 2013) and *hMSH2* (Valeri *et al.*, 2010), respectively. Following treatment with gemcitabine the levels of miR-155 have been shown to increase (Ren and Yu, 2018). Studies have also demonstrated that high miR-155 expression was associated with poor overall survival and carries out its oncogenic functions by altering anti-inflammatory signalling pathways (Baradaran, Shahbazi and Khordadmehr, 2019). Furthermore, a downregulation of the miR-200 family has been detected in gemcitabine resistant pancreatic cell lines. Wellner *et al.* has also provided evidence that miRNAs regulate the epithelial-mesenchymal transition (EMT), which control numerous types of cellular drug resistance mechanisms (Wellner *et al.*, 2009). Furthermore, members of the let-7 family are downregulated in EMT type cells resistant to gemcitabine (Ren and Yu, 2018). Let-7 families have been recognised to modulate de-differentiation, EMT and PDAC progression by acting through its targets; KRAS, HMGA1, HMGA2, that are frequently deregulated in PDACs (Baradaran, Shahbazi and Khordadmehr, 2019). One study reported that miR-1266 was significantly elevated in PaC tissues and associated with poor prognosis and chemotherapy response in patients with PaC. This miRNA was shown to prevent PaC cells from the induction of apoptosis following gemcitabine treatment by targeting SOCS3, ITCH and TNIP1, which are negative regulators resulting in the activation of the STAT3 and NF- $\kappa$ B pathways (Zhang *et al.*, 2018). Another study (Meng *et al.*, 2020) established pancreatic cancer cell lines with acquired chemoresistance to gemcitabine (GEM - GR cells), where miRNA microarray analysis demonstrated that miR-146a-5p was significantly downregulated in GR cells compared with parental cells, suggesting its potential role in modulating chemoresistance.

PDAC is characterised by significant fibrosis and stromal myofibroblasts, yet their role remains unclear. Studies have highlighted the complexity of targeting carcinoma-associated fibroblasts in PDAC. Prior to 2016, comprehensive data on miRNA expression in tumour-associated stroma (TAS) were lacking. Han et al. identified 158 miRNAs in cultured pancreatic cancer (PC) cells and 116 in TAS cells, with notable differences between them. For instance, miR-200b/-200c were highly expressed in PC, while miR-145/miR-199a/199b were predominant in TAS. Co-culturing revealed miRNA exchanges via extracellular vesicles (EVs), with selective packaging suggesting a tumour-suppressive role for TAS-derived EVs in PDAC cells.

**Table 2: Summary of miRNA and their biological functions in pancreatic cancer mentioned in the above text**

| miRNA                                       | Biological Function                                                                                                                                                                                                           |
|---------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| miR-21                                      | -Prognostic significance in pancreatic cancer, frequently up-regulated<br>-Contributes to inhibition of cell cycle arrest, apoptosis, and gemcitabine sensitivity<br>-Upregulates Bcl-2, RECK, FasL, p85 $\alpha$ , and PDCD4 |
| miR-196a, -210, -155,                       | -Potential prognostic significance in pancreatic cancer                                                                                                                                                                       |
| miR-376a, -301, -145, -221                  | -Distinguishes tumor cells from normal tissue                                                                                                                                                                                 |
| miR-96                                      | -Decreases the invasive and metastatic nature of cancer cells<br>-Slows tumour growth by downregulating the oncogene KRAS                                                                                                     |
| miR-206                                     | -Downregulated in PDAC tumours & cell lines<br>-Negative regulator of NF- $\kappa$ B signaling<br>-Targets oncogenes KRAS and ANXA2, affecting tumor vascularization and invasiveness                                         |
| miR-143                                     | -Loss associated with increased metastasis; restoration inhibits liver metastasis and growth                                                                                                                                  |
| miR-100                                     | -Expressed at significantly higher levels in metastatic pancreatic cell lines                                                                                                                                                 |
| miR-1266                                    | -Elevated in PC tissues; associated with poor prognosis and chemotherapy response<br>-Prevents apoptosis following gemcitabine treatment by targeting SOCS3, ITCH, and TNIP1                                                  |
| miR-146a-5p                                 | -Downregulated in gemcitabine-resistant cells<br>-Regulates carcinogenesis and chemoresistance by repressing TRAF6 expression                                                                                                 |
| miR-194-5p                                  | -Potentiates survival of residual tumor repopulating cells                                                                                                                                                                    |
| miR-205, -200b/c, -429, -145, -199a/b, -155 | -Shows increased expression in co-cultured tumor-associated stroma (TAS) cells                                                                                                                                                |

### **1.15 miRNAs: Multifaceted Regulators of Disease**

miRNAs have the remarkable ability to target multiple key components across various oncogenic signaling pathways, making them powerful regulators in cancer biology. Their ability to modulate pathways like PI3K/AKT/mTOR and Wnt/ $\beta$ -catenin underscores their potential as both oncogenes and tumour suppressors (Fu *et al.*, 2022). The PI3K-AKT-mTOR pathway acts downstream of the KRAS signalling pathway and is involved in the inhibition of apoptosis and promoting cell proliferation (Yonemori *et al.*, 2017). PTEN which suppresses the PI3K-AKT-mTOR signalling pathway is negatively regulated by miR-21. This multitarget effect is particularly promising for the future of treating hard-to-treat cancers, such as pancreatic cancer, where conventional therapies have seen limited success. Pancreatic cancer, notorious for its poor prognosis and resistance to treatment, often involves mutations in key driver genes like KRAS, found in approximately 90% of patients. miRNAs, by targeting such mutations, hold the potential to disrupt multiple signaling cascades that drive tumour progression and survival. Their role in simultaneously influencing several critical pathways could lead to more effective therapeutic strategies, offering new hope for combating this devastating disease.

## **2. Aims**

This research focuses on advancing pancreatic cancer treatment and understanding by addressing critical areas in drug interactions and miRNA biology. The study aims to:

- Investigate Drug Interactions in Pancreatic Cancer Treatment: Use pharmacodynamic modelling to assess interactions within the FOLFIRINOX regimen across various pancreatic cancer cell lines.
- Uncover and Explore Therapeutic Potential of Key miRNAs: Identify specific miRNAs from human pancreatic cancer tissues and patient-derived xenografts, and investigate their impact on disease progression, treatment response, and potential as therapeutic targets.
- Optimise miRNA Research Techniques: Refine molecular tools for studying miRNA effects in pancreatic cancer, addressing challenges in vector design and miRNA processing.

Through these aims, this research seeks to improve therapeutic strategies and provide deeper insights into the molecular mechanisms driving pancreatic cancer, ultimately contributing to more effective treatment approaches and targeted therapies.

### 3. Research Findings

#### **3.1 Paper 1: FOLFIRINOX Pharmacodynamic Interactions in 2D and 3D Pancreatic Cancer Cell Cultures**

Allen-Coyle TJ, Niu J, Welsch E, Conlon NT, Garner W, Clynes M, O'Sullivan F, Straubinger RM, Mager DE, Roche S. FOLFIRINOX Pharmacodynamic Interactions in 2D and 3D Pancreatic Cancer Cell Cultures. *AAPS J.* 2022 Oct 13;24(6):108. doi: 10.1208/s12248-022-00752-8. PMID: 36229752.

The PRODIGE4/ACCORD11 trial established FOLFIRINOX as a preferred first-line treatment for pancreatic cancer due to its superior outcomes over gemcitabine, but its severe side effects—including neutropenia, fatigue, diarrhoea, and neurotoxicity—limit its accessibility, particularly for older patients or those with lower performance status (Conroy *et al.*, 2011). This limitation is critical, given that the median age of pancreatic cancer diagnosis is 71 years, with many patients nearing or surpassing the age cutoff of 76 years (Mizrahi *et al.*, 2020), excluding a significant portion of patients from receiving this treatment (Conroy *et al.*, 2011). While modified-dose FOLFIRINOX (mFOLFIRINOX) has shown promise in reducing toxicity (Kang *et al.*, 2018; Mizrahi *et al.*, 2020), the optimal strategy for its use remains unclear. Despite advances in cancer treatment, pancreatic cancer remains a formidable challenge, with current therapies resulting in severe toxicity that limits their use, leaving many without viable options in a landscape sorely lacking in personalised or targeted approaches. This present study investigates the single agent and combined effects of the FOLFIRINOX agents (5-Fluorouracil (5-FU), oxaliplatin (Oxa), and SN-38, the active metabolite of irinotecan) across a panel of primary and established pancreatic cancer cells and describes a pharmacodynamic (PD) model to quantify their pharmacological interactions (Allen-Coyle *et al.*, 2022) with the aim of better understanding how to potentially optimise the current treatment options available to patients.

PD drug modelling is crucial in contemporary pharmacology for drug development providing a comprehensive view, guiding dosage decisions and minimising side effects. Due to the fields infancy studies on PD drug-drug interactions (DDIs) lack a formal evaluation framework. Most investigations focus on high-throughput *in vitro* screening, with limited testing *in vivo* or clinical trials. This gap leads to uncertainties about the translational impact of pharmacodynamic DDIs identified during drug discovery. Despite a lack of formal guidance, there's a growing need for pharmacodynamic DDI studies using advanced mathematical modelling to aid data interpretation and guide research and decision-making. PD DDIs are typically categorised as

additivity when the combined effect equals the sum of individual drug effects, synergy when the overall effect surpasses additivity, and antagonism when the combined effect is less than additive (Niu, Straubinger and Mager, 2019). This primary impact of the methodology used in the present study was the introduction of an interaction term  $\psi$  ( $\Psi$ ) to traditional Bliss Independent interaction equations allowing for assessment of interactions for three or more drugs (see table 3) (Molins and Jusko, 2018).

**Table 3: Pharmacodynamic Equations for Model Fitting** - SAS 9.4 software was used to model the effect of the single, pairwise and triple combinations on cell proliferation. The equations were applied to single (Eq. (1)), pairwise (Eq. (2)) and triple (Eq. (3)) combination data to determine pharmacodynamic interactions.  $I_{max}$  represents the maximum inhibition achieved at high concentrations.  $IC_{50}$  represents the concentration to achieve 50% of  $I_{max}$ , and  $m$  is the Hill coefficient that corresponds with the steepness of the inhibition curve.

|                                                                                                                                                                                                                                                                                               |   |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---|
| $R_i = 100\% \cdot \left( 1 - I_{max,i} \cdot \frac{C_i^{m_i}}{C_i^{m_i} + IC_{50,i}^{m_i}} \right)$                                                                                                                                                                                          | 1 |
| $R = 100\% \cdot \left( 1 - I_{max,i} \cdot \frac{C_i^{m_i}}{C_i^{m_i} + IC_{50,i}^{m_i}} \right) \cdot \left( 1 - I_{max,j} \cdot \frac{C_j^{m_j}}{C_j^{m_j} + (\psi) \cdot IC_{50,j}^{m_j}} \right)$                                                                                        | 2 |
| $R = 100\% \cdot \left( 1 - I_{max,i} \cdot \frac{C_i^{m_i}}{C_i^{m_i} + IC_{50,i}^{m_i}} \right) \cdot \left( 1 - I_{max,j} \cdot \frac{C_j^{m_j}}{C_j^{m_j} + IC_{50,j}^{m_j}} \right) \cdot \left( 1 - I_{max,k} \cdot \frac{C_k^{m_k}}{C_k^{m_k} + (\psi) \cdot IC_{50,k}^{m_k}} \right)$ | 3 |

This study used a varied panel of cell line models including well established PaC cell lines including BxPC-3, MIA PaCa-2 and PANC-1. In addition a primary patient derived xenograft cell line, PIN-127 was used. The origin of this cell line was from an invasive moderately differentiated adenocarcinoma taken from a male patient aged 72 and implanted into severe combined immunodeficiency (SCID) mice. The first generation (F1) PDX tumour was then developed into a cell line (Roche *et al.*, 2020). The final model included in this study was a non-attached 3D spheroid model of PANC-1 cells. The main finding of this study shown in Table X below, was that the triple combination of Oxaliplatin, 5-Fluorouracil and SN-38 suggested antagonistic interactions in three out of five cell lines. Of most interest was the strong antagonism observed in the primary PIN-127 cell line and the loss of synergy observed for the PANC-1 cells when moving from 2D to 3D culture (see table 4). Of the established cell lines, BxPC-3 seemed to cluster with the PIN-127 in terms of interactions observed. PIN-127 cell line has previously been shown to harbour KRAS and Phosphoinositide-3-Kinase

Regulatory Subunit 1 (PI3K31) mutations (Roche *et al.*, 2020). The BxPC-3 cell line is known to be a KRAS wildtype cell line (Deer *et al.*, 2010). Therefore, the KRAS mutation is unlikely to explain the clustering of antagonistic responses observed in these two cell lines, given the wildtype status of BxPC3.

**Table 4: Summary of the Triple Combination Across the Panel of Cell Lines** - The interaction term psi ( $\psi$ ) was estimated in combination treatments using Eq. (3) (Table 1). Interactions were categorised as synergistic ( $\psi < 1$ ), additive ( $\psi = 1$ ) or antagonistic ( $\psi > 1$ ). Red indicates antagonism, and green identifies combinations with synergistic interactions.

| Cell line  | Triple Combination | Psi Value ( $\psi$ ) | SE   | 95% CI Lower | 95% CI Higher |
|------------|--------------------|----------------------|------|--------------|---------------|
| PIN-127    | 5-FU + Oxa + SN38  | 5.57                 | 1.6  | 2.3          | 8.8           |
| BxPC-3     | 5-FU + Oxa + SN38  | 1.37                 | 2.1  | 0.4          | 0.6           |
| Mia PaCa-2 | 5-FU + Oxa + SN38  | 0.22                 | 0.05 | 0.1          | 0.3           |
| PANC1 (2D) | 5-FU + Oxa + SN38  | 0.78                 | 0.1  | 0.6          | 1.0           |
| PANC1 (3D) | 5-FU + Oxa + SN38  | 1.65                 | 0.3  | 1.0          | 2.3           |

The COMPASS trial demonstrated that Stage III/IV PDAC patients with Moffitt “classical” tumours responded better to first-line chemotherapy compared to those with “basal-like” tumours (Aung *et al.*, 2018). The main finding of the COMPASS trial was that the best progression-free survival was observed in those with classical subtype treated with modified FOLFIRINOX. However, the results described here show that the BxPC-3 cell line which has previously described as representing the classical subtype (Färber *et al.*, 2023) showed numerous areas on antagonism for both fixed and varying ratio drug combination concentrations for both the pairwise and triple combination. The cell lines PANC-1 and MIA PaCa-2, both classified as quasimesenchymal (QM), exhibit notable differences in drug sensitivity. Despite their QM classification, synergy was observed in pairwise and triple drug combinations. Collisson’s quasi-mesenchymal subtype are partially integrated into Moffitt’s basal subtype and Bailey’s classifications of squamous (Espiau-Romera *et al.*, 2020). PANC-1 cells, which undergo complete epithelial-mesenchymal transition (cEMT), are identified as “E-like” within the QM category, characterised by low but detectable ECAD and RAC1b expression (Ungefroren *et al.*, 2022; Aiello *et al.*, 2018). We show these cells displayed the lowest sensitivity to single agent oxaliplatin and SN-38. MIA PaCa-2 cells, embodying an extreme mesenchymal phenotype, lacking both ECAD and RAC1b expression (Ungefroren *et al.*, 2022) were the least responsive to single agent 5-FU. This study revealed that the pairwise

combination of oxaliplatin and SN-38 emerged as the most potent, with toxicity levels comparable to the triple combination.

In conclusion, this analysis identified various additive and antagonistic interactions within the triple regimen, raising the possibility that the clinical success of FOLFIRINOX may not solely rely on synergistic drug interactions. This suggests that re-evaluating drug scheduling or considering non-simultaneous use of these agents could potentially reduce toxicity and expand access to this regimen for a broader patient population. By leveraging pharmacodynamic modelling, this research offers valuable insights into the optimisation of current first-line treatments for pancreatic cancer. The model developed here not only evaluates combinations of three drugs but can also be adapted to include a fourth variable, such as temporal interactions or additional treatment-response biomarkers. Additionally, the following papers described in this thesis explored small non-coding RNAs as potential indicators of disease progression and treatment response, aiming to enhance understanding of patient stratification to refine therapeutic strategies in the absence of personalised or targeted therapies.

### **3.2 Paper 2: Alteration in levels of specific miRNAs and their potential protein targets between human pancreatic cancer samples, adjacent normal tissue, and xenografts derived from these tumours.**

O'Neill F, Allen-Coyle TJ, Roche S, Meiller J, Conlon NT, Swan N, Straubinger RM, Geoghegan J, Straubinger NL, Conlon K, McDermott R, O'Sullivan F, Henry M, Meleady P, McVey G, O'Connor R, Moriarty M, Clynes M. Alteration in Levels of Specific miRNAs and Their Potential Protein Targets between Human Pancreatic Cancer Samples, Adjacent Normal Tissue, and Xenografts Derived from These Tumors. *Life (Basel)*. 2023 Feb 22;13(3):608. doi: 10.3390/life13030608. PMID: 36983764; PMCID: PMC10057657.

The research described in paper 2 of this thesis delves into the critical role of miRNAs in the development and progression of pancreatic cancer. Over the past decade, research on miRNAs in pancreatic cancer has intensified, highlighting their involvement in the disease's development, progression, and prognosis (Subramani *et al.*, 2015) (Passadouro and Faneca, 2016). However, a notable gap exists in studies that simultaneously incorporate transcriptomic, proteomic, and miRNA analyses on the same sample set. As a result, protein and gene targets of significant miRNAs have often been identified using predictive software, without validation in matched samples. Despite this limitation, miRNAs hold particular promise because each miRNA can target multiple mRNAs, thereby regulating the cellular levels of corresponding proteins (Ratti *et al.*, 2020). The main aim of paper 2 was to leverage the differential expression of miRNAs to unravel the biology of human pancreatic cancer. This paper presents foundational data, summarising differentially expressed (DE) miRNAs between adjacent normal, tumour, and F1 patient-derived xenograft (PDX) samples (O'Neill *et al.*, 2023). Previous analyses of proteomic (Coleman *et al.*, 2018) and mRNA (Roche *et al.*, 2020) on these clinical and F1 have been carried out. The key strength of this multilevel profiling study, which incorporated mRNA microarrays and mass spectrometry proteomic data, addressed a common limitation of miRNA research: the reliance on prediction software without experimental validation. By integrating these data sets, we were able to tentatively validate targets and generate a focused list of miRNAs for further investigation, setting the stage for the paper 3 and 4.

The multiomic approach described in this study offers several significant benefits. First, the approach allows for comprehensive molecular characterisation by integrating multiple layers of regulation. miRNAs are key post-transcriptional regulators of gene expression, influencing both mRNA levels and protein production. Simultaneous profiling of miRNAs, mRNAs, and proteins captures the full spectrum of gene regulation, helping to identify how miRNA

alterations impact downstream targets and pathways, offering deeper insights into the molecular drivers of pancreatic cancer. This method also contextualises miRNA functions, as miRNAs often regulate multiple targets with effects that vary depending on the cellular context. Comparing miRNA alterations in tumour tissues with those in adjacent normal tissues and PDX models enhances our understanding of context-specific miRNA functions and their contributions to cancer progression. Second, this multiomic approach increased the likelihood of identifying robust biomarkers that reflect the complex biology of PaC. Finally, the validation and cross-verification of findings are crucial aspects of this research. Using multiple-omic layers allowed for cross-verification, increasing confidence in the biological relevance of identified miRNA alterations. If a miRNA is found to be deregulated, and its predicted mRNA targets and downstream proteins are also altered, this consistency across omics layers supports the validity of the findings. Moreover, multiomic integration helps to filter out false positives that might arise from single-omic analyses. A miRNA-mRNA interaction predicted by bioinformatics tools is more likely to be functionally relevant if the corresponding protein levels are also affected as expected.

miRNA prediction software tools are indispensable in studying gene regulation due to their ability to identify potential miRNA-target interactions on a large scale. These tools use algorithms based on sequence complementarity, thermodynamics, evolutionary conservation, and machine learning models to predict miRNA binding sites on mRNA (John *et al.*, 2004). The primary advantages of these tools include high-throughput prediction, initial filtering to narrow down targets for experimental validation, evolutionary conservation analysis to identify biologically relevant interactions, and integration with existing biological databases. However, these tools also have limitations. They often generate a high number of false positives due to reliance on sequence complementarity alone, which does not guarantee functional interactions (Agarwal *et al.*, 2015). Different prediction tools may produce conflicting results for the same miRNA-mRNA pair due to variations in algorithms, scoring systems, and databases used (John, Sander and Marks, 2006). Additionally, most prediction tools do not account for the cellular context in which miRNAs function, such as tissue-specific expression, RNA secondary structure, and availability of cofactors, leading to inaccurate predictions. Overemphasis on conservation may also cause non-conserved but biologically important miRNA interactions to be overlooked. While computational predictions provide valuable insights, experimental validation is crucial for confirming miRNA-mRNA interactions. This study carried out parallel mRNA profiling on matched tissue samples. An alteration in predicted target mRNA levels matched with alterations in miRNA levels supports a direct interaction. In addition, mass spectrometry was used to assess the impact of miRNAs on

protein levels. Since miRNAs can repress translation without degrading mRNA, protein assays provide direct evidence of functional interactions.

### **3.3 Paper 3: miRNA-and cell line-specific constraints on precursor miRNA processing of stably transfected pancreatic cancer and other mammalian cells**

Allen-Coyle TJ, Roca BC, Costello A, Barron N, Keenan J, Clynes M, O'Neill F, O'Sullivan F. miRNA- and Cell Line-Specific Constraints on Precursor miRNA Processing of Stably Transfected Pancreatic Cancer and Other Mammalian Cells. *Int J Mol Sci.* 2024 May 23;25(11):5666. doi: 10.3390/ijms25115666. PMID: 38891854; PMCID: PMC11172344.

To study the function of miRNAs, we need reliable methods to overexpress them both *in vitro* and *in vivo* in a way that mimics natural mechanisms. Overexpression can be achieved using either synthetic mature miRNAs or miRNA-expressing DNA vectors. These DNA vectors can be either constitutively active or designed with inducible expression systems (Costello *et al.*, 2017). Constitutively active plasmids can disrupt cellular functions, including cell death, so user-defined heterologous miRNA regulation is preferred. Inducible systems are particularly useful because they reveal the direct effects of specific genetic manipulations and are advantageous when working with mammalian cell lines governed by complex genetic networks (Kallunki *et al.*, 2019). Paper 3 highlights our findings on the structural and sequence differences between successful and non-successful vector designs, the difficulties of generating non-problematic empty vectors and leaky vector expression could help to inform future chimeric miRNA design strategies and act as a guide to other researchers on the intricate dynamics that can impact vector efficiency.

During experiments to overexpress specific miRNAs in human pancreatic cancer cells, vector designs successfully increased miR-708 expression but not that of miR-29b, -1290, -2467, and -6831. In this study, the mechanisms behind this finding were investigated by exploring three hypotheses: (1) Failed DROSHA processing of pri-miRNA? (2) Inadequate transport of pre-miRNA from the nucleus? and (3) Incomplete pre-miRNA processing in the cytoplasm? Results identified pre-miRNA accumulation of four miRNAs in the cytoplasm of transfected cells. This study showed a diversity in expression patterns in an miRNA- and cell line-specific manner. It was demonstrated that different miRNAs with the same vector in the same cell line, the same miRNA between the two vector designs, and with the same miRNA across different cell lines displayed distinct expression patterns. The results described in this chapter emphasised the complexity of miRNA processing, where factors beyond secondary structure and motifs played crucial roles and could help inform future chimeric miRNA design principles. This research provided a strong foundation for further investigation into the multifaceted mechanisms governing miRNA maturation, offering valuable insights into pre-miRNA processing and opening new avenues for exploring the intricate control mechanisms involved.

The conceptual advancements in understanding the complexity of designing miRNA-expressing vectors for *in vitro* work described here could have potential implications for the development of RNA-based therapeutics such as RNA vaccines. These insights could help inform several challenges associated with RNA vaccine design and delivery, particularly effective expression and stability. The study's findings on the intricate factors influencing miRNA expression—such as sequence, structure, and cellular context—highlight the need for precise RNA design in targeted therapies, for example, RNA vaccines must ensure that the RNA sequence is efficiently transcribed and translated within the host cells, similar to how miRNA vectors must be carefully designed to achieve desired expression levels. Understanding these complexities is essential to ensure robust and consistent expression of the target antigen. Just as miRNA vectors must be optimised to avoid issues like incomplete processing or leaky expression, RNA based therapies must be designed to remain stable and functional once delivered into the body. Insights from miRNA vector design could inform strategies to enhance RNA stability, prevent degradation by cellular enzymes, and ensure proper processing within the target cells. This might involve optimising the RNA's secondary structure or incorporating modifications to increase stability without compromising function. The challenges identified in ensuring proper miRNA processing and expression could inform the development of delivery systems for RNA therapies. For instance, delivery vehicles like lipid nanoparticles (LNPs) must be optimised to protect the RNA during delivery and facilitate its uptake by target cells, ensuring that the RNA reaches its destination intact and functional. Lessons from miRNA vector research could lead to the design of delivery systems that better navigate cellular environments, improving the efficiency of RNA vaccine delivery.

### ***3.4 Paper 4: miR-6831-5p overexpression in pancreatic cancer cell line results in changes in cell proliferation, gemcitabine sensitivity and apoptosis accompanied by expression changes at both the mRNA and protein level.***

**\*\* Manuscript under review \*\***

This paper investigated the functional significance of miR-6831-5p in pancreatic cancer, focusing on its potential to enhance therapeutic strategies, particularly in the context of gemcitabine treatment. Our study built upon the previous identification of miR-6831-5p as a key miRNA of interest in paper 1. Here, miR-6831-5p was shown to significantly reduce cell proliferation by 40% in PANC-1 pancreatic cancer cells. While its overexpression alone did not increase apoptosis, it markedly enhanced gemcitabine-induced apoptosis and improved gemcitabine sensitivity. This indicated that miR-6831-5p could be a valuable target for improving the efficacy of gemcitabine, a key chemotherapeutic agent for pancreatic cancer.

Through comprehensive multiomics analyses, including 3' QuantSeq RNA sequencing and mass spectrometry, it was uncovered that miR-6831-5p impacted critical biological pathways. It induced changes in mitochondrial bioenergetics, purine nucleotide homeostasis, and apoptosis. Specifically, the data revealed a shift away from oxidative phosphorylation (OXPHOS) towards alternative metabolic pathways, which is particularly relevant given pancreatic cancer's reliance on OXPHOS for energy production. This metabolic shift could enhance the efficacy of gemcitabine by altering cellular processes involved in drug metabolism and DNA repair. miR-6831-5p's role in enhancing gemcitabine sensitivity underscores its potential as a therapeutic target. Despite its limited prior exploration, with only five studies primarily identifying its expression patterns, our findings highlight its functional relevance in cancer treatment. This suggests that miR-6831-5p could be harnessed to overcome gemcitabine resistance and improve patient outcomes.

The study also revealed discrepancies between predicted and experimentally validated miRNA targets, emphasising the need for *in-vitro* validation to complement prediction software. Expanding research to include a broader range of cell lines and exploring miR-6831-5p's role in other cancers could further validate its therapeutic potential.

In summary, this paper significantly advanced the understanding of miR-6831-5p in pancreatic cancer, presenting it as a promising target for enhancing gemcitabine efficacy and providing new avenues for therapeutic development in this challenging disease.

### **3.5 Optimisation Chapter: Refining miRNA Investigation Techniques in Pancreatic Cancer Cells**

#### **3.5.1 Introduction**

Preliminary research described in paper 1 identified 11 miRNAs of interest through global gene expression analysis conducted at the mRNA, miRNA, and protein levels on human primary pancreatic adenocarcinomas, adjacent normal tissues, and patient-derived xenografts generated from these tumours. Among the 11 miRNAs identified—miR-21, -29b-1-5p, -125a-3p, -148-5p, -206, -378e, -615-3p, -708-5p, -1290, -2467-3p, and -6831-5p—some were less well-characterised in the context of pancreatic cancer. Specifically, miR-29b, -378e, -1290, -2467-3p, and -6831-5p have not been extensively studied for their roles in pancreatic cancer, making them of particular interest for further investigation.

miR-378e is part of the miR-378 family, which includes 11 members (Jiang *et al.*, 2017). miR-378e has been associated with various cancers, including osteosarcoma, cervical cancer, and colon cancer (Peng *et al.*, 2018) (Tan *et al.*, 2018) (Zeng *et al.*, 2017; Wang *et al.*, 2006) (Ding *et al.*, 2019). However, its role in pancreatic cancer remains underexplored. Previous studies suggest that miR-378e might function as a tumour suppressor, inhibiting migration, invasion, and promoting apoptosis in certain cancer types. miR-2467-3p is a newly discovered miRNA has shown regulatory roles in renal dysfunction in diabetic nephropathy and has been implicated in cancer-related processes, such as epithelial-mesenchymal transition (EMT) and matrix metalloproteinase expression (Xu *et al.*, 2020; Xiao and Liu, 2020; Duan, Wei and Liu, 2021; Liu and Wen, 2020). Its role in pancreatic cancer, however, is not well-defined, making it a candidate for further study. miR-6831-5p was identified in a miRNA signature associated with metastatic intramucosal gastric cancer, miR-6831-5p has been linked to pathways related to apoptosis and the PI3K/AKT/mTOR signaling axis (Kim *et al.*, 2021). In pancreatic cancer, it has shown differential expression and potential interactions with key signaling molecules like MAPK1 and TGFBR1, making it a promising target for further exploration (Usuba *et al.*, 2019; Aita *et al.*, 2021). miR-29b-1-5p, while less studied in pancreatic cancer, has shown potential as a tumour suppressor in other cancer types, such as triple-negative breast cancer and bladder cancer (Milevskiy *et al.*, 2018; Drago-Ferrante *et al.*, 2017; Xu *et al.*, 2013). Its role in reducing cell growth, invasion, and chemotherapy resistance suggests it may have similar functions in pancreatic cancer.

Unlike the others, miR-1290 is relatively well-studied, particularly for its oncogenic role in pancreatic ductal adenocarcinoma. It has been implicated in tumour-stroma interactions and has been suggested as a circulating biomarker for pancreatic cancer prognosis (Li *et al.*, 2013; Tavano *et al.*, 2018; Wei *et al.*, 2020). Several miRNAs, including miR-21, -708, and -148, demonstrated significance in pancreatic cancer and malignant transformation of intraductal papillary mucinous neoplasms (Francone *et al.*, 2021). MiR-21 targeted MAPK/ERK and PI3K/AKT pathways, promoting cell proliferation (Usuba *et al.*, 2019). MiR-708 displayed oncogenic activity in PDAC via inhibition of the expression of tumour suppressor sirt3 (Huang *et al.*, 2019). MiR-148 inhibited cancer cell migration and invasion and regulated oncogene expression (Aita *et al.*, 2021).

The initial aim of this study was to explore the biological roles of these miRNAs by manipulating their expression in pancreatic cancer cell lines. This included using overexpression and sponge vectors to assess their impact on cellular processes such as proliferation, colony formation, anoikis, and invasion. However, some intriguing results led to a shift in focus towards investigating miRNA processing dysregulation and a more detailed analysis of miR-6831-5p, which is discussed in papers 3 and 4, respectively. This chapter delves into the molecular biology techniques and optimisations that were necessary for these investigations. It also discusses the preliminary findings for the other priority miRNAs, laying the groundwork for potential future studies that could further elucidate their roles in pancreatic cancer.

### **3.5.2 Methods**

#### *3.5.2.1 Cell culture*

The human pancreatic cell line MIAPaCa-2 was obtained from the European Collection of Cell Cultures (ECACC, UK). BxPC-3, Capan-2 and PANC-1 human pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). PANC-1 and MIAPaCa-2 cells were grown in DMEM (Sigma-Aldrich, Dublin Ireland) supplemented with 5% (v/v) Fetal Bovine Serum (FBS; Gibco) and 2% L-Glutamine. BxPC-3 and Capan-2 cells were grown in RPMI-1640 Medium supplemented with 5% (v/v) FBS. Cells were grown in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C in culture flasks. Cells were sub-cultured, using PBS to wash and trypsin-EDTA (Thermo Fisher Scientific) to detach when cells reached 80-90% confluency.

#### *3.5.2.2 Vector design*

A pcDNA3.1(+)-HYG vector backbone was used for both vectors (sponge (SPG) and overexpression (OV) as shown in paper 3. The expression of the pri-miRNA (OV) or MRE (SPG) is controlled by a pTight promoter, part of an inducible TET-ON system which requires the presence of doxycycline to activate expression. Both vectors co-express the MRE or pre-miRNA with a fluorescent protein (destabilised GFP in this case) to follow transfection efficiency.

#### *3.5.2.3 Transfection & Selection of stably transfected cells*

The Mirus TransIT-X2 (Mirus MIR 6000) transfection reagent was used to transfect plasmid DNA. 24hr prior to transfection cells were seeded at a density of  $2.5 \times 10^5$  in 2mL in a 6 well plate. The following day the TransIT-X2 reagent was brought to room temperature. 250µL of DMEM/F-12 Hams medium was mixed with 1µg of plasmid DNA and 2µL of TransIT-X2. The solution was mixed gently and incubated at room temperature for 30min. The solution was then added in a dropwise fashion to the cells seeded the previous day. The cells were incubated for 48hr. Confirmation of transfection efficiency was achieved via doxycycline (100 ng/mL) induction of GFP. 48hr post transfection with plasmid DNA the cells were fed with complete media containing the selection agent, Hygromycin B (Roche) at a concentration of 400µg/mL. Cells were under selection until their growth profile matched that of the parental cell line, at this point they were determined to be resistant and RNA samples taken and cell stocks made.

#### 3.5.2.4 *miRNA mimic transfection*

The Mirus TransIT-X2 transfection reagent (Medical Supply Co Ltd, Ireland) was used to introduce miRNA mimics as per the manufacturer's instructions. Cells were seeded at a density of  $1$  or  $2 \times 10^5$  for MIA PaCa-2 and PANC-1, respectively, in 6-well plates 24 hours before transfection. A mixture of 100  $\mu$ L of serum-free DMEM/F-12 Hams medium and a 10  $\mu$ M mirVana™ miRNA mimic (Bio-Sciences Ltd, Ireland) along with 2  $\mu$ L of TransIT-X2, was gently prepared and allowed to incubate at room temperature for 30 minutes. The solution was then added in a dropwise fashion to the cells seeded the previous day. The cells were incubated for 24 hours before RNA collection for QuantSeq 3' mRNA-Seq library prep and 48 hours for phenotypic analysis.

#### 3.5.2.5 *RNA isolation*

RNA samples were prepared using Trizol, according to manufacturer's guidelines. Briefly, 1 mL of Trizol reagent was added per confluent T25 flask of cells. Per 1 mL of Trizol reagent used, 200  $\mu$ L of chloroform was added to the Eppendorf (Hamburg, Germany), sample was mixed and allowed to sit for 5 min. At 4°C samples were spun at 12,000 $\times$  g for 15 min. The aqueous phase was then separated using a micropipette and placed in a new Eppendorf. 500  $\mu$ L of isopropanol was added to the aqueous phase, allowed to sit at room temperature for 10 min, after which it was spun at 12,000 $\times$  g at 4°C for 10 min. The RNA pellet was then washed using 75% ethanol and spun at 4°C at 7500 $\times$  g for 5 min. Ethanol was removed from pellet, which were allowed to air dry, and were then reconstituted in nuclease free water. Samples were quantified using a Nanodrop (Thermo Scientific, Dublin, Ireland).

#### 3.5.2.6 *RT-qPCR for mature miRNA*

Relative quantification (RQ) of mature miRNA was assessed from a total of 10ng RNA samples using the TaqMan® Advanced miRNA Assays (ThermoFisher, A25576) as per manufacturer's recommendations. The delta delta Ct method was used to determine the RQ of each miRNA with miR-16-5p and miR-24-3p as endogenous controls (Livak and Schmittgen, 2001). The 96-well plate reactions were run in a 7500 (Applied Biosystems). Technical triplicates were run per sample, including the negative controls and no template.

### 3.5.2.7 QuantSeq 3' mRNA-Seq library prep

Three replicates per condition of total RNA was extracted from cells as described above. The QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen) was used to prepare illumina compatible cDNA libraries from 500 ng input RNA as per the manufacturer's protocol. As suggested the PCR Add-on Kit for Illumina (Lexogen) was used to calculate the optimal number of cycles to include in the endpoint PCR. Quantity and quality of the finished libraries were assessed using the Qubit fluorometer (Invitrogen) and high sensitivity DNA screen-tape analysis (Agilent Technologies, Inc.) as per manufacturer's instructions. Individually indexed libraries were pooled and processed for 150 bp paired-end sequencing on a NextSeq550 sequencer (Illumina) using the NextSeq 500 High Output v2 kit (75 cycle) (Illumina, FC-404–2005) as per the manufacturer's instructions.

### 3.5.2.8 QuantSeq 3' mRNA-Seq data analysis

The BBDuk module from the BBTools package was used to perform adapter trimming and quality filtering as recommended by the manufacturer. The STAR aligner was used to align filtered reads to the hg38 human genome build. The htseq-count tool was used to calculate the number of reads that align to each gene. Differentially expressed genes were detected using a  $\leq 1.5$  fold change and a  $\leq 0.05$  Benjamini-Hochberg adjusted P value using the DESeq2 R package (Love, Huber and Anders, 2014). Gene ontology analysis was performed using ShinyGo. The R package EnhancedVolcano (Blighe K) was used to generate volcano plot visualisations.

### 3.5.2.9 Phenotypic assays

For phenotypic assays two rounds of experiments were carried out. Round 1 assays were pre-induced with 100 ng/mL doxycycline prior to seeding and re-induced 24 hours post seeding. Results displayed variability between biological replicates and a new approach was tested, referred to round 2 in main text. In round 2 assays cells were not pre-induced before seeding and contained both a +/- Dox control. In addition everything in round 2 was compared back to empty vector control. miRNA targets and assays complete in each round are described in Table 5.

### *3.5.2.10 2D proliferation*

Stably selected MIAPaCa-2 & PANC-1 cells were seeded in 6-well plates at a density of  $5 \times 10^3$  /  $5 \times 10^4$  cells/mL, respectively and allowed to incubate overnight at 5% CO<sub>2</sub> and 37°C. Fresh medium with/without 100 ng/mL Dox and added to each well. The plates were incubated for a further 6 days until confluency was being approached in the control cells before assessment of cell proliferation using acid phosphate assays as previously described [1].

### *3.5.2.11 3D cell proliferation assay*

96 well plates were coated with 50 µL polyhema as a non-adherent coating (Sigma Aldrich S3932, 5 mg/mL in 96% ethanol) and baked at 50°C for two days. Plates are stored at room temperature (RT) until required, for up to 6 months. Stable selected MIA PaCa-2 & PANC-1 cells (100 µL/well) were seeded at  $5 \times 10^4$  cells/mL in 5% FBS DMEM incubated overnight at 37 °C. Complete media (100 µL) with/without dox was added to the wells and incubated at 37°C until day 10. 12 µL of AlamarBlue® Cell Viability reagent (10% of final volume) was added to the wells and incubated at 37 °C for 4 hrs and fluorescently measured at 535/590 nm excitation/emission wavelength on Biotek plate reader using KC4 software. Percentage viability was calculated relative to empty vector control. Each assay was performed in biological triplicate.

### *3.5.2.12 Anoikis assay*

The polyhema was prepared by adding polyHEMA (Merck, P3932-10G) to 95% EtOH slowly with gentle mixing at 50°C to a final concentration of 12.5 mg/ml. 24-well plates were coated with 200 µl/well and allowed to dry overnight. A second coat was applied and again allowed to dry overnight. Wells were rinsed with cell culture medium before use. Stably selected MIAPaCa-2 & PANC-1 cells were seeded in 24-well plates at a density of  $5 \times 10^3$  /  $5 \times 10^4$  cells/mL, respectively. Wells were loaded with 500 µl cells.

**Table 5: Phenotypic assay set-up** – the cell lines and miRNAs investigated in both rounds alongside the assays and their corresponding set-up time.

| Round 1 Assay     | Duration |
|-------------------|----------|
| 2D colony forming | 14 days  |
| 2D proliferation  | 7 days   |
| 3D proliferation  | 8 days   |
| Invasion          | 24 hrs   |

| Mia PaCa-2         | Panc-1             |
|--------------------|--------------------|
| Parental (+/- dox) | Parental (+/- dox) |
| EV                 | EV                 |
| miR-21 spng        | miR-21 spng        |
| miR-29b spng       | miR-125a spng      |
| miR-125a spng      | miR-1290 spng      |
| miR-125a OV        | miR-148 spng       |
| miR-148 OV         | miR-148 OV         |
| miR-615 OV         | miR-708 OV         |
| miR-708 OV         |                    |
| miR-206 OV         |                    |

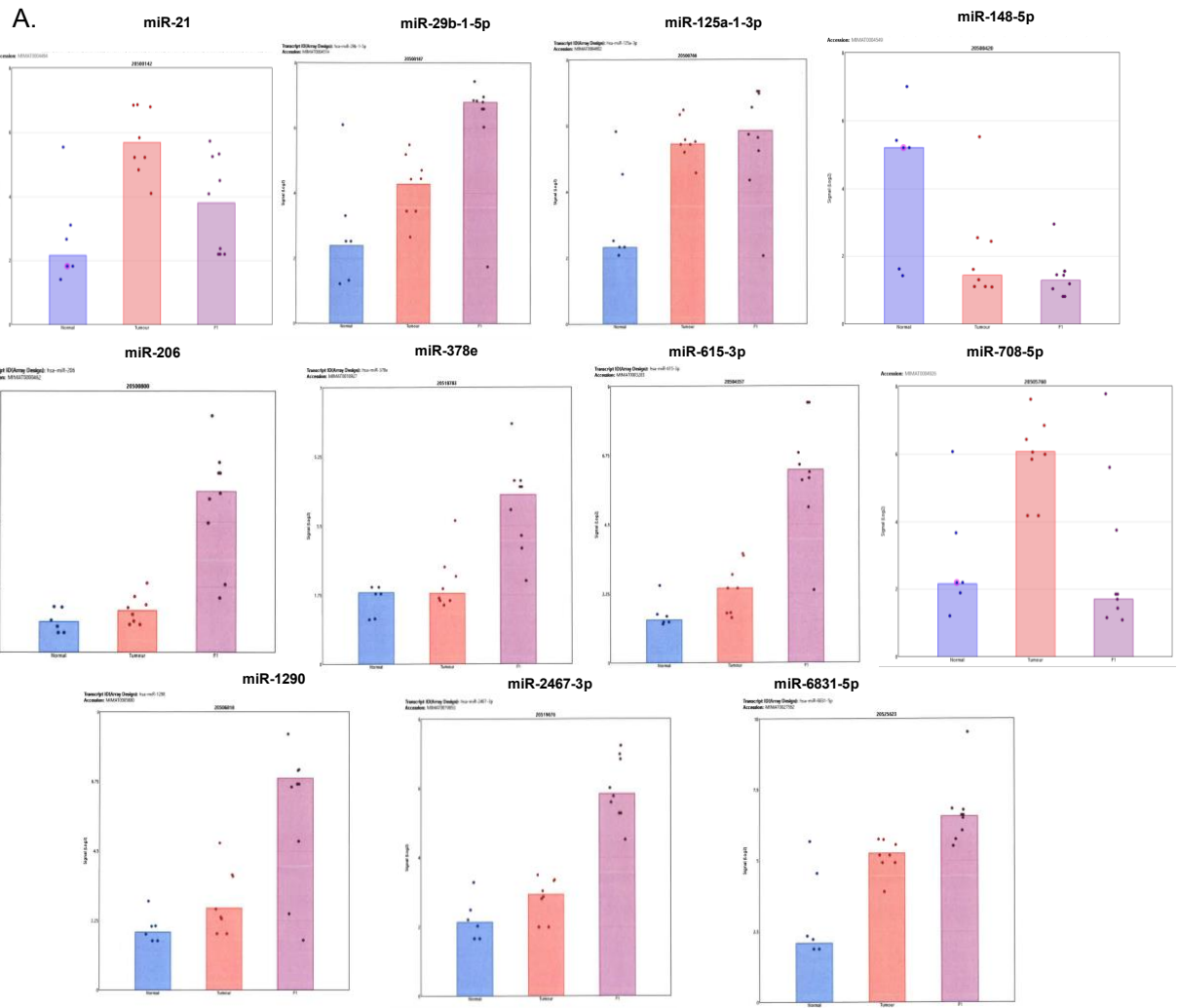
| Round 2 Assay         | Duration |
|-----------------------|----------|
| 2D colony forming     | 14 days  |
| 2D proliferation      | 7 days   |
| 3D proliferation      | 10 days  |
| Anoikis               | 48 hrs   |
| Scratch wound healing | 24 hrs   |

| Mia PaCa-2            | Panc-1                  |
|-----------------------|-------------------------|
| Parental (+/- dox)    | Parental (+/- dox)      |
| EV (+/- dox)          | EV (+/- dox)            |
| miR-21 spng (+/- dox) | miR-21 spng (+/- dox)   |
| miR-148 OV (+/- dox)  | miR-1290 spng (+/- dox) |
| miR-615 OV (+/- dox)  | miR-148 OV (+/- dox)    |

### 3.5.3 Results and Discussion

#### 3.5.3.1 In-vitro validation of miRNAs identified in microarray of adjacent normal, tumour and patient derived xenograft SCID models of pancreatic cancer

Our group previously conducted comprehensive analysis of global gene expression across three concurrent levels (mRNA, miRNA, and protein) using human surgical material obtained from primary pancreatic adenocarcinomas (Roche *et al.*, 2020; O'Neill *et al.*, 2023; Coleman *et al.*, 2020; Meleady *et al.*, 2020). Comparisons were carried out between tumour samples and adjacent "normal" tissue and matched samples of the same tumours cultivated as xenotransplants in SCID mice (F1). The primary emphasis of this project was on the miRNA data (O'Neill *et al.*, 2023). The Transcriptome Analysis Console (TAC) software was used to process the miRNA microarray data. Scatterplots were generated to visualise top-prioritised miRNAs, selected for further validation. Each target's data underwent individual scrutiny to eliminate miRNAs affected by a large differential expression due to a small number of samples with exceptionally high or low expression levels. The focus is on identifying changes shared by the majority of samples within each category. Additional in-depth analysis and discussion of the targets identified in this study are provided in paper 2. Here, Figure 5 displays the scatterplots for 11 miRNAs of interest and a Table of the corresponding log 2 fold change values in adjacent normal versus tumour and tumour versus F1. This profiling allowed the generation of various differential expression (DE) lists: Tumour vs Normal (T vs N); Tumour vs F1 (T vs F1) and of particular interest, miRNAs which increased in expression between N vs T, and again increased between T vs F1. We hypothesised that these 2 transitions might represent progressive enrichment for cancer cells, and might therefore represent a list enriched for miRNAs of functional and/or diagnostic relevance in pancreatic cancer. These DE lists are extensively discussed in paper 2. This chapter focuses on the following prioritised miRNAs; 1) Increased in T vs N and T vs F1 - miRs 29b-1-5p; 125a-3p;615-3p;1290; 6831-5p, and 2) Similar in T and N but higher in T vs F1- miRs 206, 378e,2467-3p.



**B.**

| miRNA        | N vs T –<br>log2FC | p value | T vs F1 –<br>log2FC | p value |
|--------------|--------------------|---------|---------------------|---------|
| miR-21       | 4.18               | 0.0041  | -2.14               | 0.0371  |
| miR-29b-1-5p | 2.52               | 0.2276  | 5.8                 | 0.026   |
| miR-125a-3p  | 5.46               | 0.0026  |                     |         |
| miR-148-5p   | 1.44               | 0.0079  |                     |         |
| miR-206      |                    |         | 6.8                 | 3.5E-06 |
| miR-378e     | 1.23               | 0.3952  | 4.3                 | 5.7E-04 |
| miR-615-3p   |                    |         | 6.3                 | 1.6E-06 |
| miR-708-5p   | 6.07               | 0.0042  | 1.47                | 0.0019  |
| miR-1290     | 1.39               | 0.395   | 15.4                | 8E-04   |
| miR-2467-3p  | 1.48               | 0.168   | 7.91                | 5.8E-09 |
| miR-6831-5p  | 4.82               | 0.0042  | 1.81                | 7.7E-04 |

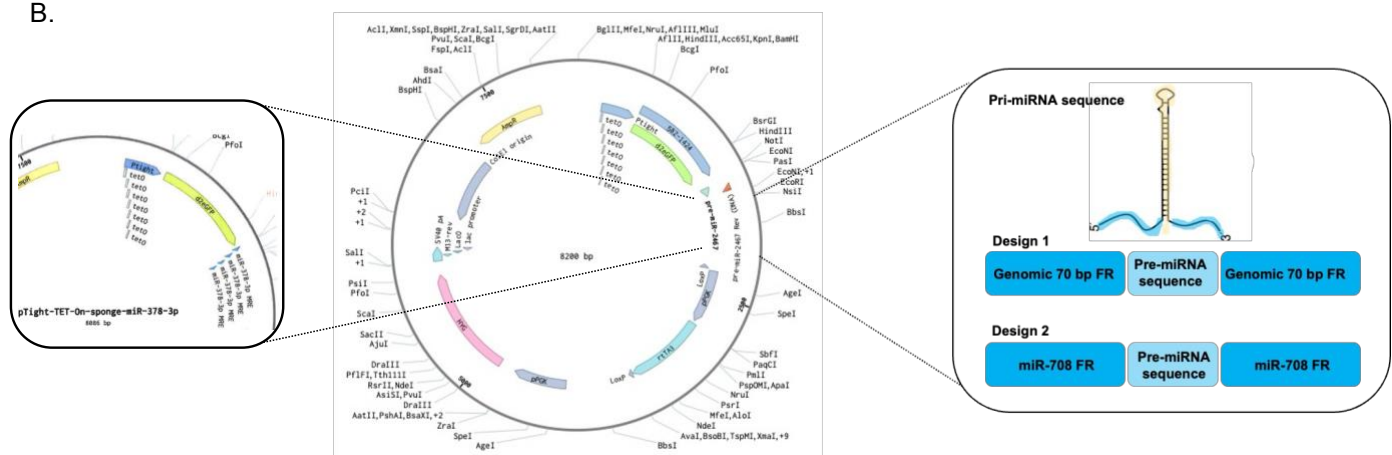
**Figure 5: Background microarray study.** A. Microarray analysis highlighting priority miRNAs based on log2 fold change values in adjacent normal, tumour and F1 pancreatic cancer samples. Scatterplots show log2FC and sample distribution for miR-21, -29b-1-5p, -125a-3p, -148-5p, -206, -378e, -615-3p, -708-5p, -1290, -2467-3p and -6831-5p and the values in B. corresponding p-values are listed in the table. Blank rows are where miRNAs did not show a significant difference in that direction.

A wide panel of pancreatic cancer cell lines were screened by RT-qPCR to check for endogenous expression levels of each of the priority miRNAs. The expression level miRNAs of interest were categories into not expressed, low, medium or highly expressed (see Figure 6A). In order to manipulate the expression of these miRNAs *in-vitro* the cloning of miRNA expressing vectors was undertaken (described in methods section 3.5.2.2). In brief, there were two original vector options of an overexpressing or sponge vector (see Figure 6B). Each vector contains a destabilised GFP reporter gene and a hygromycin b resistance gene to allow for the generation of stable cell lines. To conditionally trigger the expression of miRNAs the TET-On system was used. The vector constitutively expresses the tetracycline responsive trans-activator (rtTA3) under a weak PKG promoter. Addition of doxycycline to culture media permits binding of the rtTA3 to the tet operon (tetO) regions in the pTight promoter sequence, facilitating transcriptional activation in a doxycycline concentration dependant manner. The use of an overexpression and/or sponge vector was based on endogenous expression levels (Figure 6A). Mia PaCa-2 and Panc-1 were the cell lines chosen for further investigation based on the following criteria; short doubling time rate, high transfection efficiency and a good mixture of all expression level categories. Following transfection the RQ fold change was determined for each miRNA by RT-qPCR (Figure 6C) that the sponge vectors achieved reduction levels from 2-10 fold less expression as seen in Figure 6C. While most miRNAs of interest displayed very high levels of overexpression, some achieved no overexpression. This was hypothesised to be due to secondary structure achieved *in vitro* based on genomic flanking regions and hence these miRNAs were re-cloned into the flanking regions of miR-708 based on its high overexpression achieved. However, this did not help the problem and further investigation into this is discussed extensively in paper 3. The miRNAs where the stable vectors achieved desired results were taken for further phenotypic validation. For the miRNAs who showed no detectable levels of mature miRNAs using either vector design were further investigated using mature miRNA mimics. Following miRNA mimic transfection cells underwent transcriptomic and phenotypic analysis. This chapter will also show these results and the miRNA which came out as most interesting is discussed in detail in paper 4.

|              | Mia<br>PaCa-2 | Panc-1 | Capan-2 | SW1990 | BxPC-3 | HPAC |
|--------------|---------------|--------|---------|--------|--------|------|
| miR-21       | 23            | 21     | 20      | 22     | 21     | 22   |
| miR-29b-1-5p | 30            | 30     | 29      | 29     | 28     | 30   |
| miR-125a-3p  | 27            | 25     | 25      | 25     | 24     | 27   |
| miR-148-5p   | -             | 30     | 34      | 27     | 27     | -    |
| miR-206      | -             | -      | -       | -      | -      | -    |
| miR-378e     | -             | -      | -       | -      | -      | -    |
| miR-615-3p   | 29            | 22     | 25      | 24     | 24     | 27   |
| miR-708-5p   | -             | 35     | 33      | 27     | 23     | 30   |
| miR-1290     | -             | 33     | 33      | 34     | 34     | 33   |
| miR-2467-3p  | -             | -      | -       | -      | -      | -    |
| miR-6831-5p  | -             | -      | -       | -      | -      | -    |

| Ct value | Expression level category | Transfection protocol |
|----------|---------------------------|-----------------------|
| > 35     | Not expressed             | OV                    |
| 30-35    | Low                       | OV / spng             |
| 25-30    | Medium                    | OV / spng             |
| < 25     | High                      | Spng                  |

B.



C.

| OV       | MIA PaCa-2 | PANC-1 | SPNG     | MIA PaCa-2 | PANC-1 |
|----------|------------|--------|----------|------------|--------|
| miR-29b  | x          | x      | miR-21   | 0.5        | 0.1    |
| miR-125a | 18         | x      | miR-29b  | 0.5        | -      |
| miR-206  | 197795     | 304814 | miR-125a | 0.6        | 0.1    |
| miR-148  | 82         | 23     | miR-206  | -          | -      |
| miR-378e | x          | x      | miR-148  | -          | 0.1    |
| miR-615  | 65         | x      | miR-378e | -          | --     |
| miR-708  | 4331       | 419    | miR-615  | 0.5        | -      |
| miR-1290 | x          | x      | miR-708  | -          | -      |
| miR-2467 | x          | x      | miR-1290 | -          | 0.1    |
| miR-6831 | x          | x      | miR-2467 | -          | -      |
|          |            |        | miR-6831 | -          | -      |

**Figure 6: Cell line characterisation and miRNA overexpression and sponge vector design.** A. shows the ct values representing endogenous expression levels on priority miRNAs in panel of pancreatic cancer cell lines including Mia PaCa-2, Panc-1, Capan-2, SW1990, BxPC-3 and HPAC. miRNAs were then categorised into not expressed, low, medium or high expression groups and B. transfected with either a sponge or overexpression vector. Panel C. shows the RQ fold change values based on the delta delta ct method following transfection with either overexpression (OV) or sponge (SPNG) vectors. Data represented as three biological replicates. x represents vectors that did not achieve mature levels of miRNAs following transfection with design 1 overexpression vector in which case they were re-transfected with design 2 which contained miR-708 flanking regions but still no mature expression was detected. – represents where a sponge vector was not transfected based on endogenous expression categories.

To ensure that the lack of detectable mature miRNA levels for certain miRNAs was not due to an inability of the TaqMan probes used, the probes were tested on the original clinical tissues used for the microarray study. Figure 7A shows that expression was detectable for all probes. Following on from this, the level of doxycycline induced GFP expression and its effect on cell proliferation was tested to ensure that the induced expression lasted the length of time needed for the assays and did not have a toxic effect on the chosen cell lines (Figure 7 B-C). A range of 1-100 ng of doxycycline was tested and since 100 ng did not have any significant negative effect on cell proliferation and achieved the highest level of GFP expression this was the concentration chosen for all assays. The GFP fluorescence was monitored every 24 hours for up to 96 hours following a single induction. At 96 hours (Figure 7B) the GFP still shows strong signal. It was concluded that expression lasts for at a minimum of 96 hours post induction. From this observation cells were induced once in a 7 day assay and twice in a 14 day assay.

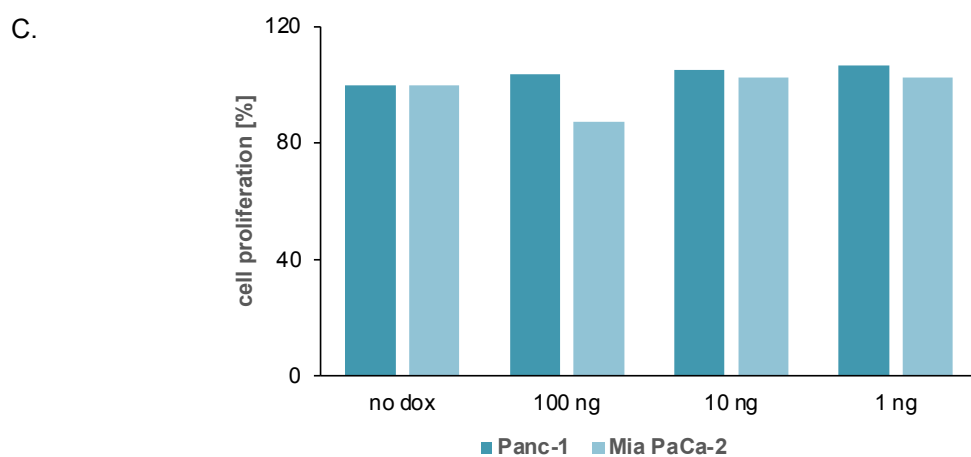
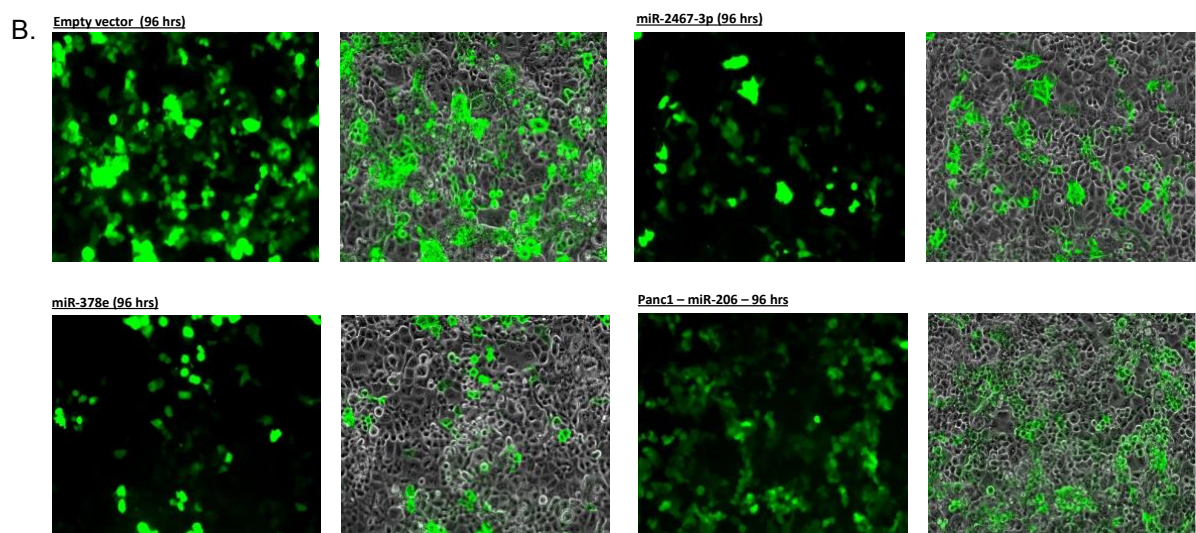
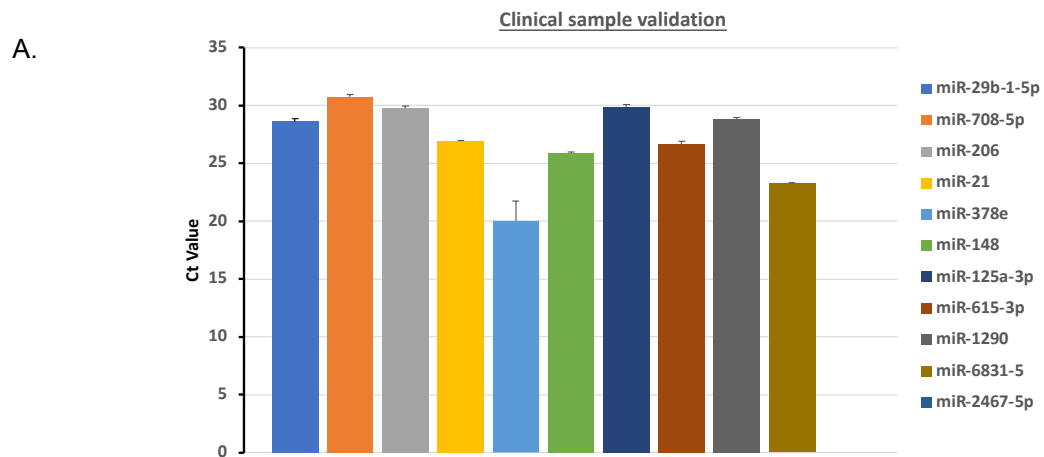


**Figure 7: TaqMan Probe Validation with Doxycycline-Induced GFP Expression imaging and impact on Cell Proliferation.** A. shows the qPCR validation of TaqMan probes on original tissue used for microarray study. B. shows GFP expression for empty vector and three additional targets at 96 hours post-induction concluding expression remains long enough for desired assay length. C. shows the effect of varying doxycycline concentrations on cell proliferation to ensure no toxic effect observed at working concentration in MIA PaCa-2 cells.

In the following section, references are made to round 1 and round 2. As some phenotypic assays were carried out for only 24 hours, cells were pre-induced with Dox to allow cells time to express sufficient levels of miRNAs prior to the assay. However, large variation was seen between biological replicates across a range of assays. The culturing for this included each flask of cells including parental +dox pre-induced for 48 hours prior to seeding for assays. These cells were then reseeded into a flask for culture and the next rep of assays the following week. This pre-induction could have led to the variability observed across reps as the constant pre-induction meant that cells seeding for the final rep were exposed to a miRNA environmental change for a longer period than the first rep. This method of culturing and seeding was discontinued, and a second round of assays was carried out. This is the difference between Round 1 and 2 results described below. The differences in conditions tested are also listed in the methods section Table 5.

#### *3.5.3.2 Unveiling the impact of an 'Empty Vector'*

A primary finding from proliferation assays both in 2D and 3D was a significant alteration in cell proliferation in the presence of the empty vector. Previous studies have shown that doxycycline (dox) can be toxic to cells in culture and its toxicity directly correlates with its concentration. Studies suggest that dox toxicity must be considered while analysing physiological effects when using TET systems (Ermak, Cancasci and Davies, 2003). Several factors contributed to eliminating the possibility of doxycycline toxicity. The experiments included a +dox parental control, and the concentration had been previously tested. While the concentration of doxycycline resulted in a modest reduction in cell proliferation in parental cells, the empty vector further decreased this effect. To eliminate that no random sequences had been integrated during vector cloning that could impact on cell proliferation the vector was re-sequenced. This confirmed that the sequence was intact. While termed an “empty vector” it is far from that, expressing genes for hygromycin and ampicillin resistance and a GFP reporter and this along with the process of transfecting can all play a role in an unfolded protein response and immune responses which can affect cell proliferation (Stepanenko and Heng, 2017). Variation was observed between the effect the empty vector had depending on whether

cells were cultured in 2D or 3D conditions (Figure 8). For Panc-1 cells the empty vector reduced proliferation compared to +doxycycline parental cells by 35% but only in the 3D condition (Figure 8C). For Mia PaCa-2 cells a reduction of 34% (2D) and 14% (3D) was observed (Figure 8 B & D). Due to this clear effect on cell proliferation following genetic manipulation via transfection of vectors, hygromycin selection and doxycycline induction, all other miRNA conditions were compared back to the empty vector control and not the +doxycycline control.

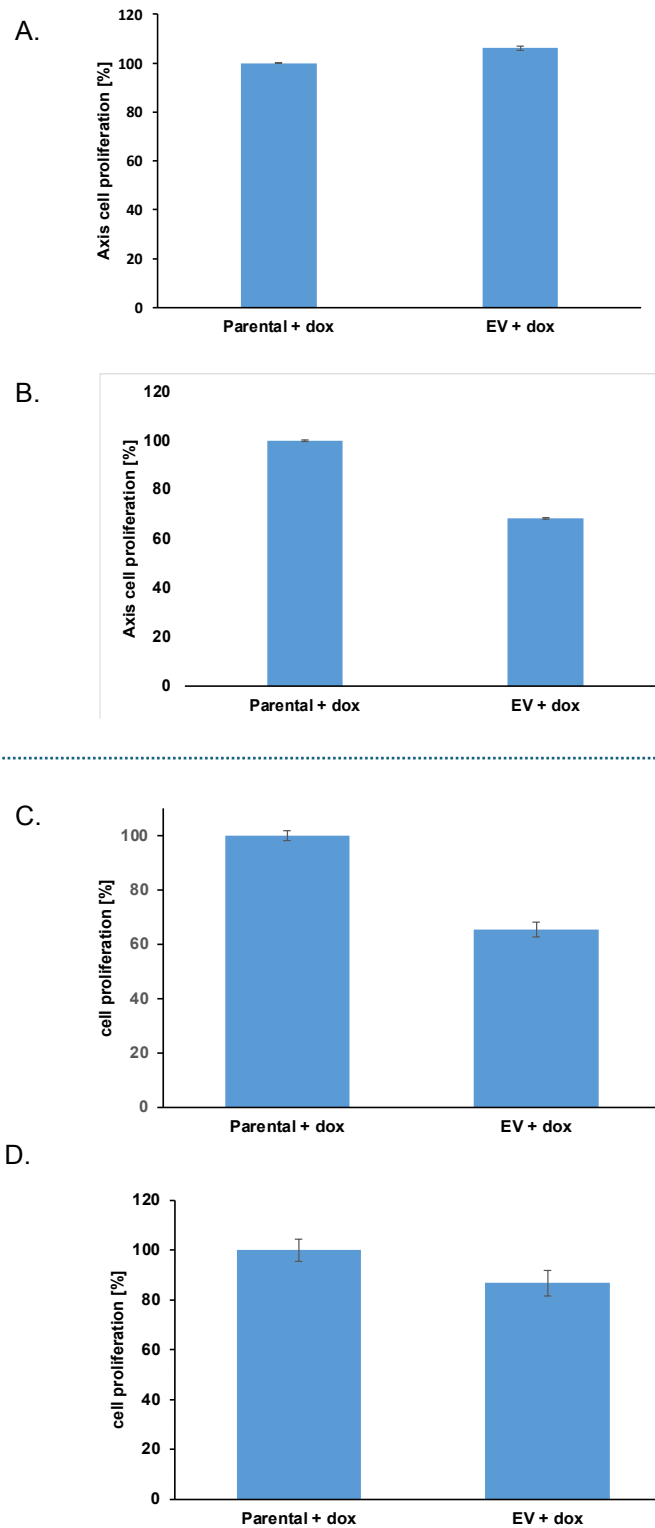


**Figure 8: Effect of empty vector on cell proliferation.** A PANC-1 2D B. MIA PaCa-2 C. PANC-1 3D D. MIA PaCa-2 3D. Results represent 3 biological replicates and corresponding standard deviation error bars.

### 3.5.3.3 Exploring the influence of dysregulated miRNA expression on cancer-related phenotypes

miR-125a exhibited an impact on 2D proliferation in both Panc-1 and Mia PaCa-2 cells. Depleting miR-125a expression in both cell lines led to a decrease in the percentage of cell proliferation, with a more pronounced effect in Panc-1, showing a 19% reduction (Figure 9 A & B). In Mia PaCa-2, the sponge-induced reduction in proliferation was 5%, but overexpression of this miRNA rescued the phenotype, resulting in a 14% increase in proliferation. The inverse phenotypic effect on proliferation between sponge and overexpression of miR-125a remained in 3D culture but reversed in the opposite direction with the overexpression causing a reduction in proliferation (46 % for Panc-1 and 37% for Mia PaCa-2), while the overexpression caused a 6% reduction in proliferation for Mia PaCa-2. recent investigation conducted by Ling *et al.*, revealing that circular RNA circRHOT1, associated with lymphatic metastasis in PDAC, was identified as a regulator influencing the proliferation, invasion, and apoptosis of PaC cells. The mechanism involved down-regulation of miR-125a-3p, leading to an increase in the expression of E2F3 (Ling *et al.*, 2020).

Regarding miR-708, there was a range of effects on proliferation in both 2D and 3D for both cell lines (Figure 9). Panc-1 showed a 5% and 22% increase in proliferation in 2D and 3D, respectively (Figure 9 A & C). MiaPaCa-2 cells had a substantial increase of 77% in 2D and a modest 7% in 3D (Figure 9 B & D). These results highlight the differences observed between 2D and 3D *in vitro* models and the necessity for a combination of both. 3D cultures face additional extracellular cues and physical conditions like rigidity, gradient of gas, varying levels of adhesion and polarity, all which can lead to differences between 2D and 3D culture results and may represent a more representative model of in-vivo conditions (Follin *et al.*, 2016). These observed effects of miR-708 are in agreement with current literature whereby miR-708-5p was shown to be upregulated in PDAC tissues and associated with poor prognosis (Huang *et al.*, 2019). They also showed that overexpression in PDAC cells resulted in promotion of cell proliferation, colony formation, cell invasion and migration. The encoded protein Sirtuin 3 (SIRT3) is found exclusively in mitochondria and eliminates reactive oxygen species and has effects on nuclear gene expression and has been related to cancer as a tumour suppressor (Kim *et al.*, 2010). They identified that SIRT3 was a direct target of miRNA-708-5p, and partly involved in miRNA-708-5p-mediated PDAC cells progression (Huang *et al.*, 2019).

Reduction of miR-29b expression in Mia PaCa-2 2D increased proliferation by 35% while reducing it by 12% in 3D (Figure 9 B & D). Numerous studies have shown that miR-29b functions as a tumour suppressor in PaC, one such study examined miR-29b in pancreatic cancer and revealed its diminished expression in pancreatic cancer tissues. In this study it

was observed that miR-29b targeted the 3'-UTR of DNA methyltransferase (DNMT) 3b, leading to an increase in its expression. The overexpression of miR-29b mimics had a significant impact, substantially hindering cell viability and promoting apoptosis in PANC-1 cells through the inhibition of DNMT3b (Wang *et al.*, 2018). In addition to this other research demonstrated that miR-29b suppressed proliferation, migration, and invasion by directly targeting SRY-Box Transcription Factor 12 (SOX12) and DNMT3b in pancreatic cancer cells, and DNMT3b might be a target gene of SOX12 (Wang *et al.*, 2019). Another study aimed to establish a lncRNA-miRNA-mRNA network potentially influencing PDAC cell proliferation and Gemcitabine-induced apoptosis, focusing on the cyclin-dependent (CDK) family member, CDK14 (Sun *et al.*, 2019). TCGA data showed a noteworthy positive correlation between the lncRNA MSC-AS1 and CDK14. In two PDAC cell lines, miR-29b-3p exhibited a negative regulatory impact on both MSC-AS1 and CDK14. They confirmed that MSC-AS1 functioned as a ceRNA for miR-29b-3p, thereby counteracting the miR-29b-mediated repression of CDK14. They demonstrated that MSC-AS1/miR-29b-3p axis modulates the cell proliferation and Gemcitabine-induced cell apoptosis in PDAC cell lines through CDK14 (Sun *et al.*, 2019).

One study showed that miR-206 directly targets the oncogenes KRAS and annexin a2 (ANXA2), acting as tumour suppressor in PDAC cells by blocking cell cycle progression, cell proliferation, migration and invasion (Keklikoglou *et al.*, 2015). Our results showed that in Mia PaCa-2 cells the overexpression of miR-206 reduced cell proliferation by 15% in 2D culture (Figure 9 B & D).

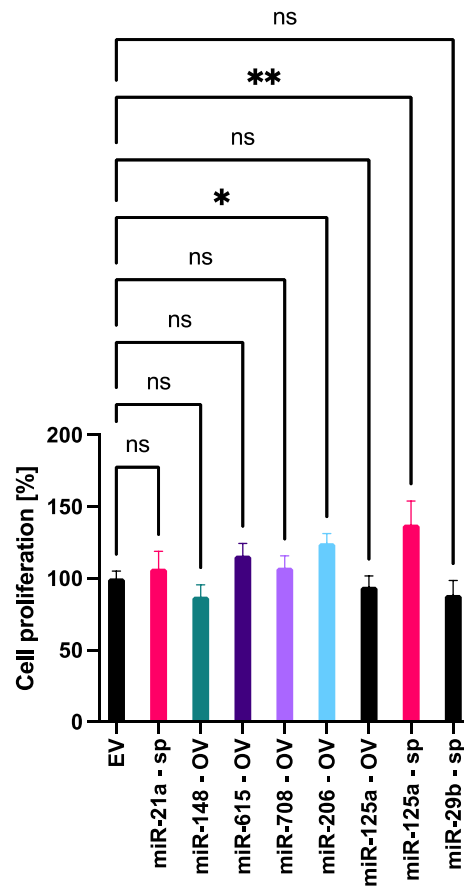
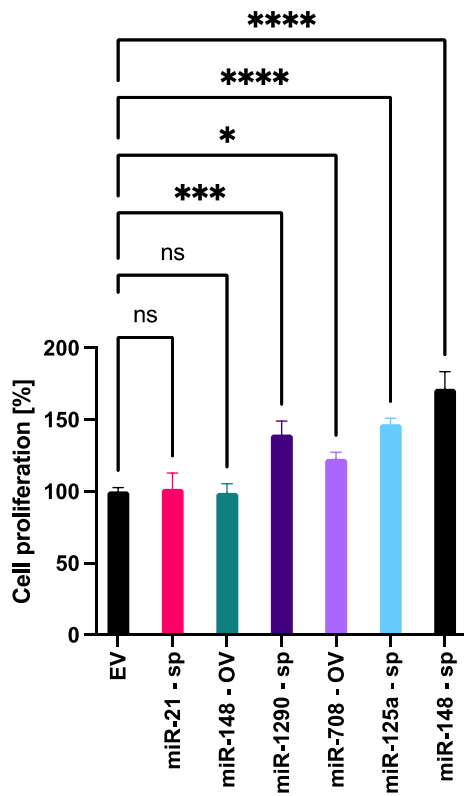
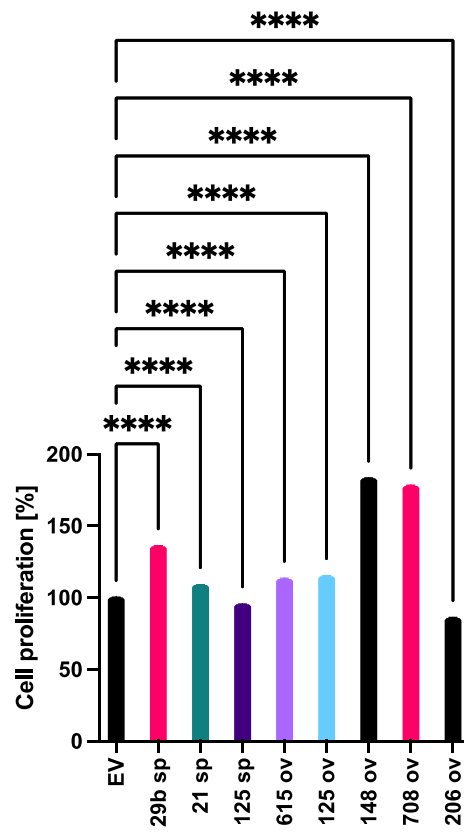
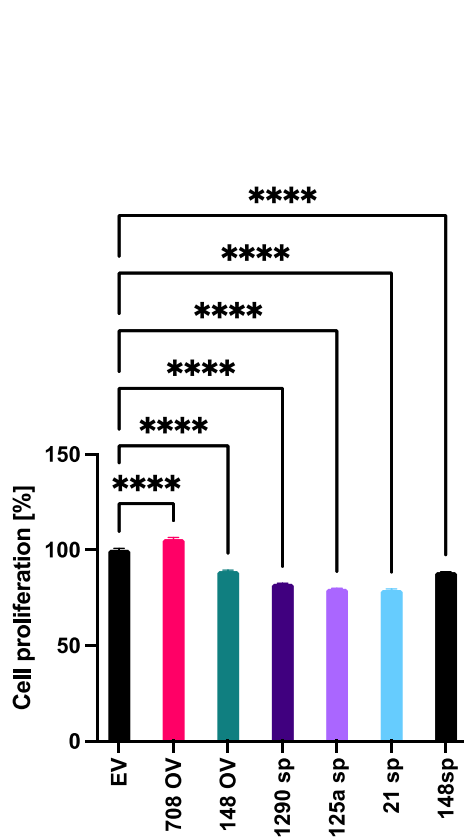


**Figure 9: Round 1 - 2D and 3D proliferation effect of miRNA panel in PANC-1 and MIA PaCa-2.**

Panel description: A. PANC-1 2D B. MIA PaCa-2 2D C. PANC-1 3D D. MIA PaCa-2 3D. Results represent three biological and statistical analysis was conducted in GraphPad Prism using One-Way Analysis of Variance (ANOVA) with Dunnett's Multiple Comparison Test and 95% Confidence Interval (CI). \* 0.0332, \*\* 0.0021, \*\*\* 0.0002, \*\*\*\* < 0.0001.

*3.5.3.4 Secondary validation of miR-21, -148, -1290 suggests regulatory roles in proliferation and anoikis resistance in pancreatic cancer cells*

Round 1 of proliferation assays highlighted miR-21, -148 and -1290 as miRNAs capable of reducing cell proliferation. As described in Table 5, a second round of assays without doxycycline pre-induction was being carried out to try reduce variation between biological replicates it was also decided that fewer targets would be carried into the second round of assays. For PANC-1 cells three targets were carried into the second round based on initial promise (Figure 10). In round 1 the overexpression of miR-148 and the sponging of miR-21 and -1290 resulted in reduced proliferation in 2D culture (Figure 9). Intriguingly, the sponging on miR-148 did not change the phenotype compared to the overexpression condition in 2D culture (Figure 9A). In 3D culture, miR-148 overexpression did not cause a reduction in cell proliferation compared to empty vector control but the sponging of this miRNA caused increased proliferation by 70% (Figure 9C). In 2D culture, miR-21 sponge caused a 20% reduction in cell proliferation, while miR-1290 sponge caused a 16% reduction in proliferation. These results were not mirrored in the 3D conditions. Round 2 of assays confirmed the reduction in cell proliferation seen for miR-148, -21 and -1290 in 2D conditions (Figure 10A). The overexpression of miR-148 caused the largest reduction in proliferation of 36.5%, followed by miR-21 spng (27.5%) and miR-1290 (16.3%) (Figure 10A). miR-148 was the only miRNA to maintain this phenotype in 3D culture with a reduction of 21.7% while the other two actually increased proliferation compared to control by 28% and 50% for miR-21 and -1290, respectively (Figure 10C). Round 2 of assays also included the addition of anoikis assays (Figure 11). Surviving detachment-induced cell death, commonly known as anoikis resistance, is increasingly recognised as a characteristic feature of metastatic malignancies. Overexpression of miR-148 resulted in a modest 4% decrease in anoikis resistance compared to control in PANC-1 cells (Figure 11A).

In Mia PaCa-2 cells miR-148 and -21 were also investigated with the addition of miR-615. Preliminary results in round 1 of assays suggested that overexpression of miR-148 and -615 and the sponging of miR-21 resulted in an increased proliferation in Mia PaCa-2 cells (Figure 9). However, round 2 which did not include pre-induction and had far less targets under investigation showed a clear reduction in proliferation similar to in the PANC-1 cells in 2D conditions (Figure 10B). For the overexpression of miR-148 and -615 this phenotype was mirrored in 3D culture with a reduced proliferation of 29 and 20%, respectively (Figure 10D). The overexpression of miR-148 resulted in a 12% decrease in anoikis resistance (Figure 11B). The overexpression of miR-615 caused a modest reduction of 3% while the sponging of miR-21 resulted in a 6% reduction in anoikis resistance compared to empty vector control (Figure 11B).

In summary round 2 confirmed the results observed for miR-21, -148 and -1290 in round 1 for PANC-1 cells with the sponging of miR-21 and -1290 and the overexpression of miR-148 resulting in reduced proliferation in 2D growth conditions. In addition, round 2 confirmed an opposing phenotypic effect for reduced miR-1290 expression resulting in a significant increase in proliferation in 3D growth conditions. Round 2 also confirmed that these miRNAs did not play such a strong oncogenic role in the MIA PaCa-2 cell line with most differences observed not reaching statistical significance. The one statistically significant observation being the overexpression of miR-148 in 3D conditions resulting in a decrease in cell proliferation which while not statistically significant was also observed in round 1.

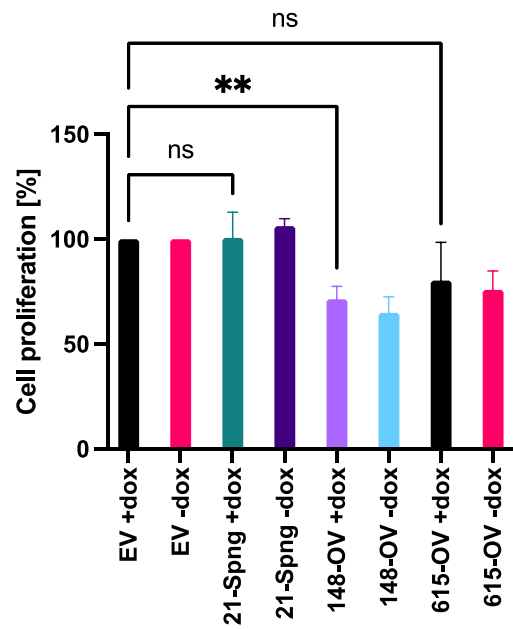
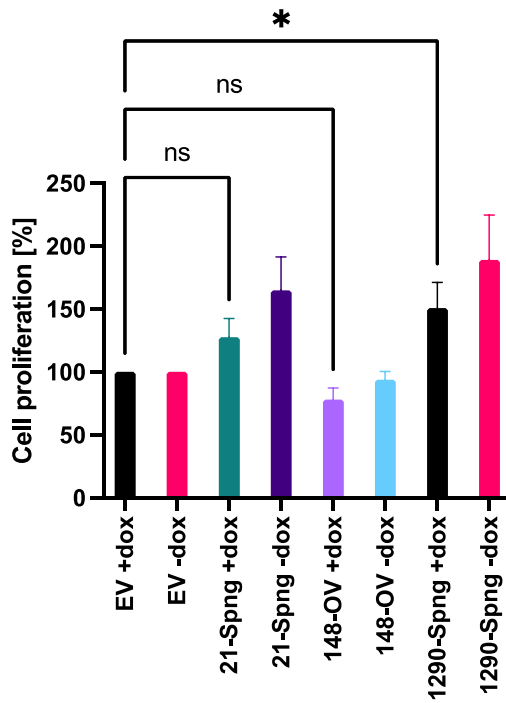
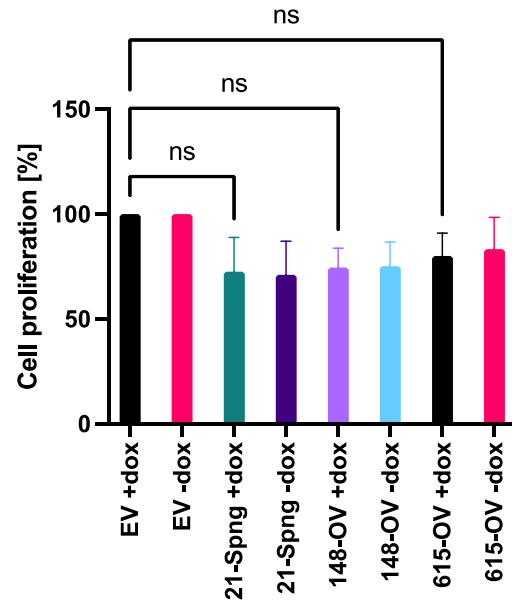
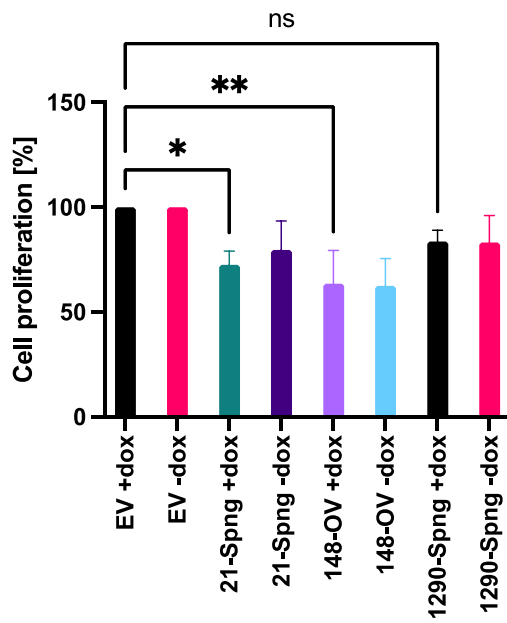


**Figure 10: Round 2 - 2D and 3D proliferation effect of miRNA panel in PANC-1 and MIA PaCa-2.**  
Panel description: A. PANC-1 2D B. MIA PaCa-2 2D C. PANC-1 3D D. MIA PaCa-2 3D. Each miRNA has a +/- doxycycline control. Results represent three biological and statistical analysis was conducted in GraphPad Prism using One-Way Analysis of Variance (ANOVA) with Dunnett's Multiple Comparison Test and 95% Confidence Interval (CI). \* 0.0332, \*\* 0.0021, \*\*\* 0.0002, \*\*\*\* < 0.0001.

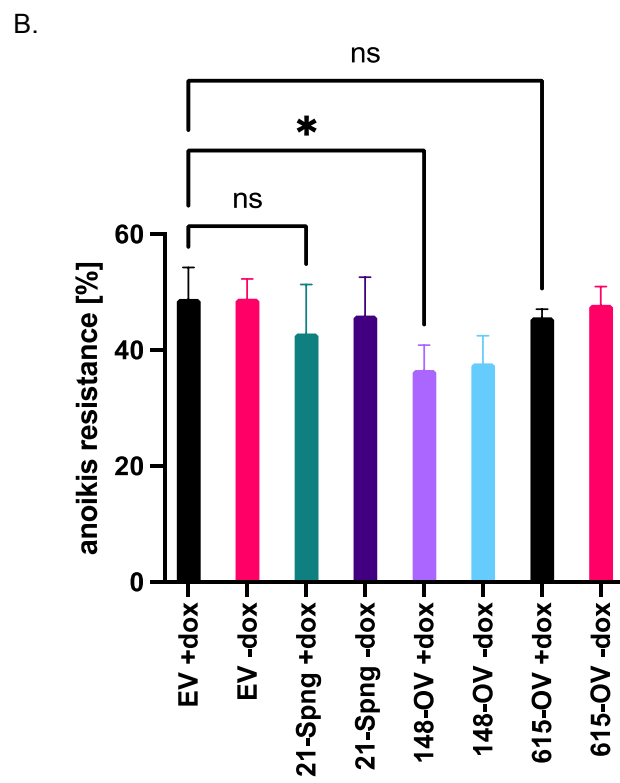
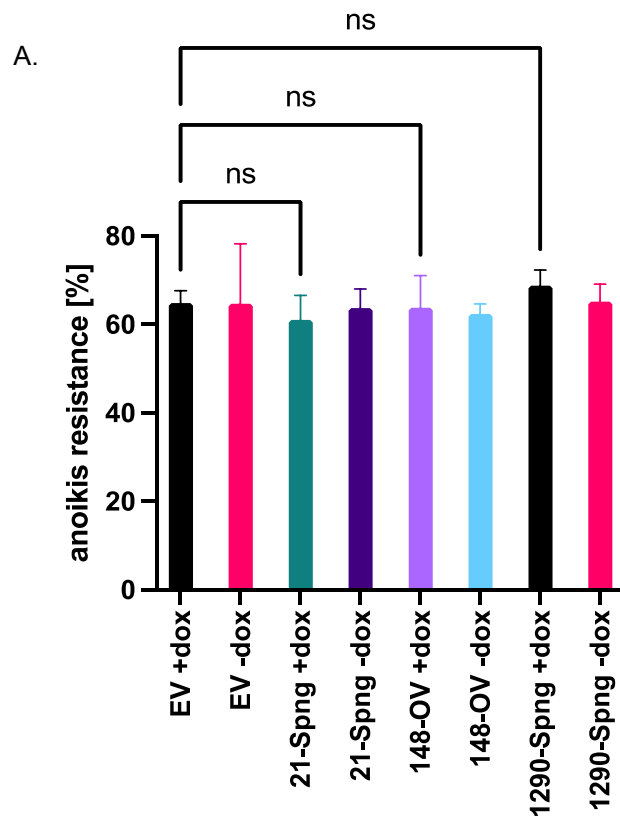


**Figure 11: Anoikis resistance of miRNA panel in PANC-1 and MIA PaCa-2.** Panel description: A. PANC-1 B. MIA PaCa-2 Results represent three biological and statistical analysis was conducted in GraphPad Prism using One-Way Analysis of Variance (ANOVA) with Dunnett's Multiple Comparison Test and 95% Confidence Interval (CI). \* 0.0332, \*\* 0.0021, \*\*\* 0.0002, \*\*\*\* < 0.0001.

The results observed here are in agreement with current literature for these miRNAs. One study showed that miR-21 targeted MAPK/ERK and PI3K/ATK signalling pathways to modulate cell proliferation. They showed that miR-21 promoted epidermal growth factor (EGF) induced proliferation, inhibited cell apoptosis and accelerated cell cycle progression (Zhao *et al.*, 2018). These results are in agreement with the effect of mi-21 sponging in PANC-1 and Mia PaCa-2 cells observed in this study.

It has been previously shown that serum levels of miR-1290 exhibited superior discriminatory power in identifying individuals with low-stage pancreatic cancer compared to CA19-9 levels. Similar to CA19-9, elevated miR-1290 levels were indicative of a less favourable prognosis for patients undergoing pancreaticoduodenectomy (Li *et al.*, 2013). NF-kB is consistently activated in most patients with PDAC and is associated with a KRAS mutation through Ikb kinase complex 1 (IKK1). One investigation explored the correlation between PDAC aggressiveness and miR-1290, and found that this miRNA played a role in promoting cancer aggressiveness by directly targeting IKK1. The combined impact of miR-1290 on the proliferation and metastasis of PDAC cells was dampened with IKK1 overexpression and heightened with IKK1 knockdown. Corresponding with these *in vitro* findings, a subcutaneous tumour mouse model demonstrated that miR-1290 significantly accelerated PDAC progression *in vivo* (Ta *et al.*, 2018). Again, this is in agreement with the results described in this study whereby the sponging on miR-1290 in PANC-1 cells reduced proliferation in 2D conditions. It was observed that the putative promoter region of miR-615-5p was hypermethylated, resulting in its silencing within PDAC cell lines (Gao *et al.*, 2015). Furthermore, the elevated expression of miR-615-5p in pancreatic cancer cells effectively inhibited cell proliferation, migration, and invasion (Gao *et al.*, 2015). Another study showed that Caudal Type Homeobox 2 (CDX2) inhibited PDAC cell proliferation via promoting tumour suppressor miR-615-5p (Jiang *et al.*, 2016). This tumour suppressor activity is also observed in the data described in this study where the overexpression of miR-615 in Mia PaCa-2 cells reduced proliferation.

Interestingly, the upregulation of miR-148 emerged as the sole miRNA effectively diminishing anoikis resistance in both cell lines. This provides one explanation for why miR-148 is the only miRNA associated with significantly reduced proliferation in 3D culture. Cell attachment to the extracellular matrix (ECM) is pivotal for structural stability and overall cellular well-being. ECM detachment in tumours precedes the abnormal dissemination of tumour cells, ultimately leading to metastasis. Programmed cell death, specifically "anoikis," is triggered during tumourigenesis to eliminate ECM-detached cells. Cancer cells deploy various mechanisms to thwart anoikis, thereby fostering increased proliferation, invasion, and eventual metastasis (Adeshakin, 2021). This is also in agreement with previous work that has shown that miR-148 significantly inhibited cancer cell migration and invasion and can regulate the expression of numerous oncogenes (Idichi *et al.*, 2018).

#### 3.5.3.5 Challenges in Vector Systems: Addressing Technical Hurdles, Leaky Vectors, and Disparities in 2D vs. 3D Cultures

Amidst the discussion of challenges posed by the empty vector, two additional hurdles emerged during these assays: leaky vectors and disparities between 2D and 3D cultures. The observed differences between +/- dox, while not substantial, were noteworthy. Live imaging revealed a significant level of leakiness, which while not a problem for assay outcome since none were dependent on time sensitive induction of the miRNA expression, prompt future steps. qPCR comparison between both +/- doxycycline conditions to assess expression levels in relation to the observed phenotypic effects may yield interesting insight. The TET-On system was created by random mutagenesis of the tetracycline repressor gene (tetR), identifying the sequence responsible for tetracycline operator (tetO) binding (Hecht, Müller and Hillen, 1993). The modified tetracycline-controlled trans-activator (tTA) was named reverse tT (rtTA) as it works in an opposing manner. The presence of Dox enables rtTA to bind to the tetO and up-regulate transcription in a dose dependant manner. Leaky expression from TET promoters is well documented (Gossen and Bujard, 1992; Gossen *et al.*, 1995). A study by Costello *et al.* addressed this by reducing the number of miRNA response elements (MRE) and the incorporation of an acting transcriptional repressor domain, however, leaky expression still persisted (Costello *et al.*, 2019). The leakiness of inducible systems may explain the variability between biological replicates using pre-induced cells and in particular the lack of phenotypic difference observed between the +/- doxycycline state.

Another notable observation from these numerous assays was inconsistency between 2D and 3D culture conditions. Though differences in both conditions had been described previously, many assays documented opposing phenotypes in 2D versus 3D conditions. Round 1 of 3D assays ran for a total of 8 days, with cells induced on day 1 after seeding. Round 2 of 3D assays ran for 10 days, inducing on day 3 after seeding to allow sufficient spheroid formation before induction. Since the cells used here were stably transfected and underwent hygromycin selection before spheroid formation, the entire spheroid should represent miRNA expression level modulation, not just the outer cells, as may be the case with transient transfections of spheroids. While doxycycline might not have penetrated the entire spheroid, consistent vector leakiness suggested cells not exposed to doxycycline in the centre likely still produced significant overexpression or sponging.

Although 3D models were more representative of in-vivo conditions, various 3D methodologies exhibited inferior efficacy, duration, reproducibility, and convenience compared to 2D systems (Kapalczyńska, 2018). Future investigations into these miRNA targets in 3D culture could employ different types of 3D models and various assay quantification reagents. While this study used AlamarBlue® Cell Viability reagent, the CellTiter-Glo® 3D Cell Viability Assay, noted for its ability to penetrate large spheroids with increased lytic capacity, might offer more accurate viability determination for future work.

#### *3.5.3.6 Exploring the miRNA mimic-mediated effects of miR-29b, -378e, -1290, -2467, and -6831 on phenotype and transcriptomic analysis in pancreatic cancer cells*

As successful stable overexpression vectors could not be generated for miR-29b, -378e, -1290, -2467, and -6831 their phenotypic and transcriptomic effects were investigated using miRNA mimics. Their expression levels were validated by RT-qPCR (Figure 12). The proliferative effects of these miRNAs were tested in 2D cultures of PANC-1 and Mia PaCa-2 cells (Figure 13). miR-29b showed no significant effect on proliferation in either cell line. It should be noted that no effect was statistically significant in the MIA PaCa-2 cell line. The overexpression of miR-378e resulted in reduced proliferation with a 37% reduction in PANC-1. miR-378e was the miRNA with the biggest effect on proliferation in PANC-1 cells (Figure 13). miR-1290 overexpression resulted in approximately 20% reduction in proliferation in both cell lines but was only statistically significant in PANC-1. miR-2467 reduced proliferation in PANC-1 cells with a 17% reduction (Figure 13A). miR-6831 overexpression resulted in a 33% reduction in cell proliferation in PANC-1 cells (Figure 13A). While a 26% reduction in proliferation is observed in Mia PaCa-2 cells it is not statistically significant as the standard

deviation is much higher than in PANC-1 cells and future work should address repeating this assay. To compliment the phenotypic assays and to understand if any gene expression programs were controlled by these miRNAs in pancreatic cancer cells global transcriptional analysis using QuantSeq 3' mRNA-Seq was performed in PANC-1 cells (Moll, 2014). The phenotypic and transcriptomic effects of miR-6831 form the basis of paper 4 and are discussed extensively there. This section addresses the findings for the other four miRNAs. Before delving into the results, the background and benefits of the QuantSeq technique will be briefly discussed. This method is a form of targeted RNA-seq that focuses on sequencing only the 3' end of transcripts as opposed to traditional RNA-seq where the entire length of the transcript is sequenced (Moll, 2014). As a result, QuantSeq requires a lower sequencing depth and is, therefore, more cost-effective. The targeted nature of QuantSeq does limit its use to measuring gene expression as unlike traditional RNA-seq it is not capable of detecting novel transcripts, splice variants and non-coding RNAs. Transcriptomic analysis revealed that of miR-29b, -378e, -1290 and -2467, miR-2467 was the only other miRNA to cause significant gene expression changes in PANC-1 cells, outside of miR-6831-5p. The top 10 up- and down-regulated genes are illustrated on the volcano plot (Figure 14B). The gene ontology analysis for both molecular function and biological processes of the up-regulated genes suggest a change in nuclear chromosome segregation and mitotic nuclear division being top hits for biological processes (Figure 15). In addition, chromatin remodelling and cell cycle control with terms such as histone, nucleosome and chromatin DNA binding being highly significant molecular function related terms (Figure 16).

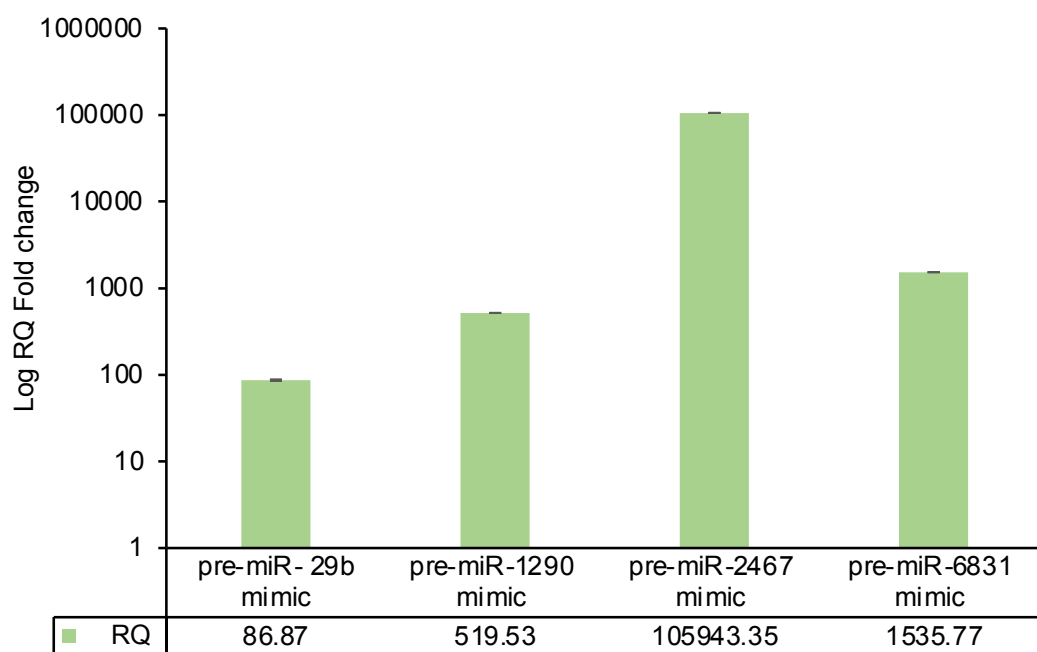


**Figure 12: Validation of miRNA mimics with RQ fold change values.** Data represents three biological replicates. The RQ fold change values calculated based on the delta delta ct method.

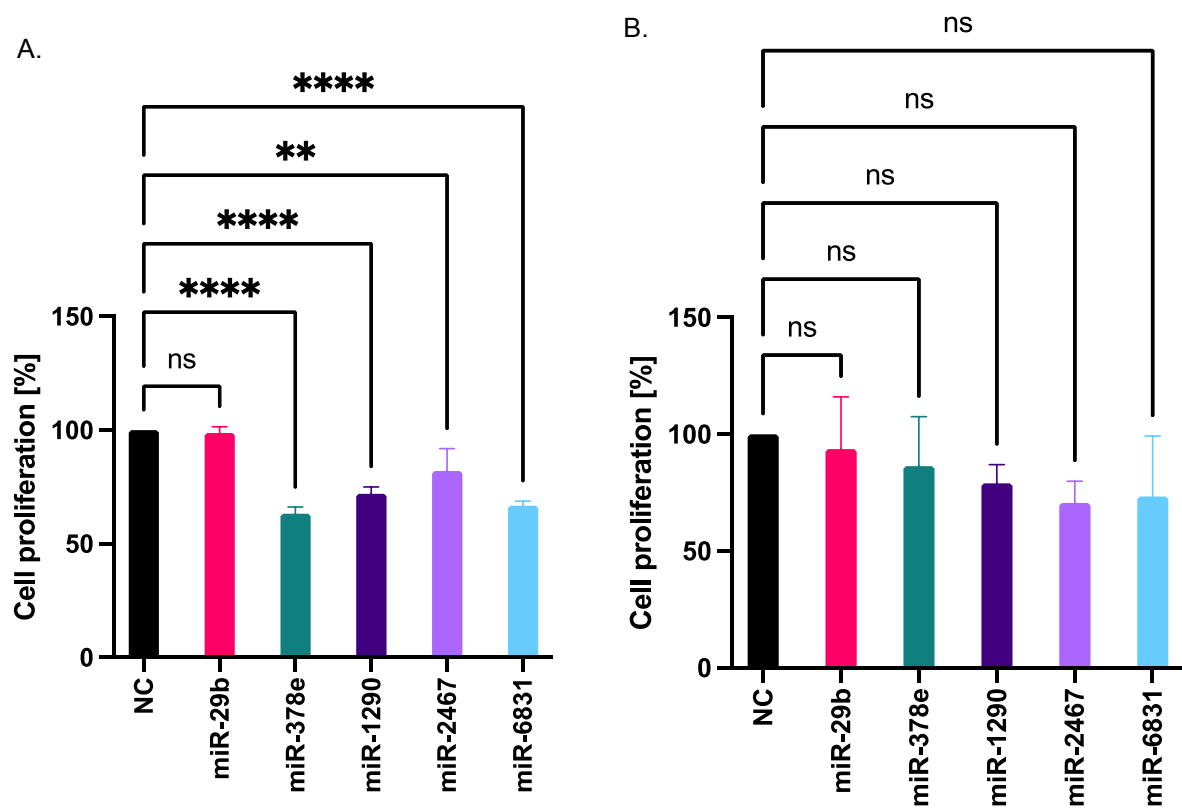
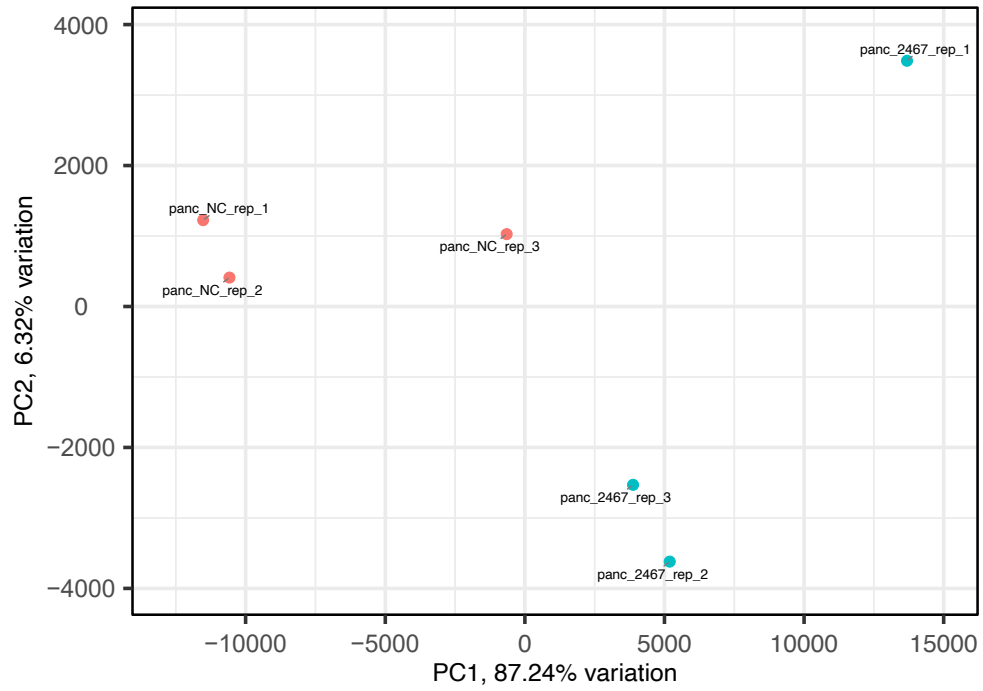


**Figure 13: 2D proliferation effect of miRNA mimics in PANC-1 and MIA PaCa-2.** Panel description: A. PANC-1 B. MIA PaCa-2. Results represent three biological and statistical analysis was conducted in GraphPad Prism using One-Way Analysis of Variance (ANOVA) with Dunnett's Multiple Comparison Test and 95% Confidence Interval (CI). \* 0.0332, \*\* 0.0021, \*\*\* 0.0002, \*\*\*\* < 0.0001.

A.



B.

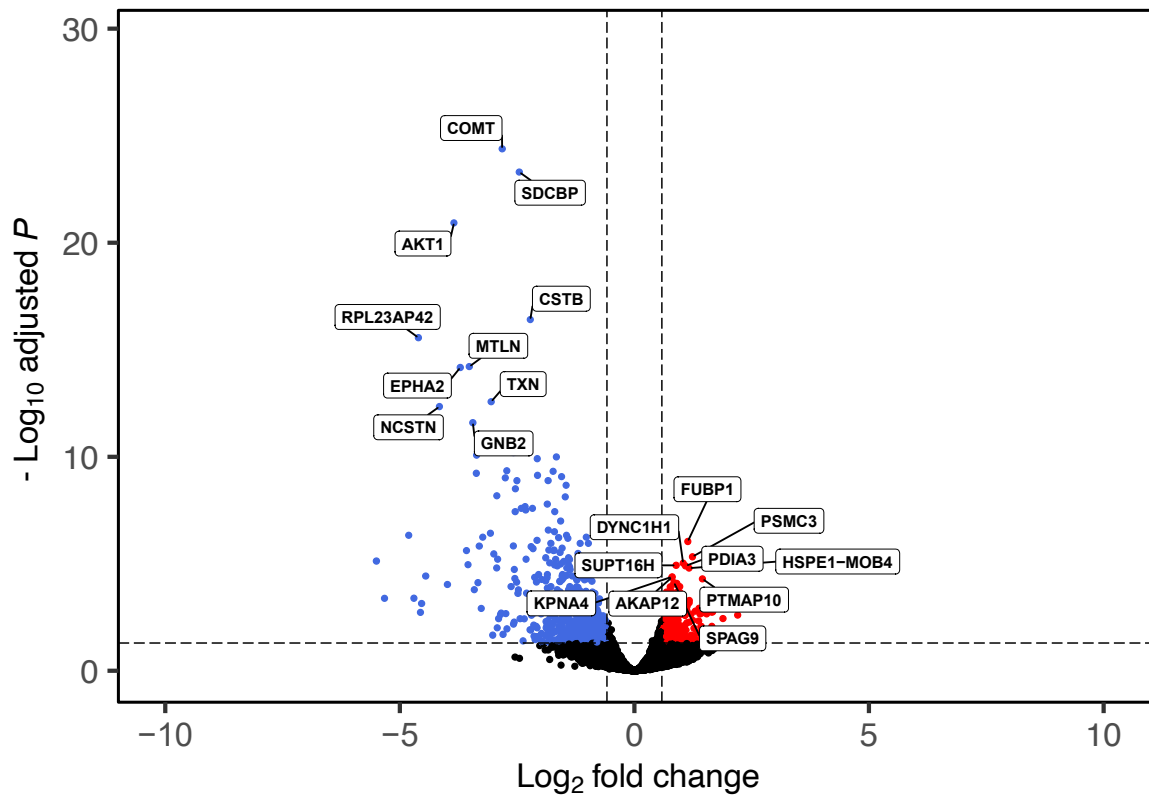
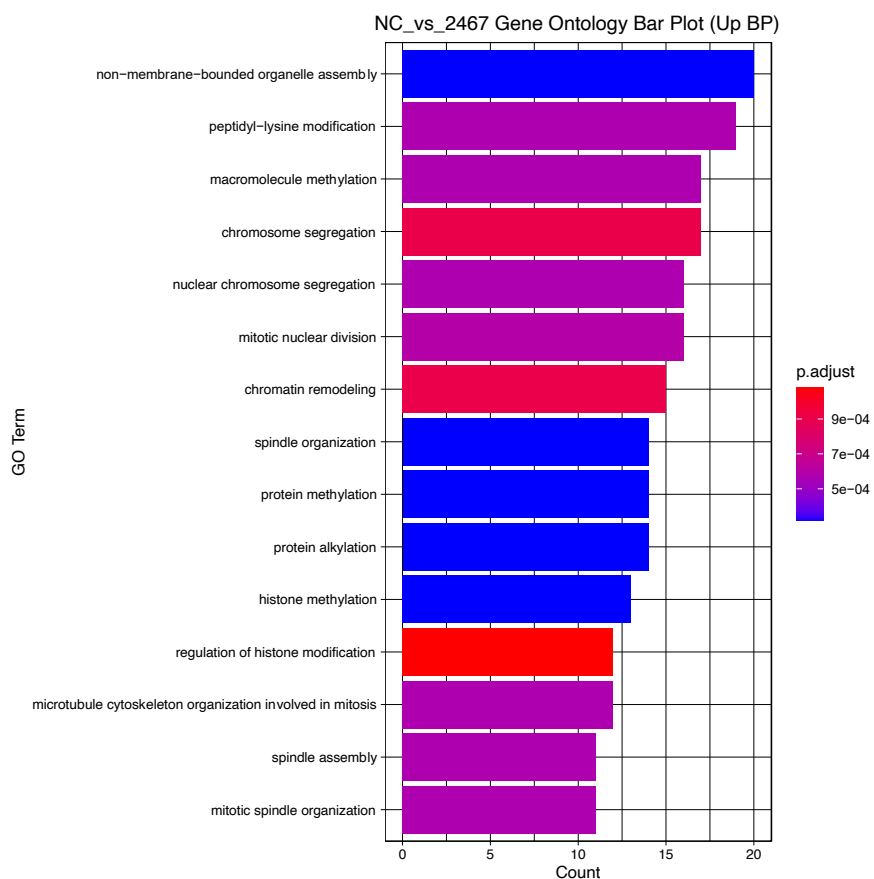


**Figure 14: Differential Expression of genes in miR-2467 overexpressing PANC-1 cells.** A. PCA plot. B. Volcano plot depicting differentially expressed genes identified by QuantSeq 3' mRNA-Sequencing in negative control vs. miR-6831 overexpressing PANC-1 cells. Red dots represent upregulated genes while blue dots represent downregulated genes in response to miR-6831 overexpression. Y-axis denotes  $-\log_{10}$  P values while X-axis shows  $\log_2$  fold change values. The R package EnhancedVolcano was used to generate volcano plot visualisation.

A.



B.

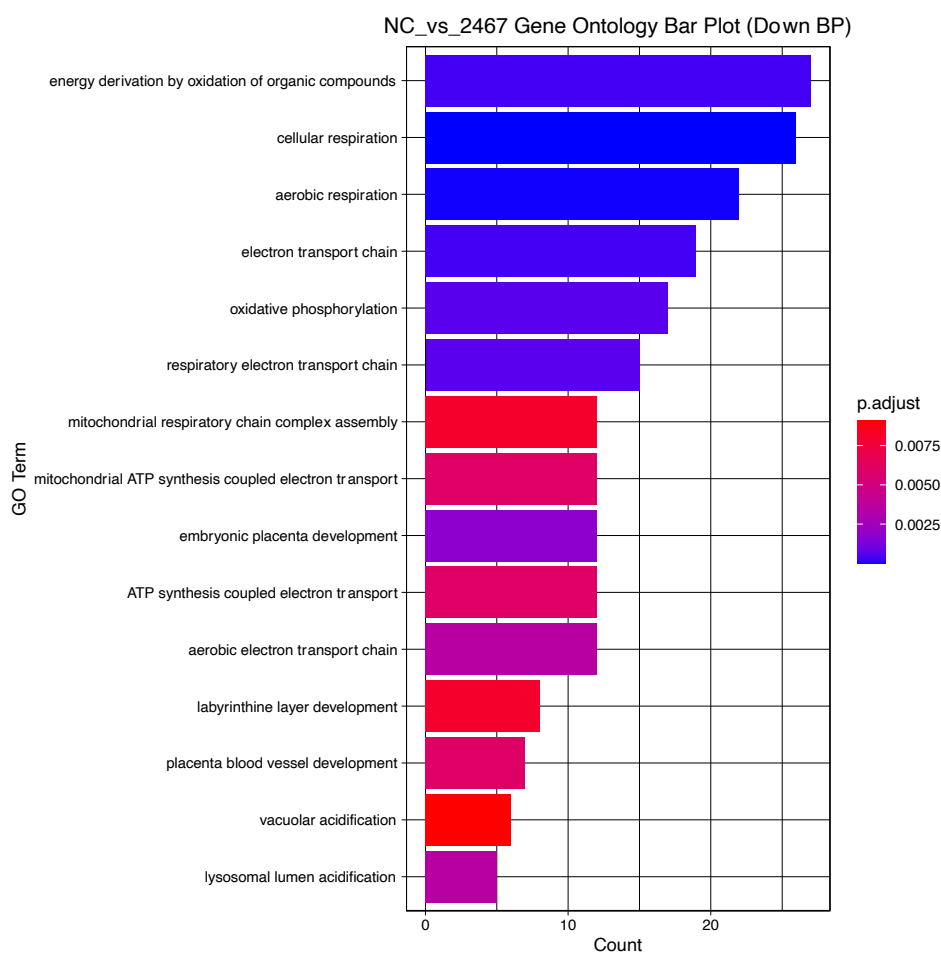
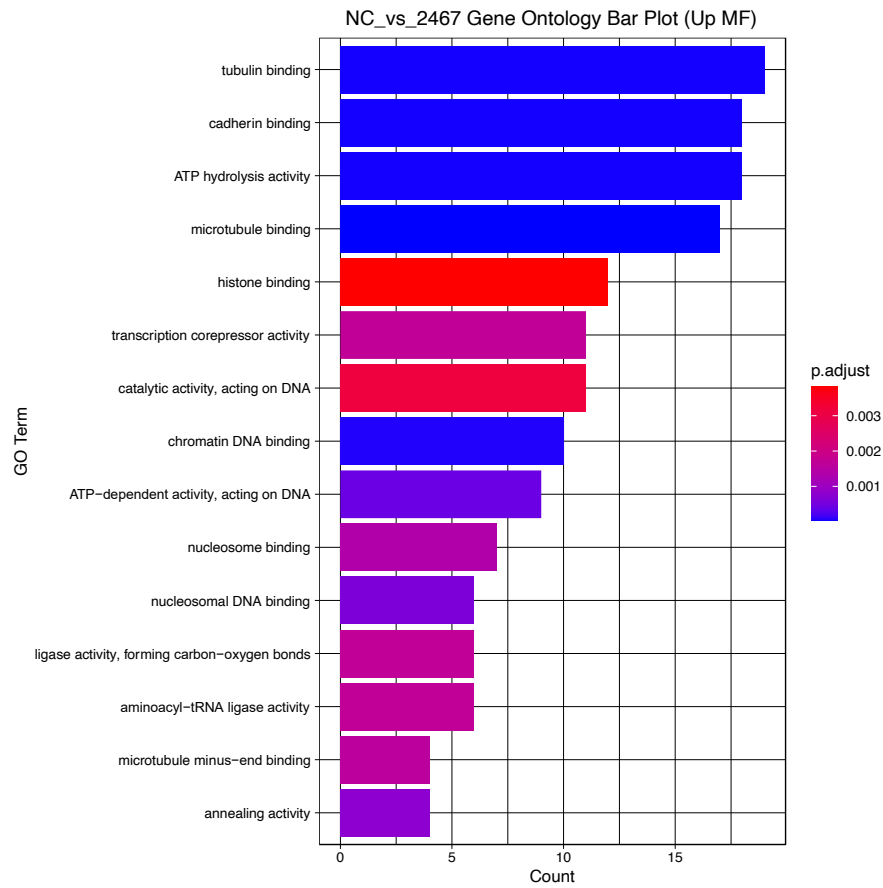


**Figure 15: Gene Ontology analysis of differentially expressed genes.** Biological processes-related pathways altered in PANC-1 cells overexpressing miR-2467. Y-axis Labels represent pathway names and significant enrichment is shown on X-axis. The count and colour of the bar represent the number of genes enriched in each pathway and their significance. Gene Ontology (GO) analysis was analysed by ShinyGo functional annotation to produce gene clusters ( $\geq 2$  genes/cluster) Panel descriptions: A. Biological processes of up-regulated genes. B. Biological processes of down-regulated genes.

A.



B.

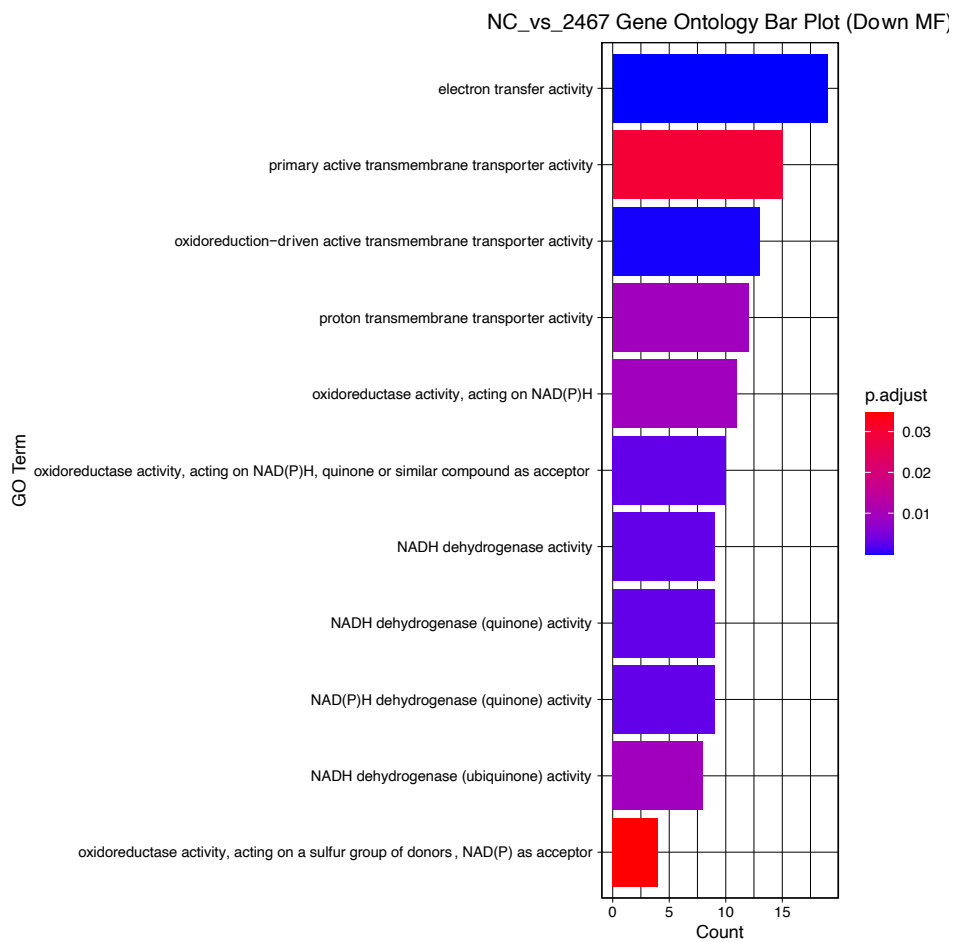
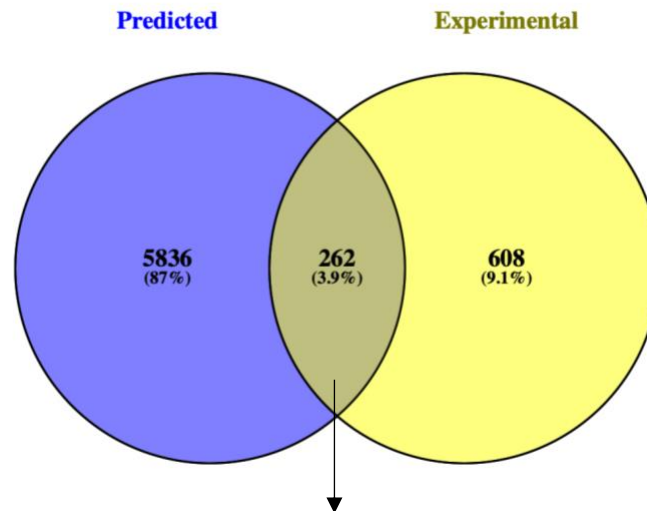


**Figure 16: Gene Ontology analysis of differentially expressed genes.** Molecular function related pathways altered in PANC-1 cells overexpressing miR-2467. Y-axis Labels represent pathway names and significant enrichment is shown on X-axis. The count and colour of the bar represent the number of genes enriched in each pathway and their significance. Gene Ontology (GO) analysis was analysed by ShinyGo functional annotation to produce gene clusters ( $\geq 2$  genes/cluster) Panel descriptions: A. Molecular function of up-regulated genes. B. Molecular function of down-regulated genes.

As miR-2467-3p is not a very well described miRNA with only 15 hits on PubMed with the earliest publication being in 2020, the gene expression changes described here were overlapped with TargetScan predicted targets to gain further insight into the molecular targets of this miRNA. Of the 6098 predicted targets, 262 overlapped with our experimentally validated 870 mRNA targets (Figure 17). To gain a better understanding of the predicted and experimentally overlapped genes, the gene ontology was investigated. The biological processes related GO terms regulation of actin filament and cytoskeleton organisation were among the top hits (Figure 18), while GO KEGG analysis showed metabolic pathways, cellular senescence, miRNAs in cancer and TNF signalling as top hits (Figure 19). The manipulation of endogenous targeting following miR-2467 overexpression offered a valuable opportunity to validate target predictions. Among the myriad prediction tools available (John *et al.*, 2004), such as miRBase Targets, miRanda, PicTar, PITA, and TargetScan, we selected TargetScan for its ability to identify messages with at least one 3'-UTR 7-8mer site conserved across mammalian species, defined as sites preserved in orthologous locations of human, mouse, rat, and dog UTRs (Agarwal *et al.*, 2015). Notably, other algorithms often include sites with mismatches or wobbles to the seed region, potentially compromising their predictive accuracy. For instance, predictions from miRBase Targets are generated using the miRanda algorithm and allow for one mismatch or wobble to the seed (John, Sander and Marks, 2006). Despite the performance of TargetScan, our findings reveal that approximately 87% of its predicted targets fail to respond to miR-2467 overexpression in PANC-1 cells, indicating a potential false-positive rate. It's worth noting that our study was limited to PANC-1 cells, and a broader panel of cell lines encompassing various cancer and non-disease cell types would offer a more comprehensive assessment of prediction specificity.

The phenotypic analysis of miR-2467 overexpression in both PANC-1 and Mia PaCa-2 cell lines revealed distinct outcomes. In Mia PaCa-2 cells, no significant effect was observed, contrasting sharply with the notable 20% decrease in proliferation observed in PANC-1 cells. Interestingly, while overexpression of miR-378e and -1290 did not induce any detectable changes in gene expression in either cell line, a significant reduction in PANC-1 proliferation was observed. This discrepancy suggests that the impact of miR-378e and -1290 on proliferation might be mediated through mechanisms other than direct gene expression modulation. Further investigations are warranted to elucidate the underlying mechanisms contributing to the observed proliferation changes. One possible avenue of exploration involves examining alterations in protein expression profiles or potential cross-talk with other miRNAs. These avenues of research will be pursued in future studies to gain a deeper understanding of the molecular mechanisms driving the observed phenotypic effects.

A.



B.

|            |          |          |          |         |          |
|------------|----------|----------|----------|---------|----------|
| TNS3       | FER      | KPNA4    | KLHDC3   | LRRC47  | CALM2    |
| SYVN1      | DPF2     | CNIH4    | PDXDC1   | LIX1L   | MAPKAPK5 |
| CX3CL1     | RAB1B    | ZDHHC8   | CTDSP2   | ARAP1   | TMEM242  |
| GPATCH8    | VPS13C   | ZFAND3   | CCDC115  | COMT    | CENPF    |
| ATP2A2     | LTBP1    | NIN      | CD164    | WDHD1   | PRPF4B   |
| ISY1-RAB43 | SLC39A13 | OLFML2A  | INPP5E   | CACNG4  | DERL1    |
| TTYH3      | RBBP4    | MFHAS1   | CTS2     | MPZL1   | WDR26    |
| CTNND1     | MYEF2    | WDR5     | CEP44    | EIF1AD  | ABCF3    |
| TMED4      | ACTN4    | KANSL3   | NRAS     | HMGN4   | ARSB     |
| PACS1      | MAP2K3   | BCL7A    | PRRC2C   | IMPA1   | PNRC1    |
| HYOU1      | NRARP    | STIL     | ARHGAP29 | CCDC47  | ID1      |
| KANSL1     | TM9SF4   | RPAP3    | TRIP12   | NDUFB4  | CDS2     |
| TNFAIP2    | BMF      | PGAM1    | SOCS3    | OCRL    | FANCM    |
| GON4L      | ZNF609   | SPIN1    | CDC5L    | ICMT    | SLAIN2   |
| ERC1       | MAFG     | CLU      | FCF1     | SUPT16H | IL17RB   |
| GSR        | PREX1    | KATNB1   | BRD2     | FUBP1   | PSMD11   |
| EIF3M      | CCNF     | BAZ1B    | MRPL10   | CLIC4   | FNBP4    |
| TPM3       | SREBF1   | OSMR     | USB1     | TPBG    | LZTFL1   |
| WNT9A      | RGP1     | IGF2R    | NT5E     | SNAI1   | TSFM     |
| ABL1       | HBS1L    | PCNA     | MANEAL   | FBXO3   | PSMA5    |
| RHOA       | MTHFD2   | CENPV    | SLC35F6  | ELK1    | UPF3B    |
| FAM168B    | PHC3     | ZFR      | SWAP70   | RBM38   | SOX18    |
| DST        | THAP4    | PXN      | ARF5     | FBXO46  | RNF214   |
| SLC41A3    | KMT2C    | THAP11   | ITPR2    | IL11RA  | ZNF706   |
| NMT1       | PGAM5    | HOXB8    | CCZ1     | SPECC1  | ENOSF1   |
| SLC25A22   | FMR1     | PLCE1    | CCZ1B    | RPA1    | YIF1A    |
| CPSF7      | MARK2    | TXNDC5   | PLP2     | DTD1    | ANXA4    |
| RDX        | PPFIA1   | RNF14    | TMEM50A  | PLAU    | CYB5A    |
| ZNF793     | TRPC4AP  | CERS6    | INF2     | HNRNPH3 | NDUFC1   |
| GNE        | CTF1     | CCNY     | RNPEP    | G6PC3   | MRPL36   |
| SLC35B2    | MYD88    | CHEK1    | C5orf15  | HS6ST1  | TRAF4    |
| MYO5A      | B4GALT4  | KCTD7    | CEBPB    | MATR3   | HIRIP3   |
| KDM5A      | RAB1A    | RACGAP1  | ZCCHC2   | KIF2A   | CAPZB    |
| MED13L     | WRAP73   | CSF1     | SGSH     | COTL1   | GMPR     |
| TRIOBP     | PKN3     | CDC42SE1 | ABCF1    | STAMBP  | CRYZ     |
| PDGFB      | ARF1     | DDB1     | AP2B1    | RFT1    | UBE2V2   |
| PHC2       | EZH1     | MAPK8IP2 | HDAC7    | FXR1    | C11orf58 |
| TMEM123    | KIF18B   | PODXL    | CHST14   | WBP1L   | COX7A2L  |
| SOX13      | REPIN1   | PIK3R3   | FBXL12   | KDM5B   | HAGH     |
| FNBP1L     | ZDHHC5   | ARFGEF2  | SPNS2    | DCTD    | NQO1     |
| SYPL1      | CSTB     | WTIP     | SRI      | SRP9    | ZFP36L2  |
| DPM1       | PHF5A    | NCKAP1   | RWDD1    |         |          |
| PPCS       | HOXB2    | ALG11    | LMNB2    |         |          |
| COA4       | ENY2     | PITPNC1  | NIF3L1   |         |          |
| PRPF6      | UBE2T    | EMC7     | NR2F6    |         |          |

**Figure 17: Overlap between predicted and experimental miR-2467 targets.** Predicted targets were taken from TargetScan and overlap between predicted with mRNA was generated using Venny 2.0 (A) with shared targets listed below Venn diagram (B).

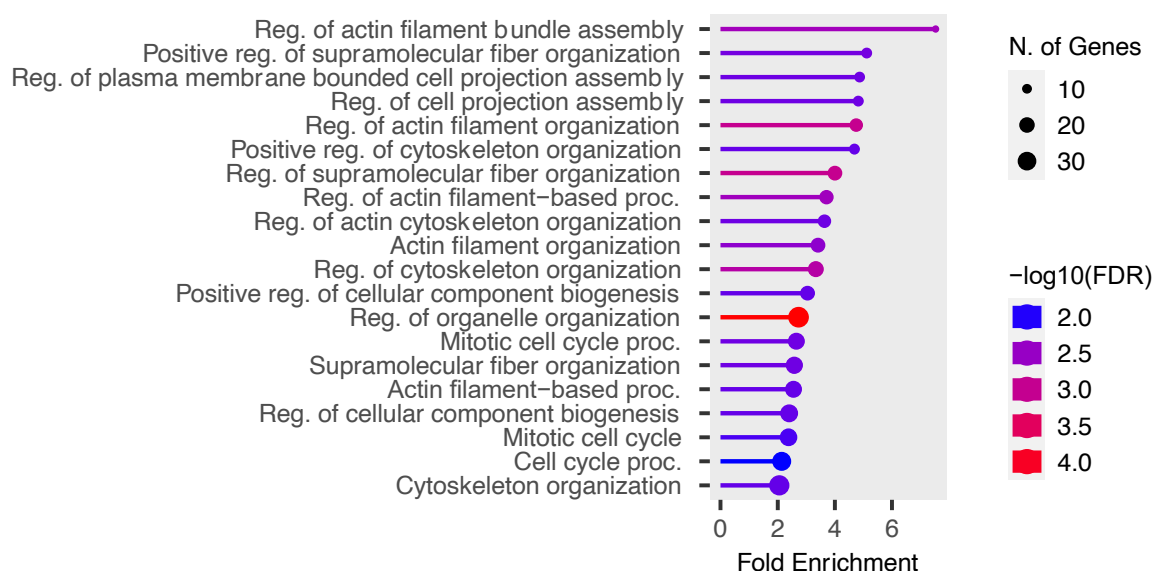


**Figure 18: Gene Ontology analysis of overlapped predicted versus experimental differentially expressed genes.** Advanced bubble plot depicts biological processes-related pathways altered in PANC-1 cells overexpressing miR-2467. Y-axis Labels represent pathway names and enrichment score is shown on X-axis. The size and colour of the bubble represent the number of genes enriched in each pathway and their enrichment significance. Gene Ontology (GO) analysis of the overlapped predicted and experimental DE genes ( $\geq 1.5$ -fold,  $P \leq 0.05$ ) was analysed by ShinyGo functional annotation to produce gene clusters ( $\geq 2$  genes/cluster).

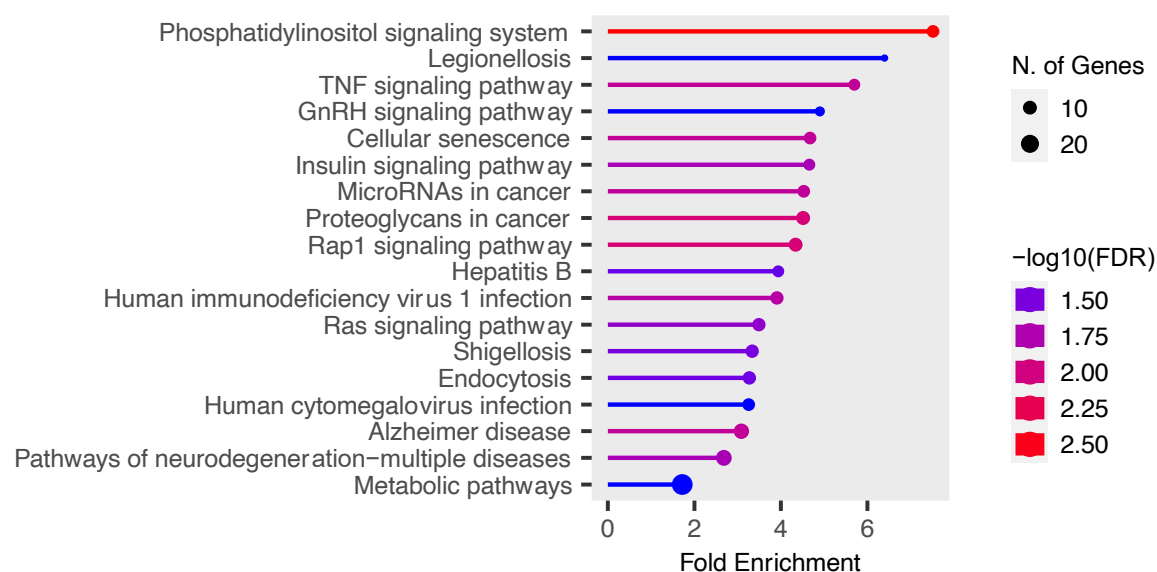


**Figure 19: Gene Ontology analysis of overlapped predicted versus experimental differentially expressed genes.** Advanced bubble plot depicts KEGG pathways altered in PANC-1 cells overexpressing miR-2467. Y-axis Labels represent pathway names and enrichment score is shown on X-axis. The size and colour of the bubble represent the number of genes enriched in each pathway and their enrichment significance. Gene Ontology (GO) analysis of the overlapped predicted and experimental DE genes ( $\geq 1.5$ -fold,  $P \leq 0.05$ ) was analysed by ShinyGo functional annotation to produce gene clusters ( $\geq 2$  genes/cluster).

### 3.5.4 Conclusion

In conclusion, this chapter highlights the molecular challenges associated with stable miRNA generation, including issues such as leaky vectors and empty vectors, which can lead to unintended phenotypic effects. However, these challenges can be effectively addressed through the use of miRNA mimics. The results presented herein demonstrate that overexpression of miR-2467 in PANC-1 cells, as analysed by 3' QuantSeq RNA sequencing, has discernible effects on gene expression. Although there is only a modest overlap with predictive target software such as TargetScan, this analysis provides valuable insights into potential targets of miR-2467. Moreover, miR-2467 overexpression has been shown to reduce proliferation in PANC-1 cells, indicating its functional relevance.

Furthermore, this chapter lays the groundwork for further investigation into miR-2467, as well as other miRNAs like miR-378e and miR-1290. Despite not eliciting significant changes in gene expression, miR-378e and miR-1290 demonstrate a reduction in proliferation, suggesting potential post-transcriptional or post-translational regulatory roles. This finding paves the way for future proteomic and small RNA analyses, as well as additional phenotypic assays, to gain a more comprehensive understanding of the mechanistic underpinnings and functional implications of these miRNAs in pancreatic cancer biology.

## **4. Future Directions**

The potential for future work in pancreatic cancer research is vast and holds significant promise for advancing both our understanding and treatment of this devastating disease. Building upon the findings detailed in previous chapters, several key areas offer rich avenues for exploration, each with the potential to make a meaningful impact.

### ***4.1 FOLFIRINOX Pharmacodynamic Interactions in Pancreatic Cancer***

Future research could delve deeper into the temporal dynamics of drug-drug interactions within the FOLFIRINOX regimen, which is crucial due to the treatment's cyclical and precisely timed nature. By exploring how the timing of drug administration affects the antagonistic interactions described in this study, this may uncover more effective treatment protocols. Furthermore, examining cell cycle distribution in conjunction with proliferation data might offer deeper insights into the mechanisms at play. The study's use of the Bliss Independent model could be enhanced by incorporating an additional interaction parameter, to address these temporal aspects of treatment (Lin *et al.*, 2021). This fourth parameter could also be adapted to further incorporate the effects of miRNAs on drug-drug interactions. By integrating miRNA influence into this pharmacodynamic model, researchers could gain a more nuanced understanding of how miRNAs modulate the efficacy of chemotherapeutic regimens. This approach could refine the modelling process and guide the development of more effective and personalised treatment strategies.

Translating these findings from *in vitro* to *in vivo* models is another critical step. Investigating these interactions in pancreatic cancer mouse models, especially focusing on treatment-mediated changes in tumour volume, could provide more clinically relevant data. Moreover, adapting a physiologically-based pharmacokinetic and pharmacodynamic model, like the one used by Zhu and colleagues for other drugs (Zhu *et al.*, 2018), could offer a more comprehensive understanding of FOLFIRINOX's effects, ultimately informing better treatment strategies that take into account both temporal dynamics and miRNA interactions.

### ***4.2 miRNA and Protein Targets in Pancreatic Cancer***

The exploration of miRNA and protein interactions opens another promising frontier. Future research could involve constructing and analysing regulatory networks involving miRNAs, mRNAs, and proteins to identify key regulatory hubs and potential therapeutic targets. This

approach could be bolstered by pathway enrichment analyses, shedding light on the biological processes common to different data sets. Furthermore, investigating the clinical relevance of identified miRNAs by correlating their expression profiles with patient outcomes and treatment responses could validate their potential as biomarkers or therapeutic targets.

### **4.3 miRNA Processing and Maturation**

- Future studies could also address the miRNA-specific constraints on precursor miRNA processing. High-throughput enzymology assays might identify optimal miRNA structures for more efficient processing (Nguyen *et al.*, 2022), while investigating the potential dysfunctions in key processing machinery could clarify why certain miRNAs fail to mature properly (Chendrimada *et al.*, 2005) (Lee *et al.*, 2006), (Davis and Hata, 2009). Exploring the dysregulation of miRNA degradation machinery, such as target-directed miRNA degradation (TDMD), could provide further insights into miRNA stability and function (Kim and Pak, 2020). Applying these findings to stable cell lines using innovative strategies, like the mMIRs expressing vectors (Fan *et al.*, 2020), could help overcome current challenges in miRNA research.

### **4.4 Role of miR-6831-5p in Pancreatic Cancer**

The identification of miR-6831-5p in pancreatic cancer and its role in metabolism and hypoxia presents another intriguing area for further investigation. Future experiments could focus on understanding its impact on mitochondrial function, ATP production, and reactive oxygen species (ROS) generation. Investigating the relationship between miR-6831-5p and hypoxia, particularly in the context of gemcitabine treatment, could provide valuable insights into its therapeutic potential. Moreover, exploring the combined effects of miR-6831-5p with other drugs, such as nab-paclitaxel, could inform more effective multi-drug regimens. Again, the application of the pharmacodynamic model described in this research could be used here to gain a better understanding on drug-drug interactions in the context of gemcitabine in combination with other drugs and miR-6831-5p mimics.

## **5. Discussion and Overall Impact**

This thesis offers a comprehensive exploration of two critical aspects of pancreatic cancer: therapeutic strategies and the molecular mechanisms underlying disease progression. By investigating the efficacy of established treatment regimens and the role of microRNAs in pancreatic cancer, the research provides valuable insights into both the treatment challenges and the biological complexities of this aggressive disease. The findings presented here are particularly significant in the context of the current pancreatic cancer landscape, where treatment options remain limited, and patient outcomes are poor.

### ***5.1 Enhancing Understanding of FOLFIRINOX Efficacy Through Pharmacodynamic Modelling***

The research begins by addressing a critical gap in the understanding of the FOLFIRINOX regimen, a first line treatment option for pancreatic cancer. Despite its widespread use, the pharmacodynamic interactions between the drugs in this combination—oxaliplatin, 5-fluorouracil, irinotecan, and leucovorin—had not been systematically evaluated. Through the application of a mathematical model, this study elucidates the complex interactions among these agents, revealing a spectrum of effects ranging from synergistic to antagonistic across various pancreatic cancer cell lines. The implications of these findings are significant. By providing a quantitative analysis of drug interactions, this research lays the groundwork for refining FOLFIRINOX treatment protocols, potentially leading to more effective regimens. Additionally, the introduction of a fourth interaction parameter in the Bliss Independent model could further enhance our understanding of temporal treatment effects and allow for the incorporation of miRNA influences on drug-drug interactions. This advancement would represent a significant step forward in the development of chemotherapeutic strategies that are tailored to the unique profile of each patient.

### ***5.2 Uncovering the Role of miRNAs in Pancreatic Cancer Progression and Treatment Resistance***

The second major focus of this thesis is the exploration of miRNAs in the context of pancreatic cancer, particularly their role in mediating resistance to gemcitabine, a first-line treatment for pancreatic cancer. By conducting a multiomics analysis of normal, tumour, and patient-derived xenograft models, this research identified a set of priority miRNAs for further investigation, offering new insights into the molecular underpinnings of treatment resistance. Among these

miRNAs, miR-6831-5p emerges as a key player, with potential therapeutic implications. The research demonstrates that miR-6831-5p may enhance sensitivity to gemcitabine, suggesting a novel approach to overcoming drug resistance in PaC. Through a combination of transcriptomic and proteomic analyses, the study provides a detailed understanding of the pathways influenced by miR-6831-5p, including mitochondrial bioenergetics, purine nucleotide homeostasis, and apoptosis. These findings are particularly relevant in light of the challenges associated with miRNA research, such as the difficulties in developing stable miRNA overexpressing cell lines. By optimising vector designs and employing miRNA mimics, this thesis not only advances the study of miRNA biology but also offers practical solutions for overcoming some of the technical hurdles that have hindered progress in this field. The insights gained from this research could inform the development of new therapeutic strategies targeting miRNAs, ultimately leading to more effective treatments for pancreatic cancer.

### ***5.3 Broader Implications***

The contributions of this thesis extend beyond the immediate findings. By integrating pharmacodynamic modelling with molecular biology, the research exemplifies a holistic approach to cancer research, one that considers both the dynamics of drug interactions and the molecular intricacies of disease progression. This approach aligns with the growing emphasis on personalised medicine. By incorporating miRNA dynamics into pharmacodynamic models, future research could develop more sophisticated and accurate predictions of treatment outcomes, leading to more effective and individualized therapeutic strategies.

In conclusion, this thesis not only advances our understanding of pancreatic cancer treatment and biology but also sets the stage for future research that could significantly impact the clinical management of this disease. By addressing key gaps in our knowledge and offering innovative solutions to existing challenges, the research contributes to the broader effort to improve outcomes for patients with pancreatic cancer, a goal that remains one of the most pressing in oncology today.

## 6. References

- Abdelfattah, A. M., Park, C. and Choi, M. Y. (2014) 'Update on non-canonical microRNAs', *Biomol Concepts*, 5(4), pp. 275-87.
- Abdellatif, A. A. H., Scagnetti, G., Younis, M. A., Bouazzaoui, A., Tawfeek, H. M., Aldosari, B. N., Almurshedi, A. S., Alsharidah, M., Rugaie, O. A., Davies, M. P. A., Liloglou, T., Ross, K. and Saleem, I. (2023) 'Non-coding RNA-directed therapeutics in lung cancer: Delivery technologies and clinical applications', *Colloids Surf B Biointerfaces*, 229, pp. 113466.
- Abreu, F. B., Liu, X. and Tsongalis, G. J. (2017) 'miRNA analysis in pancreatic cancer: the Dartmouth experience', *Clin Chem Lab Med*, 55(5), pp. 755-762.
- Adeshakin, F. O., Adeshakin, A. O., Afolabi, L. O., Yan, D., Zhang, G., & Wan, X. (2021) 'Mechanisms for Modulating Anoikis Resistance in Cancer and the Relevance of Metabolic Reprogramming', *Frontiers in oncology*, , 11 , 626577. .
- Adilakshmi, T., Sudol, I. and Tapinos, N. (2012) 'Combinatorial action of miRNAs regulates transcriptional and post-transcriptional gene silencing following in vivo PNS injury', *PLoS One*, 7(7), pp. e39674.
- Agarwal, V., Bell, G. W., Nam, J. W. and Bartel, D. P. (2015) 'Predicting effective microRNA target sites in mammalian mRNAs', *Elife*, 4.
- Aiello, N. M., Maddipati, R., Norgard, R. J., Balli, D., Li, J., Yuan, S., Yamazoe, T., Black, T., Sahmoud, A., Furth, E. E., Bar-Sagi, D. and Stanger, B. Z. (2018) 'EMT Subtype Influences Epithelial Plasticity and Mode of Cell Migration', *Dev Cell*, 45(6), pp. 681-695.e4.
- Aita, A., Millino, C., Sperti, C., Pacchioni, B., Plebani, M., Pittà, C. and Basso, D. (2021) 'Serum miRNA Profiling for Early PDAC Diagnosis and Prognosis: A Retrospective Study', *Biomedicines*, 9(7).
- Ali Syeda, Z., Langden, S. S. S., Munkhzul, C., Lee, M. and Song, S. J. (2020) 'Regulatory Mechanism of MicroRNA Expression in Cancer', *Int J Mol Sci*, 21(5).
- Allegra, D., Bilan, V., Garding, A., Döhner, H., Stilgenbauer, S., Kuchenbauer, F., Mertens, D. and Zucknick, M. (2014) 'Defective DROSHA processing contributes to downregulation of MiR-15/-16 in chronic lymphocytic leukemia', *Leukemia*, 28(1), pp. 98-107.
- Allemani, C., Matsuda, T., Di Carlo, V., Harewood, R., Matz, M., Nikšić, M., Bonaventure, A., Valkov, M., Johnson, C. J., Estève, J., Ogunbiyi, O. J., Azevedo E Silva, G., Chen, W. Q., Eser, S., Engholm, G., Stiller, C. A., Monnereau, A., Woods, R. R., Visser, O., Lim, G. H., Aitken, J., Weir, H. K., Coleman, M. P. and Group, C. W. (2018) 'Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries', *Lancet*, 391(10125), pp. 1023-1075.
- Allen-Coyle, T. J., Niu, J., Welsch, E., Conlon, N. T., Garner, W., Clynes, M., O'Sullivan, F., Straubinger, R. M., Mager, D. E. and Roche, S. (2022) 'FOLFIRINOX Pharmacodynamic Interactions in 2D and 3D Pancreatic Cancer Cell Cultures', *AAPS J*, 24(6), pp. 108.
- Allen-Coyle, T. J., Roca, B. C., Costello, A., Barron, N., Keenan, J., Clynes, M., O'Neill, F. and O'Sullivan, F. (2024) 'miRNA- and Cell Line-Specific Constraints on Precursor miRNA Processing of Stably Transfected Pancreatic Cancer and Other Mammalian Cells', *Int J Mol Sci*, 25(11).
- Almoguer, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N. and Peruch, M. (1988) 'Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes', *Cell*, 53(4), pp. 549-54.

American Cancer Society (2024) *Cancer Facts & Figures*. Available at: <https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2024.html>.

Aung, K. L., Fischer, S. E., Denroche, R. E., Jang, G. H., Dodd, A., Creighton, S., Southwood, B., Liang, S. B., Chadwick, D., Zhang, A., O'Kane, G. M., Albaba, H., Moura, S., Grant, R. C., Miller, J. K., Mbabaali, F., Pasternack, D., Lungu, I. M., Bartlett, J. M. S., Ghai, S., Lemire, M., Holter, S., Connor, A. A., Moffitt, R. A., Yeh, J. J., Timms, L., Krzyzanowski, P. M., Dhani, N., Hedley, D., Notta, F., Wilson, J. M., Moore, M. J., Gallinger, S. and Knox, J. J. (2018) 'Genomics-Driven Precision Medicine for Advanced Pancreatic Cancer: Early Results from the COMPASS Trial', *Clin Cancer Res*, 24(6), pp. 1344-1354.

Bailey, P. and Chang, D. K. and Nones, K. and Johns, A. L. and Patch, A. M. and Gingras, M. C. and Miller, D. K. and Christ, A. N. and Bruxner, T. J. and Quinn, M. C. and Nourse, C. and Murtaugh, L. C. and Harliwong, I. and Idrisoglu, S. and Manning, S. and Nourbakhsh, E. and Wani, S. and Fink, L. and Holmes, O. and Chin, V. and Anderson, M. J. and Kazakoff, S. and Leonard, C. and Newell, F. and Waddell, N. and Wood, S. and Xu, Q. and Wilson, P. J. and Cloonan, N. and Kassahn, K. S. and Taylor, D. and Quek, K. and Robertson, A. and Pantano, L. and Mincarelli, L. and Sanchez, L. N. and Evers, L. and Wu, J. and Pinese, M. and Cowley, M. J. and Jones, M. D. and Colvin, E. K. and Nagrial, A. M. and Humphrey, E. S. and Chantrill, L. A. and Mawson, A. and Humphris, J. and Chou, A. and Pajic, M. and Scarlett, C. J. and Pinho, A. V. and Giry-Laterriere, M. and Rooman, I. and Samra, J. S. and Kench, J. G. and Lovell, J. A. and Merrett, N. D. and Toon, C. W. and Epari, K. and Nguyen, N. Q. and Barbour, A. and Zeps, N. and Moran-Jones, K. and Jamieson, N. B. and Graham, J. S. and Duthie, F. and Oien, K. and Hair, J. and Grützmann, R. and Maitra, A. and Iacobuzio-Donahue, C. A. and Wolfgang, C. L. and Morgan, R. A. and Lawlor, R. T. and Corbo, V. and Bassi, C. and Rusev, B. and Capelli, P. and Salvia, R. and Tortora, G. and Mukhopadhyay, D. and Petersen, G. M. and Munzy, D. M. and Fisher, W. E. and Karim, S. A. and Eshleman, J. R. and Hruban, R. H. and Pilarsky, C. and Morton, J. P. and Sansom, O. J. and Scarpa, A. and Musgrove, E. A. and Bailey, U. M. and Hofmann, O. and Sutherland, R. L. and Wheeler, D. A. and Gill, A. J. and Gibbs, R. A. and Pearson, J. V. and Biankin, A. V. and Grimmond, S. M. and Initiative, A. P. C. G. (2016) 'Genomic analyses identify molecular subtypes of pancreatic cancer', *Nature*, 531(7592), pp. 47-52.

Baradaran, B., Shahbazi, R. and Khordadmehr, M. (2019) 'Dysregulation of key microRNAs in pancreatic cancer development', *Biomed Pharmacother*, 109, pp. 1008-1015.

Barik, S. (2008) 'An intronic microRNA silences genes that are functionally antagonistic to its host gene', *Nucleic Acids Res*, 36(16), pp. 5232-41.

Bartel, D. P. (2004) 'MicroRNAs: genomics, biogenesis, mechanism, and function', *Cell*, 116(2), pp. 281-97.

Beermann, J., Piccoli, M. T., Viereck, J. and Thum, T. (2016) 'Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches', *Physiol Rev*, 96(4), pp. 1297-325.

Ben, Q., Xu, M., Ning, X., Liu, J., Hong, S., Huang, W., Zhang, H. and Li, Z. (2011) 'Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies', *Eur J Cancer*, 47(13), pp. 1928-37.

Benhamed, M., Herbig, U., Ye, T., Dejean, A. and Bischof, O. (2012) 'Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells', *Nat Cell Biol*, 14(3), pp. 266-75.

Author *EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling*. (Version R package version 1.18.0.). Available at: <https://github.com/kevinblighe/EnhancedVolcano>.

Bloomston, M., Frankel, W. L., Petrocca, F., Volinia, S., Alder, H., Hagan, J. P., Liu, C. G., Bhatt, D., Taccioli, C. and Croce, C. M. (2007) 'MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis', *Jama*, 297(17), pp. 1901-8.

Bosetti, C., Rosato, V., Li, D., Silverman, D., Petersen, G. M., Bracci, P. M., Neale, R. E., Muscat, J., Anderson, K., Gallinger, S., Olson, S. H., Miller, A. B., Bas Bueno-de-Mesquita, H., Scelo, G., Janout, V., Holcatova, I., Laggiou, P., Serraino, D., Lucenteforte, E., Fabianova, E., Baghurst, P. A., Zatonski, W., Foretova, L., Fontham, E., Bamlet, W. R., Holly, E. A., Negri, E., Hassan, M., Prizment, A., Cotterchio, M., Cleary, S., Kurtz, R. C., Maisonneuve, P., Trichopoulos, D., Polesel, J., Duell, E. J., Boffetta, P., La Vecchia, C. and Ghadirian, P. (2014) 'Diabetes, antidiabetic medications, and pancreatic cancer risk: an analysis from the International Pancreatic Cancer Case-Control Consortium', *Ann Oncol*, 25(10), pp. 2065-2072.

Castel, S. E. and Martienssen, R. A. (2013) 'RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond', *Nat Rev Genet*, 14(2), pp. 100-12.

Catalanotto, C., Cogoni, C. and Zardo, G. (2016) 'MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions', *Int J Mol Sci*, 17(10).

Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K. and Shiekhattar, R. (2005) 'TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing', *Nature*, 436(7051), pp. 740-4.

Chiorean, E. G. and Covelev, A. L. (2015) 'Pancreatic cancer: optimizing treatment options, new, and emerging targeted therapies', *Drug Des Devel Ther*, 9, pp. 3529-45.

Coleman, O., Henry, M., O'Neill, F., Roche, S., Swan, N., Boyle, L., Murphy, J., Meiller, J., Conlon, N. T., Geoghegan, J., Conlon, K. C., Lynch, V., Straubinger, N. L., Straubinger, R. M., McVey, G., Moriarty, M., Meleady, P. and Clynes, M. (2018) 'A Comparative Quantitative LC-MS/MS Profiling Analysis of Human Pancreatic Adenocarcinoma, Adjacent-Normal Tissue, and Patient-Derived Tumour Xenografts', *Proteomes*, 6(4).

Coleman, O., Henry, M., O'Neill, F., Roche, S., Swan, N., Geoghegan, J., Conlon, K., McVey, G., Moriarty, M., Meleady, P. and Clynes, M. (2020) 'Proteomic Analysis of Cell Lines and Primary Tumors in Pancreatic Cancer Identifies Proteins Expressed Only In Vitro and Only In Vivo', *Pancreas*, 49(8), pp. 1109-1116.

Collienne, M., Loghmani, H., Heineman, T. C. and Arnold, D. (2022) 'GOBLET: a phase I/II study of pelareorep and atezolizumab +/- chemo in advanced or metastatic gastrointestinal cancers', *Future Oncol*, 18(26), pp. 2871-2878.

Collisson, E. A., Sadanandam, A., Olson, P., Gibb, W. J., Truitt, M., Gu, S., Cooc, J., Weinkle, J., Kim, G. E., Jakkula, L., Feiler, H. S., Ko, A. H., Olshen, A. B., Danenberg, K. L., Tempero, M. A., Spellman, P. T., Hanahan, D. and Gray, J. W. (2011) 'Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy', *Nat Med*, 17(4), pp. 500-3.

Conroy, T., Desseigne, F., Ychou, M., Bouche, O., Guimbaud, R., Becouarn, Y., Adenis, A., Raoul, J. L., Gourgou-Bourgade, S., de la Fouchardiere, C., Bannouna, J., Bachet, J. B., Khemissa-Akouz, F., Pere-Verge, D., Delbaldo, C., Assenat, E., Chauffert, B., Michel, P., Montoto-Grillot, C. and Ducreux, M. (2011) 'FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer', *N Engl J Med*, 364(19), pp. 1817-25.

Costello, A., Lao, N., Clynes, M. and Barron, N. (2017) 'Conditional Knockdown of Endogenous MicroRNAs in CHO Cells Using TET-ON-SanDI Sponge Vectors', *Methods Mol Biol*, 1603, pp. 87-100.

Costello, A., Lao, N. T., Gallagher, C., Capella Roca, B., Julius, L. A. N., Suda, S., Ducrée, J., King, D., Wagner, R., Barron, N. and Clynes, M. (2019) 'Leaky Expression of the TET-On

System Hinders Control of Endogenous miRNA Abundance', *Biotechnol J*, 14(3), pp. e1800219.

Daemen, A., Peterson, D., Sahu, N., McCord, R., Du, X., Liu, B., Kowanetz, K., Hong, R., Moffat, J., Gao, M., Boudreau, A., Mroue, R., Corson, L., O'Brien, T., Qing, J., Sampath, D., Merchant, M., Yauch, R., Manning, G., Settleman, J., Hatzivassiliou, G. and Evangelista, M. (2015) 'Metabolite profiling stratifies pancreatic ductal adenocarcinomas into subtypes with distinct sensitivities to metabolic inhibitors', *Proc Natl Acad Sci U S A*, 112(32), pp. E4410-7.

Davis, B. N. and Hata, A. (2009) 'Regulation of MicroRNA Biogenesis: A miRiad of mechanisms', *Cell Commun Signal*, 7, pp. 18.

Deer, E. L., González-Hernández, J., Coursen, J. D., Shea, J. E., Ngatia, J., Scaife, C. L., Firpo, M. A. and Mulvihill, S. J. (2010) 'Phenotype and genotype of pancreatic cancer cell lines', *Pancreas*, 39(4), pp. 425-35.

Ding, C., Wu, Z., You, H., Ge, H., Zheng, S., Lin, Y., Wu, X., Lin, Z. and Kang, D. (2019) 'CircNFIX promotes progression of glioma through regulating miR-378e/RPN2 axis', *J Exp Clin Cancer Res*, 38(1), pp. 506.

Distler, M., Aust, D., Weitz, J., Pilarsky, C. and Grützmann, R. (2014) 'Precursor lesions for sporadic pancreatic cancer: PanIN, IPMN, and MCN', *Biomed Res Int*, 2014, pp. 474905.

Drago-Ferrante, R., Pentimalli, F., Carlisi, D., De Blasio, A., Saliba, C., Baldacchino, S., Degaetano, J., Debono, J., Caruana-Dingli, G., Grech, G., Scerri, C., Tesoriere, G., Giordano, A., Vento, R. and Di Fiore, R. (2017) 'Suppressive role exerted by microRNA-29b-1-5p in triple negative breast cancer through SPIN1 regulation', *Oncotarget*, 8(17), pp. 28939-28958.

Duan, Z., Wei, S. and Liu, Y. (2021) 'Circ\_0074027 contributes to non-small cell lung cancer progression through positively modulating RHOA via sequestering miR-2467-3p', *J Bioenerg Biomembr*, 53(2), pp. 223-233.

Ermak, G., Cancasci, V. J. and Davies, K. J. (2003) 'Cytotoxic effect of doxycycline and its implications for tet-on gene expression systems', *Anal Biochem*, 318(1), pp. 152-4.

Espiau-Romera, P., Courtois, S., Parejo-Alonso, B. and Sancho, P. (2020) 'Molecular and Metabolic Subtypes Correspondence for Pancreatic Ductal Adenocarcinoma Classification', *J Clin Med*, 9(12).

Eto, K., Iwatsuki, M., Watanabe, M., Ida, S., Ishimoto, T., Iwagami, S., Baba, Y., Sakamoto, Y., Miyamoto, Y., Yoshida, N. and Baba, H. (2014) 'The microRNA-21/PTEN pathway regulates the sensitivity of HER2-positive gastric cancer cells to trastuzumab', *Ann Surg Oncol*, 21(1), pp. 343-50.

European Cancer Information System (2022).

**European Commission, J. R. C. (2022) *Cancer Estimates 2022 Factsheet*. Available at: Retrieved from [https://ecis.jrc.ec.europa.eu/pdf/CancerEstimates2022\\_factsheet.pdf](https://ecis.jrc.ec.europa.eu/pdf/CancerEstimates2022_factsheet.pdf).**

Eyres, M., Lanfredini, S., Xu, H., Burns, A., Blake, A., Willenbrock, F., Goldin, R., Hughes, D., Hughes, S., Thapa, A., Vavoulis, D., Hubert, A., D'Costa, Z., Sabbagh, A., Abraham, A. G., Blancher, C., Jones, S., Verrill, C., Silva, M., Soonawalla, Z., Maughan, T., Schuh, A., Mukherjee, S. and O'Neill, E. (2021) 'TET2 Drives 5hmc Marking of GATA6 and Epigenetically Defines Pancreatic Ductal Adenocarcinoma Transcriptional Subtypes', *Gastroenterology*, 161(2), pp. 653-668.e16.

Fan, J., Feng, Y., Zhang, R., Zhang, W., Shu, Y., Zeng, Z., Huang, S., Zhang, L., Huang, B., Wu, D., Zhang, B., Wang, X., Lei, Y., Ye, Z., Zhao, L., Cao, D., Yang, L., Chen, X., Liu, B., Wagstaff, W., He, F., Wu, X., Zhang, J., Moriatis Wolf, J., Lee, M. J., Haydon, R. C., Luu, H. H., Huang, A., He, T. C. and Yan, S. (2020) 'A simplified system for the effective expression and delivery of functional mature microRNAs in mammalian cells', *Cancer Gene Ther*, 27(6), pp. 424-437.

Farias, A. J., Wu, A. H., Porcel, J., Marchand, L. L., Wilkens, L. R., Monroe, K. R., Maskarinec, G., Pandol, S. J. and Setiawan, V. W. (2020) 'Diabetes-Related Complications

and Pancreatic Cancer Incidence in the Multiethnic Cohort', *JNCI Cancer Spectr*, 4(5), pp. pkaa035.

Follin, B., Juhl, M., Cohen, S., Pedersen, A. E., Kastrup, J. and Ekblond, A. (2016) 'Increased Paracrine Immunomodulatory Potential of Mesenchymal Stromal Cells in Three-Dimensional Culture', *Tissue Eng Part B Rev*, 22(4), pp. 322-9.

Forman, J. J. and Collier, H. A. (2010) 'The code within the code: microRNAs target coding regions', *Cell Cycle*, 9(8), pp. 1533-41.

Francone, E., Gentili, S., Santori, G., Stabilini, C., Fornaro, R. and Frascio, M. (2021) 'MicroRNAs differential expression profile in metastatic colorectal cancer: A pilot study with literature review', *Surg Oncol*, 37, pp. 101524.

Fu, D., Hu, Z., Xu, X., Dai, X. and Liu, Z. (2022) 'Key signal transduction pathways and crosstalk in cancer: Biological and therapeutic opportunities', *Transl Oncol*, 26, pp. 101510.

Färber, B., Lapshyna, O., Künstner, A., Kohl, M., Sauer, T., Bichmann, K., Heckelmann, B., Watzelt, J., Honselmann, K., Bolm, L., Ten Winkel, M., Busch, H., Ungefroren, H., Keck, T., Gemoll, T., Wellner, U. F. and Braun, R. (2023) 'Molecular profiling and specific targeting of gemcitabine-resistant subclones in heterogeneous pancreatic cancer cell populations', *Front Oncol*, 13, pp. 1230382.

Gagnon, K. T., Li, L., Chu, Y., Janowski, B. A. and Corey, D. R. (2014) 'RNAi factors are present and active in human cell nuclei', *Cell Rep*, 6(1), pp. 211-21.

Gao, W., Gu, Y., Li, Z., Cai, H., Peng, Q., Tu, M., Kondo, Y., Shinjo, K., Zhu, Y., Zhang, J., Sekido, Y., Han, B., Qian, Z. and Miao, Y. (2015) 'miR-615-5p is epigenetically inactivated and functions as a tumor suppressor in pancreatic ductal adenocarcinoma', *Oncogene*, 34(13), pp. 1629-40.

Gibiansky, L. G., E · Kakkar, T · Ma, P. (2008) 'Pharmacokinetic–Pharmacodynamic Modelling: History and Perspectives', *Journal of Pharmacokinetics and Pharmacodynamics*, 35, pp. 573-591.

Glinge, C., Clauss, S., Boddum, K., Jabbari, R., Jabbari, J., Risgaard, B., Tomsits, P., Hildebrand, B., Kääb, S., Wakili, R., Jespersen, T. and Tfelt-Hansen, J. (2017) 'Stability of Circulating Blood-Based MicroRNAs - Pre-Analytic Methodological Considerations', *PLoS One*, 12(2), pp. e0167969.

Gonzalez, S., Pisano, D. G. and Serrano, M. (2008) 'Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs', *Cell Cycle*, 7(16), pp. 2601-8.

Gossen, M. and Bujard, H. (1992) 'Tight control of gene expression in mammalian cells by tetracycline-responsive promoters', *Proc Natl Acad Sci U S A*, 89(12), pp. 5547-51.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. and Bujard, H. (1995) 'Transcriptional activation by tetracyclines in mammalian cells', *Science*, 268(5218), pp. 1766-9.

Halbrook, C. J., Lyssiotis, C. A., Pasca di Magliano, M. and Maitra, A. (2023) 'Pancreatic cancer: Advances and challenges', *Cell*, 186(8), pp. 1729-1754.

Han, Y., Shin, S. H., Lim, C. G., Heo, Y. H., Choi, I. Y. and Kim, H. H. (2023) 'Synthetic RNA Therapeutics in Cancer', *J Pharmacol Exp Ther*, 386(2), pp. 212-223.

Havens, M. A., Reich, A. A., Duelli, D. M. and Hastings, M. L. (2012) 'Biogenesis of mammalian microRNAs by a non-canonical processing pathway', *Nucleic Acids Res*, 40(10), pp. 4626-40.

He, L., He, X., Lim, L. P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A. L., Linsley, P. S., Chen, C., Lowe, S. W., Cleary, M. A. and Hannon, G. J. (2007) 'A microRNA component of the p53 tumour suppressor network', *Nature*, 447(7148), pp. 1130-4.

Hecht, B., Müller, G. and Hillen, W. (1993) 'Noninducible Tet repressor mutations map from the operator binding motif to the C terminus', *J Bacteriol*, 175(4), pp. 1206-10.

- Hruban, R. H., Goggins, M., Parsons, J. and Kern, S. E. (2000) 'Progression model for pancreatic cancer', *Clin Cancer Res*, 6(8), pp. 2969-72.
- Hruban, R. H., Maitra, A. and Goggins, M. (2008) 'Update on pancreatic intraepithelial neoplasia', *Int J Clin Exp Pathol*, 1(4), pp. 306-16.
- Hu, E. A., Pan, A., Malik, V. and Sun, Q. (2012) 'White rice consumption and risk of type 2 diabetes: meta-analysis and systematic review', *Bmj*, 344, pp. e1454.
- Hu, J. X., Zhao, C. F., Chen, W. B., Liu, Q. C., Li, Q. W., Lin, Y. Y. and Gao, F. (2021) 'Pancreatic cancer: A review of epidemiology, trend, and risk factors', *World J Gastroenterol*, 27(27), pp. 4298-4321.
- Huang, S., Guo, H., Cao, Y. and Xiong, J. (2019) 'MiR-708-5p inhibits the progression of pancreatic ductal adenocarcinoma by targeting Sirt3', *Pathol Res Pract*, 215(4), pp. 794-800.
- Huntzinger, E. and Izaurralde, E. (2011) 'Gene silencing by microRNAs: contributions of translational repression and mRNA decay', *Nat Rev Genet*, 12(2), pp. 99-110.
- Hwang, H. W., Wentzel, E. A. and Mendell, J. T. (2007) 'A hexanucleotide element directs microRNA nuclear import', *Science*, 315(5808), pp. 97-100.
- Idichi, T., Seki, N., Kurahara, H., Fukuhisa, H., Toda, H., Shimonosono, M., Okato, A., Arai, T., Kita, Y., Mataka, Y., Kijima, Y., Maemura, K. and Natsugoe, S. (2018) 'Molecular pathogenesis of pancreatic ductal adenocarcinoma: Impact of passenger strand of pre-miR-148a on gene regulation', *Cancer Sci*, 109(6), pp. 2013-2026.
- Ijee, S., Chambayil, K., Chaudhury, A. D., Bagchi, A., Modak, K., Das, S., Benjamin, E. S. B., Rani, S., Paul, D. Z., Nath, A., Roy, D., Palani, D., Priyanka, S., Ravichandran, R., Kumary, B. K., Sivamani, Y., S, V., Babu, D., Nakamura, Y., Thamodaran, V., Balasubramanian, P. and Velayudhan, S. R. (2023) 'Efficient deletion of microRNAs using CRISPR/Cas9 with dual guide RNAs', *Front Mol Biosci*, 10, pp. 1295507.
- Ikezawa, K., Urabe, M., Kai, Y., Takada, R., Akita, H., Nagata, S. and Ohkawa, K. (2024) 'Comprehensive review of pancreatic acinar cell carcinoma: epidemiology, diagnosis, molecular features and treatment', *Jpn J Clin Oncol*, 54(3), pp. 271-281.
- Iliou, M. S., da Silva-Diz, V., Carmona, F. J., Ramalho-Carvalho, J., Heyn, H., Villanueva, A., Muñoz, P. and Esteller, M. (2014) 'Impaired DICER1 function promotes stemness and metastasis in colon cancer', *Oncogene*, 33(30), pp. 4003-15.
- Iodice, S., Gandini, S., Maisonneuve, P. and Lowenfels, A. B. (2008) 'Tobacco and the risk of pancreatic cancer: a review and meta-analysis', *Langenbecks Arch Surg*, 393(4), pp. 535-45.
- Jacobetz, M. A., Chan, D. S., Neesse, A., Bapiro, T. E., Cook, N., Frese, K. K., Feig, C., Nakagawa, T., Caldwell, M. E., Zecchini, H. I., Lolkema, M. P., Jiang, P., Kultti, A., Thompson, C. B., Maneval, D. C., Jodrell, D. I., Frost, G. I., Shepard, H. M., Skepper, J. N. and Tuveson, D. A. (2013) 'Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer', *Gut*, 62(1), pp. 112-20.
- Javaid, D., Ganie, S. Y., Hajam, Y. A. and Reshi, M. S. (2022) 'CRISPR/Cas9 system: a reliable and facile genome editing tool in modern biology', *Mol Biol Rep*, 49(12), pp. 12133-12150.
- Jiang, X. P., Ai, W. B., Wan, L. Y., Zhang, Y. Q. and Wu, J. F. (2017) 'The roles of microRNA families in hepatic fibrosis', *Cell Biosci*, 7, pp. 34.
- Jiang, Y., Zhang, Y., Li, F., Du, X. and Zhang, J. (2016) 'CDX2 inhibits pancreatic adenocarcinoma cell proliferation via promoting tumor suppressor miR-615-5p', *Tumour Biol*, 37(1), pp. 1041-9.
- Jo, M. H., Shin, S., Jung, S. R., Kim, E., Song, J. J. and Hohng, S. (2015) 'Human Argonaute 2 Has Diverse Reaction Pathways on Target RNAs', *Mol Cell*, 59(1), pp. 117-24.
- John, B., Enright, A. J., Aravin, A., Tuschl, T., Sander, C. and Marks, D. S. (2004) 'Human MicroRNA targets', *PLoS Biol*, 2(11), pp. e363.

- John, B., Sander, C. and Marks, D. S. (2006) 'Prediction of human microRNA targets', *Methods Mol Biol*, 342, pp. 101-13.
- Kadera, B. E., Li, L., Toste, P. A., Wu, N., Adams, C., Dawson, D. W. and Donahue, T. R. (2013) 'MicroRNA-21 in pancreatic ductal adenocarcinoma tumor-associated fibroblasts promotes metastasis', *PLoS One*, 8(8), pp. e71978.
- Kallunki, T., Barisic, M., Jaattela, M. and Liu, B. (2019) 'How to Choose the Right Inducible Gene Expression System for Mammalian Studies?', *Cells*, 8(8).
- Kang, H., Jo, J. H., Lee, H. S., Chung, M. J., Bang, S., Park, S. W., Song, S. Y. and Park, J. Y. (2018) 'Comparison of efficacy and safety between standard-dose and modified-dose FOLFIRINOX as a first-line treatment of pancreatic cancer', *World J Gastrointest Oncol*, 10(11), pp. 421-430.
- Kapałczyńska, M., Kolenda, T., Przybyła, W., Zajączkowska, M., Teresiak, A., Filas, V., Ibbs, M., Bliźniak, R., Łuczewski, Ł., & Lamperska, K. (2018) '2D and 3D cell cultures - a comparison of different types of cancer cell cultures. ', *Archives of medical science : AMS*, , 14 (4), 910–919. .
- Keklikoglou, I., Hosaka, K., Bender, C., Bott, A., Koerner, C., Mitra, D., Will, R., Woerner, A., Muenstermann, E., Wilhelm, H., Cao, Y. and Wiemann, S. (2015) 'MicroRNA-206 functions as a pleiotropic modulator of cell proliferation, invasion and lymphangiogenesis in pancreatic adenocarcinoma by targeting ANXA2 and KRAS genes', *Oncogene*, 34(37), pp. 4867-78.
- Khan, M. A., Zubair, H., Srivastava, S. K., Singh, S. and Singh, A. P. (2015) 'Insights into the Role of microRNAs in Pancreatic Cancer Pathogenesis: Potential for Diagnosis, Prognosis, and Therapy', *Adv Exp Med Biol*, 889, pp. 71-87.
- Khudayberdiev, S. A., Zampa, F., Rajman, M. and Schratt, G. (2013) 'A comprehensive characterization of the nuclear microRNA repertoire of post-mitotic neurons', *Front Mol Neurosci*, 6, pp. 43.
- Kim, C. K. and Pak, T. R. (2020) 'miRNA degradation in the mammalian brain', *Am J Physiol Cell Physiol*, 319(4), pp. C624-C629.
- Kim, D. H., Saetrom, P., Snøve, O. and Rossi, J. J. (2008) 'MicroRNA-directed transcriptional gene silencing in mammalian cells', *Proc Natl Acad Sci U S A*, 105(42), pp. 16230-5.
- Kim, H. S., Patel, K., Muldoon-Jacobs, K., Bisht, K. S., Aykin-Burns, N., Pennington, J. D., van der Meer, R., Nguyen, P., Savage, J., Owens, K. M., Vassilopoulos, A., Ozden, O., Park, S. H., Singh, K. K., Abdulkadir, S. A., Spitz, D. R., Deng, C. X. and Gius, D. (2010) 'SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress', *Cancer Cell*, 17(1), pp. 41-52.
- Kim, S., Bae, W. J., Ahn, J. M., Heo, J. H., Kim, K. M., Choi, K. W., Sung, C. O. and Lee, D. (2021) 'MicroRNA signatures associated with lymph node metastasis in intramucosal gastric cancer', *Mod Pathol*, 34(3), pp. 672-683.
- Klein, A. P. (2021) 'Pancreatic cancer epidemiology: understanding the role of lifestyle and inherited risk factors', *Nat Rev Gastroenterol Hepatol*, 18(7), pp. 493-502.
- Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y. and Ochiya, T. (2010) 'Secretory mechanisms and intercellular transfer of microRNAs in living cells', *J Biol Chem*, 285(23), pp. 17442-52.
- Lee, E. J., Baek, M., Gusev, Y., Brackett, D. J., Nuovo, G. J. and Schmittgen, T. D. (2008) 'Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors', *Rna*, 14(1), pp. 35-42.
- Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993) 'The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14', *Cell*, 75(5), pp. 843-54.

Lee, Y., Hur, I., Park, S. Y., Kim, Y. K., Suh, M. R. and Kim, V. N. (2006) 'The role of PACT in the RNA silencing pathway', *EMBO J*, 25(3), pp. 522-32.

Li, A., Yu, J., Kim, H., Wolfgang, C. L., Canto, M. I., Hruban, R. H. and Goggins, M. (2013) 'MicroRNA array analysis finds elevated serum miR-1290 accurately distinguishes patients with low-stage pancreatic cancer from healthy and disease controls', *Clin Cancer Res*, 19(13), pp. 3600-10.

Lin, Q., Qian, Z., Jusko, W. J., Mager, D. E., Ma, W. W. and Straubinger, R. M. (2021) 'Synergistic Pharmacodynamic Effects of Gemcitabine and Fibroblast Growth Factor Receptor Inhibitors on Pancreatic Cancer Cell Cycle Kinetics and Proliferation', *J Pharmacol Exp Ther*, 377(3), pp. 370-384.

Lin, S. and Gregory, R. I. (2015) 'MicroRNA biogenesis pathways in cancer', *Nat Rev Cancer*, 15(6), pp. 321-33.

Ling, S., He, Y., Li, X., Hu, M., Ma, Y., Li, Y., Lu, Z., Shen, S., Kong, B., Zou, X., Jiang, K. and Huang, P. (2020) 'CircRHOT1 mediated cell proliferation, apoptosis and invasion of pancreatic cancer cells by sponging miR-125a-3p', *J Cell Mol Med*, 24(17), pp. 9881-9889.

Liu, A. K. N. and Lefresne, S. (2023) 'Palliative radiotherapy in pancreatic cancer: a retrospective study of 100 cases and regional patterns of practice', *Ann Palliat Med*, 12(5), pp. 891-899.

Liu, F. and Wen, C. (2020) 'LINC01410 Knockdown Suppresses Cervical Cancer Growth and Invasion via Targeting miR-2467-3p/VOPP1 Axis', *Cancer Manag Res*, 12, pp. 855-861.

Livak, K. J. and Schmittgen, T. D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method', *Methods*, 25(4), pp. 402-8.

Love, M. I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biol*, 15(12), pp. 550.

Matthaei, H., Schulick, R. D., Hruban, R. H. and Maitra, A. (2011) 'Cystic precursors to invasive pancreatic cancer', *Nat Rev Gastroenterol Hepatol*, 8(3), pp. 141-50.

Meleady, P., Abdul Rahman, R., Henry, M., Moriarty, M. and Clynes, M. (2020) 'Proteomic analysis of pancreatic ductal adenocarcinoma', *Expert Rev Proteomics*, 17(6), pp. 453-467.

Melo, S. A., Moutinho, C., Ropero, S., Calin, G. A., Rossi, S., Spizzo, R., Fernandez, A. F., Davalos, V., Villanueva, A., Montoya, G., Yamamoto, H., Schwartz, S., Jr. and Esteller, M. (2010) 'A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells', *Cancer Cell*, 18(4), pp. 303-15.

Meng, Q., Liang, C., Hua, J., Zhang, B., Liu, J., Zhang, Y., Wei, M., Yu, X., Xu, J. and Shi, S. (2020) 'A miR-146a-5p/TRAF6/NF-kB p65 axis regulates pancreatic cancer chemoresistance: functional validation and clinical significance', *Theranostics*, 10(9), pp. 3967-3979.

Miao, L., Yao, H., Li, C., Pu, M., Yao, X., Yang, H., Qi, X., Ren, J. and Wang, Y. (2016) 'A dual inhibition: microRNA-552 suppresses both transcription and translation of cytochrome P450 2E1', *Biochim Biophys Acta*, 1859(4), pp. 650-62.

Milevskiy, M. J. G., Sandhu, G. K., Wronski, A., Korbie, D., Brewster, B. L., Shewan, A., Edwards, S. L., French, J. D. and Brown, M. A. (2018) 'MiR-29b-1-5p is altered in BRCA1 mutant tumours and is a biomarker in basal-like breast cancer', *Oncotarget*, 9(71), pp. 33577-33588.

Mizrahi, J. D., Rogers, J. E., Hess, K. R., Wolff, R. A., Varadhachary, G. R., Javle, M. M., Shroff, R. T., Ho, L., Fogelman, D. R., Raghav, K. P. S., Overman, M. J. and Pant, S. (2020) 'Modified FOLFIRINOX in pancreatic cancer patients Age 75 or older', *Pancreatology*, 20(3), pp. 501-504.

Moffitt, R. A., Marayati, R., Flate, E. L., Volmar, K. E., Loeza, S. G., Hoadley, K. A., Rashid, N. U., Williams, L. A., Eaton, S. C., Chung, A. H., Smyla, J. K., Anderson, J. M., Kim, H. J., Bentrem, D. J., Talamonti, M. S., Iacobuzio-Donahue, C. A., Hollingsworth, M.

A. and Yeh, J. J. (2015) 'Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma', *Nat Genet*, 47(10), pp. 1168-78.

Molins, E. A. G. and Jusko, W. J. (2018) 'Assessment of Three-Drug Combination Pharmacodynamic Interactions in Pancreatic Cancer Cells', *Aaps j*, 20(5), pp. 80.

Moll, P., Ante, M., Seitz, A. *et al.* (2014) 'QuantSeq 3' mRNA sequencing for RNA quantification.', *Nature Methods* 11.

Mucileanu, A., Chira, R. and Mircea, P. A. (2021) 'PD-1/PD-L1 expression in pancreatic cancer and its implication in novel therapies', *Med Pharm Rep*, 94(4), pp. 402-410.

National Cancer Institute (2024a) *Cancer Stat Facts: Pancreatic Neuroendocrine Tumors*. Available at: <https://seer.cancer.gov/statfacts/html/pancreas.html>.

National Cancer Institute (2024b) *Pancreatic cancer treatment (PDQ®)–patient version*. Available at: <https://www.cancer.gov/types/pancreatic/patient/pancreatic-treatment-pdq#:~:text=Pancreatic%20cancer%20is%20a%20type,is%20difficult%20to%20diagnose%20early>.

National Cancer Registry Ireland (2023) *Cancer in Ireland 1994-2021: Annual statistical report of the National Cancer Registry Ireland*.

Naudin, S., Li, K., Jaouen, T., Assi, N., Kyrø, C., Tjønneland, A., Overvad, K., Boutron-Ruault, M. C., Rebours, V., Védié, A. L., Boeing, H., Kaaks, R., Katzke, V., Bamia, C., Naska, A., Trichopoulou, A., Berrino, F., Tagliabue, G., Palli, D., Panico, S., Tumino, R., Sacerdote, C., Peeters, P. H., Bueno-de-Mesquita, H. B. A., Weiderpass, E., Gram, I. T., Skeie, G., Chirlaque, M. D., Rodríguez-Barranco, M., Barricarte, A., Quirós, J. R., Dorronsoro, M., Johansson, I., Sund, M., Sternby, H., Bradbury, K. E., Wareham, N., Riboli, E., Gunter, M., Brennan, P., Duell, E. J. and Ferrari, P. (2018) 'Lifetime and baseline alcohol intakes and risk of pancreatic cancer in the European Prospective Investigation into Cancer and Nutrition study', *Int J Cancer*, 143(4), pp. 801-812.

Neoptolemos, J. P., Stocken, D. D., Friess, H., Bassi, C., Dunn, J. A., Hickey, H., Beger, H., Fernandez-Cruz, L., Dervenis, C., Lacaine, F., Falconi, M., Pederzoli, P., Pap, A., Spooner, D., Kerr, D. J., Büchler, M. W. and Cancer, E. S. G. f. P. (2004) 'A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer', *N Engl J Med*, 350(12), pp. 1200-10.

Neoptolemos, J. P., Stocken, D. D., Tudur Smith, C., Bassi, C., Ghaneh, P., Owen, E., Moore, M., Padbury, R., Doi, R., Smith, D. and Büchler, M. W. (2009) 'Adjuvant 5-fluorouracil and folinic acid vs observation for pancreatic cancer: composite data from the ESPAC-1 and -3(v1) trials', *Br J Cancer*, 100(2), pp. 246-50.

Nguyen, T. D., Trinh, T. A., Bao, S. and Nguyen, T. A. (2022) 'Secondary structure RNA elements control the cleavage activity of DICER', *Nat Commun*, 13(1), pp. 2138.

Niu, J., Straubinger, R. M. and Mager, D. E. (2019) 'Pharmacodynamic Drug-Drug Interactions', *Clin Pharmacol Ther*, 105(6), pp. 1395-1406.

Nywenning, T. M., Wang-Gillam, A., Sanford, D. E., Belt, B. A., Panni, R. Z., Cusworth, B. M., Toriola, A. T., Nieman, R. K., Worley, L. A., Yano, M., Fowler, K. J., Lockhart, A. C., Suresh, R., Tan, B. R., Lim, K. H., Fields, R. C., Strasberg, S. M., Hawkins, W. G., DeNardo, D. G., Goedegebuure, S. P. and Linehan, D. C. (2016) 'Targeting tumour-associated macrophages with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer: a single-centre, open-label, dose-finding, non-randomised, phase 1b trial', *Lancet Oncol*, 17(5), pp. 651-62.

O'Brien, J., Hayder, H., Zayed, Y. and Peng, C. (2018) 'Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation', *Front Endocrinol (Lausanne)*, 9, pp. 402.

O'Carroll, D. and Schaefer, A. (2013) 'General principals of miRNA biogenesis and regulation in the brain', *Neuropsychopharmacology*, 38(1), pp. 39-54.

O'Neill, F., Allen-Coyle, T. J., Roche, S., Meiller, J., Conlon, N. T., Swan, N., Straubinger, R. M., Geoghegan, J., Straubinger, N. L., Conlon, K., McDermott, R., O'Sullivan, F., Henry, M., Meleady, P., McVey, G., O'Connor, R., Moriarty, M. and Clynes, M. (2023) 'Alteration in Levels of Specific miRNAs and Their Potential Protein Targets between Human Pancreatic Cancer Samples, Adjacent Normal Tissue, and Xenografts Derived from These Tumors', *Life (Basel)*, 13(3).

Padrón, L. J., Maurer, D. M., O'Hara, M. H., O'Reilly, E. M., Wolff, R. A., Wainberg, Z. A., Ko, A. H., Fisher, G., Rahma, O., Lyman, J. P., Cabanski, C. R., Yu, J. X., Pfeiffer, S. M., Spasic, M., Xu, J., Gherardini, P. F., Karakunnel, J., Mick, R., Alanio, C., Byrne, K. T., Hollmann, T. J., Moore, J. S., Jones, D. D., Tognetti, M., Chen, R. O., Yang, X., Salvador, L., Wherry, E. J., Dugan, U., O'Donnell-Tormey, J., Butterfield, L. H., Hubbard-Lucey, V. M., Ibrahim, R., Fairchild, J., Bucktrout, S., LaVallee, T. M. and Vonderheide, R. H. (2022) 'Sotigalimab and/or nivolumab with chemotherapy in first-line metastatic pancreatic cancer: clinical and immunologic analyses from the randomized phase 2 PRINCE trial', *Nat Med*, 28(6), pp. 1167-1177.

Paramythiotis, D., Karlafti, E., Fotiadou, G., Charalampidou, M., Karakatsanis, A., Ioannidis, A. and Michalopoulos, A. (2023) 'Pancreatic Intraductal Papillary Mucinous Neoplasms: A Narrative Review', *Acta Med Litu*, 30(1), pp. 53-65.

Partyka, O., Pajewska, M., Kwaśniewska, D., Czerw, A., Deptała, A., Budzik, M., Cipora, E., Gaska, I., Gazdowicz, L., Mielnik, A., Sygit, K., Sygit, M., Krzych-Falta, E., Schneider-Matyka, D., Grochans, S., Cybulska, A. M., Drobnik, J., Bandurska, E., Ciećko, W., Ratajczak, P., Kamecka, K., Marczak, M. and Kozłowski, R. (2023) 'Overview of Pancreatic Cancer Epidemiology in Europe and Recommendations for Screening in High-Risk Populations', *Cancers (Basel)*, 15(14).

Passadouro, M. and Faneca, H. (2016) 'Managing Pancreatic Adenocarcinoma: A Special Focus in MicroRNA Gene Therapy', *Int J Mol Sci*, 17(5).

Peng, N., Miao, Z., Wang, L., Liu, B., Wang, G. and Guo, X. (2018) 'MiR-378 promotes the cell proliferation of osteosarcoma through down-regulating the expression of Kruppel-like factor 9', *Biochem Cell Biol*, 96(5), pp. 515-521.

Peng, Y. and Croce, C. M. (2016) 'The role of MicroRNAs in human cancer', *Signal Transduct Target Ther*, 1, pp. 15004.

Perera, R. M. and Bardeesy, N. (2015) 'Pancreatic Cancer Metabolism: Breaking It Down to Build It Back Up', *Cancer Discov*, 5(12), pp. 1247-61.

Place, R. F., Li, L. C., Pookot, D., Noonan, E. J. and Dahiya, R. (2008) 'MicroRNA-373 induces expression of genes with complementary promoter sequences', *Proc Natl Acad Sci U S A*, 105(5), pp. 1608-13.

Ratti, M., Lampis, A., Ghidini, M., Salati, M., Mirchev, M. B., Valeri, N. and Hahne, J. C. (2020) 'MicroRNAs (miRNAs) and Long Non-Coding RNAs (lncRNAs) as New Tools for Cancer Therapy: First Steps from Bench to Bedside', *Target Oncol*, 15(3), pp. 261-278.

Ren, L. and Yu, Y. (2018) 'The role of miRNAs in the diagnosis, chemoresistance, and prognosis of pancreatic ductal adenocarcinoma', *Ther Clin Risk Manag*, 14, pp. 179-187.

Riedl, J. M., Posch, F., Horvath, L., Gantschnigg, A., Renneberg, F., Schwarzenbacher, E., Moik, F., Barth, D. A., Rossmann, C. H., Stotz, M., Schaberl-Moser, R., Pichler, M., Stöger, H., Greil, R., Djanani, A., Schlick, K. and Gerger, A. (2021) 'Gemcitabine/nab-Paclitaxel versus FOLFIRINOX for palliative first-line treatment of advanced pancreatic cancer: A propensity score analysis', *Eur J Cancer*, 151, pp. 3-13.

Roche, S., O'Neill, F., Murphy, J., Swan, N., Meiller, J., Conlon, N. T., Geoghegan, J., Conlon, K., McDermott, R., Rahman, R., Toomey, S., Straubinger, N. L., Straubinger, R. M., O'Connor, R., McVey, G., Moriarty, M. and Clynes, M. (2020) 'Establishment and

Characterisation by Expression Microarray of Patient-Derived Xenograft Panel of Human Pancreatic Adenocarcinoma Patients', *Int J Mol Sci*, 21(3).

Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L. and Bradley, A. (2004) 'Identification of mammalian microRNA host genes and transcription units', *Genome Res*, 14(10A), pp. 1902-10.

Rojas, L. A., Sethna, Z., Soares, K. C., Olcese, C., Pang, N., Patterson, E., Lihm, J., Ceglia, N., Guasp, P., Chu, A., Yu, R., Chandra, A. K., Waters, T., Ruan, J., Amisaki, M., Zebboudj, A., Odgerel, Z., Payne, G., Derhovanessian, E., Müller, F., Rhee, I., Yadav, M., Dobrin, A., Sadelain, M., Łuksza, M., Cohen, N., Tang, L., Basturk, O., Gönen, M., Katz, S., Do, R. K., Epstein, A. S., Momtaz, P., Park, W., Sugarman, R., Varghese, A. M., Won, E., Desai, A., Wei, A. C., D'Angelica, M. I., Kingham, T. P., Mellman, I., Merghoub, T., Wolchok, J. D., Sahin, U., Türeci, Ö., Greenbaum, B. D., Jarnagin, W. R., Drebin, J., O'Reilly, E. M. and Balachandran, V. P. (2023) 'Personalized RNA neoantigen vaccines stimulate T cells in pancreatic cancer', *Nature*, 618(7963), pp. 144-150.

Ruby, J. G., Jan, C. H. and Bartel, D. P. (2007) 'Intronic microRNA precursors that bypass Drosha processing', *Nature*, 448(7149), pp. 83-6.

Salamekh, S., Gottumukkala, S., Park, C., Lin, M. H. and Sanford, N. N. (2022) 'Radiotherapy for Pancreatic Adenocarcinoma: Recent Developments and Advances on the Horizon', *Hematol Oncol Clin North Am*, 36(5), pp. 995-1009.

Semaan, A., Bernard, V., Wong, J. W., Makino, Y., Swartzlander, D. B., Rajapakshe, K. I., Lee, J. J., Officer, A., Schmidt, C. M., Wu, H. H., Scaife, C. L., Affolter, K., Nachmanson, D., Firpo, M. A., Yip-Schneider, M., Lowy, A. M., Harismendy, O., Sen, S., Maitra, A., Jakubek, Y. A. and Guerrero, P. A. (2023) 'Integrated Molecular Characterization of Intraductal Papillary Mucinous Neoplasms: An NCI Cancer Moonshot Precancer Atlas Pilot Project', *Cancer Res Commun*.

Shigeyasu, K., Okugawa, Y., Toden, S., Boland, C. R. and Goel, A. (2017) 'Exportin-5 Functions as an Oncogene and a Potential Therapeutic Target in Colorectal Cancer', *Clin Cancer Res*, 23(5), pp. 1312-1322.

Si, W., Shen, J., Zheng, H. and Fan, W. (2019) 'The role and mechanisms of action of microRNAs in cancer drug resistance', *Clin Epigenetics*, 11(1), pp. 25.

Sipos, B., Frank, S., Gress, T., Hahn, S. and Klöppel, G. (2009) 'Pancreatic intraepithelial neoplasia revisited and updated', *Pancreatology*, 9(1-2), pp. 45-54.

Slotwinski, R., Lech, G. and Slotwinska, S. M. (2018) 'MicroRNAs in pancreatic cancer diagnosis and therapy', *Cent Eur J Immunol*, 43(3), pp. 314-324.

Srivastava, S. K., Arora, S., Singh, S., Bhardwaj, A., Averett, C. and Singh, A. P. (2014) 'MicroRNAs in pancreatic malignancy: progress and promises', *Cancer Lett*, 347(2), pp. 167-74.

Stavast, C. J. and Erkeland, S. J. (2019) 'The Non-Canonical Aspects of MicroRNAs: Many Roads to Gene Regulation', *Cells*, 8(11).

Stepanenko, A. A. and Heng, H. H. (2017) 'Transient and stable vector transfection: Pitfalls, off-target effects, artifacts', *Mutat Res Rev Mutat Res*, 773, pp. 91-103.

Strobel, O., Neoptolemos, J., Jager, D. and Buchler, M. W. (2019) 'Optimizing the outcomes of pancreatic cancer surgery', *Nat Rev Clin Oncol*, 16(1), pp. 11-26.

Subramani, R., Gangwani, L., Nandy, S. B., Arumugam, A., Chattopadhyay, M. and Lakshmanaswamy, R. (2015) 'Emerging roles of microRNAs in pancreatic cancer diagnosis, therapy and prognosis (Review)', *Int J Oncol*, 47(4), pp. 1203-10.

Sun, X., Zhou, X., Zhang, Y., Zhu, X. and Liu, H. (2018) 'Systematic Review and Meta-Analysis of Diagnostic Accuracy of miRNAs in Patients with Pancreatic Cancer', *Dis Markers*, 2018, pp. 6292396.

Sun, Y., Wang, P., Yang, W., Shan, Y., Zhang, Q. and Wu, H. (2019) 'The role of lncRNA MSC-AS1/miR-29b-3p axis-mediated CDK14 modulation in pancreatic cancer proliferation and Gemcitabine-induced apoptosis', *Cancer Biol Ther*, 20(6), pp. 729-739.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. and Bray, F. (2021) 'Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries', *CA Cancer J Clin*, 71(3), pp. 209-249.

Suzuki, H., Zuo, Y., Wang, J., Zhang, M. Q., Malhotra, A. and Mayeda, A. (2006) 'Characterization of RNase R-digested cellular RNA source that consists of lariat and circular RNAs from pre-mRNA splicing', *Nucleic Acids Res*, 34(8), pp. e63.

T. Conroy, P. P., V. Vilgrain, A. Lamarca, T. Seufferlein, E.M. O'Reilly, T. Hackert, T. Golan, G. Prager, K. Haustermans, A. Vogel, M. Ducreux, on behalf of the ESMO Guidelines Committee (2023) 'Pancreatic cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up'.

Ta, N., Huang, X., Zheng, K., Zhang, Y., Gao, Y., Deng, L., Zhang, B., Jiang, H. and Zheng, J. (2018) 'miRNA-1290 Promotes Aggressiveness in Pancreatic Ductal Adenocarcinoma by Targeting IKK1', *Cell Physiol Biochem*, 51(2), pp. 711-728.

Tan, D., Zhou, C., Han, S., Hou, X., Kang, S. and Zhang, Y. (2018) 'MicroRNA-378 enhances migration and invasion in cervical cancer by directly targeting autophagy-related protein 12', *Mol Med Rep*, 17(5), pp. 6319-6326.

Tavano, F., Gioffreda, D., Valvano, M. R., Palmieri, O., Tardio, M., Latiano, T. P., Piepoli, A., Maiello, E., Pirozzi, F. and Andriulli, A. (2018) 'Droplet digital PCR quantification of miR-1290 as a circulating biomarker for pancreatic cancer', *Sci Rep*, 8(1), pp. 16389.

Torphy, R. J., Fujiwara, Y. and Schulick, R. D. (2020) 'Pancreatic cancer treatment: better, but a long way to go', *Surg Today*, 50(10), pp. 1117-1125.

Téoule, P., Rasbach, E., Oweira, H., Otto, M., Rahbari, N. N., Reissfelder, C., Rückert, F. and Birgin, E. (2020) 'Obesity and Pancreatic Cancer: A Matched-Pair Survival Analysis', *J Clin Med*, 9(11).

Ungefroren, H., Thürling, I., Färber, B., Kowalke, T., Fischer, T., De Assis, L. V. M., Braun, R., Castven, D., Oster, H., Konukiewitz, B., Wellner, U. F., Lehnert, H. and Marquardt, J. U. (2022) 'The Quasimesenchymal Pancreatic Ductal Epithelial Cell Line PANC-1-A Useful Model to Study Clonal Heterogeneity and EMT Subtype Shifting', *Cancers (Basel)*, 14(9).

Usuba, W., Urabe, F., Yamamoto, Y., Matsuzaki, J., Sasaki, H., Ichikawa, M., Takizawa, S., Aoki, Y., Niida, S., Kato, K., Egawa, S., Chikaraishi, T., Fujimoto, H. and Ochiya, T. (2019) 'Circulating miRNA panels for specific and early detection in bladder cancer', *Cancer Sci*, 110(1), pp. 408-419.

Vaksman, O., Hetland, T. E., Trope, C. G., Reich, R. and Davidson, B. (2012) 'Argonaute, Dicer, and Drosha are up-regulated along tumor progression in serous ovarian carcinoma', *Hum Pathol*, 43(11), pp. 2062-9.

Valeri, N., Gasparini, P., Braconi, C., Paone, A., Lovat, F., Fabbri, M., Sumani, K. M., Alder, H., Amadori, D., Patel, T., Nuovo, G. J., Fishel, R. and Croce, C. M. (2010) 'MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2)', *Proc Natl Acad Sci U S A*, 107(49), pp. 21098-103.

Von Hoff, D. D., Ervin, T., Arena, F. P., Chiorean, E. G., Infante, J., Moore, M., Seay, T., Tjulandin, S. A., Ma, W. W., Saleh, M. N., Harris, M., Reni, M., Dowden, S., Laheru, D., Bahary, N., Ramanathan, R. K., Tabernero, J., Hidalgo, M., Goldstein, D., Van Cutsem, E., Wei, X., Iglesias, J. and Renschler, M. F. (2013) 'Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine', *N Engl J Med*, 369(18), pp. 1691-703.

Vreeland, T. J., McAllister, F., Javadi, S., Prakash, L. R., Fogelman, D. R., Ho, L., Varadhachary, G., Aloia, T. A., Vauthey, J. N., Lee, J. E., Kim, M. P., Katz, M. H. G. and Tzeng, C. D. (2019) 'Benefit of Gemcitabine/Nab-Paclitaxel Rescue of Patients With

Borderline Resectable or Locally Advanced Pancreatic Adenocarcinoma After Early Failure of FOLFIRINOX', *Pancreas*, 48(6), pp. 837-843.

Wainberg, Z. A., Hochster, H. S., Kim, E. J., George, B., Kaylan, A., Chiorean, E. G., Waterhouse, D. M., Guitierrez, M., Parikh, A., Jain, R., Carrizosa, D. R., Soliman, H. H., Lila, T., Reiss, D. J., Pierce, D. W., Bhore, R., Banerjee, S., Lyons, L., Louis, C. U., Ong, T. J. and O'Dwyer, P. J. (2020) 'Open-label, Phase I Study of Nivolumab Combined with', *Clin Cancer Res*, 26(18), pp. 4814-4822.

Wang, C., Harris, W. S., Chung, M., Lichtenstein, A. H., Balk, E. M., Kupelnick, B., Jordan, H. S. and Lau, J. (2006) 'n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review', *Am J Clin Nutr*, 84(1), pp. 5-17.

Wang, J., Chen, J., Chang, P., LeBlanc, A., Li, D., Abbruzzese, J. L., Frazier, M. L., Killary, A. M. and Sen, S. (2009) 'MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease', *Cancer Prev Res (Phila)*, 2(9), pp. 807-13.

Wang, L., Wang, Z., Huang, L., Wu, C. and Zhang, B. (2019) 'MiR-29b suppresses proliferation and mobility by targeting SOX12 and DNMT3b in pancreatic cancer', *Anticancer Drugs*, 30(3), pp. 281-288.

Wang, L. H., Huang, J., Wu, C. R., Huang, L. Y., Cui, J., Xing, Z. Z. and Zhao, C. Y. (2018) 'Downregulation of miR-29b targets DNMT3b to suppress cellular apoptosis and enhance proliferation in pancreatic cancer', *Mol Med Rep*, 17(2), pp. 2113-2120.

Wang, P., Zhuang, L., Zhang, J., Fan, J., Luo, J., Chen, H., Wang, K., Liu, L., Chen, Z. and Meng, Z. (2013) 'The serum miR-21 level serves as a predictor for the chemosensitivity of advanced pancreatic cancer, and miR-21 expression confers chemoresistance by targeting FasL', *Mol Oncol*, 7(3), pp. 334-45.

Wei, J., Yang, L., Wu, Y. N. and Xu, J. (2020) 'Serum miR-1290 and miR-1246 as Potential Diagnostic Biomarkers of Human Pancreatic Cancer', *J Cancer*, 11(6), pp. 1325-1333.

Weinmann, L., Höck, J., Ivacevic, T., Ohrt, T., Mütze, J., Schwille, P., Kremmer, E., Benes, V., Urlaub, H. and Meister, G. (2009) 'Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs', *Cell*, 136(3), pp. 496-507.

Wellner, U., Schubert, J., Burk, U. C., Schmalhofer, O., Zhu, F., Sonntag, A., Waldvogel, B., Vannier, C., Darling, D., zur Hausen, A., Brunton, V. G., Morton, J., Sansom, O., Schuler, J., Stemmler, M. P., Herzberger, C., Hopt, U., Keck, T., Brabletz, S. and Brabletz, T. (2009) 'The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs', *Nat Cell Biol*, 11(12), pp. 1487-95.

Wong, A. C. H. and Rasko, J. E. J. (2021) 'Splice and Dice: Intronic microRNAs, Splicing and Cancer', *Biomedicines*, 9(9).

Wörmann, S. M., Diakopoulos, K. N., Lesina, M. and Algül, H. (2014) 'The immune network in pancreatic cancer development and progression', *Oncogene*, 33(23), pp. 2956-67.

Xiao, H. and Liu, M. (2020) 'Circular RNA hsa\_circ\_0053277 promotes the development of colorectal cancer by upregulating matrix metalloproteinase 14 via miR-2467-3p sequestration', *J Cell Physiol*, 235(3), pp. 2881-2890.

Xu, F., Zhang, Q., Cheng, W., Zhang, Z., Wang, J. and Ge, J. (2013) 'Effect of miR-29b-1\* and miR-29c knockdown on cell growth of the bladder cancer cell line T24', *J Int Med Res*, 41(6), pp. 1803-10.

Xu, Y., Ouyang, C., Lyu, D., Lin, Z., Zheng, W., Xiao, F., Xu, Z. and Ding, L. (2020) 'Diabetic nephropathy exacerbates epithelial-to-mesenchymal transition (EMT) via miR-2467-3p/Twist1 pathway', *Biomed Pharmacother*, 125, pp. 109920.

Yakar, D., Bozkırlı, B. O. and Ceyhan, G. O. (2022) 'Genetic landscape of pancreatic cancer: a narrative review', *Chin Clin Oncol*, 11(1), pp. 5.

- Yonemori, K., Kurahara, H., Maemura, K. and Natsugoe, S. (2017) 'MicroRNA in pancreatic cancer', *J Hum Genet*, 62(1), pp. 33-40.
- Younger, S. T. and Corey, D. R. (2011) 'Transcriptional regulation by miRNA mimics that target sequences downstream of gene termini', *Mol Biosyst*, 7(8), pp. 2383-8.
- Younger, S. T., Pertsemlidis, A. and Corey, D. R. (2009) 'Predicting potential miRNA target sites within gene promoters', *Bioorg Med Chem Lett*, 19(14), pp. 3791-4.
- Yu, J., Li, A., Hong, S. M., Hruban, R. H. and Goggins, M. (2012) 'MicroRNA alterations of pancreatic intraepithelial neoplasias', *Clin Cancer Res*, 18(4), pp. 981-92.
- Yu, S., Lu, Z., Liu, C., Meng, Y., Ma, Y., Zhao, W., Liu, J., Yu, J. and Chen, J. (2010) 'miRNA-96 suppresses KRAS and functions as a tumor suppressor gene in pancreatic cancer', *Cancer Res*, 70(14), pp. 6015-25.
- Yu, S. N., Ma, Y. H., Zhao, W. G., Jin, X. L., Yang, H. Y., Liu, P. P. and Chen, J. (2016) 'KRAS-related noncoding RNAs in pancreatic ductal adenocarcinoma', *Chronic Dis Transl Med*, 2(4), pp. 215-222.
- Zardo, G., Ciolfi, A., Vian, L., Starnes, L. M., Billi, M., Racanicchi, S., Maresca, C., Fazi, F., Travaglini, L., Noguera, N., Mancini, M., Nanni, M., Cimino, G., Lo-Coco, F., Grignani, F. and Nervi, C. (2012) 'Polycombs and microRNA-223 regulate human granulopoiesis by transcriptional control of target gene expression', *Blood*, 119(17), pp. 4034-46.
- Zeng, M., Zhu, L., Li, L. and Kang, C. (2017) 'miR-378 suppresses the proliferation, migration and invasion of colon cancer cells by inhibiting SDAD1', *Cell Mol Biol Lett*, 22, pp. 12.
- Zhang, H., Feng, X., Zhang, M., Liu, A., Tian, L., Bo, W., Wang, H. and Hu, Y. (2019) 'Long non-coding RNA CASC2 upregulates PTEN to suppress pancreatic carcinoma cell metastasis by downregulating miR-21', *Cancer Cell Int*, 19, pp. 18.
- Zhang, X., Ren, D., Wu, X., Lin, X., Ye, L., Lin, C., Wu, S., Zhu, J., Peng, X. and Song, L. (2018) 'miR-1266 Contributes to Pancreatic Cancer Progression and Chemoresistance by the STAT3 and NF-kappaB Signaling Pathways', *Mol Ther Nucleic Acids*, 11, pp. 142-158.
- Zhao, L., Singh, V., Ricca, A. and Lee, P. (2022) 'Survival Benefit of Pembrolizumab for Patients With Pancreatic Adenocarcinoma: A Case Series', *J Med Cases*, 13(5), pp. 240-243.
- Zhao, Q., Chen, S., Zhu, Z., Yu, L., Ren, Y., Jiang, M., Weng, J. and Li, B. (2018) 'miR-21 promotes EGF-induced pancreatic cancer cell proliferation by targeting Spry2', *Cell Death Dis*, 9(12), pp. 1157.
- Zhao, Y., Yin, J., Zhang, L., Zhang, Y. and Chen, X. (2023) 'Drug-drug interaction prediction: databases, web servers and computational models', *Brief Bioinform*, 25(1).
- Zheng, L. (2017) 'PD-L1 Expression in Pancreatic Cancer', *J Natl Cancer Inst*, 109(6).
- Zhu, X., Trueman, S., Straubinger, R. M. and Jusko, W. J. (2018) 'Physiologically-based pharmacokinetic and pharmacodynamic models for gemcitabine and birinapant in pancreatic cancer xenografts', *J Pharmacokinet Pharmacodyn*, 45(5), pp. 733-746.

## **7.Appendix**



# FOLFIRINOX Pharmacodynamic Interactions in 2D and 3D Pancreatic Cancer Cell Cultures

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## Abstract

The multi-drug combination regime, FOLFIRINOX, is a standard of care chemotherapeutic therapy for pancreatic cancer patients. However, systematic evaluation of potential pharmacodynamic interactions among multi-drug therapy has not been reported previously. Here, pharmacodynamic interactions of the FOLFIRINOX agents (5-fluorouracil (5-FU), oxaliplatin (Oxa) and SN-38, the active metabolite of irinotecan) were assessed across a panel of primary and established pancreatic cancer cells. Inhibition of cell proliferation was quantified for each drug, alone and in combination, to obtain quantitative, drug-specific interaction parameters and assess the nature of drug interactions. The experimental data were analysed assuming Bliss independent interactions, and nonlinear regression model fitting was conducted in SAS. Estimates of the drug interaction term,  $\psi$  ( $\psi$ ), revealed that the Oxa/SN-38 combination appeared synergistic in PANC-1 ( $\psi = 0.6$ , 95% CI = 0.4, 0.9) and modestly synergistic, close to additive, in MIAPaCa-2 ( $\psi = 0.8$ , 95% CI = 0.6, 1.0) in 2D assays. The triple combination was strongly synergistic in MIAPaCa-2 ( $\psi = 0.2$ , 95% CI = 0.1, 0.3) and modestly synergistic/borderline additive in PANC-1 2D ( $\psi = 0.8$ , 95% CI = 0.6, 1.0). The triple combination showed antagonistic interactions in the primary PIN-127 and 3D PANC-1 model ( $\psi > 1$ ). Quantitative pharmacodynamic interactions have not been described for the FOLFIRINOX regimen; this analysis suggests a complex interplay among the three chemotherapeutic agents. Extension of this pharmacodynamic analysis approach to clinical/translational studies of the FOLFIRINOX combination could reveal additional pharmacodynamic interactions and guide further refinement of this regimen to achieve optimal clinical responses.

**Keywords** FOLFIRINOX · pancreatic cancer · pharmacodynamic modelling

## Introduction

Pancreatic adenocarcinoma (PDAC) has one of the highest mortality rates, with a 5-year survival rate of approximately 9% and an average survival time of less than 6 months (1). The poor prognosis of PDAC is due to a combination of factors including frequent diagnosis at a late stage, when it is locally advanced or metastatic, and its asymptomatic progression (2). Because of the combined effect of genetic and epigenetic modifications in pancreatic tumours, they exhibit an aggressive nature, with therapeutic resistance from an early stage (3, 4).

Surgery remains the only curative option for pancreatic cancer patients; however, less than one in five patients are eligible for surgery. Patients often receive neoadjuvant therapy to down-stage tumour burden and allow resection (5). There is limited data on regimen selection, with the most common therapeutic regimes selected for the metastatic disease being

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FOLFIRINOX (5-fluorouracil (5-FU)/leucovorin (LV), irinotecan and oxaliplatin (6)) or gemcitabine/nab-paclitaxel. There is even less guidance in relation to second-line regimens. Studies comparing the efficacy of FOLFIRINOX versus gemcitabine/nab-paclitaxel are also confounded based on comparisons of different patient populations (7). FOLFIRINOX is the preferred first-line option for patients with metastatic PDAC younger than 76 years and who have good performance status (ECOG 0 or 1). To increase tolerability and decrease side effects, modified FOLFIRINOX regimens typically eliminate the 5-FU bolus loading dose (8, 9). Patients also receive these agents as adjuvant (10) and palliative treatment settings (11, 12).

Empirical clinical optimisation of multi-drug treatment regimens is often hampered by a lack of understanding of pharmacodynamic (PD) drug-drug interactions (DDIs) (13). DDIs can be additive, synergistic or antagonistic (14). With additive interactions, the overall effect of a drug combination is the sum of the pharmacological effects of the individual agents (15). Synergistic interactions have an overall effect that is greater than additive, and conversely, antagonism represents an overall response that is less than additive. However, the approaches to evaluate PD DDIs vary, and these terms are frequently misused (13). PD DDI studies require the assessment of interactions between multiple concentrations of each single agent in the combination, which decreases the feasibility of studying PD interactions in animal models and clinical trials. With the clinical proliferation of combination therapies, there is an increasing opportunity for the application of mathematical modelling strategies to evaluate therapeutic combination regimens (16), which could ultimately enable therapeutic optimisation by modelling and simulation. Improvements in high-throughput experimental and computational techniques are contributing to the growing rationale for using PD DDI modelling in clinical trials (17).

*In vitro* cellular models of PDAC often fail to represent the heterogeneity of the tumour and the tumour microenvironment. In recent years, a shift to 3D spheroid or organoid models to recapitulate more physiologically relevant morphologies has occurred. Nelson et al. described a method for the establishment of organoids and isogenetically matched 2D primary cell lines from PDAC patient-derived xenograft (PDX) tumour samples (including PIN-127) and recapitulation of the cell lines to cell-line organoids that were shown to be morphologically, molecularly and transcriptionally similar to their PDX-derived tumour organoids (18). Another study compared a large drug panel across 2D and 3D cultures, finding that most cytotoxic agents were less active in 3D, but a few drugs showed greater cytotoxicity in 3D models compared to 2D culture, which proved to be both cell and drug dependent (19). Ait-Oudhia et al. described a 3D and dynamic cellular system suitable for *in*

*vitro* pharmacokinetic (PK) and PD analysis of combination therapies and compared this to static 2D culture, showing that 2D data successfully predicted the 3D data profiles (20). These studies highlight the importance of incorporating multiple cellular models into drug screening experiments.

Here, we investigated PD DDIs of the drugs in the FOLFIRINOX regimen to determine the nature of their interactions, using both primary and established pancreatic cancer cell lines cultured as 2-D and 3-D models. The three cytotoxic agents of FOLFIRINOX were evaluated alone, in pairwise combinations, and together over ranges of concentrations. For each combination, a quantitative DDI term,  $\psi$  ( $\psi$ ), was derived to express the nature and magnitude of the interaction. Established pharmacodynamic modelling strategies for pairwise comparisons of drug interactions were extended to include triple drug combinations.

## Materials and Methods

### Cell Culture

The human pancreatic cell-line MIAPaCa-2 was obtained from the European Collection of Cell Cultures (ECACC, UK). BxPC-3 and PANC-1 human pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). PIN-127 cells were derived in-house from a patient-derived xenograft model as previously described (21).

PANC-1 and MIAPaCa-2 cells were grown in DMEM (Sigma-Aldrich, Dublin, Ireland) supplemented with 5% (v/v) fetal bovine serum (FBS) (Thermo Fischer Scientific, UK) and 2% L-glutamine. BxPC-3 cells were grown in RPMI-1640 medium supplemented with 5% (v/v) FBS. PIN-127 cells were cultured in DMEM/F-12 Hams medium supplemented with 10% (v/v) FBS (Corning, SA). Cells were grown in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C in culture flasks. Cells were sub-cultured, using PBS to wash and trypsin-EDTA (Bio-Sciences Ltd, Ireland) to detach when cells reached 80–90% confluency.

### Reagents

SN-38, the active metabolite of irinotecan, was obtained from Stratech Scientific Ltd (Ely UK). A 10 mM SN-38 stock solution was prepared in DMSO and stored as aliquots at –20°C. Leucovorin was purchased from Sigma-Aldrich (Dublin, Ireland), and 10 mM stocks were prepared in water. 5-Fluorouracil (5-FU) and oxaliplatin (Oxa) were supplied as clinical formulations from St. Vincent's University Hospital, Dublin, at stock concentrations of 192 mM and 12.5 mM, respectively, and stored at room temperature. All

reagent aliquots were dated upon reconstitution and assessed to ensure efficiency was consistent between replicates.

## 2D Proliferation Assay

MIAPaCa-2, PANC-1, BxPC-3 and PIN-127 cells were seeded in 96-well plates at a density of 1 cells/mL, 2 cells/mL, 3 cells/mL, and  $5 \times 10^4$  cells/mL, respectively, and incubated overnight at 5% CO<sub>2</sub> and 37°C. The following day, freshly prepared drug dilutions were added to each well. Two approaches were taken for drug exposure concentrations. (1) **Fixed ratio**: cells were treated with single, pairwise and triple combinations at a fixed ratio, or (2) **Varying ratio**: pairwise drug combinations at 5 concentrations of each drug, resulting in 25 combinations were tested. The plates were incubated for a further 6 days until control cells approached confluency. Following incubation with the drug, the media was removed, and cell proliferation was quantified using an acid phosphate assay as previously described (22). Briefly, the wells were washed with phosphate-buffered saline (PBS). Paranitrophenol phosphate (PNP) (ThermoFisher) substrate (10 mM PNP in sodium acetate buffer, pH 5.5) was added to each well and incubated (37°C) for 1–2 h; 50 µL 1 M NaOH was added, and the absorbance was read at 405 nm (reference – 620 nm).

Both methods required different analysis software. Schema (1) in which cells were treated with single, pairwise and triple combinations at fixed ratio were analysed using SAS 9.4 software as described in the ‘[Mathematical Modelling](#)’ section. Schema (2) in which cells were treated with varying ratios of pairwise combinations at 5 concentrations of each drug was analysed using Combeneft™, as described in the ‘[Analysis of Varying Pairwise Ranges Using Combeneft™ Software](#)’ section.

## Analysis of Varying Pairwise Ranges Using Combeneft™ Software

As described in the ‘[2D proliferation assay](#)’ section, BxPC-3 and PIN-127 cells were examined using a varying pairwise concentration proliferation assay. *In vitro* experimental data were analysed using a surface approach, in which data were compared to mathematical models of concentration response for additive combinations. To keep results comparable, the Bliss model was selected. In brief, an experimental concentration–response surface that describes the combination effect in a concentration space was derived from a matrix of percentage of control values across concentrations. The effect of the single agents was extracted from the data by curve fitting. Based on the single agent curves, a model-based pairwise combination concentration–response surface was composed, providing a ‘reference’ surface for additivity.

Experimental and model-generated combination concentration–response surfaces were then compared, resulting in a synergy distribution in concentration space (23).

## 3D Cell Proliferation Assay

96 well plates coated with 50 µL polyhema (Sigma-Aldrich S3932, 5 mg/mL in 96% ethanol) were baked at 50°C for two days. Plates were stored at room temperature for up to 6 months. PANC-1 cells (90 µL/well) were seeded at  $2 \times 10^4$  cells/mL in 5% FBS DMEM with Matrigel™ (Sigma-Aldrich) (2%) and incubated overnight at 37°C. Serial drug dilutions in complete media (30 µL) or drug-free media (control) were added to the wells and incubated at 37°C. After 9 days, cells were quantified using alamarBlue® Cell Viability assay (Invitrogen) to avoid the washing steps required for the acid phosphate assay. Briefly, 12 µL of alamarBlue® reagent (10% of final volume) was added to the wells and incubated at 37°C for 4 h, and fluorescence was measured at 535/590 nm excitation/emission wavelength on a Biotek plate reader using KC4 software. The blank for the background consisted of media and 2% Matrigel™. Percent viability was calculated relative to untreated control. Each assay was performed in biological triplicate.

## Mathematical Modelling

### Single-drug Studies

For single-agent 5-FU, oxaliplatin and SN-38, the IC<sub>50</sub> (half maximal inhibitory concentration) values were determined using the Hill equation (Table I, Eq. (1)). In this equation,  $R_i$  represents the viability of cells after 5 days of exposure to drug  $i$ ;  $I_{\max}$  represents the maximum inhibition achieved at a high concentration;  $C$  represents the concentration used for testing;  $IC_{50}$  represents the concentration to achieve 50% of  $I_{\max}$ ; and  $m$  is the Hill coefficient that corresponds with the steepness of the inhibition curve. These parameters provided the foundation for further pairwise and triple drug combination studies and associated modelling. The data were analysed by nonlinear regression to provide a statistical analysis of the estimated parameters.

### Pairwise Drug Combinations

A universal response surface approach to fit all data simultaneously was applied to the Bliss independent model to analyse cell viability data for PANC-1 (2D and 3D), MIA-PaCa-2, BxPC-3 and PIN-127 (24, 25). In Eq. (2) (Table I),  $i$  and  $j$  represent the two drugs in a combination pair; and  $\psi$  is the drug interaction term for combination treatments. The interaction term  $\psi$  is introduced into the equation to quantify interaction in combination groups. If  $\psi < 1$ , the

**Table I** Pharmacodynamic Equations for Model Fitting

|                                                                                                                                                                                                                                                                                                  |   |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---|
| $R_i = 100\% \cdot \left( 1 - I_{\max,i} \cdot \frac{C_i^{m_i}}{C_i^{m_i} + IC_{50,i}^{m_i}} \right)$                                                                                                                                                                                            | 1 |
| $R = 100\% \cdot \left( 1 - I_{\max,i} \cdot \frac{C_i^{m_i}}{C_i^{m_i} + IC_{50,i}^{m_i}} \right) \cdot \left( 1 - I_{\max,j} \cdot \frac{C_j^{m_j}}{C_j^{m_j} + (\psi) \cdot IC_{50,j}^{m_j}} \right)$                                                                                         | 2 |
| $R = 100\% \cdot \left( 1 - I_{\max,i} \cdot \frac{C_i^{m_i}}{C_i^{m_i} + IC_{50,i}^{m_i}} \right) \cdot \left( 1 - I_{\max,j} \cdot \frac{C_j^{m_j}}{C_j^{m_j} + IC_{50,j}^{m_j}} \right) \cdot \left( 1 - I_{\max,k} \cdot \frac{C_k^{m_k}}{C_k^{m_k} + (\psi) \cdot IC_{50,k}^{m_k}} \right)$ | 3 |

SAS 9.4 software was used to model the effect of the single, pairwise and triple combinations on cell proliferation. The equations were applied to single (Eq. (1)), pairwise (Eq. (2)) and triple (Eq. (3)) combination data to determine pharmacodynamic interactions.  $I_{\max}$  represents the maximum inhibition achieved at high concentrations.  $IC_{50}$  represents the concentration to achieve 50% of  $I_{\max}$ , and  $m$  is the Hill coefficient that corresponds with the steepness of the inhibition curve

interaction is synergistic, additive if  $\psi = 1$ , and antagonistic if  $\psi > 1$ . Upper and lower 95% confidence intervals were used to show statistically significant interaction indexes. In fitting the data,  $\psi$  was estimated separately for both drugs to evaluate the sensitivity of the calculated value.

### Triple Drug Combination

The FOLFIRINOX combination includes leucovorin (LV) as a potential sensitiser of tumour cells to 5-FU (26, 27). *In vitro* analysis of the 5-FU/LV combination showed no significant synergistic effect (Supplementary Fig. S2). Therefore, further analyses included only the three cytotoxic FOLFIRINOX drugs. For pharmacodynamic modelling of the triple drug combination, Eq. (2) was expanded, as shown in Eq. (3) (Table I). In estimating  $\psi$ , the fitted value was assigned to the  $IC_{50}$  of each cytotoxic agent as follows: 5-FU (MIAPaCa-2/BxPC-3), SN-38 (PANC-1 2D/3D) and 5-FU and Oxa (PIN-127).

### Model Fitting

Model fitting was conducted in SAS 9.4 (Cary, NC) using the nonlinear regression procedure ‘nlin’ with the MARQUARDT method (Fig. 1). The models were weighted by the square of the fitted values (i.e. inverse of the variance). The theoretical additive responses were calculated by Monte-Carlo simulation considering the uncertainty of parameter estimation and random experimental error [28].

## Results

### Effect of the Single FOLFIRINOX Agents in Pancreatic Cancer Cells

Concentration-effect assays of each single agent were performed to determine  $IC_{50}$  values. The 2D assays employed

an acid phosphatase end-point assay, whereas the 3D assays employed alamarBlue to estimate cell proliferation. alamarBlue measures cellular metabolic activity; the active ingredient resazurin is reduced to the resorufin product within viable cells and as such is an indirect measure of cell proliferation. To ensure comparability, a comparison of the alamarBlue and acid phosphatase proliferation assays was performed for the triple combination in PANC-1 cells, and the results were statistically equivalent (Supplementary Fig. S1).

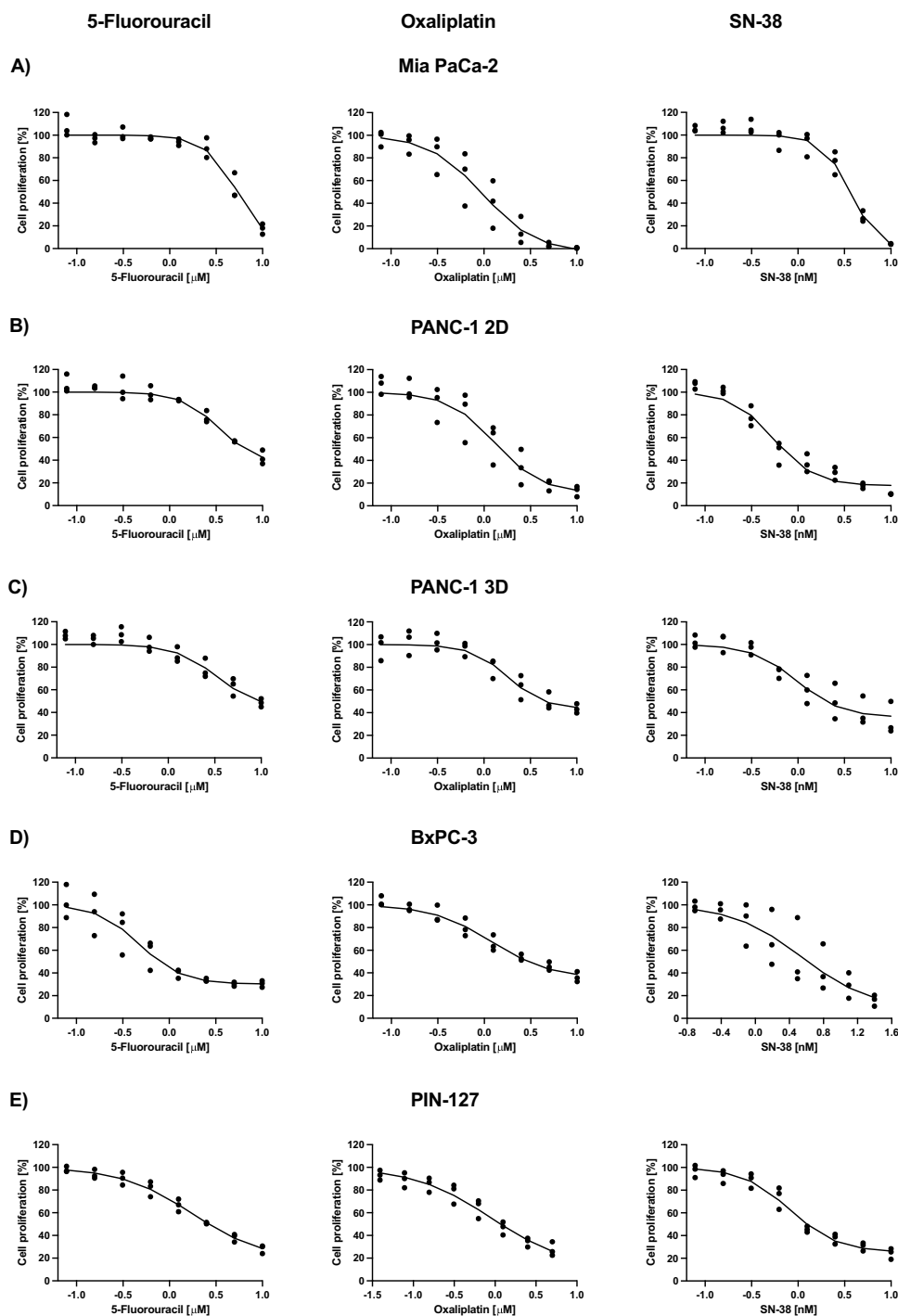
Model-estimated parameters  $I_{\max}$ ,  $IC_{50}$  and  $m$  (Table II) were consistent with the experimental data in Fig. 1. Varying responses to each single agent were observed for each cell line (Table II). BxPC-3 cells were the most sensitive to 5-FU ( $IC_{50} = 0.5 \mu\text{M}$ ), and MIAPaCa-2 cells were the least sensitive ( $IC_{50} = 5.4 \mu\text{M}$ ). The primary cell-line PIN-127 was the most sensitive to oxaliplatin and to SN-38. PANC-1 cells were the least sensitive to SN-38 in these *in vitro* studies in both 2D ( $IC_{50} = 7.6 \text{ nM}$ ) and 3D ( $IC_{50} = 42.9 \text{ nM}$ ) cultures.

Leucovorin (LV), a potentiator of 5-FU efficacy (26), was tested alone and combined with 5-FU across the panel of cell lines. Supplementary Fig. S2 shows that single-agent LV had no effect on cell proliferation and no statistically significant potentiating effect in combination with 5-FU. Therefore, the remaining evaluation of 2- and 3-drug effects on the cell-line panel included only the 3 cytotoxic agents of the FOLFIRINOX combination.

### Combination of Oxaliplatin and SN-38 Synergistically Inhibits Cell Proliferation Across Multiple Pancreatic Cancer Cell Lines

Analysis of the pairwise pharmacodynamic interactions of each agent of the FOLFIRINOX regimen demonstrated that oxaliplatin and SN-38 was the only combination that showed synergistic interactions across two cell lines. This synergy was observed in MIAPaCa-2 (2D) and PANC-1 (2D), as shown in Table III. This suggests that oxaliplatin

**Fig. 1** Experimental versus model-fitted responses. SAS 9.4 software was used for nonlinear regression to generate the model-fitted values for the single-drug experiments in all cell lines. The points on the graphs represent the observed data, and the solid sigmoidal lines represent the model-fitted curves. Columns 1–3 represent 5-FU, oxaliplatin and SN-38. Cell lines are separated into rows A–E: MIAPaCa-2 (A), PANC-1 2D (B), PANC-1 3D (C), BxPC-3 (D) and PIN-127 (E)



and SN-38 were the most potent pairwise combination. Borderline additive interactions were observed between 5-FU and oxaliplatin in the MIAPaCa-2 cell line, whereas interactions trending towards antagonism were observed across all other cell lines. SN-38 and 5-FU interactions were additive in both the MIAPaCa-2 and PANC-1 cell lines but trended towards antagonistic in the PIN-127, BxPC-3 and PANC-1 3D cells, with  $\psi$  values ranging from 1 to 8.4 (Table III). These results suggest that 5-FU/Oxa and SN-38/5-FU show

less beneficial DDI than oxaliplatin/SN-38 in this panel of pancreatic cancer cells.

Each of the pairwise drug combinations displayed antagonistic interactions in the primary PIN-127 and KRAS wild-type BxPC-3 cell lines, with  $\psi$  values listed in Table III. To evaluate these drug interactions further, BxPC-3 (Fig. 2) and PIN-127 (Fig. 3) cell proliferation was evaluated in a  $5 \times 5$  grid of concentrations. Experimental response to these varying concentrations as percentage proliferation compared to

**Table II** Model-estimated Pharmacodynamic Parameters for the Single Agent Chemosensitivity Experiments Across All Cell Lines

| Cell line          | Drug | I <sub>max</sub> | SE  | IC <sub>50</sub> | SE  | m   | SE  |
|--------------------|------|------------------|-----|------------------|-----|-----|-----|
| <b>PIN-127</b>     | 5-FU | 0.8              | 0.1 | 1.8              | 0.3 | 1.1 | 0.2 |
|                    | Oxal | 0.9              | 0.1 | 0.9              | 0.3 | 0.9 | 0.1 |
|                    | SN38 | 0.7              | 0.0 | 1.7              | 0.0 | 1.7 | 0.2 |
| <b>BxPC-3</b>      | 5-FU | 0.7              | 0.0 | 0.5              | 0.1 | 1.9 | 0.5 |
|                    | Oxal | 0.6              | 0.1 | 1.2              | 0.4 | 1.4 | 0.4 |
|                    | SN38 | 0.9              | 0.1 | 5.1              | 0.0 | 1.1 | 0.3 |
| <b>MIA PaCa-2</b>  | 5-FU | 1.0              | 0.2 | 5.4              | 1.2 | 2.4 | 0.7 |
|                    | Oxal | 1.0              | 0.1 | 1.0              | 0.1 | 1.5 | 0.2 |
|                    | SN38 | 1.0              | 0.1 | 1.8              | 0.0 | 2.8 | 0.5 |
| <b>PANC-1 (2D)</b> | 5-FU | 0.6              | 0.1 | 3.4              | 0.8 | 2.1 | 0.7 |
|                    | Oxal | 0.9              | 0.0 | 1.3              | 0.2 | 1.7 | 0.3 |
|                    | SN38 | 0.8              | 0.0 | 7.6              | 0.0 | 2.0 | 0.3 |
| <b>PANC-1 (3D)</b> | 5-FU | 0.6              | 0.1 | 3.3              | 1.1 | 1.9 | 0.7 |
|                    | Oxal | 0.6              | 0.1 | 1.8              | 0.3 | 2.2 | 0.6 |
|                    | SN38 | 0.6              | 0.0 | 42.9             | 0.0 | 1.8 | 0.4 |

Parameter values were derived by nonlinear regression using SAS 9.4, and parameter abbreviations are defined in Table I

MG, model generated; SE, standard error

**Table III** Summary of the Pairwise Combinations Across the Cell Lines

| Cell line          | Pairwise Combination | Psi Value ( $\psi$ ) | SE  | 95% CI Lower | 95% CI Higher |
|--------------------|----------------------|----------------------|-----|--------------|---------------|
| <b>PIN-127</b>     | 5-FU + Oxa           | 3.9                  | 0.9 | 2.1          | 5.7           |
|                    | Oxa + SN-38          | 4.2                  | 1.5 | 1.2          | 7.3           |
|                    | SN-38 + 5-FU         | 1.9                  | 0.3 | 1.2          | 2.6           |
| <b>BxPC-3</b>      | 5-FU + Oxa           | 1.2                  | 0.2 | 0.8          | 1.6           |
|                    | Oxa + SN-38          | 2.2                  | 1.0 | 0.1          | 3.3           |
|                    | SN-38 + 5-FU         | 1.7                  | 0.5 | 0.6          | 2.7           |
| <b>MiaPaCa-2</b>   | 5-FU + Oxa           | 1.0                  | 0.1 | 0.8          | 1.2           |
|                    | Oxa + SN-38          | 0.8                  | 0.1 | 0.6          | 1.0           |
|                    | SN-38 + 5-FU         | 1.0                  | 0.0 | 0.9          | 1.1           |
| <b>PANC-1 (2D)</b> | 5-FU + Oxa           | 1.3                  | 0.1 | 1.0          | 1.6           |
|                    | Oxa + SN-38          | 0.6                  | 0.1 | 0.4          | 0.9           |
|                    | SN-38 + 5-FU         | 1.0                  | 0.1 | 0.6          | 1.2           |
| <b>PANC-1 (3D)</b> | 5-FU + Oxa           | 1.1                  | 0.2 | 0.6          | 1.6           |
|                    | Oxa + SN-38          | 1.0                  | 0.2 | 0.4          | 1.5           |
|                    | SN-38 + 5-FU         | 8.4                  | 2.3 | 3.6          | 13.1          |

The psi ( $\psi$ ) interaction term was estimated for the combinations using Eq. (2) (Table I). Interactions were categorised as synergistic ( $\psi < 1$ ), additive ( $\psi = 1$ ) or antagonistic ( $\psi > 1$ ). Red indicates antagonism, orange indicates additivity, and green identifies combinations with synergistic interactions

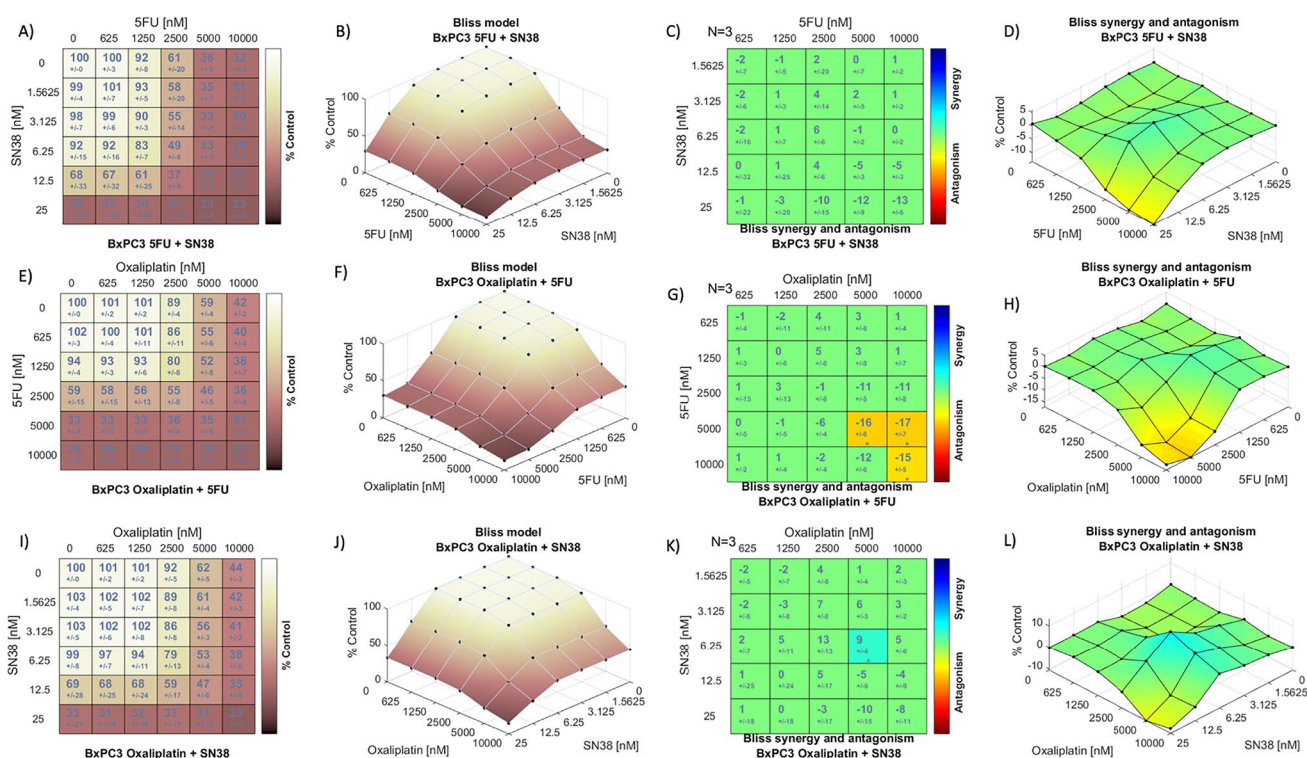


**Fig. 2** Pairwise combinations produce varying areas of strong antagonism and synergy in BxPC-3 cells. **A–D**: response to 5-FU/SN-38, **E–H**: response to Oxa/5-FU, **I–L**: response to Oxa/SN-38. Analysis of altering concentration–response represented in matrix format (**A**, **E**, **I**) and surface plot (**B**, **F**, **J**) as a percentage of proliferation compared to media control. **Synergy levels in matrix format** (**C**, **G**, **K**) represent the synergy/antagonism score with the

standard deviation number listed below; only coloured synergy levels were significant following a one-sample *t*-test (\**p*-value  $< 5 \times 10^{-2}$ ; \*\**p*-value  $< 10^{-3}$ ; \*\*\**p*-value  $< 10^{-4}$ ). This is generated by CombeneFit™ according to the **Bliss independent model** in comparison to the reference concentration–response surface (**D**, **H**, **L**). Data, mean  $\pm$  SD, *n* = 3

control is shown in Fig. 2A, E, I (BxPC-3) and Fig. 3A, E, I (PIN-127). Bliss scores generated by CombeneFit™ are coloured when greater than 0 to indicate statistically significant synergistic effects. The experimental combination concentration–response surface, with an overlay of synergy levels, illustrated the inhibition of proliferation achieved within the concentration space (Fig. 2B, F, J: BxPC-3 and Fig. 3B, F, J: PIN-127). Figure 2K, L shows an identified area of synergy for BxPC-3 cells, but it was narrowly confined at 5  $\mu$ M oxaliplatin and 6.25 nM SN-38. Substantial antagonism was observed for oxaliplatin and 5-FU at higher drug concentrations, with antagonism scores ranging from  $-15$  to  $-17$ , as shown in Fig. 2G, H. Importantly, the overall interactions remained additive or antagonistic in agreement with the model fitting results shown in Table III. This highlights how pharmacodynamic modelling could be used to identify potential opportunities to increase efficacy and minimise overlapping toxicity by utilising lower drug concentrations.

In PIN-127 cells, the combination of 5-FU and SN-38 showed numerous antagonistic combination ratios at the higher concentration range for both drugs (Fig. 3C, D). Antagonism scores ranged from  $-8$  to  $-17$ ; 5-FU (2.5  $\mu$ M,

5  $\mu$ M, and 10  $\mu$ M) combined with SN-38 (5 nM and 10 nM) were antagonistic, with the highest antagonism observed between 5  $\mu$ M and 10  $\mu$ M 5-FU and 5 nM SN-38. Figure 3G, H displays the antagonism observed between 5  $\mu$ M oxaliplatin and 10  $\mu$ M 5-FU, with an antagonism score of  $-9$  (*p*-value  $< 0.05$ ). At the higher concentration ratios, antagonism was observed for the pairwise combination of oxaliplatin and SN-38; antagonism scores of  $-14$  and  $-10$  were observed at 5  $\mu$ M Oxa/5 nM SN-38 and 2.5  $\mu$ M Oxa/10 nM SN-38, respectively. These antagonistic interactions are concordant with the findings from fixed-concentration treatment experiments shown in Table III. These trends of antagonism between the pairwise combinations in the BxPC-3 and PIN-127 cell lines were mirrored in the triple combination.

### A Range of Antagonistic Interactions Found Among the 3-Drug Combination

MIAPaCa-2 was the most sensitive to the triple combination of 5-FU, oxaliplatin and SN-28, showing strongly synergistic interactions (Table IV). Whereas PANC-1 cells displayed marginally synergistic interactions in

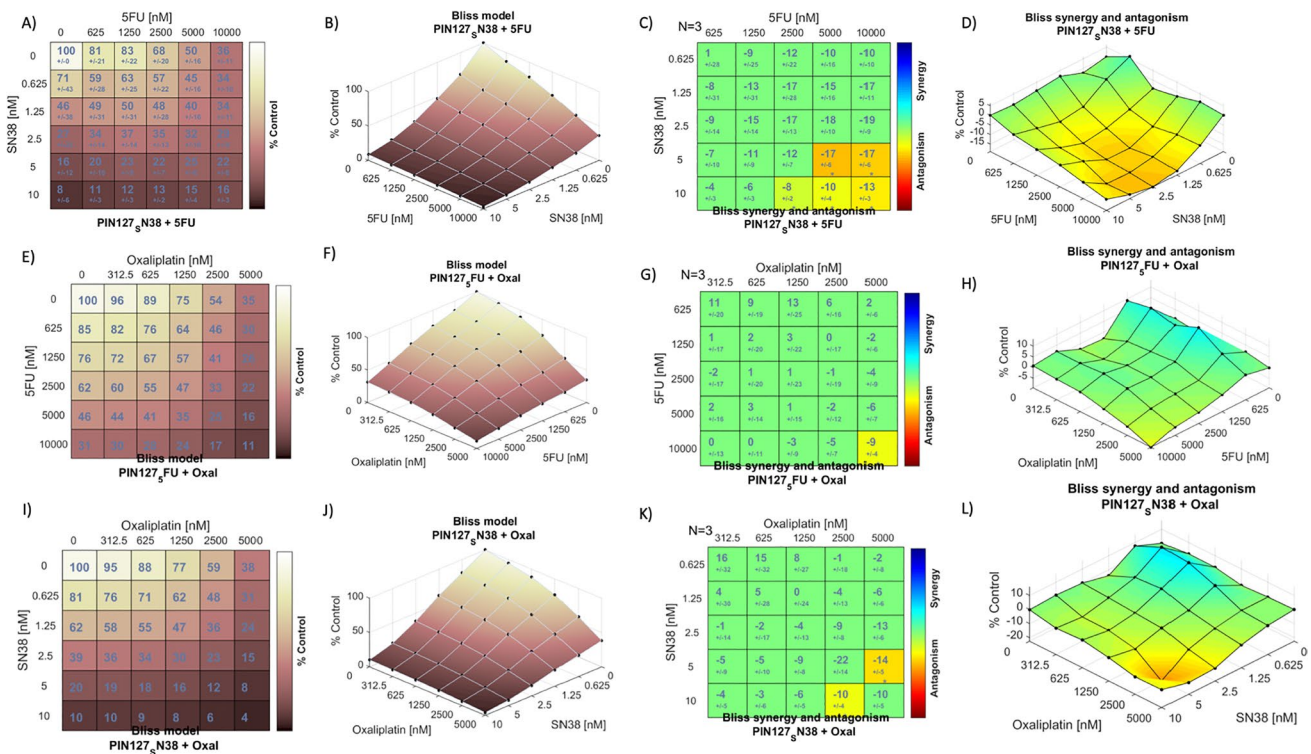


**Fig. 3** Pairwise combinations produce areas of strong antagonism in primary PIN-127 pancreatic cancer cells. **A–D**: response to 5-FU/SN-38, **E–H**: response to Oxa/5-FU, **I–L**: response to Oxa/SN-38. Analysis of altering concentration–response represented in matrix format (**A**, **E**, **I**) and surface plot (**B**, **F**, **J**) as a percentage of proliferation compared to media control. Synergy levels in matrix format (**C**, **G**, **K**) represent the synergy/antagonism score with the

standard deviation number listed below; only coloured synergy levels were significant following a one-sample *t*-test (\**p*-value  $< 5 \times 10^{-2}$ ; \*\**p*-value  $< 10^{-3}$ ; \*\*\**p*-value  $< 10^{-4}$ ). This is generated by Combenefit™ according to the Bliss independent model in comparison to the reference concentration–response surface (**D**, **H**, **L**). Data, mean  $\pm$  SD, *n* = 3

**Table IV** Summary of the Triple Combination Across the Panel of Cell Lines

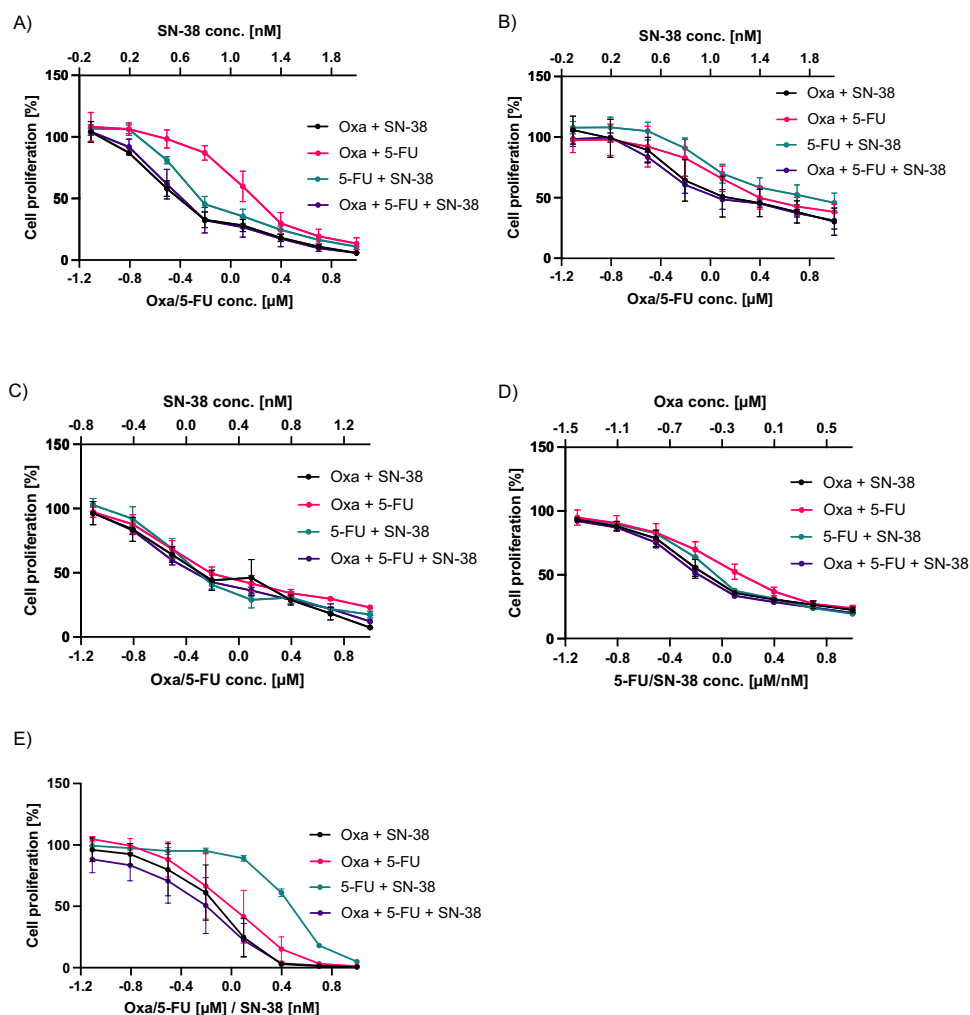
| Cell line         | Triple Combination | Psi Value ( $\psi$ ) | SE   | 95% CI Lower | 95% CI Higher |
|-------------------|--------------------|----------------------|------|--------------|---------------|
| <b>PIN-127</b>    | 5-FU + Oxa + SN38  | <b>5.6</b>           | 1.6  | 2.3          | 8.8           |
| <b>BxPC-3</b>     | 5-FU + Oxa + SN38  | <b>1.4</b>           | 2.1  | 0.4          | 1.6           |
| <b>Mia PaCa-2</b> | 5-FU + Oxa + SN38  | <b>0.2</b>           | 0.05 | 0.1          | 0.3           |
| <b>PANC1 (2D)</b> | 5-FU + Oxa + SN38  | <b>0.8</b>           | 0.1  | 0.6          | 1.0           |
| <b>PANC1 (3D)</b> | 5-FU + Oxa + SN38  | <b>1.6</b>           | 0.3  | 1.0          | 2.3           |

The interaction term psi ( $\psi$ ) was estimated in combination treatments using Eq. (3) (Table I). Red indicates antagonism, and green identifies combinations with synergistic interactions

2D, the  $\psi$  value changed from 0.8 to 1.6 in 3D conditions, indicating additivity trending towards antagonism. Although synergy/additivity was observed in MIAPaCa-2 and PANC-1, respectively, simultaneous exposure to the triple combination resulted in antagonistic interactions

in the other cell lines (Table IV): PIN-127 ( $\psi = 5.6$ ), BxPC-3 ( $\psi = 1.4$ ) and PANC-1 in 3D ( $\psi = 1.6$ ). Nevertheless, despite indications of antagonism, the triple combination resulted in the greatest overall reduction in cell proliferation compared to each pairwise combination

**Fig. 4** Effect of double and triple combination agents. The response was assessed as a % reduction in cell proliferation compared to untreated (y-axis). Combinations tested were 5-FU, Oxa and SN-38 in pairwise vs triple combinations. The x-axis represents drug concentration in  $\mu\text{M}$  (5-FU/Oxa) or nM (SN-38). Cell lines are displayed as follows: **A** PANC-1 2D, **B** PANC-1 3D, **C** BxPC-3, **D** PIN-127 and **E** MIAPaCa-2. Data represents the average and standard deviation of three biological replicates



(Fig. 4), except for the BxPC-3 cell line, where oxaliplatin plus SN-38 mediated the greatest reduction in proliferation (Fig. 4C). Oxaliplatin plus SN-38 was also the second-most effective combination at reducing proliferation across the remaining cell lines and was shown to be the only combination demonstrating synergistic interactions.

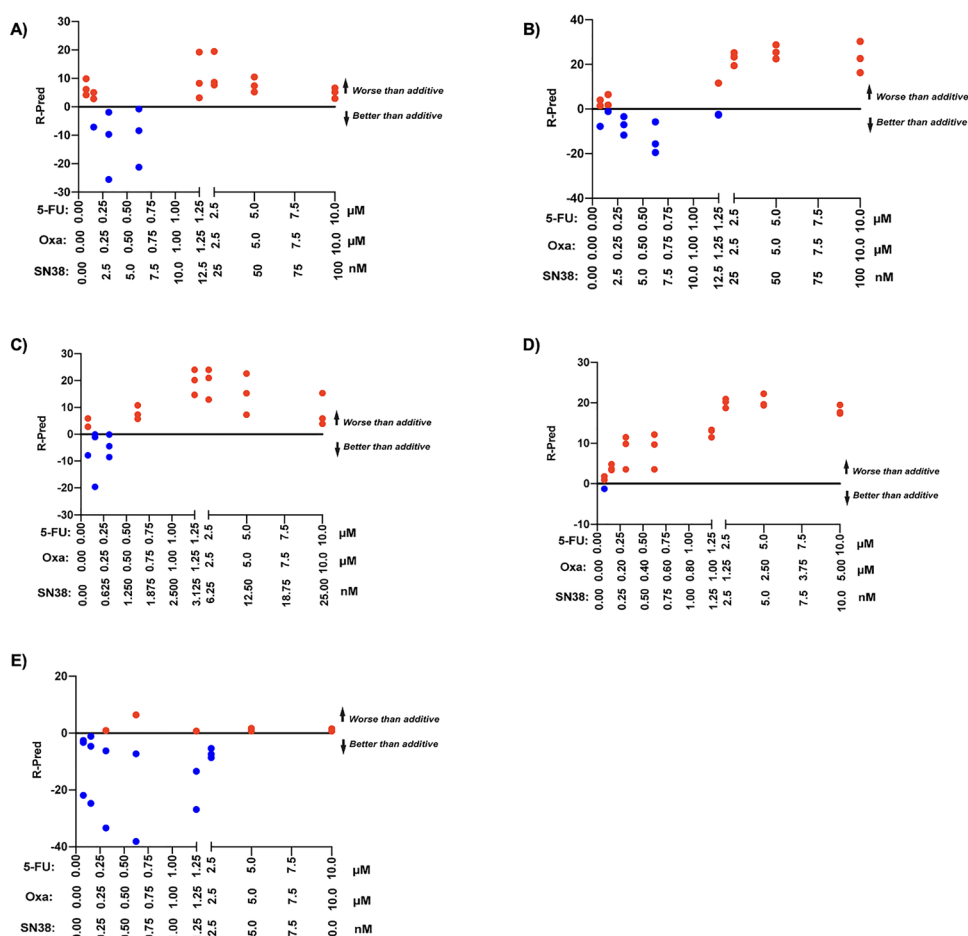
Figure 5 displays the drug interaction score for multiple concentrations combinations of the triple drug combination for each cell line. In agreement with the antagonism of the triple combination, the majority of experimental points displayed in Fig. 5 are above the y-axis horizontal line (model-predicted additivity) and are coloured red symbolising antagonistic interactions. Lower concentrations of the triple combination were better than additive in both PANC-1 2D and 3D cultures (Fig. 5A, B). Figure 5E shows that this combination is strongly synergistic in MIAPaCa-2 cells, as the majority of points are below the y-axis horizontal even at relatively low drug concentrations.

## Discussion

Despite the clinical superiority of FOLFIRINOX over gemcitabine as first-line therapy in patients with metastatic pancreatic cancer, the regime is associated with a greater toxicity profile. Patient selection and monitoring is of prime importance (6). The high observed toxicity raised the question as to how the regime could be optimised to increase its availability to more patients. Optimisation of the regime requires a better understanding of the pharmacodynamic drug-drug interactions among the agents of the FOLFIRINOX regimen. In the present study, we investigated the interaction between Oxa, 5-FU and the active metabolite of irinotecan, SN-38, to obtain *in vitro* evidence of potential opportunities for optimising the combination of these chemotherapeutics for the treatment of pancreatic cancer.

As listed in Table II,  $\text{IC}_{50}$  values for each drug were established for a range of pancreatic cancer cell lines

**Fig. 5 Drug interactions among triple combination agents.** The X horizontal line (R-Pred) that intercepts  $Y=0$  represents predicted additivity (Pred), while all points above or below this line represent the experimental response (R). Each dot represents a biological replicate. All dots above the x horizontal represent drug concentrations which are worse than additive and are coloured red to represent antagonism. All dots below the x represent better than additive and are coloured blue to signify synergistic interactions. Cell lines are represented as follows: **A** PANC-1 2D, **B** PANC-1 3D, **C** BxPC-3, **D** PIN127 and **E** MIAPaCa-2



(MIAPaCa-2, PANC-1 – 2D and 3D, BxPC-3) and primary cells (PIN-127). As single agents, SN-38 was the most potent on all cell lines, with only nm concentrations required for 50% cell growth inhibition. Of the two agents used at  $\mu\text{M}$  concentrations, cell lines were most sensitive to Oxa, except for BxPC-3 which was most sensitive to 5-FU. In a panel of human PDAC cell lines similar to those used in this study, Chowbay et al. found that all were most sensitive to single-agent oxaliplatin (29). We found that overall, the PANC-1 cell line was least sensitive to the single agents, with  $\text{IC}_{50}$  values increasing significantly in 3D cultures. Others have also found increased resistance to chemotherapeutics in PANC-1 cells (29, 30).

Our study found that LV combined with 5-FU had no significant potentiating effect *in vitro* and thus was not carried further into triple combination studies. LV's mechanism of action *in vivo* is the stabilisation of the ternary complex of thymidylate synthase, 5-fluoro-2'-deoxyuridine-5'-monophosphate and the folate cofactor 5,10-methylene tetrahydrofolate (31). LV increases levels of reduced folates in tissues overcoming the short half-life of 5-FU (31, 32). This complex interaction is not replicated in cell culture models that lack contributions of liver catabolism. Others

have found in *in vitro* and *in vivo* experiments that cell lines with high thymidylate synthase expression display greater enhancement of 5FU cytotoxicity by LV (33, 34). Nakamura et al. demonstrated that in cell lines with low expression levels of thymidylate synthase mRNA, LV could not enhance the cytotoxicity of 5FU (35). Furthermore, the enhancement of 5FU cytotoxicity may be saturated by the folic acid content of the cell culture medium and serum because of limited thymidylate synthase expression of the cells investigated. Here, BxPC-3 showed the greatest LV enhancement of 5-FU (Supplementary Fig. S1A). These cells were cultured in RPMI-1640 medium, which has the lowest concentration of folic acid compared to other media: 0.001 g/L.

Oxaliplatin plus SN-38 was the only pairwise combination to show synergistic interactions across multiple cell lines (MIAPaCa-2 and PANC-1 2D; Table III), however, the  $\psi$  determined for PANC-1 ( $\psi = 0.6$ ,  $\text{CI} = 0.4\text{--}0.9$ ) was the only interaction where the CI values did not overlap with the additive interaction range. Interestingly, this combination trended towards antagonistic at fixed-concentration combinations in BxPC-3, but demonstrated areas of synergy in a  $5 \times 5$  concentration grid. For example, high-concentration oxaliplatin with low-concentration SN-38

resulted in a synergy score of 9 (Fig. 2K,L). This suggests that investigating concentration ratios, in combination with pharmacodynamic modelling, can provide valuable insight into multi-drug chemotherapeutic regimens. Previous studies demonstrated that SN-38 and Oxa reciprocally affect the cellular response to DNA damage, resulting in synergy between these drugs (36). A recent study developed polymeric micelles bearing the active forms of irinotecan and Oxa (SN-38/m and DACHPt/m), which showed remarkable cytotoxicity in human pancreatic cancer cell lines (37). Like the current study, their results highlighted the importance of drug ratios to achieve synergy. They found that the clinically used drug weight ratio (irinotecan: Oxa = 2:1) was the least effective for the combination of the active compounds SN-38/m and DACHPt/m. Here, SN-38 was used in nm concentrations at high drug weight ratios for Oxa:SN-38, with the highest ratio (Oxa:SN-38 = 800:1), resulting in synergy in BxPC3 cells. The alteration of drug concentration ratios in the primary PIN-127 cell line did not result in a synergistic shift, but instead brought attention to the antagonistic relationship between the agents in this cell line, particularly at higher concentrations of both drugs (Fig. 3). The PIN-127 cell line was isolated from a PDX model and has previously been shown to harbour KRAS and phosphoinositide-3-kinase regulatory subunit 1 (PI3K31) mutations (21). BxPC3 cell line is a KRAS wild-type cell line (38). Therefore, the KRAS mutation is unlikely to explain the clustering of antagonistic responses observed in these two cell lines.

Oxa and 5-FU pairwise combination was found to be antagonistic in 4 out of 5 cell lines examined. PIN-127 showed strong synergy ( $\psi = 3.9$ , 95% CI = 2.1, 5.7) and was the only cell line where  $\psi$  was outside an additive CI range. MIAPaCa-2 displayed additive interactions. Yao et al. have shown using FACS analysis that MIAPaCa-2 cells have the lowest proportion (0.03%) of side-population cells compared to other pancreatic cancer cells used: BxPC-3 (0.79%) and PANC-1 (7.75%) (39). Side-population cells share similarities to cancer stem cells (CSCs) and exhibit properties of pluripotential differentiation (both *in vivo* and *in vitro*) and higher levels of chemoresistance (39, 40). Qin et al. (41) evaluated interactions between 5-FU and Oxa based on the median-effect principle of Chou and Talalay (42) in human gastric cancer cell lines. Unlike the results described in Table III, which showed additivity or antagonism in pancreatic cancer cells, they found synergy. Interestingly, their results showed that the synergy was sequence dependent: simultaneous and sequential treatments of Oxa, followed by 5-FU produced synergistic interaction, with the opposite sequence yielding clear antagonism. Another study showed that synergistic interactions between 5-FU and Oxa were dependent on sequential exposure, with Oxa, followed by 5-FU, resulting in the most cytotoxic effect in multiple colorectal cancer cell lines [43]. Thus, a sequence-dependent

effect of Oxa and SN-38 may play an important role in addition to the drug weight ratio in achieving synergistic interactions between Oxa and SN-38 and better outcomes (41, 43). Simultaneous exposure of 5-FU and Oxa could explain the antagonistic interactions observed here (Table III).

The main novel finding of this study was that the triple combination of Oxa, 5-FU and SN-38 displayed antagonistic interactions in BxPC-3, PANC-1 (3D culture) and primary PIN-127 cells. PIN-127 cells showed strong antagonism, with a drug interaction term of 5.6. Patient-derived PDAC cell lines are a useful *in vitro* tool as they better reflect the heterogeneity of patient response to drug regimens, as seen in a recent report (44) where a panel of patient-derived PDAC cell lines exhibited differential sensitivity to FOLFIRINOX. It is important to note that additive DDIs can be highly beneficial clinically and that synergy is not a requirement for useful treatment regimens. While additivity is clinically useful, further evaluating the non-simultaneous use of the agents could potentially reduce toxicity, allowing access to this multi-drug regimen to more patients. The results shown here suggest that two-drug regimens such as Oxa and SN-38 could be equally effective and support the need for further preclinical *in vivo* studies.

Another interesting finding in PANC-1 was the shift from trending towards synergy in 2D towards antagonism in 3D. We describe this as trending towards antagonism as both conditions had confidence intervals that overlapped with additivity. Many studies suggest a molecular and phenotypic association between increased chemoresistance and the presence of an EMT-like phenotype of cancer cells (45–47). This suggests a hypothesis for the loss of synergy observed in the 3D growth of PANC-1, as Matrigel can modulate an EMT phenotype (48, 49). EMT involves a change in the extracellular matrix as cells detach from the basement membrane (BM) and engage the interstitial matrix (50, 51). Matrigel is a reconstituted BM isolated from Engelbreth-Holm-Swarm mouse sarcomas containing a complex mixture of macromolecules including laminin, type IV collagen and enactin that mimic BM *in vitro* (48). Puls et al. have also shown matrix-dependent PANC-1 cell sensitivity to gemcitabine, with increasing IC<sub>50</sub> values for cells grown in Matrigel (48). These observations are consistent with the hypothesis that extracellular matrix composition and tumour microenvironment are critical determinants of PDAC drug response and sensitivity (3, 4, 52).

The switch from synergy to antagonism observed in PANC-1 cells when grown in 3D compared to 2D could also result from reduced drug penetration into the centre of cell spheroids (53, 54). Cells grown in 3D may also be more secretory than their monolayer counterparts, potentially increasing paracrine signalling, resulting in differential drug responses. Spheroids face additional extracellular cues and physical conditions such as rigidity and limited adhesion,

which can affect drug response (55). Hypoxia, a common characteristic of pancreatic cancers, can alter tumour cell phenotype, which adapts their metabolism to sustain growth (56) and can result in genetic alterations, giving rise to multi-drug resistance (57). Spheroids such as those described in this study can have increased levels of hypoxia, which could explain the antagonistic switch observed (58).

The results shown here confirm that the agents of the FOLFIRINOX regimen can have various DDIs *in vitro* depending on the cell line tested. While the triple combination appeared antagonistic in many cell lines, meaning the net effect is less than the additive effect of individual agents, it still resulted in greater cell kill than any agent alone. Therefore, a better understanding of their pharmacodynamic interactions is warranted, particularly *in vivo*.

Early pharmacodynamic modelling of combination therapies (42) was successful in overcoming the assumptions and limitations implicit in the classical fractional inhibitory concentration index or checkerboard approach (59, 60). However, interaction parameters must be interpreted cautiously in that marginal values, although within objective definitions of synergy and antagonism, will not directly translate into clinically meaningful interactions.

This study was conducted *in vitro*, and the nature of interactions may differ *in vivo*. The results shown in this study demonstrated how LV enhancement of 5-FU was not statistically significant when carried out *in vitro* due to the lack of catabolic activity present in culture conditions. The use of *in vivo* PDX models could overcome this limitation and provide more evidence for the DDIs observed here. This study suggests the necessity of further investigation into the complex interactions among the FOLFIRINOX agents as a route to enhance their efficacy in pancreatic cancer.

## Conclusion

Application of a mathematical model-estimated drug interaction parameter  $\psi$  reveals that pairwise and triple combinations of the FOLFIRINOX agents exert a range of pharmacodynamic drug-drug interactions across a panel of pancreatic cancer cell lines, with numerous combinations being antagonistic. Extension of this modelling analysis approach to *in vivo* models could provide greater insight into the optimisation of this first-line pancreatic cancer treatment regimen. Interestingly, analysis of the primary PDX-derived PIN-127 cell line showed antagonistic interactions among all drug combinations. These results suggest a complex interplay among the three agents that may be dependent upon specific tumour cell characteristics, and future work will use the PD data to assess whether lower concentrations and/or alternate schedules would be both efficacious and less toxic. This pharmacodynamic, model-informed approach provides

a quantitative platform to increase our understanding of complex drug interactions, with the objective of identifying a more efficacious and tolerable FOLFIRINOX regimen that would be feasible for a greater fraction of PDAC patients.

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Eva Welsch – formal analysis, investigation and methodology, writing – review.

Neil T. Conlon – formal analysis, writing – review and editing.

Weylon Garner – formal analysis, writing – early alternative draft preparation.

Martin Clynes – resources, supervision, writing – review and editing.

Finbarr O'Sullivan – resources, supervision, writing – review and editing.

Robert M. Straubinger – conceptualisation, resources, supervision, writing – review and editing.

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## Declarations

**Conflict of Interest** The authors declare no competing interests.

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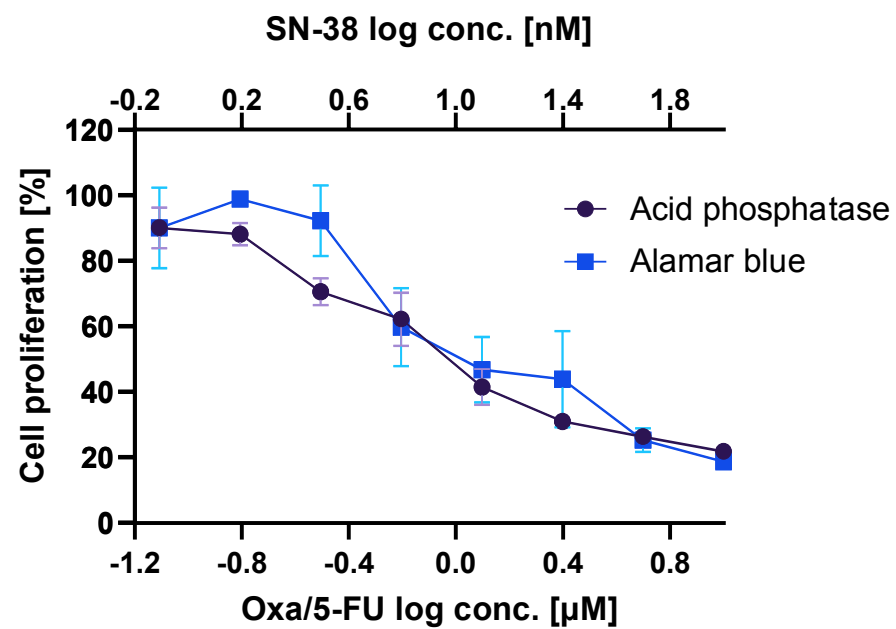
## References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin.* 2020;70(1):7–30. <https://doi.org/10.3322/caac.21590>.
2. Evans J, Chapple A, Salisbury H, Corrie P, Ziebland S. ‘It can’t be very important because it comes and goes’—patients’ accounts of intermittent symptoms preceding a pancreatic cancer diagnosis:

- a qualitative study. *BMJ Open*. 2014;4(2): e004215. <https://doi.org/10.1136/bmjopen-2013-004215>.
3. Adamska A, Elaskalani O, Emmanouilidi A, Kim M, Abdol Razak NB, Metharom P, et al. Molecular and cellular mechanisms of chemoresistance in pancreatic cancer. *Adv Biol Regul*. 2018;68:77–87. <https://doi.org/10.1016/j.bior.2017.11.007>.
  4. Murakami T, Hiroshima Y, Matsuyama R, Homma Y, Hoffman RM, Endo I. Role of the tumor microenvironment in pancreatic cancer. *Ann Gastroenterol Surg*. 2019;3(2):130–7. <https://doi.org/10.1002/ags3.12225>.
  5. Andrén-Sandberg A. Pancreatic cancer: chemotherapy and radiotherapy. *N Am J Med Sci*. 2011;3(1):1–12. <https://doi.org/10.4297/najms.2011.31>.
  6. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011;364(19):1817–25. <https://doi.org/10.1056/NEJMoa1011923>.
  7. Vreeland TJ, McAllister F, Javadi S, Prakash LR, Fogelman DR, Ho L, et al. Benefit of gemcitabine/nab-paclitaxel rescue of patients with borderline resectable or locally advanced pancreatic adenocarcinoma after early failure of FOLFIRINOX. *Pancreas*. 2019;48(6):837–43. <https://doi.org/10.1097/MPA.0000000000001345>.
  8. Cavanna L, Stroppa EM, Citterio C, Mordenti P, Di Nunzio C, Peveri S, et al. Modified FOLFIRINOX for unresectable locally advanced/metastatic pancreatic cancer. A real-world comparison of an attenuated with a full dose in a single center experience. *Onco Targets Ther*. 2019;12:3077–85. <https://doi.org/10.2147/ott.s200754>.
  9. Yang F, Jin C, Fu DL, Warshaw AL. Modified FOLFIRINOX for resected pancreatic cancer: opportunities and challenges. *World J Gastroenterol*. 2019;25(23):2839–45. <https://doi.org/10.3748/wjg.v25.i23.2839>.
  10. Ueno H, Kosuge T. Adjuvant treatments for resectable pancreatic cancer. *J Hepatobiliary Pancreat Surg*. 2008;15(5):468–72. <https://doi.org/10.1007/s00534-008-1357-3>.
  11. Abbassi R, Algül H. Palliative chemotherapy in pancreatic cancer-treatment sequences. *Transl Gastroenterol Hepatol*. 2019;4:56. <https://doi.org/10.21037/tgh.2019.06.09>.
  12. Riedl JM, Posch F, Horvath L, Gantschnigg A, Renneberg F, Schwarzenbacher E, et al. Gemcitabine/nab-paclitaxel versus FOLFIRINOX for palliative first-line treatment of advanced pancreatic cancer: a propensity score analysis. *Eur J Cancer*. 2021;151:3–13. <https://doi.org/10.1016/j.ejca.2021.03.040>.
  13. Niu J, Straubinger RM, Mager DE. Pharmacodynamic drug-drug interactions. *Clin Pharmacol Ther*. 2019;105(6):1395–406. <https://doi.org/10.1002/cpt.1434>.
  14. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. *Pharmacol Res Perspect*. 2015;3(3): e00149. <https://doi.org/10.1002/prp2.149>.
  15. Molins EAG, Jusko WJ. Assessment of three-drug combination pharmacodynamic interactions in pancreatic cancer cells. *Aaps j*. 2018;20(5):80. <https://doi.org/10.1208/s12248-018-0235-4>.
  16. Mager DE, Jusko WJ. Development of translational pharmacokinetic-pharmacodynamic models. *Clin Pharmacol Ther*. 2008;83(6):909–12. <https://doi.org/10.1038/clpt.2008.52>.
  17. Zimmer A, Katzir I, Dekel E, Mayo AE, Alon U. Prediction of multidimensional drug dose responses based on measurements of drug pairs. *Proc Natl Acad Sci U S A*. 2016;113(37):10442–7. <https://doi.org/10.1073/pnas.1606301113>.
  18. Nelson SR, Zhang C, Roche S, O'Neill F, Swan N, Luo Y, et al. Modelling of pancreatic cancer biology: transcriptomic signature for 3D PDX-derived organoids and primary cell line organoid development. *Sci Rep*. 2020;10(1):2778. <https://doi.org/10.1038/s41598-020-59368-7>.
  19. Hou S, Tiriach H, Sridharan BP, Scampavia L, Madoux F, Seldin J, et al. Advanced development of primary pancreatic organoid tumor models for high-throughput phenotypic drug screening. *SLAS Discov*. 2018;23(6):574–84. <https://doi.org/10.1177/2472555218766842>.
  20. Ande A, Vaidya TR, Tran BN, Vicchiarelli M, Brown AN, Ait-Oudhia S. Utility of a Novel three-dimensional and dynamic (3DD) cell culture system for PK/PD studies: evaluation of a triple combination therapy at overcoming anti-HER2 Treatment resistance in breast cancer. *Front Pharmacol*. 2018;9:403. <https://doi.org/10.3389/fphar.2018.00403>.
  21. Roche S, O'Neill F, Murphy J, Swan N, Meiller J, Conlon NT, et al. Establishment and characterisation by expression microarray of patient-derived xenograft panel of human pancreatic adenocarcinoma patients. *Int J Mol Sci*. 2020;21(3). <https://doi.org/10.3390/ijms21030962>.
  22. Martin A, Clynes M. Comparison of 5 microplate colorimetric assays for in vitro cytotoxicity testing and cell proliferation assays. *Cytotechnology*. 1993;11(1):49–58. <https://doi.org/10.1007/bf00749057>.
  23. Y. Di Veroli G, Fornari C, Wang D, Mollard Sv, Bramhall JL, M. Richards F, et al. Combeneft: an interactive platform for the analysis and visualisation of drug combinations. *Bioinformatics*. 2016;32 2866–8.
  24. Bliss CI. The toxicity of poisons applied jointly. *Annals of Applied Biology* 1939. p. 585–615.
  25. Webb JL. Effect of more than one inhibitor. *Enzyme and Metabolic Inhibitors* 1963. p. 66–79.
  26. Bruckner HW, Spigelman M. Leucovorin as a clinical potentiator of 5-fluorouracil toxicity and anticancer efficacy. *Mt Sinai J Med*. 1988;55(5):390–4.
  27. Showalter SL, Showalter TN, Witkiewicz A, Havens R, Kennedy EP, Hucl T, et al. Evaluating the drug-target relationship between thymidylate synthase expression and tumor response to 5-fluorouracil. Is it time to move forward? *Cancer Biol Ther*. 2008;7(7):986–94. <https://doi.org/10.4161/cbt.7.7.6181>.
  28. Harrison RL. Introduction to Monte Carlo simulation. *AIP Conf Proc*. 2010;1204:17–21. <https://doi.org/10.1063/1.3295638>.
  29. Chowbay B XJ, Wong JXG, Marvalim C, Ng SL, Sutiman N, Leong WF. Effects of oxaliplatin, capecitabine and irinotecan (OXIRI) on PDAC cell lines carrying different *Kras* mutational status ASCO annual meeting: *Journal of Clinical Oncology*; 2016.
  30. Kim Y, Han D, Min H, Jin J, Yi EC. Comparative proteomic profiling of pancreatic ductal adenocarcinoma cell lines. *Mol Cells*. 2014;37(12):888–98. <https://doi.org/10.14348/molcells.2014.0207>.
  31. Peters GJ, van Groeningen CJ, van der Wilt CL, Meijer S, Smid K, Laurensse E, et al. Time course of inhibition of thymidylate synthase in patients treated with fluorouracil and leucovorin. *Semin Oncol*. 1992;19(2 Suppl 3):26–35.
  32. Van der Wilt CL, Pinedo HM, Smid K, Peters GJ. Elevation of thymidylate synthase following 5-fluorouracil treatment is prevented by the addition of leucovorin in murine colon tumors. *Cancer Res*. 1992;52(18):4922–8.
  33. Tsujimoto H, Tsukioka S, Ono S, Sakamoto E, Sakamoto K, Tsuta K, et al. Effect of leucovorin on the antitumor efficacy of the 5-FU prodrug, tegafur-uracil, in human colorectal cancer xenografts with various expression levels of thymidylate synthase. *Oncol Lett*. 2010;1(6):973–80. <https://doi.org/10.3892/ol.2010.172>.
  34. Sakamoto E, Tsukioka S, Oie S, Kobunai T, Tsujimoto H, Sakamoto K, et al. Folylpolyglutamate synthase and gamma-glutamyl hydrolase regulate leucovorin-enhanced 5-fluorouracil anticancer activity. *Biochem Biophys Res Commun*. 2008;365(4):801–7. <https://doi.org/10.1016/j.bbrc.2007.11.043>.
  35. Nakamura A, Nakajima G, Okuyama R, Kuramochi H, Kondoh Y, Kanemura T, et al. Enhancement of 5-fluorouracil-induced

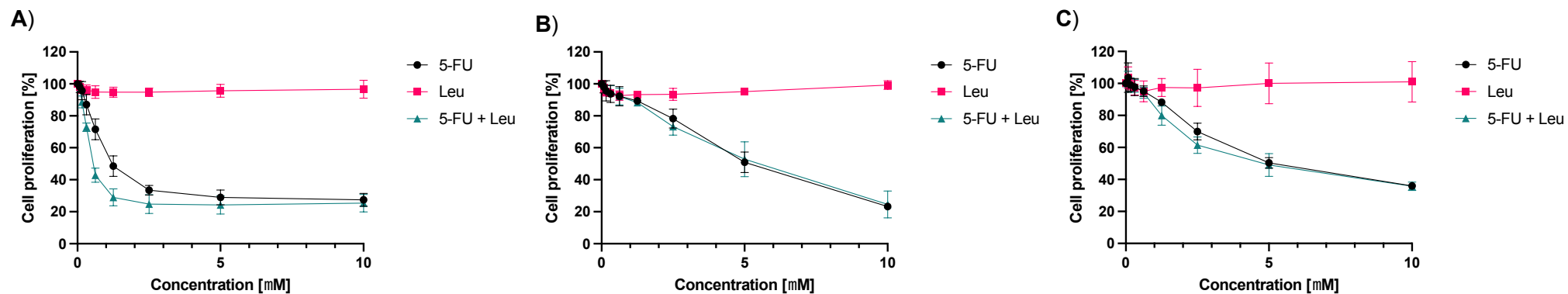
- cytotoxicity by leucovorin in 5-fluorouracil-resistant gastric cancer cells with upregulated expression of thymidylate synthase. *Gastric Cancer*. 2014;17(1):188–95. <https://doi.org/10.1007/s10120-013-0249-7>.
36. Zeghari-Squalli N, Raymond E, Cvitkovic E, Goldwasser F. Cellular pharmacology of the combination of the DNA topoisomerase I inhibitor SN-38 and the diaminocyclohexane platinum derivative oxaliplatin. *Clin Cancer Res*. 1999;5(5):1189–96.
  37. Lei F, Xi X, Rachagani S, Seshacharyulu P, Talmon GA, Pon-nusamy MP, et al. Nanoscale platform for delivery of active IRINOX to combat pancreatic cancer. *J Control Release*. 2021;330:1229–43. <https://doi.org/10.1016/j.jconrel.2020.11.029>.
  38. Deer EL, González-Hernández J, Coursen JD, Shea JE, Ngatia J, Scaife CL, et al. Phenotype and genotype of pancreatic cancer cell lines. *Pancreas*. 2010;39(4):425–35. <https://doi.org/10.1097/MPA.0b013e3181c15963>.
  39. Yao J, Cai HH, Wei JS, An Y, Ji ZL, Lu ZP, et al. Side population in the pancreatic cancer cell lines SW1990 and CFPAC-1 is enriched with cancer stem-like cells. *Oncol Rep*. 2010;23(5):1375–82. [https://doi.org/10.3892/or\\_00000774](https://doi.org/10.3892/or_00000774).
  40. Rao CV, Mohammed A. New insights into pancreatic cancer stem cells. *World J Stem Cells*. 2015;7(3):547–55. <https://doi.org/10.4252/wjsc.v7.i3.547>.
  41. Qin B, Tanaka R, Shibata Y, Arita S, Ariyama H, Kusaba H, et al. In-vitro schedule-dependent interaction between oxaliplatin and 5-fluorouracil in human gastric cancer cell lines. *Anticancer Drugs*. 2006;17(4):445–53. <https://doi.org/10.1097/01.cad.0000198912.98442.cd>.
  42. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*. 1984;22:27–55.
  43. Failli A, Consolini R, Legitimo A, Orsini G, Romanini A, Spisni R, et al. Evaluation of in vitro cytotoxicity of oxaliplatin and 5-fluorouracil in human colon cancer cell lines: combination versus sequential exposure. *J Biol Regul Homeost Agents*. 2011;25(4):575–88.
  44. Begg SKS, Birnbaum DJ, Clark JW, Mino-Kenudson M, Wellner UF, Schilling O, et al. FOLFIRINOX. *Anticancer Res*. 2020;40(7):3659–67. <https://doi.org/10.21873/anticancer.14355>.
  45. Martinelli P, Carrillo-de Santa Pau E, Cox T, Sainz B, Jr., Dusetti N, Greenhalf W, et al. GATA6 regulates EMT and tumour dissemination, and is a marker of response to adjuvant chemotherapy in pancreatic cancer. *Gut*. 2017;66(9):1665–76. <https://doi.org/10.1136/gutjnl-2015-311256>.
  46. Quiñero F, Mesas C, Doello K, Cabeza L, Perazzoli G, Jimenez-Luna C, et al. The challenge of drug resistance in pancreatic ductal adenocarcinoma: a current overview. *Cancer Biol Med*. 2019;16(4):688–99. <https://doi.org/10.20892/j.issn.2095-3941.2019.0252>.
  47. Gaianigo N, Melisi D, Carbone C. EMT and treatment resistance in pancreatic cancer. *Cancers (Basel)*. 2017;9(9). <https://doi.org/10.3390/cancers9090122>.
  48. Puls TJ, Tan X, Whittington CF, Voytik-Harbin SL. 3D collagen fibrillar microstructure guides pancreatic cancer cell phenotype and serves as a critical design parameter for phenotypic models of EMT. *PLoS ONE*. 2017;12(11): e0188870. <https://doi.org/10.1371/journal.pone.0188870>.
  49. Shichi Y, Sasaki N, Michishita M, Hasegawa F, Matsuda Y, Arai T, et al. Enhanced morphological and functional differences of pancreatic cancer with epithelial or mesenchymal characteristics in 3D culture. *Sci Rep*. 2019;9(1):10871. <https://doi.org/10.1038/s41598-019-47416-w>.
  50. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol*. 2014;15(3):178–96. <https://doi.org/10.1038/nrm3758>.
  51. Walma DAC, Yamada KM. The extracellular matrix in development. *Development*. 2020;147(10). <https://doi.org/10.1242/dev.175596>.
  52. Shaked Y. The pro-tumorigenic host response to cancer therapies. *Nat Rev Cancer*. 2019. <https://doi.org/10.1038/s41568-019-0209-6>.
  53. Vinci M, Gowen S, Boxall F, Patterson L, Zimmermann M, Court W, et al. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol*. 2012;10:29. <https://doi.org/10.1186/1741-7007-10-29>.
  54. Al-Abd AM, Lee JH, Kim SY, Kun N, Kuh HJ. Novel application of multicellular layers culture for in situ evaluation of cytotoxicity and penetration of paclitaxel. *Cancer Sci*. 2008;99(2):423–31. <https://doi.org/10.1111/j.1349-7006.2007.00700.x>.
  55. Follin B, Juhl M, Cohen S, Pedersen AE, Kastrup J, Ekblond A. Increased paracrine immunomodulatory potential of mesenchymal stromal cells in three-dimensional culture. *Tissue Eng Part B Rev*. 2016;22(4):322–9. <https://doi.org/10.1089/ten.TEB.2015.0532>.
  56. Shah VM, Sheppard BC, Sears RC, Alani AW. Hypoxia: friend or foe for drug delivery in pancreatic cancer. *Cancer Lett*. 2020;492:63–70. <https://doi.org/10.1016/j.canlet.2020.07.041>.
  57. Erler JT, Cawthorne CJ, Williams KJ, Koritzinsky M, Wouters BG, Wilson C, et al. Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. *Mol Cell Biol*. 2004;24(7):2875–89. <https://doi.org/10.1128/mcb.24.7.2875-2889.2004>.
  58. Riffle S, Hegde RS. Modeling tumor cell adaptations to hypoxia in multicellular tumor spheroids. *J Exp Clin Cancer Res*. 2017;36(1):102. <https://doi.org/10.1186/s13046-017-0570-9>.
  59. Odds FC. Synergy, antagonism, and what the checkerboard puts between them. *J Antimicrob Chemother*. 2003;52(1):1. <https://doi.org/10.1093/jac/dkg301>.
  60. Fratini F, Mancini S, Turchi B, Friscia E, Pistelli L, Giusti G, et al. A novel interpretation of the fractional inhibitory concentration index: the case *Origanum vulgare* L. and *Leptospermum scoparium* J. R. et G. Forst essential oils against *Staphylococcus aureus* strains. *Microbiol Res*. 2017;195:11–7. <https://doi.org/10.1016/j.micres.2016.11.005>.

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|                  | IC50   | p-value |
|------------------|--------|---------|
| Acid phosphatase | 0.654  | 0.743   |
| Alamar blue      | 0.8573 |         |

Supplementary Figure 1: Comparison of acid phosphatase and alamarBlue proliferation assays



**Supplementary Figure 2: Effect of Leucovorin in addition to 5-FU**

## Article

# Alteration in Levels of Specific miRNAs and Their Potential Protein Targets between Human Pancreatic Cancer Samples, Adjacent Normal Tissue, and Xenografts Derived from These Tumors

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**Abstract:** Herein, we describe the global comparison of miRNAs in human pancreatic cancer tumors, adjacent normal tissue, and matched patient-derived xenograft models using microarray screening. RNA was extracted from seven tumor, five adjacent normal, and eight FI PDX tumor samples and analyzed by Affymetrix GeneChip miRNA 4.0 array. A transcriptome analysis console (TAC) was used to generate comparative lists of up- and downregulated miRNAs for the comparisons, tumor vs. normal and F1 PDX vs. tumor. Particular attention was paid to miRNAs that were changed in the same direction in both comparisons. We identified the involvement in pancreatic tumor tissue of several miRNAs, including miR4534, miR3154, and miR4742, not previously highlighted as being involved in this type of cancer. Investigation in the parallel mRNA and protein lists from the same samples allowed the elimination of proteins where altered expression correlated with corresponding mRNA levels and was thus less likely to be miRNA regulated. Using the remaining differential expression protein lists for proteins predicted to be targeted for differentially expressed miRNA on our list, we were able to tentatively ascribe specific protein changes to individual miRNA. Particularly interesting target proteins for miRs 615-3p, 2467-3p, 4742-5p, 509-5p, and 605-3p were identified. Prominent among the protein targets are enzymes involved in aldehyde metabolism and membrane transport and trafficking. These results may help to uncover vulnerabilities that could enable novel approaches to treating pancreatic cancer.

**Keywords:** pancreatic cancer; miRNA; PDX; tumour

## 1. Introduction

Survival rates are very poor in pancreatic cancer, owing in part to the early disease being frequently asymptomatic. Diagnosis frequently occurs at an advanced stage when the tumor has invaded locally and often metastasized. While the majority of pancreatic adenocarcinomas (PDAC) are K-ras mutants [1], and there is as yet no clinically demonstrated way of selectively targeting it without also inhibiting wild-type Ras, which is essential for the survival of normal cells [2]. However, recent promising results with the covalent KRAS-G12C inhibitors sotorasib and adagrasib in the treatment of non-small cell lung

cancer patients that harbor the mutation [3]. Many other mutations in proteins of signaling pathways have been identified in a proportion of pancreatic cancers, but to date, targeted therapies of the type that are so effective in, for example, molecularly defined subsets of breast cancer have not been defined in pancreatic cancer. A complex microenvironment with molecular crosstalk, for example, between stellate cells and cancer cells, complicates attempts to understand and treat pancreatic cancer [4]. There has been no fundamental advance in the treatment of pancreatic cancer in the past 20 years, although better medical management of side effects and improvements in surgery, pain control, and improved management of palliative quality of life indicators are all very important. However, a significant improvement in outcomes is still lacking [5]. This somewhat gloomy background underlines the need for a better understanding of the biology of pancreatic cancer in the hope of discovering new vulnerabilities that can be exploited in new treatments [6].

MicroRNAs are short, non-coding RNAs, typically of 20–24 nucleotides in length, that can be responsible for the regulation of biological pathways in cells. Since their discovery in the nematode *C. elegans* in 1993, these RNAs have received considerable attention due to their roles in diverse biological functions, including the regulation of cell development, differentiation, and survival. Approximately one-third of all human genes are thought to be regulated by miRNAs, with approximately 2500 miRNAs identified to date [7]. Dysregulation of miRNA profiles has been found to be associated with the development of various human cancers. In pancreatic cancer specifically, there has been a significant increase in interest in the expression of miRNAs and their possible roles as diagnostic or prognostic markers [8–14]. Some of these studies have highlighted particular miRNAs as being of particular interest, including miR21, miR217, and miR222. Furthermore, there has been an interest in the targeting of these miRNAs with antimirs as potential targets, although this has so far been met with limited success [15–17]. The stability of miRNAs in bodily fluids such as plasma or serum and tissue samples is a major advantage for their use as potential biomarkers, but lack of specificity may be a limitation [18].

In the work detailed in this paper, we have applied a multilevel approach looking at datasets from proteomic [19], mRNA microarray [20] or and miRNA microarray analysis from the same tumor-adjacent normal tissue (5 samples), tumor samples (7 samples) and F1 patient-derived xenograft (F1) tumor (8 samples) material cohorts. Applying this multilevel approach has allowed us to overcome one drawback often associated with miRNA studies. We have been able to identify effects on predicted targets of miRNAs using both mRNA and proteomic expression and have not had to rely on computational algorithms.

## 2. Materials and Methods

### 2.1. Acquisition and Generation of Patient-Derived Xenograft Models

Pancreatic tumor sample acquisition and generation of PDX models were described by our group [20]. Briefly, pancreatic tumor tissue (T) and adjacent normal tissue (N) were obtained from patients undergoing pancreatic ductal adenocarcinoma (PDAC) surgical resection at St. Vincent's University Hospital Dublin. After initial macroscopic pathological confirmation, the material remaining after diagnostic sampling was cold transferred to DCU. The collection of patient material was approved by St Vincent's University Hospital and Dublin City University (DCU) Research Ethics Committee. All animal work received ethical approval from the DCU Research Ethics Committee (DCUREC/2012/202) and was licensed by the Irish Department of Health (license number B100-4501). Severe combined immunodeficiency (SCID), CB17/lcr-Prkdc<sup>scid</sup>/lcrCrl mice (Charles River, U.K.) were implanted subcutaneously with fresh patient tumor material. Mice were monitored for tumor development for up to 1 year post-implantation. Animal welfare monitoring criteria included tumor volume, tumor axis, body weight, and condition. The tumor volume and tumor axis limits were set as <2000 mm<sup>3</sup> and <20 mm, respectively. Following the humane euthanasia of the mouse, the tumor was divided for cryopreservation, formalin-fixed paraffin embedding (FFPE), and snap frozen (F1). For snap freezing, a piece of tumor was

minced with a scalpel, placed in a cryovial, and placed directly into liquid nitrogen (LN<sub>2</sub>), then stored at −80 °C.

## 2.2. Proteomics Analysis

Proteomic analysis of PDAC tumors and normal tissue was previously described by our group [19]. Briefly, tissue samples were enriched for membrane proteins using MemPER plus membrane protein extraction kit as per the manufacturer's instructions (Thermo Fisher, Hemel Hempstead, UK). Two subcellular fractions were generated, cytosolic and solubilized membrane/membrane-associated proteins. Nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) was carried out on a Dionex Ultimate 3000 nano-RSLC (Thermo Scientific, Hemel Hempstead, UK) coupled in-line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). The LC-MS/MS parameters were previously [21] described using a 140 min separation gradient per sample. Protein identification was carried out using Proteome Discoverer Version 2.2 software (Thermo Scientific) with the SEQUEST HT algorithm. Raw MS files were searched against the UniProt/SwissProt human protein database (downloaded on 29 August 2018, containing 20,325 sequences). The following criteria were applied: (1) precursor mass tolerance set to 10 ppm; (2) fragment mass tolerance set to 0.6 Da; (3) oxidation of methionine set as a dynamic modification; (4) carbamidomethylation of cysteine set as a static modification; and (5) a maximum of 2 missed cleavage sites were allowed. False-discovery rates were applied to peptide-spectrum matches using Percolator, and only those with a false-discovery rate of less than 1% were allowed. In the case of F1 samples, identified proteins could be of human or murine origin. From scrutiny of the peptides used to identify the proteins, we can divide the detected proteins into 3 categories: (i) human not murine, (ii) murine not human, (iii) could be murine or human. To simplify the analysis, we have confined our list to the first category—protein lists presented here are human not murine.

## 2.3. mRNA and miRNA Microarray Analysis

Sample preparation and mRNA microarray analysis was as previously described [20]. Briefly snap frozen samples of F1 tumors, original patient tumor material, and adjacent normal tissue were prepared for RNA extraction by grinding samples under LN<sub>2</sub> using a mortar and pestle. The tissue sample was placed in a metal mortar with a small volume of LN<sub>2</sub> and ground quickly. When powdered, the powder was transferred to a clean microcentrifuge tube. This was performed before the LN<sub>2</sub> completely evaporated and while the powder was still frozen. Once homogenized, RNA samples were prepared using Trizol (Sigma, Merck KGaA, Darmstadt, Germany), according to the manufacturer's guidelines. Samples were quantified using a Nanodrop (Thermo Scientific), and quality was determined using Agilent Bioanalyser (Agilent, Santa Clara, Ca, USA). Samples with RIN numbers greater than 8 were accepted as most suitable for microarray analysis.

Briefly, the preparation of cRNA, hybridization, and scanning of microarrays was performed according to the manufacturer's protocol (Affymetrix, Thermo-Fischer, Santa Clara, CA, USA). A total of 300 ng of total RNA extracted from homogenized patient samples isolated using Trizol was converted into double-stranded cDNA by reverse transcription. Biotin-labeled cRNA was generated by converting the cDNA sample using the Genechip WT plus reagent kit (Affymetrix). Labeled cRNA was hybridized to the Affymetrix GeneChip<sup>®</sup> Human Gene 2.0 ST Array while rotating at 60 rpm for 16 h at 45 °C. After hybridization, the microarray was washed using the Affymetrix Fluidics Station according to the manufacturer's protocol. The chips were scanned in an Affymetrix 3000 7G scanner.

miRNA arrays were performed according to the manufacturer's protocol (Affymetrix). A total of 1000 ng of total RNA was extracted from homogenized patient samples and isolated using Trizol. Following this, samples underwent Poly(A) tailing followed by flash-tag biotin HSR ligation. Labeled samples were hybridized to the Affymetrix GeneChip<sup>®</sup> miRNA 4.0 Array while rotating at 60 rpm for 16–18 h at 48 °C. After hybridization, the mi-

croarray was washed using the Affymetrix Fluidics Station according to the manufacturer's protocol. The chips were scanned in an Affymetrix 3000 7G scanner.

#### 2.4. Bioinformatic Analysis

Differential gene analysis expression was carried out using Applied Biosystems Transcriptome Analysis Console (TAC) software 4.0.2. The software was integrated with the established LIMMA package, which allowed for improved inference at both the gene and gene set levels in small experiments. Resulting gene signal values are expressed as biweight robust averages shown on the log<sub>2</sub> scale. Fold change values are calculated from the log<sub>2</sub> signal values. Resulting gene lists were filtered for  $\pm 1.5$  fold changes, a  $p$ -value  $< 0.05$  and an overall FDR F-Test:  $< 0.005$ . The miRNA 4.0 chip comprises of all mature miRNA sequences in the miRBase release 20, including miRNA transcripts from humans, mice, and rats. As they are suitable for multi-species analysis, an additional filter of transcript ID containing only hsa was also included.

The miRNA and mRNA cel files are deposited on the GEO repository, submission GSE141873 (mRNA) and GSE207345 (miRNA).

miRwalk was used for predicted protein identification [22]. Filters applied to the results were a binding probability value of 0.95, a binding site position of 3-UTR, and a validated interaction with an miRTarbase entry.

### 3. Results

Bioinformatics analysis of the miRNA data from the tumor (T), adjacent normal (N), and patient-derived xenograft tumors (F1) identified 100 differentially expressed (DE) miRNA (87 upregulated and 13 downregulated in the tumor sample) in the tumor versus normal tissue samples (Supplementary Table S1). In the F1 vs. tumor comparison, 214 DE miRNA were identified (67 upregulated and 147 downregulated in the F1 samples (Supplementary Table S2). Identifications were based on a  $p$ -value  $< 0.05$  and a fold change of  $\pm 1.5$ . A fold change of  $\pm 1.5$  was selected as previous profiling studies have shown that even subtle changes in miRNA expression can have an impact on cell biology [23]. In comparisons where a very large fold change is recorded, it generally flags that the expression level is very low in one of the conditions rather than being a strictly accurate assessment of the ratio of the number of molecules present in each condition. These particular arrays (miRNA 4.0 array) have transcripts relating to an extensive range of species as well as humans, including mice and rats. There are a total of 4603 miRNA transcripts represented on the arrays that are determined to be expressed in humans. Due to the size and homogeneity of the miRNA sequences, it is not possible to say with certainty in the case of the F1 miRNA whether any particular miRNA is of human or mouse origin.

There are many ways in which the data could be presented and in which the three profile data strands could be analyzed. In this paper, we focus on (1) the changes of greatest magnitude up and down in the two comparisons (Tables 1 and 2), (2) miRNAs where expression changed in the same direction in both comparisons (Table 3), and (3) changes in expression of individual proteins, which could be explained by our miRNA data (Tables 4–6).

#### 3.1. Examination of miRNA Expression Changes in Pancreatic Cancer Tumor Samples and Adjacent Normal Tissue

Table 1 shows the top 10 up- and downregulated miRNAs in the comparison of the patient tumor samples and adjacent normal tissue. Figure 1 shows a visual representation of each of the miRNA expressions in all three conditions within this study.

**Table 1.** Priority miRNA from miRNA array analysis with corresponding fold changes and *p*-values for TvN comparison.

| Transcript ID (Array Design) | TvN         |                       |
|------------------------------|-------------|-----------------------|
|                              | Fold Change | <i>p</i> -Values      |
| hsa-miR-21-3p                | 12.89       | $1.20 \times 10^{-3}$ |
| hsa-miR-708-5p               | 9.15        | $6.70 \times 10^{-3}$ |
| hsa-miR-181c-5p              | 8.52        | $3.00 \times 10^{-3}$ |
| hsa-miR-125b-1-3p            | 6.95        | $3.18 \times 10^{-2}$ |
| hsa-miR-21-5p                | 6.89        | $1.58 \times 10^{-2}$ |
| hsa-miR-331-5p               | 6.81        | $3.60 \times 10^{-3}$ |
| hsa-miR-210-3p               | 6.73        | $6.60 \times 10^{-3}$ |
| hsa-miR-181d-5p              | 6.51        | $2.28 \times 10^{-2}$ |
| hsa-miR-214-5p               | 6.3         | $1.02 \times 10^{-2}$ |
| hsa-miR-143-5p               | 5.55        | $2.00 \times 10^{-4}$ |
| hsa-mir-375                  | −1.67       | $3.16 \times 10^{-2}$ |
| hsa-miR-3618                 | −1.76       | $4.70 \times 10^{-2}$ |
| hsa-miR-509-5p               | −1.85       | $1.30 \times 10^{-3}$ |
| hsa-mir-6722                 | −2          | $4.62 \times 10^{-2}$ |
| hsa-miR-4742-5p              | −2.06       | $2.15 \times 10^{-2}$ |
| hsa-mir-139                  | −2.2        | $4.92 \times 10^{-2}$ |
| hsa-miR-451a                 | −2.93       | $3.50 \times 10^{-3}$ |
| hsa-miR-139-5p               | −4.51       | $1.20 \times 10^{-3}$ |
| hsa-miR-486-5p               | −4.65       | $1.33 \times 10^{-2}$ |
| hsa-miR-148a-5p              | −8.15       | $4.08 \times 10^{-2}$ |

**Table 2.** Priority miRNA from miRNA array analysis with corresponding fold changes and *p*-values for F1vT comparison.

| Transcript ID (Array Design) | F1vT        |                       |
|------------------------------|-------------|-----------------------|
|                              | Fold Change | <i>p</i> -Values      |
| hsa-miR-206                  | 26.01       | $8.59 \times 10^{-5}$ |
| hsa-miR-4521                 | 24.27       | $4.48 \times 10^{-6}$ |
| hsa-miR-6872-5p              | 21.19       | $1.35 \times 10^{-7}$ |
| hsa-miR-1290                 | 15.4        | $6.80 \times 10^{-3}$ |
| hsa-miR-486-5p               | 12.48       | $1.40 \times 10^{-3}$ |
| hsa-miR-615-3p               | 10.81       | $5.59 \times 10^{-5}$ |
| hsa-miR-203a                 | 9.43        | $2.30 \times 10^{-3}$ |
| hsa-miR-6778-5p              | 8.56        | $5.42 \times 10^{-8}$ |
| hsa-miR-2467-3p              | 7.91        | $2.01 \times 10^{-8}$ |
| hsa-miR-139-5p               | 6.7         | $2.78 \times 10^{-6}$ |
| hsa-miR-409-3p               | −11.45      | $1.47 \times 10^{-6}$ |
| hsa-miR-146b-3p              | −12.7       | $6.39 \times 10^{-6}$ |
| hsa-miR-217                  | −13.39      | $1.98 \times 10^{-2}$ |
| hsa-miR-503-5p               | −14.24      | $2.30 \times 10^{-3}$ |
| hsa-miR-1271-5p              | −17.01      | $1.88 \times 10^{-6}$ |
| hsa-miR-125b-2-3p            | −21.33      | $6.58 \times 10^{-7}$ |
| hsa-miR-708-5p               | −21.66      | $2.00 \times 10^{-3}$ |
| hsa-miR-487b-3p              | −34.33      | $1.31 \times 10^{-9}$ |
| hsa-miR-424-3p               | −56.66      | $2.27 \times 10^{-6}$ |
| hsa-miR-432-5p               | −113.44     | $2.10 \times 10^{13}$ |

**Table 3.** Priority miRNA from miRNA array analysis with corresponding fold changes and *p*-values for shown to be differentially expressed in both comparisons in the same direction.

| Transcript ID<br>(Array Design) | TvN         |                       | F1vT        |                       |
|---------------------------------|-------------|-----------------------|-------------|-----------------------|
|                                 | Fold Change | <i>p</i> -Val         | Fold Change | <i>p</i> -Values      |
| hsa-miR-6831-5p                 | 4.82        | $2.16 \times 10^{-2}$ | 1.81        | $1.32 \times 10^{-2}$ |
| hsa-miR-222-3p                  | 1.99        | $7.10 \times 10^{-3}$ | 2.27        | $2.41 \times 10^{-2}$ |
| hsa-miR-4534                    | 1.92        | $4.76 \times 10^{-2}$ | 1.83        | $3.49 \times 10^{-2}$ |
| hsa-miR-4743-5p                 | 1.87        | $1.12 \times 10^{-2}$ | 1.94        | $2.20 \times 10^{-3}$ |
| hsa-miR-3154                    | 1.86        | $3.12 \times 10^{-2}$ | 5.14        | $1.60 \times 10^{-5}$ |
| hsa-miR-3935                    | 1.85        | $2.99 \times 10^{-2}$ | 2.5         | $1.59 \times 10^{-2}$ |

### 3.2. Examination of miRNA Expression Changes in Pancreatic Cancer Tumor Samples and Patient-Derived Xenograft Models

Table 2 shows the top 10 up- and downregulated miRNAs in the comparison of the patient tumor samples and adjacent normal tissue. Figure 2 shows a visual representation of each of the miRNA expressions in all three conditions within this study.

### 3.3. Examination of miRNA Expression Changes in Pancreatic Cancer, Adjacent Normal Tissue, and Patient-Derived Xenograft Models of Pancreatic Cancer

Table 3 shows the priority miRNA that was shown to be differentially expressed in both comparisons in the same direction. Figure 3 show a visual representation of each of the miRNA expression in all three conditions within this study. While the comparisons TvN and F1vT are clearly interesting in their own right, we have also searched for changes that happen in both comparisons but in the same direction. These may be of special interest because the F1 tumor may be due to cell selection following transplantation, have a higher proportion of tumor cells than the original tumor sample (a mixture of normal and tumor cells), and our proteomics analysis identifies only human and not murine proteins. Of course, other phenomena are also involved in these transitions; for example, major changes in the cellular composition of the stroma.

### 3.4. Investigation of Predicted miRNA Target Proteins in Proteomic and Transcription Datasets

Since miRNAs target multiple mRNAs and proteins, we hypothesized that these DE expression lists might lead us to pathways as well as individual proteins of functional, diagnostic, or therapeutic significance. We are particularly well placed to investigate this network in detail in this study as we have performed global mRNA [20] and proteomic [19,24] profiling on the same clinical and F1 samples. Protein changes that do not have a corresponding same direction mRNA change could be strong candidates for regulation by miRNAs. Histological examples, as well as clinical descriptions of the samples, were previously described previously [20].

Table 4 (TvN), Table 5 (F1vT), and Table 6 (DE in the same direction in both) list predicted protein targets of specific miRNAs, which, as well as being predicted, were also found in the proteomic dataset to be different from the expressed in the appropriate direction (i.e., inverse to the direction of difference of expression of the miRNA). Note that only proteins for which the mass spectrometry data indicated definitively human, and not murine, origin are included in our analysis to avoid confusion in the interpretation of the F1 proteomic data.

The predicted protein list was generated from miRWalk, which was filtered to only include validated interactions with an entry on miRTarbase [22].

Of the 39 miRNAs investigated from the priority list, 7 miRNAs are enriched in the list by targeting multiple proteins. miRs 2467, 4742, 509, 615-3p, 4534, 222, and 206 are all shown to target two or more proteins across the two comparisons.

**Table 4.** Priority miRNA fold changes and *p*-values and corresponding predicted protein target expression identified in LC-MS/MS dataset with *p*-value for the TvN comparison.

| Transcript ID<br>(Array Design) | TvN   |                       | F1vT |                  | Gene<br>Symbol | TvN (Membrane) |                       | TvN (Cytosolic) |                       | F1vT (Membrane) |                       | F1vT (Cytocolic) |                       |
|---------------------------------|-------|-----------------------|------|------------------|----------------|----------------|-----------------------|-----------------|-----------------------|-----------------|-----------------------|------------------|-----------------------|
|                                 | FC    | <i>p</i> -Val         | FC   | <i>p</i> -Values |                | FC             | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC               | <i>p</i> -Values      |
| hsa-miR-143-5p                  | 5.55  | $2.00 \times 10^{-4}$ |      |                  | GSTM3          | −2.04          | $6.48 \times 10^{-3}$ |                 |                       |                 |                       | −15.16           | $6.46 \times 10^{-3}$ |
| hsa-miR-181c-5p                 | 8.52  | $3.00 \times 10^{-3}$ |      |                  | PEBP1          | −3.20          | $1.19 \times 10^{-2}$ | −2.90           | $1.33 \times 10^{-3}$ | −2.10           | $1.04 \times 10^{-2}$ | −3.50            | $3.77 \times 10^{-5}$ |
| hsa-miR-181d-5p                 | 6.51  | $2.28 \times 10^{-2}$ |      |                  | PEBP1          | −3.20          | $1.19 \times 10^{-2}$ | −2.90           | $1.33 \times 10^{-3}$ | −2.10           | $1.04 \times 10^{-2}$ | −3.50            | $3.77 \times 10^{-5}$ |
| hsa-miR-210-3p                  | 6.73  | $6.60 \times 10^{-3}$ |      |                  | ERP27          | −21.19         | $9.29 \times 10^{-4}$ |                 |                       |                 |                       |                  |                       |
| hsa-miR-214-5p                  | 6.3   | $1.02 \times 10^{-2}$ |      |                  | TBL2           |                |                       | −13.95          | $4.01 \times 10^{-3}$ |                 |                       |                  |                       |
| hsa-miR-21-5p                   | 6.89  | $1.58 \times 10^{-2}$ |      |                  | DDAH1          | −2.13          | $1.92 \times 10^{-3}$ |                 |                       |                 |                       | 4.16             | $3.84 \times 10^{-4}$ |
| hsa-miR-331-5p                  | 6.81  | $3.60 \times 10^{-3}$ |      |                  | DDAH1          | −2.13          | $1.92 \times 10^{-3}$ |                 |                       |                 |                       | 4.16             | $3.84 \times 10^{-4}$ |
| hsa-miR-4742-5p                 | −2.06 | $2.15 \times 10^{-2}$ |      |                  | CSRP1          | 3.11           | $1.39 \times 10^{-3}$ | 2.34            | $1.31 \times 10^{-2}$ |                 |                       |                  |                       |
| hsa-miR-4742-5p                 | −2.06 | $2.15 \times 10^{-2}$ |      |                  | S100A16        | 4.20           | $1.12 \times 10^{-2}$ |                 |                       | 3.57            | $8.51 \times 10^{-4}$ |                  |                       |
| hsa-miR-4742-5p                 | −2.06 | $2.15 \times 10^{-2}$ |      |                  | SOD2           | 2.29           | $5.88 \times 10^{-3}$ |                 |                       | −4.84           | $5.06 \times 10^{-6}$ | −22.02           | $2.29 \times 10^{-9}$ |
| hsa-miR-509-5p                  | −1.85 | $1.30 \times 10^{-3}$ |      |                  | COTL1          | 2.23           | $3.83 \times 10^{-2}$ |                 |                       |                 |                       | 8.77             | $1.61 \times 10^{-4}$ |
| hsa-miR-509-5p                  | −1.85 | $1.30 \times 10^{-3}$ |      |                  | LRPAP1         | 2.21           | $3.10 \times 10^{-3}$ |                 |                       | −2.37           | $1.53 \times 10^{-3}$ |                  |                       |
| hsa-miR-509-5p                  | −1.85 | $1.30 \times 10^{-3}$ |      |                  | SLC4A2         | 6.03           | $3.11 \times 10^{-3}$ |                 |                       | −1.98           | $5.14 \times 10^{-3}$ |                  |                       |

List of predicted target proteins for priority miRNAs in Table 1 list, which also showed inverse DE with the miRNA in the proteomics analysis.

**Table 5.** Priority miRNA fold changes and *p*-values and corresponding predicted protein target expression identified in LC-MS/MS dataset with *p*-value for the F1vT comparison.

| Transcript ID<br>(Array Design) | TvN |                  | F1vT  |                       | Gene<br>Symbol | TvN (Membrane) |                       | TvN (Cytosolic) |                       | F1vT (Membrane) |                       | F1vT (Cytocolic) |                       |
|---------------------------------|-----|------------------|-------|-----------------------|----------------|----------------|-----------------------|-----------------|-----------------------|-----------------|-----------------------|------------------|-----------------------|
|                                 | FC  | <i>p</i> -Values | FC    | <i>p</i> -Values      |                | FC             | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC               | <i>p</i> -Values      |
| hsa-miR-125b-2-3p               |     |                  | −21.3 | $6.58 \times 10^{-7}$ | SUGT1          |                |                       |                 |                       |                 |                       | 3.19             | $9.56 \times 10^{-4}$ |
| hsa-miR-206                     |     |                  | 26.01 | $8.59 \times 10^{-5}$ | PPIB           |                |                       | −3.55           | $5.45 \times 10^{-3}$ | −1.94           | $2.55 \times 10^{-2}$ | −3.30            | $1.64 \times 10^{-2}$ |
| hsa-miR-206                     |     |                  | 26.01 | $8.59 \times 10^{-5}$ | SLC25A22       | −2.77          | $1.19 \times 10^{-2}$ |                 |                       |                 |                       |                  |                       |
| hsa-miR-615-3p                  |     |                  | 10.81 | $5.59 \times 10^{-5}$ | DPP3           | 2.53           | $2.66 \times 10^{-3}$ |                 |                       | −1.91           | $1.20 \times 10^{-2}$ | 2.25             | $3.86 \times 10^{-4}$ |
| hsa-miR-615-3p                  |     |                  | 10.81 | $5.59 \times 10^{-5}$ | GANAB          | 1.67           | $7.26 \times 10^{-3}$ |                 |                       | −2.04           | $2.12 \times 10^{-4}$ |                  |                       |
| hsa-miR-708-5p                  |     |                  | −21.7 | $2.00 \times 10^{-3}$ | RANBP2         |                |                       |                 |                       |                 |                       | 1.93             | $8.16 \times 10^{-3}$ |
| hsa-miR-2467-3p                 |     |                  | 7.91  | $2.01 \times 10^{-8}$ | AKR7A2         |                |                       | −2.09           | $1.56 \times 10^{-3}$ | −1.81           | $1.01 \times 10^{-2}$ |                  |                       |
| hsa-miR-2467-3p                 |     |                  | 7.91  | $2.01 \times 10^{-8}$ | ATP1B3         | 1.88           | $3.03 \times 10^{-2}$ |                 |                       | −2.26           | $1.59 \times 10^{-2}$ |                  |                       |
| hsa-miR-2467-3p                 |     |                  | 7.91  | $2.01 \times 10^{-8}$ | CPM            |                |                       |                 |                       | −5.55           | $1.31 \times 10^{-2}$ |                  |                       |

Table 5. Cont.

| Transcript ID<br>(Array Design) | TvN |                  | F1vT |                       | Gene<br>Symbol | TvN (Membrane) |                       | TvN (Cytosolic) |                       | F1vT (Membrane) |                       | F1vT (Cytocolic) |                  |
|---------------------------------|-----|------------------|------|-----------------------|----------------|----------------|-----------------------|-----------------|-----------------------|-----------------|-----------------------|------------------|------------------|
|                                 | FC  | <i>p</i> -Values | FC   | <i>p</i> -Values      |                | FC             | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC               | <i>p</i> -Values |
| hsa-miR-2467-3p                 |     |                  | 7.91 | $2.01 \times 10^{-8}$ | FKBP15         |                |                       |                 |                       | −4.64           | $5.12 \times 10^{-3}$ |                  |                  |
| hsa-miR-2467-3p                 |     |                  | 7.91 | $2.01 \times 10^{-8}$ | RAB1A          |                |                       | −3.33           | $4.87 \times 10^{-3}$ |                 |                       |                  |                  |
| hsa-miR-2467-3p                 |     |                  | 7.91 | $2.01 \times 10^{-8}$ | UGGT1          |                |                       |                 |                       | −1.80           | $1.91 \times 10^{-2}$ |                  |                  |
| hsa-miR-378e                    |     |                  | 4.2  | $1.10 \times 10^{-3}$ | TXNL1          |                |                       |                 |                       | −2.54           | $2.30 \times 10^{-3}$ |                  |                  |
| hsa-miR-6778-5p                 |     |                  | 8.56 | $5.42 \times 10^{-8}$ | ATP5F1A        | −2.19          | $9.76 \times 10^{-3}$ | −2.70           | $1.53 \times 10^{-3}$ |                 |                       |                  |                  |

List of predicted target proteins for priority miRNAs in Table 2 list, which also showed inverse DE with the miRNA in the proteomics analysis.

**Table 6.** Priority miRNA fold changes and *p*-values and corresponding predicted protein target expression identified in LC-MS/MS dataset with *p*-value for miRNA that were DE expressed in the same direction in both comparisons.

| Transcript ID<br>(Array Design) | TvN  |                       | F1vT |                       | Gene<br>Symbol | TvN (Membrane) |                       | TvN (Cytosolic) |                  | F1vT (Membrane) |                       | F1vT (Cytocolic) |                  |
|---------------------------------|------|-----------------------|------|-----------------------|----------------|----------------|-----------------------|-----------------|------------------|-----------------|-----------------------|------------------|------------------|
|                                 | FC   | <i>p</i> -Values      | FC   | <i>p</i> -Values      |                | FC             | <i>p</i> -Values      | FC              | <i>p</i> -Values | FC              | <i>p</i> -Val         | FC               | <i>p</i> -Values |
| hsa-miR-4534                    | 1.92 | $4.76 \times 10^{-2}$ | 1.83 | $3.49 \times 10^{-2}$ | CALR           |                |                       |                 |                  | −2.18           | $1.52 \times 10^{-4}$ |                  |                  |
| hsa-miR-4534                    | 1.92 | $4.76 \times 10^{-2}$ | 1.83 | $3.49 \times 10^{-2}$ | CAPZA1         |                |                       |                 |                  | −2.05           | $2.49 \times 10^{-3}$ |                  |                  |
| hsa-miR-4743-5p                 | 1.87 | $1.12 \times 10^{-2}$ | 1.94 | $2.20 \times 10^{-3}$ | CYP20A1        |                |                       |                 |                  | −2.50           | $6.46 \times 10^{-3}$ |                  |                  |
| hsa-miR-222-3p                  | 1.99 | $7.10 \times 10^{-3}$ | 2.27 | $2.41 \times 10^{-2}$ | GNAI3          |                |                       |                 |                  | −2.56           | $3.85 \times 10^{-4}$ |                  |                  |
| hsa-miR-3154                    | 1.86 | $3.12 \times 10^{-2}$ | 5.14 | $1.60 \times 10^{-5}$ | HSP90B1        | −6.63          | $4.34 \times 10^{-3}$ |                 |                  | −1.72           | $1.11 \times 10^{-2}$ | −2.71            |                  |
| hsa-miR-4534                    | 1.92 | $4.76 \times 10^{-2}$ | 1.83 | $3.49 \times 10^{-2}$ | OLA1           | −4.41          | $3.21 \times 10^{-4}$ |                 |                  |                 |                       |                  |                  |
| hsa-miR-6831-5p                 | 4.82 | $2.16 \times 10^{-2}$ | 1.81 | $1.32 \times 10^{-2}$ | PLD3           |                |                       |                 |                  | −3.55           | $1.13 \times 10^{-3}$ | −6.41            |                  |
| hsa-miR-222-3p                  | 1.99 | $7.10 \times 10^{-3}$ | 2.27 | $2.41 \times 10^{-2}$ | RECK           |                |                       |                 |                  | −12.65          | $9.53 \times 10^{-8}$ |                  |                  |
| hsa-miR-222-3p                  | 1.99 | $7.10 \times 10^{-3}$ | 2.27 | $2.41 \times 10^{-2}$ | SOD2           | 2.29           | $5.88 \times 10^{-3}$ |                 |                  | −4.84           | $5.06 \times 10^{-6}$ | −22.02           |                  |
| hsa-miR-222-3p                  | 1.99 | $7.10 \times 10^{-3}$ | 2.27 | $2.41 \times 10^{-2}$ | TOM1           |                |                       |                 |                  | −1.93           | $3.90 \times 10^{-2}$ |                  |                  |

List of predicted target proteins for priority miRNAs in Table 3 list, which also showed inverse DE with the miRNA in the proteomics analysis.

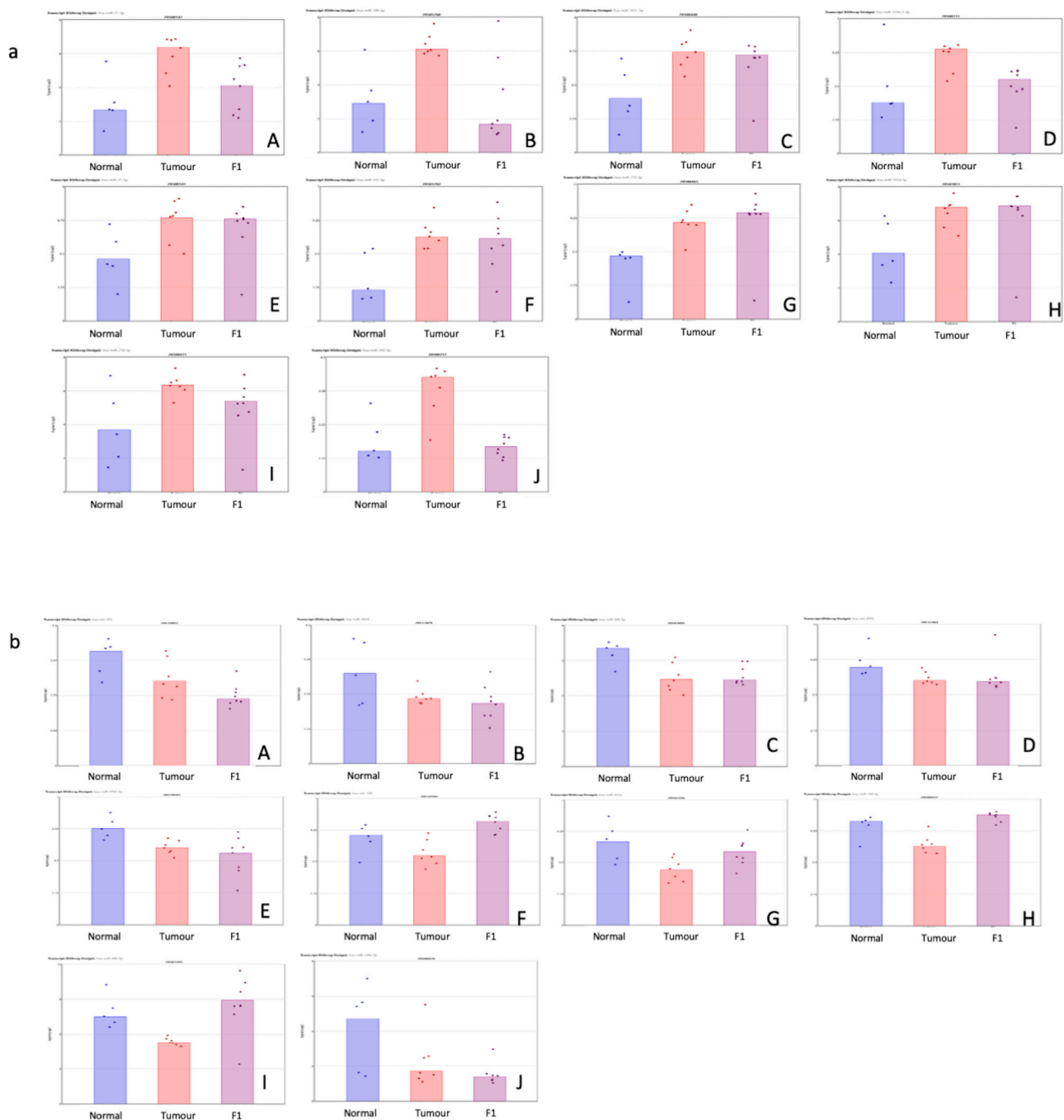


**Figure 1.** (a,b) Scatterplot representation of data from Table 1 (TvN comparison) to provide a visual representation of the interpatient variation of the expression of each miRNA in each of the 3 tissue cohorts. Column 1 represents adjacent normal tissue, column 2 represents the patient tumor material, and column 3 represents the F1 tissue. (a) Log2 expression scatterplots in normal, tumour and F1 conditions of miRNAs shown to be upregulated in TvN comparison (Table 1). (A) miR21-3p, (B) miR708-3p, (C) miR181c-5p, (D) miR125b-1-5p, (E) miR21-5p, (F) miR331-5p, (G) miR210-3p, (H) miR181d-5p, (I) miR214-5p and (J) miR143-5p. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA. (b) Log2 expression scatterplots in normal, tumour and F1 conditions of miRNAs shown to be downregulated in TvN comparison (Table 1). (A) mir375, (B) miR3618, (C) miR509-5p, (D) mir6722, (E) miR4742-5p, (F) mir139, (G) miR451a, (H) miR139-5p, (I) miR486-5p and (J) miR148a-5p. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA.

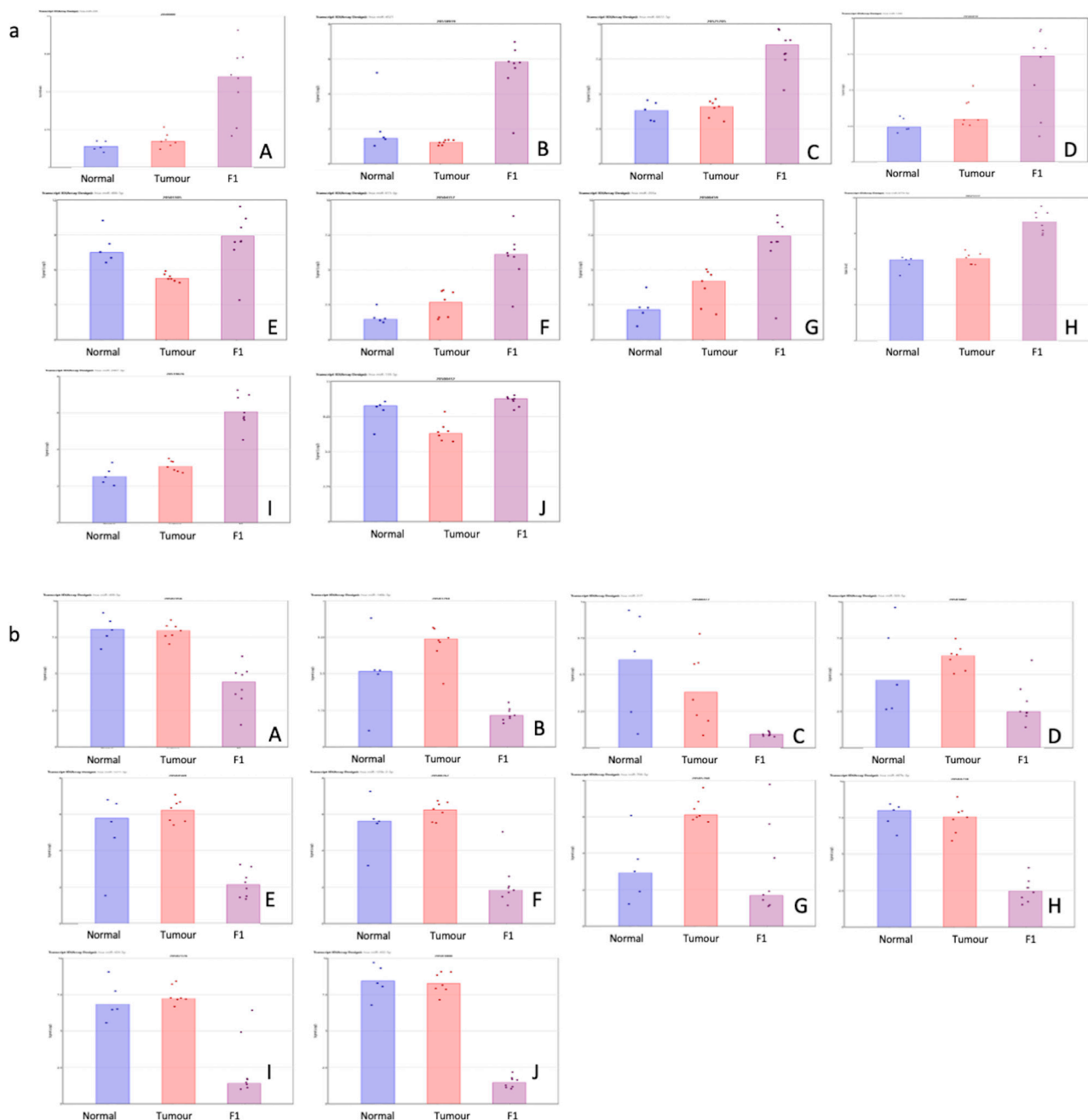


**Figure 2.** (a,b) Scatterplot representation of data from Table 1 (F1vT comparison) to provide a visual representation of the interpatient variation of the expression of each miRNA in each of the 3 tissue cohorts. Column 1 represents adjacent normal tissue, column 2 represents the patient tumor material, and column 3 represents the F1 tissue. (a) Log2 expression scatterplots in normal, tumour and F1 conditions of miRNAs shown to be upregulated in F1vT comparison (Table 2). (A) miR206, (B) miR4521, (C) miR6872-5P, (D) miR1290, (E) miR486-5p, (F) miR6153p, (G) miR203A, (H) miR6778-5p, (I) miR2467-3p and (J) miR139-5p. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA. (b) Log2 expression scatterplots in normal, tumour and F1 conditions of miRNAs shown to be downregulated in F1vT comparison (Table 2). (A) miR409-3p, (B) miR146b-3p, (C) miR217, (D) miR503-5p, (E) miR1271-5p, (F) miR125b-2-3p, (G) miR708-5p, (H) miR487-3p, (I) miR424-5p and (J) miR432-5p. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA.

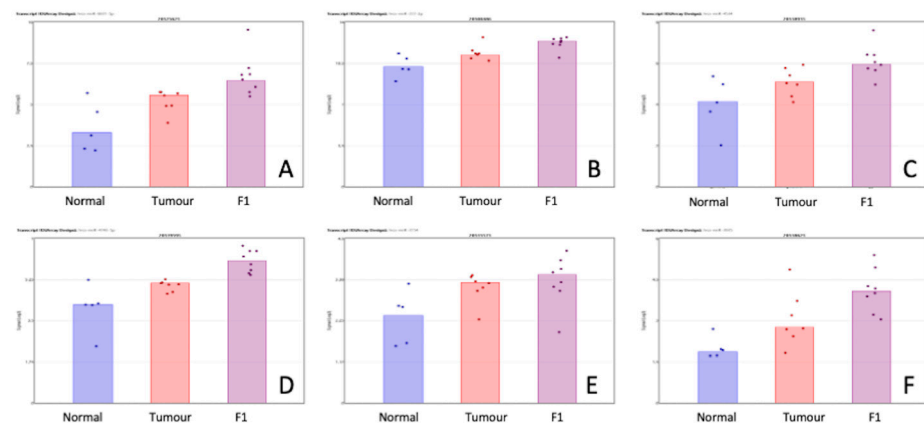


**Figure 3.** Scatterplot representation of data from Table 3 (miRNA shown to be differentially expressed in both the TvN and F1vT comparison) to provide a visual representation of the interpatient variation of the expression of each miRNA in each of the 3 tissue cohorts. Column 1 represents adjacent normal tissue, column 2 represents the patient tumor material, and column 3 represents the F1 tissue. Log2 expression scatterplots in normal tumour and F1 conditions of miRNAs shown to be upregulated in both the TvN and the F1vT comparison (Table 3). (A) miR6831-5p, (B) miR222-3p, (C) miR4534, (D) miR4743-5p, (E) miR3154, (F) miR3935. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA.

#### 4. Discussion

The core concept of this study was to use the differential expression of miRNAs as a tool to further unravel the biology of human pancreatic cancer in the hope of uncovering new targets or vulnerabilities that might be exploited therapeutically; and/or finding new biomarkers or combinations of biomarkers that might contribute to better or earlier diagnosis [25]. We believe that the study of miRNAs may be particularly fruitful since each miRNA targets multiple mRNAs, regulating the cellular levels of the corresponding proteins.

The data presented here can be viewed in a number of different ways, simple lists of miRNAs DE between tumor and normal and between F1 and tumors (and in some cases both), and because of our previously published information on DE mRNAs and proteins, it is also possible to tentatively identify predicted protein targets of specific DE miRNAs.

Previous analyses of these proteomic [19,24] and mRNA [20] or datasets on these clinical and F1 samples have looked at each dataset individually. mRNA analysis [20] showed an enrichment of genes associated with proliferation, cell cycle, and mitotic processes. Label-free LC-MS-based proteomic analysis carried out on this sample set identified a number of membrane proteins in both comparisons (TvN, F1vT) that had the potential to be utilized either as biomarkers or ADC drug conjugate targets [24].

The study of miRNAs in the development, progression, and prognosis of PDAC has intensified in the last decade. Interestingly there are few studies including transcriptomic and proteomic analysis as well as miRNA analysis on the same sample set. This limitation has meant that protein and gene targets of interesting miRNAs have predominantly been identified using predictive software and have not been validated in matched samples [8,25–29]. Mattie et al. [30] performed miRNA analysis on matched tumor vs. PDX samples; however, this study was complicated by a high degree of homology with mouse miRNA sequences. Hanoun et al. [31] initially published a study looking at the silencing of miR148a as a potential therapeutic target in PDAC. However, following further studies in which they looked at the role this miRNA had in the modulation of protein expression, they discovered that miR148a was responsible for only minimal protein regulation and were unable to identify protein targets in cell lines [32]. This indicates the importance of looking at parallel analysis where possible, as it may help to reduce the number of false leads emerging from in silico studies [33] miR148a was shown to be significantly downregulated in our patient tumor when compared with the normal-adjacent samples fold change of  $-8.15$ , it was,

however, unchanged in the F1vT comparison. The miRWalk prediction software identified nine targets for this miRNA; however, when compared with the protein list, none of these were inversely expressed.

The investigation of miRNAs, with mRNA and proteomic investigations in parallel from the same samples, as we propose, may lead to the identification of proteins that could be druggable targets. Furthermore, RNA therapeutics are now a reality, and miRNA manipulation has the potential to become a useful addition to the therapeutic armamentarium against pancreatic cancer [15,34].

#### 4.1. Tumor vs. Adjacent Normal Tissue Comparison

Examination of the dysregulation of miRNA in our dataset provided a short list of interesting miRNA; the top 10 up and down differentially expressed miRNAs in the (a) TvN comparison (Table 1), (b) F1vT comparisons (Table 2), and (c) miRNAs that are differentially expressed in the same direction in both comparisons (Table 3). Of the 39 miRNAs within this priority list, 7 of them predicted an effect on multiple proteins, which were found to be inversely expressed in the corresponding protein dataset (Tables 4–6). Several studies in gastric cancer [35], triple-negative breast cancer [36], and lung cancer [37] have indicated roles for miR509. This miRNA, however, has not been implicated in the literature on pancreatic cancer. Our data indicated that miR509 was shown to be downregulated in the patient tumors when compared to the normal adjacent, with a fold change of  $-1.85$  (Supplementary Table S1). It was predicted to target a number of proteins in our list, including COTL1, LRPAP1, and SL4A2 (Table 4). Predicted targets of the remaining miRNAs were shown to be differentially expressed in the TvN comparisons, although none of their predicted targets were found to be inversely expressed in the proteomic dataset, but alterations in the expression of miR708 [38], miR210 [39,40], and miR214 [41,42] have all been studied in pancreatic cancer.

In the TvN comparison, it would be expected that we would see the alteration in the expression of proteins and miRNAs associated with tumorigenesis and proliferation in the pathways associated with this transition. miR21 has been extensively studied in PDAC, with high expression levels being linked with shorter overall survival [43–45], while miR331-5p has not been reported in any cancer-linked study. miR21-5p and miR331-5p were shown to target Dimethylarginine Dimethylaminohydrolase 1 (DDAH1). Reduced levels of DDAH1 have been shown to mediate cell invasion and metastasis in gastric cancer through the WNT signaling pathway. Additionally, downregulation was linked with poor prognosis [46]. These miRNAs were both shown to be upregulated in the TvN comparison, and the expression of DDAH1 was shown to be downregulated in the membrane protein fraction of the same comparison. Our data suggest that both miR181c and miR181d target phosphatidylethanolamine binding protein 1 (PEBP1) with a reduction in expression in the TvN in both the membrane and cytoplasmic fraction. Loss of expression of PEBP1 (also known as raf kinase inhibitor protein (RKIP), an endogenous inhibitor of the MAPK pathway) has been shown to be linked with PDAC metastasis and poor overall survival [47,48].

miR210-3p was predicted to target endoplasmic reticulum protein 27 (ERP27), and the protein was shown to be inversely expressed in the membrane fraction (Table 4). Transcriptomic analysis of the cancer genome atlas has identified ERP27 as a pivotal gene in the pathogenesis and progression of PDAC [49]. Increased glutathione S-transferase M3 (GSTM3) expression has been linked with improved overall survival in PDAC [50]. This protein was inversely regulated by miR143-5p, which was found to be upregulated in the tumor samples when compared with the normal-adjacent samples. Literature searches showed no previous studies linked with cancer and the expression of miR4742. Our proteomic data shows that miR4742 targets a number of proteins within the dataset, including superoxide dismutase 2 (SOD2), cysteine and glycine-rich protein 1 (CSRP1), and protein S100-A16 (S100A16). SOD2 is involved in oxidative phosphorylation and the regulation of ROS (reactive oxygen species), and upregulation in its expression has

been linked with acquired resistance to ROS-inducing anticancer drugs and potential irradiation in pancreatic cancer cell lines [51]. While little is known about CSRP1 in pancreatic cancer, S100A16 has been extensively studied in PDAC. Roles in metastasis [52], epithelial–mesenchymal transition (EMT) [53], and increased expression linked with poor prognosis have all been reported in the literature. In our protein data, we do see an increased expression in the membrane fraction in both the TvN and F1vT comparison of S100A16.

#### 4.2. F1 Xenograft vs. Tumor Comparison

In the case of the F1vT comparison, our previous studies on mRNA and protein expression changes on the samples that were used here have highlighted the importance of proteins and mRNA that drive the important process of tumor engraftment in tumorigenesis. As described by Coleman et al. [24], the selection pressure that is exerted on the tumor cells during the engraftment process may result in the altered expression of proteins that drive tumor growth. The upregulation of miR125a in the PDAC samples agrees with what is already shown in the literature [54], and it has been shown that its expression can contribute to cell survival [55]. One of its predicted targets is SGT1 homolog (SUGT1), a highly conserved protein generally found in the nucleus, involved in the kinetochore function and essential for the G1/S and G2M transition, which was shown to be increased in expression in the cytosolic fraction of the tumors in comparison to the tumor sample. The expression of this protein has been potentially linked with a poor prognosis in colorectal cancer [56].

Keklikoglou et al., 2015, found that the expression of miR206 was downregulated in pancreatic tumors vs. non-malignant normal tissue [57]. In our study, miR206 was not differentially expressed in the TvN comparison; however, it was significantly upregulated in the F1vT comparison with a fold change of 26.01. Wu et al. showed that the expression of miR-206 inhibited hepatocellular carcinoma cell migration and invasion while promoting apoptosis by targeting Peptidylprolyl Isomerase B (PPIB) [58]. Another study evaluated the expression of 41 genes as a function of in vitro radioresistance in the NCI-60 cancer cell line panel and found that PPIB had the strongest direct correlation. They also showed that siRNA downregulation of PPIB leads to radio-sensitization of the cancer cells [59]. PPIB expression was shown to be downregulated in both comparisons (TvN and F1vT) in our proteomic data.

Levels of miR2467 were shown to be significantly increased in the F1 samples in comparison to the original patient tumor. This miRNA was predicted to target a large number of proteins, and when examined in our protein dataset, a large number of proteins showed inverse expression. There are limited studies in the literature on this miRNA, with one indicating a downregulation of the miRNA in colorectal tumor samples compared to tumor-adjacent normal tissue [60]. We observed a decrease in the expression of Aflatoxin B1 aldehyde reductase member 2 (AKR7A2) in both of our comparisons, not reported in the proteomic analysis by Cui et al. [61]. Furthermore, this protein was found to be targeted by two miRNAs in our analysis (miR2467 and miR1290). The latter has been suggested as a biomarker in PDAC [62]. Wei et al. [63] and Tavaano et al. [64] both examined the expression of miR1290, with and without CA19-9 in serum and plasma, respectively, and noted that while the expression was found to be higher in PDAC patients, it alone was insufficient to select patients at risk of developing PDAC. Both studies indicated that combining the expression of miR1290 with Ca19-9 was more effective. Whole miRNome and proteome profiling revealed miR1290 as a novel hypoxia-associated microRNA, which was highly abundant in hypoxic extracellular vesicles (EVs) and also exhibited a signature consisting of six proteins including AKR7A2, which was significantly associated with a poor prognosis for melanoma patients [65].

miR615 overexpression has been shown to inhibit cell proliferation, migration, and invasion in vitro [66]. miR615 was increased in the F1 samples compared to the original patient tumors, and a number of predicted target proteins were found to be inversely

expressed in the matching transition in proteomic data. These include several enzymes involved in the reduction and oxidation of aldehydes (AKR7A2, aldehyde dehydrogenase 1 family member B1 (ALDH1B1). Aldehyde dehydrogenase (ALDH2)) and aldehyde dehydrogenases are often highly expressed in cancer stem cells. ALDH1B1 may have a role in cell proliferation [67]. It has also been suggested as a potential biomarker of colon cancer [68]. It was another predicted target of miR615 found in our proteomic dataset to be downregulated in the F1vT comparison as well as in the cytosolic fraction of the TvN comparison. Calnexin (CANX), a member of the calnexin family of molecular chaperones, is a calcium-binding, endoplasmic reticulum (ER)-associated protein that interacts transiently with newly synthesized N-linked glycoproteins, facilitating protein folding and assembly. It may also play a central role in the quality control of protein folding by retaining incorrectly folded protein subunits within the ER for degradation. This protein has the potential as a biomarker in breast [69] and lung patients [70]. Several of the other miR615-3p targets identified in our analysis showed consistent changes across both comparisons and are involved in processing membrane protein targets to ER (SECG1A1, SPC53, TM95F2).

The miR-378 family has been associated with numerous cancers, including: osteosarcoma [71], cervical [72], and colon [73]. MiR-378e has not yet been extensively studied for an oncogenic/oncosuppressive role. Wang et al. [73] have reported an association between miR-378e and colorectal cancer, as the survival rate of patients in the high-expression group was significantly higher than that in the low-expression group. In our dataset, it was shown to be differentially upregulated in the F1 samples vs. the patient samples. miR409 expression, which was downregulated in the F1 tumors vs. patient tumor samples, has been linked with PDAC cell proliferation, invasion, and migration [74] proteins. Predicted to be targeted by miR409, Catenin Delta 1 (CTNND1) along with Catenin alpha 1 (CTNNA1) and Catenin Beta 1 (CTNNB1) expression was shown to indicate a poor prognosis for pancreatic cancer patients [75]. In the F1 samples, this protein was shown to be differentially upregulated versus the patient samples (Table 5).

#### 4.3. miRNA Altered in Both Comparisons

Across the two transitions, from normal to the tumor and then tumor to F1 models, there was a small cohort of miRNAs that were shown to be DE, namely miR4534, miR4743, miR222, miR3154, and miR6831. miR-6831-5p had been previously identified as being differentially expressed in PDAC vs. normal control serum samples, as shown by Aita et al. [76]. KEGG pathway analysis showed enrichment for the pancreatic cancer term. Dysregulation of miR6831-5p has been associated with gastric [77] and colorectal cancer [78]. Additionally, PLD3, which we found to be downregulated in both the cytoplasmic and membrane protein fraction of the F1 tumor samples compared to the patient material, has been shown to be a potential marker of senescence [79]. Limited studies have been completed on miR4534; however, one study indicated the potential roles of miR4534 in prostate cancer through the regulation of PTEN [80]. miR3154 has been investigated in leukemia, with its expression shown to be higher at initial diagnosis compared to complete remission [81]. miR222 has been widely studied in pancreatic cancer. Elevated levels of these miRNAs have been associated with poor survival [82–85].

Of course, a caveat with any research of this kind using tissue extracts is the fact that the samples consist of a heterogeneous mixture of cell types and connective tissue, so we cannot, for example, be certain that an alteration observed in the tumor samples reflects an alteration in expression in the tumor cells (for definitive attribution some form of *in situ* hybridization would be needed for miRNAs and mRNAs, and immunohistochemistry in the case of proteins).

## 5. Conclusions

This profiling study complements the data currently available on the expression of miRNA in pancreatic cancer. The availability of the mRNA microarrays and LC-MS-MS proteomic data has allowed for the tentative validation of what would typically have only

been predicted targets. By combining the three datasets, we have been able to identify connections between the expression of proteins and their miRNA regulators that have not been identified in pancreatic cancer previously. Analysis of the F1 samples may also allow for the identification of miRNA, which has roles in key networks associated with tumor engraftment and proliferation. The combined analysis has provided a shortlist of protein and miRNAs that warrant further investigation; this list has a mixture of established miRNAs associated with PDAC progression, in particular, miR21, miR2467, miR615 but also a number of miRs that have either limited or no studies in pancreatic cancer such as miR4534, miR3154, and miR4742. Nevertheless, some of these may have potential as biomarkers or for the development of therapeutic targets with further validation. Further analysis of these three parallel data strands that we have placed in the public domain by others using new bioinformatic tools could discover additional connections and networks that we could not uncover by our analysis. The lists we make available here will then be available to other groups for histochemical investigation and for validation in a variety of cancer-relevant in vitro assays for functional assignment.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/life13030608/s1>, Table S1: List of differentially expressed (DE) miRNA in tumour vs adjacent normal tissue comparison, Table S2: List of differentially expressed (DE) miRNA in F1 vs. tumour comparison.

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## References

- Waters, A.M.; Der, C.J. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harb. Perspect. Med.* **2018**, *8*, a031435. [\[CrossRef\]](#)
- Pant, S.; Hubbard, J.; Martinelli, E.; Bekaii-Saab, T. Clinical Update on K-Ras Targeted Therapy in Gastrointestinal Cancers. *Crit. Rev. Oncol. Hematol.* **2018**, *130*, 78–91. [\[CrossRef\]](#)
- Liu, J.; Kang, R.; Tang, D. The KRAS-G12C Inhibitor: Activity and Resistance. *Cancer Gene* **2022**, *29*, 875–878. [\[CrossRef\]](#)
- Ho, W.J.; Jaffee, E.M.; Zheng, L. The Tumour Microenvironment in Pancreatic Cancer—Clinical Challenges and Opportunities. *Nat. Rev. Clin. Oncol.* **2020**, *17*, 527. [\[CrossRef\]](#)
- Guo, S.; Fesler, A.; Wang, H.; Ju, J. MicroRNA Based Prognostic Biomarkers in Pancreatic Cancer. *Biomark. Res.* **2018**, *6*, 18. [\[CrossRef\]](#)
- Chen, L.; Ma, C.; Bian, Y.; Shao, C.; Wang, T.; Li, J.; Chong, X.; Su, L.; Lu, J. Aberrant Expression of STYK1 and E-Cadherin Confer a Poor Prognosis for Pancreatic Cancer Patients. *Oncotarget* **2017**, *8*, 111333–111345. [\[CrossRef\]](#)
- Khan, M.A.; Zubair, H.; Srivastava, S.K.; Singh, S.; Singh, A.P. Insights into the Role of MicroRNAs in Pancreatic Cancer Pathogenesis: Potential for Diagnosis, Prognosis, and Therapy. *Adv. Exp. Med. Biol.* **2015**, *889*, 71–87. [\[CrossRef\]](#)
- Bloomston, M.; Frankel, W.L.; Petrocca, F.; Volinia, S.; Alder, H.; Hagan, J.P.; Liu, C.-G.; Bhatt, D.; Taccioli, C.; Croce, C.M. MicroRNA Expression Patterns to Differentiate Pancreatic Adenocarcinoma From Normal Pancreas and Chronic Pancreatitis. *JAMA* **2007**, *297*, 1901. [\[CrossRef\]](#)
- Iorio, M.V.; Croce, C.M. MicroRNA Dysregulation in Cancer: Diagnostics, Monitoring and Therapeutics. A Comprehensive Review. *EMBO Mol. Med.* **2012**, *4*, 143. [\[CrossRef\]](#)
- Jamieson, N.B.; Morran, D.C.; Morton, J.P.; Ali, A.; Dickson, E.J.; Carter, C.R.; Sansom, O.J.; Evans, T.R.J.; McKay, C.J.; Oien, K.A. MicroRNA Molecular Profiles Associated with Diagnosis, Clinicopathologic Criteria, and Overall Survival in Patients with Resectable Pancreatic Ductal Adenocarcinoma. *Clin. Cancer Res.* **2012**, *18*, 534–545. [\[CrossRef\]](#)
- Steele, C.W.; Oien, K.A.; McKay, C.J.; Jamieson, N.B. Clinical Potential of MicroRNAs in Pancreatic Ductal Adenocarcinoma. *Pancreas* **2011**, *40*, 1165–1171. [\[CrossRef\]](#)
- Fathi, M.; Ghafouri-Fard, S.; Abak, A.; Taheri, M. Emerging Roles of MiRNAs in the Development of Pancreatic Cancer. *Biomed. Pharmacother.* **2021**, *141*, 111914. [\[CrossRef\]](#)
- Smolarz, B.; Durczyński, A.; Romanowicz, H.; Hogendorf, P. The Role of MicroRNA in Pancreatic Cancer. *Biomedicines* **2021**, *9*, 1322. [\[CrossRef\]](#)
- Park, W.; Chawla, A.; O'Reilly, E.M. Pancreatic Cancer: A Review. *JAMA* **2021**, *326*, 851–862. [\[CrossRef\]](#)
- Gilles, M.E.; Hao, L.; Huang, L.; Rupaimoole, R.; Lopez-Casas, P.P.; Pulver, E.; Jeong, J.C.; Muthuswamy, S.K.; Hidalgo, M.; Bhatia, S.N.; et al. Personalized RNA Medicine for Pancreatic Cancer. *Clin. Cancer Res.* **2018**, *24*, 1734–1747. [\[CrossRef\]](#)
- Sicard, F.; Gayral, M.; Lulka, H.; Buscail, L.; Cordelier, P. Targeting MiR-21 for the Therapy of Pancreatic Cancer. *Mol. Ther. J. Am. Soc. Gene Ther.* **2013**, *21*, 986–994. [\[CrossRef\]](#)
- Lu, T.; Prakash, J. Nanomedicine Strategies to Enhance Tumor Drug Penetration in Pancreatic Cancer. *Int. J. Nanomed.* **2021**, *16*, 6313–6328. [\[CrossRef\]](#)
- O'Brien, J.; Hayder, H.; Zayed, Y.; Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front. Endocrinol.* **2018**, *9*, 402. [\[CrossRef\]](#)
- Coleman, O.; Henry, M.; O'Neill, F.; Roche, S.; Swan, N.; Geoghegan, J.; Conlon, K.; McVey, G.; Moriarty, M.; Meleady, P.; et al. Proteomic Analysis of Cell Lines and Primary Tumors in Pancreatic Cancer Identifies Proteins Expressed Only In Vitro and Only In Vivo. *Pancreas* **2020**, *49*, 1109–1116. [\[CrossRef\]](#)
- Roche, S.; O'Neill, F.; Murphy, J.; Swan, N.; Meiller, J.; Conlon, N.T.; Geoghegan, J.; Conlon, K.; McDermott, R.; Rahman, R.; et al. Establishment and Characterisation by Expression Microarray of Patient-Derived Xenograft Panel of Human Pancreatic Adenocarcinoma Patients. *Int. J. Mol. Sci.* **2020**, *21*, 962. [\[CrossRef\]](#)
- Kaushik, P.; Henry, M.; Clynes, M.; Meleady, P. The Expression Pattern of the Phosphoproteome Is Significantly Changed During the Growth Phases of Recombinant CHO Cell Culture. *Biotechnol. J.* **2018**, *13*, 1700221. [\[CrossRef\]](#)
- Sticht, C.; Torre, C.D.L.; Parveen, A.; Gretz, N. MiRWalk: An Online Resource for Prediction of MicroRNA Binding Sites. *PLoS ONE* **2018**, *13*, e0206239. [\[CrossRef\]](#)
- Mestdagh, P.; Van Vlierberghe, P.; De Weer, A.; Muth, D.; Westermann, F.; Speleman, F.; Vandesompele, J. A Novel and Universal Method for MicroRNA RT-QPCR Data Normalization. *Genome Biol.* **2009**, *10*, R64. [\[CrossRef\]](#)
- Coleman, O.; Henry, M.; O'Neill, F.; Roche, S.; Swan, N.; Boyle, L.; Murphy, J.; Meiller, J.; Conlon, N.T.; Geoghegan, J.; et al. A Comparative Quantitative LC-MS/MS Profiling Analysis of Human Pancreatic Adenocarcinoma, Adjacent-Normal Tissue, and Patient-Derived Tumour Xenografts. *Proteomes* **2018**, *6*, 45. [\[CrossRef\]](#)
- Naderi, E.; Mostafaei, M.; Pourshams, A.; Mohamadkhani, A. Network of MicroRNAs-MRNAs Interactions in Pancreatic Cancer. *BioMed. Res. Int.* **2014**, *2014*, 1–8. [\[CrossRef\]](#)
- Shams, R.; Saberi, S.; Zali, M.; Sadeghi, A.; Ghafouri-Fard, S.; Aghdaei, H.A. Identification of Potential MicroRNA Panels for Pancreatic Cancer Diagnosis Using Microarray Datasets and Bioinformatics Methods. *Sci. Rep.* **2020**, *10*, 7559. [\[CrossRef\]](#)
- Daoud, A.Z.; Mulholland, E.J.; Cole, G.; McCarthy, H.O. MicroRNAs in Pancreatic Cancer: Biomarkers, Prognostic, and Therapeutic Modulators. *BMC Cancer* **2019**, *19*, 1–13. [\[CrossRef\]](#)

28. Kent, O.A.; Mullendore, M.; Wentzel, E.A.; López-Romero, P.; Tan, A.C.; Alvarez, H.; West, K.; Ochs, M.F.; Hidalgo, M.; Arking, D.E.; et al. A Resource for Analysis of MicroRNA Expression and Function in Pancreatic Ductal Adenocarcinoma Cells. *Cancer Biol. Ther.* **2009**, *8*, 2013–2024. [\[CrossRef\]](#)
29. Eun, J.L.; Gusev, Y.; Jiang, J.; Nuovo, G.J.; Lerner, M.R.; Frankel, W.L.; Morgan, D.L.; Postier, R.G.; Brackett, D.J.; Schmittgen, T.D. Expression Profiling Identifies MicroRNA Signature in Pancreatic Cancer. *Int. J. Cancer* **2007**, *120*, 1046–1054. [\[CrossRef\]](#)
30. Mattie, M.; Christensen, A.; Chang, M.S.; Yeh, W.; Said, S.; Shostak, Y.; Capo, L.; Verlinsky, A.; An, Z.; Joseph, I.; et al. Molecular Characterization of Patient-Derived Human Pancreatic Tumor Xenograft Models for Preclinical and Translational Development of Cancer Therapeutics. *Neoplasia* **2013**, *15*, 1138–1150. [\[CrossRef\]](#)
31. Hanoun, N.; Delpu, Y.; Suriawinata, A.A.; Bournet, B.; Bureau, C.; Selves, J.; Tsongalis, G.J.; Dufresne, M.; Buscail, L.; Cordelier, P.; et al. The Silencing of MicroRNA 148a Production by DNA Hypermethylation Is an Early Event in Pancreatic Carcinogenesis. *Clin. Chem.* **2010**, *56*, 1107–1118. [\[CrossRef\]](#)
32. Delpu, Y.; Lulka, H.; Sicard, F.; Saint-Laurent, N.; Lopez, F.; Hanoun, N.; Buscail, L.; Cordelier, P.; Torrisani, J. The Rescue of MiR-148a Expression in Pancreatic Cancer: An Inappropriate Therapeutic Tool. *PLoS ONE* **2013**, *8*, e55513. [\[CrossRef\]](#)
33. Clarke, C.; Henry, M.; Doolan, P.; Kelly, S.; Aherne, S.; Sanchez, N.; Kelly, P.; Kinsella, P.; Breen, L.; Madden, S.F.; et al. Integrated MiRNA, mRNA and Protein Expression Analysis Reveals the Role of Post-Transcriptional Regulation in Controlling CHO Cell Growth Rate. *BMC Genom.* **2012**, *13*, 656. [\[CrossRef\]](#)
34. Passadouro, M.; Faneca, H. Managing Pancreatic Adenocarcinoma: A Special Focus in MicroRNA Gene Therapy. *Int. J. Mol. Sci.* **2016**, *17*, 718. [\[CrossRef\]](#)
35. Fattahi, S.; Nikbakhsh, N.; Ranaei, M.; Sabour, D.; Akhavan-Niaki, H. Association of Sonic Hedgehog Signaling Pathway Genes IHH, BOC, RAB23a and MIR195-5p, MIR509-3-5p, MIR6738-3p with Gastric Cancer Stage. *Sci. Rep.* **2021**, *11*, 16027.
36. Zhang, G.; Liu, Z.; Han, Y.; Wang, X.; Yang, Z. Overexpression of MiR-509 Increases Apoptosis and Inhibits Invasion via Suppression of Tumor Necrosis Factor- $\alpha$  in Triple-Negative Breast Cancer Hs578T Cells. *Oncol. Res.* **2016**, *24*, 233–238. [\[CrossRef\]](#)
37. Liang, J.-J.; Wang, J.-Y.; Zhang, T.-J.; An, G.-S.; Ni, J.-H.; Li, S.-Y.; Jia, H.-T. MiR-509-3-5p-NONHSAT112228.2 Axis Regulates P21 and Suppresses Proliferation and Migration of Lung Cancer Cells. *Curr. Top. Med. Chem.* **2020**, *20*, 835–846. [\[CrossRef\]](#)
38. Yu, J.; Li, A.; Hong, S.M.; Hruban, R.H.; Goggins, M. MicroRNA Alterations of Pancreatic Intraepithelial Neoplasias. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **2012**, *18*, 981–992. [\[CrossRef\]](#)
39. Sun, F.B.; Lin, Y.; Li, S.J.; Gao, J.; Han, B.; Zhang, C.S. MiR-210 Knockdown Promotes the Development of Pancreatic Cancer via Upregulating E2F3 Expression. *Eur. Rev. Med. Pharmacol. Sci.* **2018**, *22*, 8640–8648. [\[CrossRef\]](#)
40. Liu, G.; Shao, C.; Li, A.; Zhang, X.; Guo, X.; Li, J. Diagnostic Value of Plasma MiR-181b, MiR-196a, and MiR-210 Combination in Pancreatic Cancer. *Gastroenterol. Res. Pract.* **2020**, *2020*, 6073150. [\[CrossRef\]](#)
41. Cao, T.H.; Ling, X.; Chen, C.; Tang, W.; Hu, D.M.; Yin, G.J. Role of MiR-214-5p in the Migration and Invasion of Pancreatic Cancer Cells. *Eur. Rev. Med. Pharmacol. Sci.* **2018**, *22*, 7214–7221. [\[CrossRef\]](#)
42. Deng, S.; Wang, J.; Xu, J.; Li, J.; Zhang, L.; Jin, Y. Expression of MiR-214 in Pancreatic Cancer and Its Effect on the Biological Function of Pancreatic Cancer Cells. *J. B.U.ON. Off. J. Balk. Union Oncol.* **2021**, *26*, 1111–1120.
43. Zhao, F.; Wei, C.; Cui, M.Y.; Xia, Q.Q.; Wang, S.B.; Zhang, Y. Prognostic Value of MicroRNAs in Pancreatic Cancer: A Meta-Analysis. *Aging* **2020**, *12*, 9380–9404. [\[CrossRef\]](#)
44. Vila-Casadesus, M.; Vila-Navarro, E.; Raimondi, G.; Fillat, C.; Castells, A.; Lozano, J.J.; Gironella, M.; Vila-Casadesus, M.; Vila-Navarro, E.; Raimondi, G.; et al. Deciphering MicroRNA Targets in Pancreatic Cancer Using MiRComb R Package. *Oncotarget* **2018**, *9*, 6499–6517. [\[CrossRef\]](#)
45. Frampton, A.E.; Krell, J.; Jamieson, N.B.; Gall, T.M.H.; Giovannetti, E.; Funel, N.; Prado, M.M.; Krell, D.; Habib, N.A.; Castellano, L.; et al. MicroRNAs with Prognostic Significance in Pancreatic Ductal Adenocarcinoma: A Meta-Analysis. *Eur. J. Cancer (Oxf. Engl. 1990)* **2015**, *51*, 1389–1404. [\[CrossRef\]](#)
46. Ye, J.; Xu, J.; Li, Y.; Huang, Q.; Huang, J.; Wang, J.; Zhong, W.; Lin, X.; Chen, W.; Lin, X. DDAH1 Mediates Gastric Cancer Cell Invasion and Metastasis via Wnt/ $\beta$ -Catenin Signaling Pathway. *Mol. Oncol.* **2017**, *11*, 1208–1224. [\[CrossRef\]](#)
47. Kim, H.S.; Kim, G.Y.; Lim, S.J.; Kim, Y.W. Loss of Raf-1 Kinase Inhibitory Protein in Pancreatic Ductal Adenocarcinoma. *Pathology* **2010**, *42*, 655–660. [\[CrossRef\]](#)
48. Karamitopoulou, E.; Zlobec, I.; Gloor, B.; Kondi-Pafiti, A.; Lugli, A.; Perren, A. Loss of Raf-1 Kinase Inhibitor Protein (RKIP) Is Strongly Associated with High-Grade Tumor Budding and Correlates with an Aggressive Phenotype in Pancreatic Ductal Adenocarcinoma (PDAC). *J. Transl. Med.* **2013**, *11*, 1–11. [\[CrossRef\]](#)
49. Chang, X.; Yang, M.F.; Fan, W.; Wang, L.S.; Yao, J.; Li, Z.S.; Li, D.F. Bioinformatic Analysis Suggests That Three Hub Genes May Be a Vital Prognostic Biomarker in Pancreatic Ductal Adenocarcinoma. *J. Comput. Biol. A J. Comput. Mol. Cell Biol.* **2020**, *27*, 1595–1609. [\[CrossRef\]](#)
50. Wang, S.; Yang, J.; Ding, C.; Li, J.; You, L.; Dai, M.; Zhao, Y. Glutathione S-Transferase Mu-3 Predicts a Better Prognosis and Inhibits Malignant Behavior and Glycolysis in Pancreatic Cancer. *Front. Oncol.* **2020**, *10*, 1539. [\[CrossRef\]](#)
51. Zhou, J.; Du, Y. Acquisition of Resistance of Pancreatic Cancer Cells to 2-Methoxyestradiol Is Associated with the Upregulation of Manganese Superoxide Dismutase. *Mol. Cancer Res. MCR* **2012**, *10*, 768–777. [\[CrossRef\]](#)
52. Fang, D.; Zhang, C.; Xu, P.; Liu, Y.; Mo, X.; Sun, Q.; Abdelatty, A.; Hu, C.; Xu, H.; Zhou, G.; et al. S100A16 Promotes Metastasis and Progression of Pancreatic Cancer through FGF19-Mediated AKT and ERK1/2 Pathways. *Cell Biol. Toxicol.* **2021**, *37*, 555–571. [\[CrossRef\]](#)

53. Li, T.; Ren, T.; Huang, C.; Li, Y.; Yang, P.; Che, G.; Luo, L.; Chen, Y.; Peng, S.; Lin, Y.; et al. S100A16 Induces Epithelial-Mesenchymal Transition in Human PDAC Cells and Is a New Therapeutic Target for Pancreatic Cancer Treatment That Synergizes with Gemcitabine. *Biochem. Pharmacol.* **2021**, *189*, 114396. [\[CrossRef\]](#)
54. Pan, L.; Zhou, L.; Yin, W.; Bai, J.; Liu, R. MiR-125a Induces Apoptosis, Metabolism Disorder and Migration Impairment in Pancreatic Cancer Cells by Targeting Mfn2-Related Mitochondrial Fission. *Int. J. Oncol.* **2018**, *53*, 124–136. [\[CrossRef\]](#)
55. Jia, C.-W.; Sun, Y.; Zhang, T.-T.; Lu, Z.-H.; Chen, J. Effects of MiR-125a-5p on Cell Proliferation, Apoptosis and Cell Cycle of Pancreatic Cancer Cells. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* **2016**, *38*, 415–421. [\[CrossRef\]](#)
56. Iwatsuki, M.; Mimori, K.; Sato, T.; Toh, H.; Yokobori, T.; Tanaka, F.; Ishikawa, K.; Baba, H.; Mori, M. Overexpression of SUGT1 in Human Colorectal Cancer and Its Clinicopathological Significance. *Int. J. Oncol.* **2010**, *36*, 569–575. [\[CrossRef\]](#)
57. Keklikoglou, I.; Hosaka, K.; Bender, C.; Bott, A.; Koerner, C.; Mitra, D.; Will, R.; Woerner, A.; Muenstermann, E.; Wilhelm, H.; et al. MicroRNA-206 Functions as a Pleiotropic Modulator of Cell Proliferation, Invasion and Lymphangiogenesis in Pancreatic Adenocarcinoma by Targeting ANXA2 and KRAS Genes. *Oncogene* **2015**, *34*, 4867–4878. [\[CrossRef\]](#)
58. Wu, H.; Xie, D.; Yang, Y.; Yang, Q.; Shi, X.; Yang, R. Ultrasound-Targeted Microbubble Destruction-Mediated MiR-206 Overexpression Promotes Apoptosis and Inhibits Metastasis of Hepatocellular Carcinoma Cells Via Targeting PPIB. *Technol. Cancer Res. Treat.* **2020**, *19*, 1533033820959355. [\[CrossRef\]](#)
59. Williams, P.D.; Owens, C.R.; Dziegielewska, J.; Moskaluk, C.A.; Read, P.W.; Larner, J.M.; Story, M.D.; Brock, W.A.; Amundson, S.A.; Lee, J.K.; et al. Cyclophilin B Expression Is Associated with in Vitro Radioresistance and Clinical Outcome after Radiotherapy. *Neoplasia* **2011**, *13*, 1122–1131. [\[CrossRef\]](#)
60. Chen, L.; Chen, W.; Zhao, C.; Jiang, Q. LINC01224 Promotes Colorectal Cancer Progression by Sponging MiR-2467. *Cancer Manag. Res.* **2021**, *13*, 733. [\[CrossRef\]](#)
61. Cui, Y.; Tian, M.; Zong, M.; Teng, M.; Chen, Y.; Lu, J.; Jiang, J.; Liu, X.; Han, J. Proteomic Analysis of Pancreatic Ductal Adenocarcinoma Compared with Normal Adjacent Pancreatic Tissue and Pancreatic Benign Cystadenoma. *Pancreatol. Off. J. Int. Assoc. Pancreatol. (IAP) [Et Al.]* **2009**, *9*, 89–98. [\[CrossRef\]](#)
62. Ta, N.; Huang, X.; Zheng, K.; Zhang, Y.; Gao, Y.; Deng, L.; Zhang, B.; Jiang, H.; Zheng, J. MiRNA-1290 Promotes Aggressiveness in Pancreatic Ductal Adenocarcinoma by Targeting IKK1. *Cell. Physiol. Biochem.* **2018**, *51*, 711–728. [\[CrossRef\]](#)
63. Wei, J.; Yang, L.; Wu, Y.N.; Xu, J. Serum MiR-1290 and MiR-1246 as Potential Diagnostic Biomarkers of Human Pancreatic Cancer. *J. Cancer* **2020**, *11*, 1325–1333. [\[CrossRef\]](#)
64. Tavano, F.; Gioffreda, D.; Valvano, M.R.; Palmieri, O.; Tardio, M.; Latiano, T.P.; Piepoli, A.; Maiello, E.; Pirozzi, F.; Andriulli, A. Droplet Digital PCR Quantification of MiR-1290 as a Circulating Biomarker for Pancreatic Cancer. *Sci. Rep.* **2018**, *8*, 16389. [\[CrossRef\]](#)
65. Walbrecht, G.; Lecha, O.; Gaigneaux, A.; Fougères, M.R.; Philippidou, D.; Margue, C.; Nomigni, M.T.; Bernardin, F.; Dittmar, G.; Behrmann, I.; et al. Hypoxia-Induced Adaptations of MiRNomes and Proteomes in Melanoma Cells and Their Secreted Extracellular Vesicles. *Cancers* **2020**, *12*, 692. [\[CrossRef\]](#)
66. Gao, W.; Gu, Y.; Li, Z.; Cai, H.; Peng, Q.; Tu, M.; Kondo, Y.; Shinjo, K.; Zhu, Y.; Zhang, J.; et al. MiR-615-5p Is Epigenetically Inactivated and Functions as a Tumor Suppressor in Pancreatic Ductal Adenocarcinoma. *Oncogene* **2015**, *34*, 1629–1640. [\[CrossRef\]](#)
67. Singh, S.; Arcaroli, J.J.; Orlicky, D.J.; Chen, Y.; Messersmith, W.A.; Bagby, S.; Purkey, A.; Quackenbush, K.S.; Thompson, D.C.; Vasiliou, V. Aldehyde Dehydrogenase 1B1 as a Modulator of Pancreatic Adenocarcinoma. *Pancreas* **2016**, *45*, 117–122. [\[CrossRef\]](#)
68. Chen, Y.; Orlicky, D.J.; Matsumoto, A.; Singh, S.; Thompson, D.C.; Vasiliou, V. Aldehyde Dehydrogenase 1B1 (ALDH1B1) Is a Potential Biomarker for Human Colon Cancer. *Biochem. Biophys. Res. Commun.* **2011**, *405*, 173–179. [\[CrossRef\]](#)
69. Moradpoor, R.; Gharebaghian, A.; Shahi, F.; Mousavi, A.; Salari, S.; Akbari, M.E.; Ajdari, S.; Salimi, M. Identification and Validation of Stage-Associated PBMC Biomarkers in Breast Cancer Using MS-Based Proteomics. *Front. Oncol.* **2020**, *10*, 1101. [\[CrossRef\]](#)
70. Kobayashi, M.; Nagashio, R.; Jiang, S.X.; Saito, K.; Tsuchiya, B.; Ryuge, S.; Katono, K.; Nakashima, H.; Fukuda, E.; Goshima, N.; et al. Calnexin Is a Novel Sero-Diagnostic Marker for Lung Cancer. *Lung Cancer (Amst. Neth.)* **2015**, *90*, 342–345. [\[CrossRef\]](#)
71. Peng, N.; Miao, Z.; Wang, L.; Liu, B.; Wang, G.; Guo, X. MiR-378 Promotes the Cell Proliferation of Osteosarcoma through down-Regulating the Expression of Kruppel-like Factor 9. *Biochem. Cell Biol.* **2018**, *96*, 515–521. [\[CrossRef\]](#)
72. Tan, D.; Zhou, C.; Han, S.; Hou, X.; Kang, S.; Zhang, Y. MicroRNA-378 Enhances Migration and Invasion in Cervical Cancer by Directly Targeting Autophagy-Related Protein 12. *Mol. Med. Rep.* **2018**, *17*, 6319–6326. [\[CrossRef\]](#)
73. Wang, Y.; Gao, X.; Wei, F.; Zhang, X.; Yu, J.; Zhao, H.; Sun, Q.; Yan, F.; Yan, C.; Li, H.; et al. Diagnostic and Prognostic Value of Circulating MiR-21 for Cancer: A Systematic Review and Meta-Analysis. *Gene* **2014**, *533*, 389–397. [\[CrossRef\]](#)
74. Long, K.; Dong, Q.; Zeng, W. The Clinical Significance of MicroRNA-409 in Pancreatic Carcinoma and Associated Tumor Cellular Functions. *Bioengineered* **2021**, *12*, 4633–4642. [\[CrossRef\]](#)
75. Zheng, H.; Ding, B.; Xue, K.; Yu, J.; Lou, W. Construction of a LncRNA/Pseudogene-Hsa-MiR-30d-5p-GJA1 Regulatory Network Related to Metastasis of Pancreatic Cancer. *Genomics* **2021**, *113*, 1742–1753. [\[CrossRef\]](#)
76. Aita, A.; Millino, C.; Sperti, C.; Pacchioni, B.; Plebani, M.; Pittà, C.D.; Basso, D. Serum MiRNA Profiling for Early PDAC Diagnosis and Prognosis: A Retrospective Study. *Biomedicines* **2021**, *9*, 845. [\[CrossRef\]](#)
77. Kim, S.; Bae, W.J.; Ahn, J.M.; Heo, J.-H.; Kim, K.-M.; Choi, K.W.; Sung, C.O.; Lee, D. MicroRNA Signatures Associated with Lymph Node Metastasis in Intramucosal Gastric Cancer. *Mod. Pathol.* **2021**, *34*, 672–683. [\[CrossRef\]](#)

78. Francone, E.; Gentili, S.; Santori, G.; Stabilini, C.; Fornaro, R.; Frascio, M. MicroRNAs Differential Expression Profile in Metastatic Colorectal Cancer: A Pilot Study with Literature Review. *Surg. Oncol.* **2021**, *37*, 101524. [[CrossRef](#)]
79. Althubiti, M.; Lezina, L.; Carrera, S.; Jukes-Jones, R.; Giblett, S.M.; Antonov, A.; Barlev, N.; Saldanha, G.S.; Pritchard, C.A.; Cain, K.; et al. Characterization of Novel Markers of Senescence and Their Prognostic Potential in Cancer. *Cell Death Dis.* **2014**, *5*, e1528. [[CrossRef](#)]
80. Nip, H.; Dar, A.A.; Saini, S.; Colden, M.; Varahram, S.; Chowdhary, H.; Yamamura, S.; Mitsui, Y.; Tanaka, Y.; Kato, T.; et al. Oncogenic MicroRNA-4534 Regulates PTEN Pathway in Prostate Cancer. *Oncotarget* **2016**, *7*, 68371–68384. [[CrossRef](#)]
81. Lee, Y.G.; Kim, I.; Oh, S.; Shin, D.Y.; Koh, Y.; Lee, K.W. Small RNA Sequencing Profiles of Mir-181 and Mir-221, the Most Relevant MicroRNAs in Acute Myeloid Leukemia. *Korean J. Intern. Med.* **2019**, *34*, 178–183. [[CrossRef](#)]
82. Li, Z.; Tao, Y.; Wang, X.; Jiang, P.; Li, J.; Peng, M.; Zhang, X.; Chen, K.; Liu, H.; Zhen, P.; et al. Tumor-Secreted Exosomal MiR-222 Promotes Tumor Progression via Regulating P27 Expression and Re-Localization in Pancreatic Cancer. *Cell. Physiol. Biochem.* **2018**, *51*, 610–629. [[CrossRef](#)]
83. Lee, C.; He, H.; Jiang, Y.; Di, Y.; Yang, F.; Li, J.; Jin, C.; Fu, D. Elevated Expression of Tumor MiR-222 in Pancreatic Cancer Is Associated with Ki67 and Poor Prognosis. *Med. Oncol.* **2013**, *30*, 700. [[CrossRef](#)]
84. Greither, T.; Grochola, L.F.; Udelnow, A.; Lautenschläger, C.; Würfl, P.; Taubert, H. Elevated Expression of MicroRNAs 155, 203, 210 and 222 in Pancreatic Tumors Is Associated with Poorer Survival. *Int. J. Cancer* **2010**, *126*, 73–80. [[CrossRef](#)]
85. Park, J.Y.; Helm, J.; Coppola, D.; Kim, D.; Malafa, M.; Kim, S.J. MicroRNAs in Pancreatic Ductal Adenocarcinoma. *World J. Gastroenterol.* **2011**, *17*, 817–827. [[CrossRef](#)]

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## Article

# Alteration in Levels of Specific miRNAs and Their Potential Protein Targets between Human Pancreatic Cancer Samples, Adjacent Normal Tissue, and Xenografts Derived from These Tumors

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**Abstract:** Herein, we describe the global comparison of miRNAs in human pancreatic cancer tumors, adjacent normal tissue, and matched patient-derived xenograft models using microarray screening. RNA was extracted from seven tumor, five adjacent normal, and eight FI PDX tumor samples and analyzed by Affymetrix GeneChip miRNA 4.0 array. A transcriptome analysis console (TAC) was used to generate comparative lists of up- and downregulated miRNAs for the comparisons, tumor vs. normal and F1 PDX vs. tumor. Particular attention was paid to miRNAs that were changed in the same direction in both comparisons. We identified the involvement in pancreatic tumor tissue of several miRNAs, including miR4534, miR3154, and miR4742, not previously highlighted as being involved in this type of cancer. Investigation in the parallel mRNA and protein lists from the same samples allowed the elimination of proteins where altered expression correlated with corresponding mRNA levels and was thus less likely to be miRNA regulated. Using the remaining differential expression protein lists for proteins predicted to be targeted for differentially expressed miRNA on our list, we were able to tentatively ascribe specific protein changes to individual miRNA. Particularly interesting target proteins for miRs 615-3p, 2467-3p, 4742-5p, 509-5p, and 605-3p were identified. Prominent among the protein targets are enzymes involved in aldehyde metabolism and membrane transport and trafficking. These results may help to uncover vulnerabilities that could enable novel approaches to treating pancreatic cancer.

**Keywords:** pancreatic cancer; miRNA; PDX; tumour

## 1. Introduction

Survival rates are very poor in pancreatic cancer, owing in part to the early disease being frequently asymptomatic. Diagnosis frequently occurs at an advanced stage when the tumor has invaded locally and often metastasized. While the majority of pancreatic adenocarcinomas (PDAC) are K-ras mutants [1], and there is as yet no clinically demonstrated way of selectively targeting it without also inhibiting wild-type Ras, which is essential for the survival of normal cells [2]. However, recent promising results with the covalent KRAS-G12C inhibitors sotorasib and adagrasib in the treatment of non-small cell lung

cancer patients that harbor the mutation [3]. Many other mutations in proteins of signaling pathways have been identified in a proportion of pancreatic cancers, but to date, targeted therapies of the type that are so effective in, for example, molecularly defined subsets of breast cancer have not been defined in pancreatic cancer. A complex microenvironment with molecular crosstalk, for example, between stellate cells and cancer cells, complicates attempts to understand and treat pancreatic cancer [4]. There has been no fundamental advance in the treatment of pancreatic cancer in the past 20 years, although better medical management of side effects and improvements in surgery, pain control, and improved management of palliative quality of life indicators are all very important. However, a significant improvement in outcomes is still lacking [5]. This somewhat gloomy background underlines the need for a better understanding of the biology of pancreatic cancer in the hope of discovering new vulnerabilities that can be exploited in new treatments [6].

MicroRNAs are short, non-coding RNAs, typically of 20–24 nucleotides in length, that can be responsible for the regulation of biological pathways in cells. Since their discovery in the nematode *C. elegans* in 1993, these RNAs have received considerable attention due to their roles in diverse biological functions, including the regulation of cell development, differentiation, and survival. Approximately one-third of all human genes are thought to be regulated by miRNAs, with approximately 2500 miRNAs identified to date [7]. Dysregulation of miRNA profiles has been found to be associated with the development of various human cancers. In pancreatic cancer specifically, there has been a significant increase in interest in the expression of miRNAs and their possible roles as diagnostic or prognostic markers [8–14]. Some of these studies have highlighted particular miRNAs as being of particular interest, including miR21, miR217, and miR222. Furthermore, there has been an interest in the targeting of these miRNAs with antimirs as potential targets, although this has so far been met with limited success [15–17]. The stability of miRNAs in bodily fluids such as plasma or serum and tissue samples is a major advantage for their use as potential biomarkers, but lack of specificity may be a limitation [18].

In the work detailed in this paper, we have applied a multilevel approach looking at datasets from proteomic [19], mRNA microarray [20] or and miRNA microarray analysis from the same tumor-adjacent normal tissue (5 samples), tumor samples (7 samples) and F1 patient-derived xenograft (F1) tumor (8 samples) material cohorts. Applying this multilevel approach has allowed us to overcome one drawback often associated with miRNA studies. We have been able to identify effects on predicted targets of miRNAs using both mRNA and proteomic expression and have not had to rely on computational algorithms.

## 2. Materials and Methods

### 2.1. Acquisition and Generation of Patient-Derived Xenograft Models

Pancreatic tumor sample acquisition and generation of PDX models were described by our group [20]. Briefly, pancreatic tumor tissue (T) and adjacent normal tissue (N) were obtained from patients undergoing pancreatic ductal adenocarcinoma (PDAC) surgical resection at St. Vincent's University Hospital Dublin. After initial macroscopic pathological confirmation, the material remaining after diagnostic sampling was cold transferred to DCU. The collection of patient material was approved by St Vincent's University Hospital and Dublin City University (DCU) Research Ethics Committee. All animal work received ethical approval from the DCU Research Ethics Committee (DCUREC/2012/202) and was licensed by the Irish Department of Health (license number B100-4501). Severe combined immunodeficiency (SCID), CB17/lcr-Prkdc<sup>scid</sup>/lcrCrl mice (Charles River, U.K.) were implanted subcutaneously with fresh patient tumor material. Mice were monitored for tumor development for up to 1 year post-implantation. Animal welfare monitoring criteria included tumor volume, tumor axis, body weight, and condition. The tumor volume and tumor axis limits were set as <2000 mm<sup>3</sup> and <20 mm, respectively. Following the humane euthanasia of the mouse, the tumor was divided for cryopreservation, formalin-fixed paraffin embedding (FFPE), and snap frozen (F1). For snap freezing, a piece of tumor was

minced with a scalpel, placed in a cryovial, and placed directly into liquid nitrogen (LN<sub>2</sub>), then stored at −80 °C.

## 2.2. Proteomics Analysis

Proteomic analysis of PDAC tumors and normal tissue was previously described by our group [19]. Briefly, tissue samples were enriched for membrane proteins using MemPER plus membrane protein extraction kit as per the manufacturer's instructions (Thermo Fisher, Hemel Hempstead, UK). Two subcellular fractions were generated, cytosolic and solubilized membrane/membrane-associated proteins. Nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) was carried out on a Dionex Ultimate 3000 nano-RSLC (Thermo Scientific, Hemel Hempstead, UK) coupled in-line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). The LC-MS/MS parameters were previously [21] described using a 140 min separation gradient per sample. Protein identification was carried out using Proteome Discoverer Version 2.2 software (Thermo Scientific) with the SEQUEST HT algorithm. Raw MS files were searched against the UniProt/SwissProt human protein database (downloaded on 29 August 2018, containing 20,325 sequences). The following criteria were applied: (1) precursor mass tolerance set to 10 ppm; (2) fragment mass tolerance set to 0.6 Da; (3) oxidation of methionine set as a dynamic modification; (4) carbamidomethylation of cysteine set as a static modification; and (5) a maximum of 2 missed cleavage sites were allowed. False-discovery rates were applied to peptide-spectrum matches using Percolator, and only those with a false-discovery rate of less than 1% were allowed. In the case of F1 samples, identified proteins could be of human or murine origin. From scrutiny of the peptides used to identify the proteins, we can divide the detected proteins into 3 categories: (i) human not murine, (ii) murine not human, (iii) could be murine or human. To simplify the analysis, we have confined our list to the first category—protein lists presented here are human not murine.

## 2.3. mRNA and miRNA Microarray Analysis

Sample preparation and mRNA microarray analysis was as previously described [20]. Briefly snap frozen samples of F1 tumors, original patient tumor material, and adjacent normal tissue were prepared for RNA extraction by grinding samples under LN<sub>2</sub> using a mortar and pestle. The tissue sample was placed in a metal mortar with a small volume of LN<sub>2</sub> and ground quickly. When powdered, the powder was transferred to a clean microcentrifuge tube. This was performed before the LN<sub>2</sub> completely evaporated and while the powder was still frozen. Once homogenized, RNA samples were prepared using Trizol (Sigma, Merck KGaA, Darmstadt, Germany), according to the manufacturer's guidelines. Samples were quantified using a Nanodrop (Thermo Scientific), and quality was determined using Agilent Bioanalyser (Agilent, Santa Clara, Ca, USA). Samples with RIN numbers greater than 8 were accepted as most suitable for microarray analysis.

Briefly, the preparation of cRNA, hybridization, and scanning of microarrays was performed according to the manufacturer's protocol (Affymetrix, Thermo-Fischer, Santa Clara, CA, USA). A total of 300 ng of total RNA extracted from homogenized patient samples isolated using Trizol was converted into double-stranded cDNA by reverse transcription. Biotin-labeled cRNA was generated by converting the cDNA sample using the Genechip WT plus reagent kit (Affymetrix). Labeled cRNA was hybridized to the Affymetrix GeneChip<sup>®</sup> Human Gene 2.0 ST Array while rotating at 60 rpm for 16 h at 45 °C. After hybridization, the microarray was washed using the Affymetrix Fluidics Station according to the manufacturer's protocol. The chips were scanned in an Affymetrix 3000 7G scanner.

miRNA arrays were performed according to the manufacturer's protocol (Affymetrix). A total of 1000 ng of total RNA was extracted from homogenized patient samples and isolated using Trizol. Following this, samples underwent Poly(A) tailing followed by flash-tag biotin HSR ligation. Labeled samples were hybridized to the Affymetrix GeneChip<sup>®</sup> miRNA 4.0 Array while rotating at 60 rpm for 16–18 h at 48 °C. After hybridization, the mi-

croarray was washed using the Affymetrix Fluidics Station according to the manufacturer's protocol. The chips were scanned in an Affymetrix 3000 7G scanner.

#### 2.4. Bioinformatic Analysis

Differential gene analysis expression was carried out using Applied Biosystems Transcriptome Analysis Console (TAC) software 4.0.2. The software was integrated with the established LIMMA package, which allowed for improved inference at both the gene and gene set levels in small experiments. Resulting gene signal values are expressed as biweight robust averages shown on the log<sub>2</sub> scale. Fold change values are calculated from the log<sub>2</sub> signal values. Resulting gene lists were filtered for  $\pm 1.5$  fold changes, a  $p$ -value  $< 0.05$  and an overall FDR F-Test:  $< 0.005$ . The miRNA 4.0 chip comprises of all mature miRNA sequences in the miRBase release 20, including miRNA transcripts from humans, mice, and rats. As they are suitable for multi-species analysis, an additional filter of transcript ID containing only hsa was also included.

The miRNA and mRNA cel files are deposited on the GEO repository, submission GSE141873 (mRNA) and GSE207345 (miRNA).

miRwalk was used for predicted protein identification [22]. Filters applied to the results were a binding probability value of 0.95, a binding site position of 3-UTR, and a validated interaction with an miRTarbase entry.

### 3. Results

Bioinformatics analysis of the miRNA data from the tumor (T), adjacent normal (N), and patient-derived xenograft tumors (F1) identified 100 differentially expressed (DE) miRNA (87 upregulated and 13 downregulated in the tumor sample) in the tumor versus normal tissue samples (Supplementary Table S1). In the F1 vs. tumor comparison, 214 DE miRNA were identified (67 upregulated and 147 downregulated in the F1 samples (Supplementary Table S2). Identifications were based on a  $p$ -value  $< 0.05$  and a fold change of  $\pm 1.5$ . A fold change of  $\pm 1.5$  was selected as previous profiling studies have shown that even subtle changes in miRNA expression can have an impact on cell biology [23]. In comparisons where a very large fold change is recorded, it generally flags that the expression level is very low in one of the conditions rather than being a strictly accurate assessment of the ratio of the number of molecules present in each condition. These particular arrays (miRNA 4.0 array) have transcripts relating to an extensive range of species as well as humans, including mice and rats. There are a total of 4603 miRNA transcripts represented on the arrays that are determined to be expressed in humans. Due to the size and homogeneity of the miRNA sequences, it is not possible to say with certainty in the case of the F1 miRNA whether any particular miRNA is of human or mouse origin.

There are many ways in which the data could be presented and in which the three profile data strands could be analyzed. In this paper, we focus on (1) the changes of greatest magnitude up and down in the two comparisons (Tables 1 and 2), (2) miRNAs where expression changed in the same direction in both comparisons (Table 3), and (3) changes in expression of individual proteins, which could be explained by our miRNA data (Tables 4–6).

#### 3.1. Examination of miRNA Expression Changes in Pancreatic Cancer Tumor Samples and Adjacent Normal Tissue

Table 1 shows the top 10 up- and downregulated miRNAs in the comparison of the patient tumor samples and adjacent normal tissue. Figure 1 shows a visual representation of each of the miRNA expressions in all three conditions within this study.

**Table 1.** Priority miRNA from miRNA array analysis with corresponding fold changes and *p*-values for TvN comparison.

| Transcript ID (Array Design) | TvN         |                       |
|------------------------------|-------------|-----------------------|
|                              | Fold Change | <i>p</i> -Values      |
| hsa-miR-21-3p                | 12.89       | $1.20 \times 10^{-3}$ |
| hsa-miR-708-5p               | 9.15        | $6.70 \times 10^{-3}$ |
| hsa-miR-181c-5p              | 8.52        | $3.00 \times 10^{-3}$ |
| hsa-miR-125b-1-3p            | 6.95        | $3.18 \times 10^{-2}$ |
| hsa-miR-21-5p                | 6.89        | $1.58 \times 10^{-2}$ |
| hsa-miR-331-5p               | 6.81        | $3.60 \times 10^{-3}$ |
| hsa-miR-210-3p               | 6.73        | $6.60 \times 10^{-3}$ |
| hsa-miR-181d-5p              | 6.51        | $2.28 \times 10^{-2}$ |
| hsa-miR-214-5p               | 6.3         | $1.02 \times 10^{-2}$ |
| hsa-miR-143-5p               | 5.55        | $2.00 \times 10^{-4}$ |
| hsa-mir-375                  | −1.67       | $3.16 \times 10^{-2}$ |
| hsa-miR-3618                 | −1.76       | $4.70 \times 10^{-2}$ |
| hsa-miR-509-5p               | −1.85       | $1.30 \times 10^{-3}$ |
| hsa-mir-6722                 | −2          | $4.62 \times 10^{-2}$ |
| hsa-miR-4742-5p              | −2.06       | $2.15 \times 10^{-2}$ |
| hsa-mir-139                  | −2.2        | $4.92 \times 10^{-2}$ |
| hsa-miR-451a                 | −2.93       | $3.50 \times 10^{-3}$ |
| hsa-miR-139-5p               | −4.51       | $1.20 \times 10^{-3}$ |
| hsa-miR-486-5p               | −4.65       | $1.33 \times 10^{-2}$ |
| hsa-miR-148a-5p              | −8.15       | $4.08 \times 10^{-2}$ |

**Table 2.** Priority miRNA from miRNA array analysis with corresponding fold changes and *p*-values for F1vT comparison.

| Transcript ID (Array Design) | F1vT        |                       |
|------------------------------|-------------|-----------------------|
|                              | Fold Change | <i>p</i> -Values      |
| hsa-miR-206                  | 26.01       | $8.59 \times 10^{-5}$ |
| hsa-miR-4521                 | 24.27       | $4.48 \times 10^{-6}$ |
| hsa-miR-6872-5p              | 21.19       | $1.35 \times 10^{-7}$ |
| hsa-miR-1290                 | 15.4        | $6.80 \times 10^{-3}$ |
| hsa-miR-486-5p               | 12.48       | $1.40 \times 10^{-3}$ |
| hsa-miR-615-3p               | 10.81       | $5.59 \times 10^{-5}$ |
| hsa-miR-203a                 | 9.43        | $2.30 \times 10^{-3}$ |
| hsa-miR-6778-5p              | 8.56        | $5.42 \times 10^{-8}$ |
| hsa-miR-2467-3p              | 7.91        | $2.01 \times 10^{-8}$ |
| hsa-miR-139-5p               | 6.7         | $2.78 \times 10^{-6}$ |
| hsa-miR-409-3p               | −11.45      | $1.47 \times 10^{-6}$ |
| hsa-miR-146b-3p              | −12.7       | $6.39 \times 10^{-6}$ |
| hsa-miR-217                  | −13.39      | $1.98 \times 10^{-2}$ |
| hsa-miR-503-5p               | −14.24      | $2.30 \times 10^{-3}$ |
| hsa-miR-1271-5p              | −17.01      | $1.88 \times 10^{-6}$ |
| hsa-miR-125b-2-3p            | −21.33      | $6.58 \times 10^{-7}$ |
| hsa-miR-708-5p               | −21.66      | $2.00 \times 10^{-3}$ |
| hsa-miR-487b-3p              | −34.33      | $1.31 \times 10^{-9}$ |
| hsa-miR-424-3p               | −56.66      | $2.27 \times 10^{-6}$ |
| hsa-miR-432-5p               | −113.44     | $2.10 \times 10^{13}$ |

**Table 3.** Priority miRNA from miRNA array analysis with corresponding fold changes and *p*-values for shown to be differentially expressed in both comparisons in the same direction.

| Transcript ID<br>(Array Design) | TvN         |                       | F1vT        |                       |
|---------------------------------|-------------|-----------------------|-------------|-----------------------|
|                                 | Fold Change | <i>p</i> -Val         | Fold Change | <i>p</i> -Values      |
| hsa-miR-6831-5p                 | 4.82        | $2.16 \times 10^{-2}$ | 1.81        | $1.32 \times 10^{-2}$ |
| hsa-miR-222-3p                  | 1.99        | $7.10 \times 10^{-3}$ | 2.27        | $2.41 \times 10^{-2}$ |
| hsa-miR-4534                    | 1.92        | $4.76 \times 10^{-2}$ | 1.83        | $3.49 \times 10^{-2}$ |
| hsa-miR-4743-5p                 | 1.87        | $1.12 \times 10^{-2}$ | 1.94        | $2.20 \times 10^{-3}$ |
| hsa-miR-3154                    | 1.86        | $3.12 \times 10^{-2}$ | 5.14        | $1.60 \times 10^{-5}$ |
| hsa-miR-3935                    | 1.85        | $2.99 \times 10^{-2}$ | 2.5         | $1.59 \times 10^{-2}$ |

### 3.2. Examination of miRNA Expression Changes in Pancreatic Cancer Tumor Samples and Patient-Derived Xenograft Models

Table 2 shows the top 10 up- and downregulated miRNAs in the comparison of the patient tumor samples and adjacent normal tissue. Figure 2 shows a visual representation of each of the miRNA expressions in all three conditions within this study.

### 3.3. Examination of miRNA Expression Changes in Pancreatic Cancer, Adjacent Normal Tissue, and Patient-Derived Xenograft Models of Pancreatic Cancer

Table 3 shows the priority miRNA that was shown to be differentially expressed in both comparisons in the same direction. Figure 3 show a visual representation of each of the miRNA expression in all three conditions within this study. While the comparisons TvN and F1vT are clearly interesting in their own right, we have also searched for changes that happen in both comparisons but in the same direction. These may be of special interest because the F1 tumor may be due to cell selection following transplantation, have a higher proportion of tumor cells than the original tumor sample (a mixture of normal and tumor cells), and our proteomics analysis identifies only human and not murine proteins. Of course, other phenomena are also involved in these transitions; for example, major changes in the cellular composition of the stroma.

### 3.4. Investigation of Predicted miRNA Target Proteins in Proteomic and Transcription Datasets

Since miRNAs target multiple mRNAs and proteins, we hypothesized that these DE expression lists might lead us to pathways as well as individual proteins of functional, diagnostic, or therapeutic significance. We are particularly well placed to investigate this network in detail in this study as we have performed global mRNA [20] and proteomic [19,24] profiling on the same clinical and F1 samples. Protein changes that do not have a corresponding same direction mRNA change could be strong candidates for regulation by miRNAs. Histological examples, as well as clinical descriptions of the samples, were previously described previously [20].

Table 4 (TvN), Table 5 (F1vT), and Table 6 (DE in the same direction in both) list predicted protein targets of specific miRNAs, which, as well as being predicted, were also found in the proteomic dataset to be different from the expressed in the appropriate direction (i.e., inverse to the direction of difference of expression of the miRNA). Note that only proteins for which the mass spectrometry data indicated definitively human, and not murine, origin are included in our analysis to avoid confusion in the interpretation of the F1 proteomic data.

The predicted protein list was generated from miRWalk, which was filtered to only include validated interactions with an entry on miRTarbase [22].

Of the 39 miRNAs investigated from the priority list, 7 miRNAs are enriched in the list by targeting multiple proteins. miRs 2467, 4742, 509, 615-3p, 4534, 222, and 206 are all shown to target two or more proteins across the two comparisons.

**Table 4.** Priority miRNA fold changes and *p*-values and corresponding predicted protein target expression identified in LC-MS/MS dataset with *p*-value for the TvN comparison.

| Transcript ID<br>(Array Design) | TvN   |                       | F1vT |                  | Gene<br>Symbol | TvN (Membrane) |                       | TvN (Cytosolic) |                       | F1vT (Membrane) |                       | F1vT (Cytocolic) |                       |
|---------------------------------|-------|-----------------------|------|------------------|----------------|----------------|-----------------------|-----------------|-----------------------|-----------------|-----------------------|------------------|-----------------------|
|                                 | FC    | <i>p</i> -Val         | FC   | <i>p</i> -Values |                | FC             | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC               | <i>p</i> -Values      |
| hsa-miR-143-5p                  | 5.55  | $2.00 \times 10^{-4}$ |      |                  | GSTM3          | −2.04          | $6.48 \times 10^{-3}$ |                 |                       |                 |                       | −15.16           | $6.46 \times 10^{-3}$ |
| hsa-miR-181c-5p                 | 8.52  | $3.00 \times 10^{-3}$ |      |                  | PEBP1          | −3.20          | $1.19 \times 10^{-2}$ | −2.90           | $1.33 \times 10^{-3}$ | −2.10           | $1.04 \times 10^{-2}$ | −3.50            | $3.77 \times 10^{-5}$ |
| hsa-miR-181d-5p                 | 6.51  | $2.28 \times 10^{-2}$ |      |                  | PEBP1          | −3.20          | $1.19 \times 10^{-2}$ | −2.90           | $1.33 \times 10^{-3}$ | −2.10           | $1.04 \times 10^{-2}$ | −3.50            | $3.77 \times 10^{-5}$ |
| hsa-miR-210-3p                  | 6.73  | $6.60 \times 10^{-3}$ |      |                  | ERP27          | −21.19         | $9.29 \times 10^{-4}$ |                 |                       |                 |                       |                  |                       |
| hsa-miR-214-5p                  | 6.3   | $1.02 \times 10^{-2}$ |      |                  | TBL2           |                |                       | −13.95          | $4.01 \times 10^{-3}$ |                 |                       |                  |                       |
| hsa-miR-21-5p                   | 6.89  | $1.58 \times 10^{-2}$ |      |                  | DDAH1          | −2.13          | $1.92 \times 10^{-3}$ |                 |                       |                 |                       | 4.16             | $3.84 \times 10^{-4}$ |
| hsa-miR-331-5p                  | 6.81  | $3.60 \times 10^{-3}$ |      |                  | DDAH1          | −2.13          | $1.92 \times 10^{-3}$ |                 |                       |                 |                       | 4.16             | $3.84 \times 10^{-4}$ |
| hsa-miR-4742-5p                 | −2.06 | $2.15 \times 10^{-2}$ |      |                  | CSRP1          | 3.11           | $1.39 \times 10^{-3}$ | 2.34            | $1.31 \times 10^{-2}$ |                 |                       |                  |                       |
| hsa-miR-4742-5p                 | −2.06 | $2.15 \times 10^{-2}$ |      |                  | S100A16        | 4.20           | $1.12 \times 10^{-2}$ |                 |                       | 3.57            | $8.51 \times 10^{-4}$ |                  |                       |
| hsa-miR-4742-5p                 | −2.06 | $2.15 \times 10^{-2}$ |      |                  | SOD2           | 2.29           | $5.88 \times 10^{-3}$ |                 |                       | −4.84           | $5.06 \times 10^{-6}$ | −22.02           | $2.29 \times 10^{-9}$ |
| hsa-miR-509-5p                  | −1.85 | $1.30 \times 10^{-3}$ |      |                  | COTL1          | 2.23           | $3.83 \times 10^{-2}$ |                 |                       |                 |                       | 8.77             | $1.61 \times 10^{-4}$ |
| hsa-miR-509-5p                  | −1.85 | $1.30 \times 10^{-3}$ |      |                  | LRPAP1         | 2.21           | $3.10 \times 10^{-3}$ |                 |                       | −2.37           | $1.53 \times 10^{-3}$ |                  |                       |
| hsa-miR-509-5p                  | −1.85 | $1.30 \times 10^{-3}$ |      |                  | SLC4A2         | 6.03           | $3.11 \times 10^{-3}$ |                 |                       | −1.98           | $5.14 \times 10^{-3}$ |                  |                       |

List of predicted target proteins for priority miRNAs in Table 1 list, which also showed inverse DE with the miRNA in the proteomics analysis.

**Table 5.** Priority miRNA fold changes and *p*-values and corresponding predicted protein target expression identified in LC-MS/MS dataset with *p*-value for the F1vT comparison.

| Transcript ID<br>(Array Design) | TvN |                  | F1vT  |                       | Gene<br>Symbol | TvN (Membrane) |                       | TvN (Cytosolic) |                       | F1vT (Membrane) |                       | F1vT (Cytocolic) |                       |
|---------------------------------|-----|------------------|-------|-----------------------|----------------|----------------|-----------------------|-----------------|-----------------------|-----------------|-----------------------|------------------|-----------------------|
|                                 | FC  | <i>p</i> -Values | FC    | <i>p</i> -Values      |                | FC             | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC               | <i>p</i> -Values      |
| hsa-miR-125b-2-3p               |     |                  | −21.3 | $6.58 \times 10^{-7}$ | SUGT1          |                |                       |                 |                       |                 |                       | 3.19             | $9.56 \times 10^{-4}$ |
| hsa-miR-206                     |     |                  | 26.01 | $8.59 \times 10^{-5}$ | PPIB           |                |                       | −3.55           | $5.45 \times 10^{-3}$ | −1.94           | $2.55 \times 10^{-2}$ | −3.30            | $1.64 \times 10^{-2}$ |
| hsa-miR-206                     |     |                  | 26.01 | $8.59 \times 10^{-5}$ | SLC25A22       | −2.77          | $1.19 \times 10^{-2}$ |                 |                       |                 |                       |                  |                       |
| hsa-miR-615-3p                  |     |                  | 10.81 | $5.59 \times 10^{-5}$ | DPP3           | 2.53           | $2.66 \times 10^{-3}$ |                 |                       | −1.91           | $1.20 \times 10^{-2}$ | 2.25             | $3.86 \times 10^{-4}$ |
| hsa-miR-615-3p                  |     |                  | 10.81 | $5.59 \times 10^{-5}$ | GANAB          | 1.67           | $7.26 \times 10^{-3}$ |                 |                       | −2.04           | $2.12 \times 10^{-4}$ |                  |                       |
| hsa-miR-708-5p                  |     |                  | −21.7 | $2.00 \times 10^{-3}$ | RANBP2         |                |                       |                 |                       |                 |                       | 1.93             | $8.16 \times 10^{-3}$ |
| hsa-miR-2467-3p                 |     |                  | 7.91  | $2.01 \times 10^{-8}$ | AKR7A2         |                |                       | −2.09           | $1.56 \times 10^{-3}$ | −1.81           | $1.01 \times 10^{-2}$ |                  |                       |
| hsa-miR-2467-3p                 |     |                  | 7.91  | $2.01 \times 10^{-8}$ | ATP1B3         | 1.88           | $3.03 \times 10^{-2}$ |                 |                       | −2.26           | $1.59 \times 10^{-2}$ |                  |                       |
| hsa-miR-2467-3p                 |     |                  | 7.91  | $2.01 \times 10^{-8}$ | CPM            |                |                       |                 |                       | −5.55           | $1.31 \times 10^{-2}$ |                  |                       |

Table 5. Cont.

| Transcript ID<br>(Array Design) | TvN |                  | F1vT |                       | Gene<br>Symbol | TvN (Membrane) |                       | TvN (Cytosolic) |                       | F1vT (Membrane) |                       | F1vT (Cytocolic) |                  |
|---------------------------------|-----|------------------|------|-----------------------|----------------|----------------|-----------------------|-----------------|-----------------------|-----------------|-----------------------|------------------|------------------|
|                                 | FC  | <i>p</i> -Values | FC   | <i>p</i> -Values      |                | FC             | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC              | <i>p</i> -Values      | FC               | <i>p</i> -Values |
| hsa-miR-2467-3p                 |     |                  | 7.91 | $2.01 \times 10^{-8}$ | FKBP15         |                |                       |                 |                       | −4.64           | $5.12 \times 10^{-3}$ |                  |                  |
| hsa-miR-2467-3p                 |     |                  | 7.91 | $2.01 \times 10^{-8}$ | RAB1A          |                |                       | −3.33           | $4.87 \times 10^{-3}$ |                 |                       |                  |                  |
| hsa-miR-2467-3p                 |     |                  | 7.91 | $2.01 \times 10^{-8}$ | UGGT1          |                |                       |                 |                       | −1.80           | $1.91 \times 10^{-2}$ |                  |                  |
| hsa-miR-378e                    |     |                  | 4.2  | $1.10 \times 10^{-3}$ | TXNL1          |                |                       |                 |                       | −2.54           | $2.30 \times 10^{-3}$ |                  |                  |
| hsa-miR-6778-5p                 |     |                  | 8.56 | $5.42 \times 10^{-8}$ | ATP5F1A        | −2.19          | $9.76 \times 10^{-3}$ | −2.70           | $1.53 \times 10^{-3}$ |                 |                       |                  |                  |

List of predicted target proteins for priority miRNAs in Table 2 list, which also showed inverse DE with the miRNA in the proteomics analysis.

**Table 6.** Priority miRNA fold changes and *p*-values and corresponding predicted protein target expression identified in LC-MS/MS dataset with *p*-value for miRNA that were DE expressed in the same direction in both comparisons.

| Transcript ID<br>(Array Design) | TvN  |                       | F1vT |                       | Gene<br>Symbol | TvN (Membrane) |                       | TvN (Cytosolic) |                  | F1vT (Membrane) |                       | F1vT (Cytocolic) |                  |
|---------------------------------|------|-----------------------|------|-----------------------|----------------|----------------|-----------------------|-----------------|------------------|-----------------|-----------------------|------------------|------------------|
|                                 | FC   | <i>p</i> -Values      | FC   | <i>p</i> -Values      |                | FC             | <i>p</i> -Values      | FC              | <i>p</i> -Values | FC              | <i>p</i> -Val         | FC               | <i>p</i> -Values |
| hsa-miR-4534                    | 1.92 | $4.76 \times 10^{-2}$ | 1.83 | $3.49 \times 10^{-2}$ | CALR           |                |                       |                 |                  | −2.18           | $1.52 \times 10^{-4}$ |                  |                  |
| hsa-miR-4534                    | 1.92 | $4.76 \times 10^{-2}$ | 1.83 | $3.49 \times 10^{-2}$ | CAPZA1         |                |                       |                 |                  | −2.05           | $2.49 \times 10^{-3}$ |                  |                  |
| hsa-miR-4743-5p                 | 1.87 | $1.12 \times 10^{-2}$ | 1.94 | $2.20 \times 10^{-3}$ | CYP20A1        |                |                       |                 |                  | −2.50           | $6.46 \times 10^{-3}$ |                  |                  |
| hsa-miR-222-3p                  | 1.99 | $7.10 \times 10^{-3}$ | 2.27 | $2.41 \times 10^{-2}$ | GNAI3          |                |                       |                 |                  | −2.56           | $3.85 \times 10^{-4}$ |                  |                  |
| hsa-miR-3154                    | 1.86 | $3.12 \times 10^{-2}$ | 5.14 | $1.60 \times 10^{-5}$ | HSP90B1        | −6.63          | $4.34 \times 10^{-3}$ |                 |                  | −1.72           | $1.11 \times 10^{-2}$ | −2.71            |                  |
| hsa-miR-4534                    | 1.92 | $4.76 \times 10^{-2}$ | 1.83 | $3.49 \times 10^{-2}$ | OLA1           | −4.41          | $3.21 \times 10^{-4}$ |                 |                  |                 |                       |                  |                  |
| hsa-miR-6831-5p                 | 4.82 | $2.16 \times 10^{-2}$ | 1.81 | $1.32 \times 10^{-2}$ | PLD3           |                |                       |                 |                  | −3.55           | $1.13 \times 10^{-3}$ | −6.41            |                  |
| hsa-miR-222-3p                  | 1.99 | $7.10 \times 10^{-3}$ | 2.27 | $2.41 \times 10^{-2}$ | RECK           |                |                       |                 |                  | −12.65          | $9.53 \times 10^{-8}$ |                  |                  |
| hsa-miR-222-3p                  | 1.99 | $7.10 \times 10^{-3}$ | 2.27 | $2.41 \times 10^{-2}$ | SOD2           | 2.29           | $5.88 \times 10^{-3}$ |                 |                  | −4.84           | $5.06 \times 10^{-6}$ | −22.02           |                  |
| hsa-miR-222-3p                  | 1.99 | $7.10 \times 10^{-3}$ | 2.27 | $2.41 \times 10^{-2}$ | TOM1           |                |                       |                 |                  | −1.93           | $3.90 \times 10^{-2}$ |                  |                  |

List of predicted target proteins for priority miRNAs in Table 3 list, which also showed inverse DE with the miRNA in the proteomics analysis.

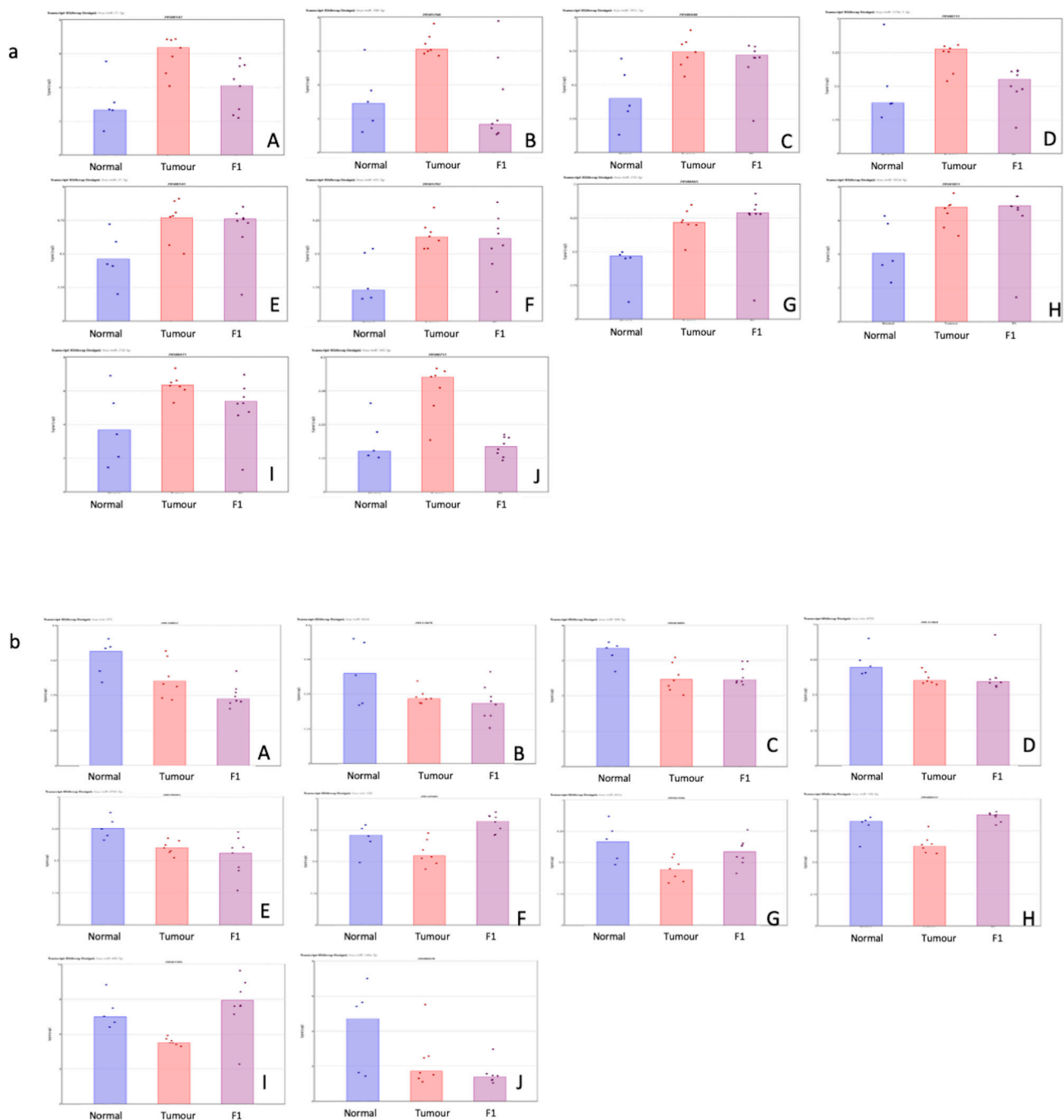


**Figure 1.** (a,b) Scatterplot representation of data from Table 1 (TvN comparison) to provide a visual representation of the interpatient variation of the expression of each miRNA in each of the 3 tissue cohorts. Column 1 represents adjacent normal tissue, column 2 represents the patient tumor material, and column 3 represents the F1 tissue. (a) Log2 expression scatterplots in normal, tumour and F1 conditions of miRNAs shown to be upregulated in TvN comparison (Table 1). (A) miR21-3p, (B) miR708-3p, (C) miR181c-5p, (D) miR125b-1-5p, (E) miR21-5p, (F) miR331-5p, (G) miR210-3p, (H) miR181d-5p, (I) miR214-5p and (J) miR143-5p. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA. (b) Log2 expression scatterplots in normal, tumour and F1 conditions of miRNAs shown to be downregulated in TvN comparison (Table 1). (A) mir375, (B) miR3618, (C) miR509-5p, (D) mir6722, (E) miR4742-5p, (F) mir139, (G) miR451a, (H) miR139-5p, (I) miR486-5p and (J) miR148a-5p. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA.

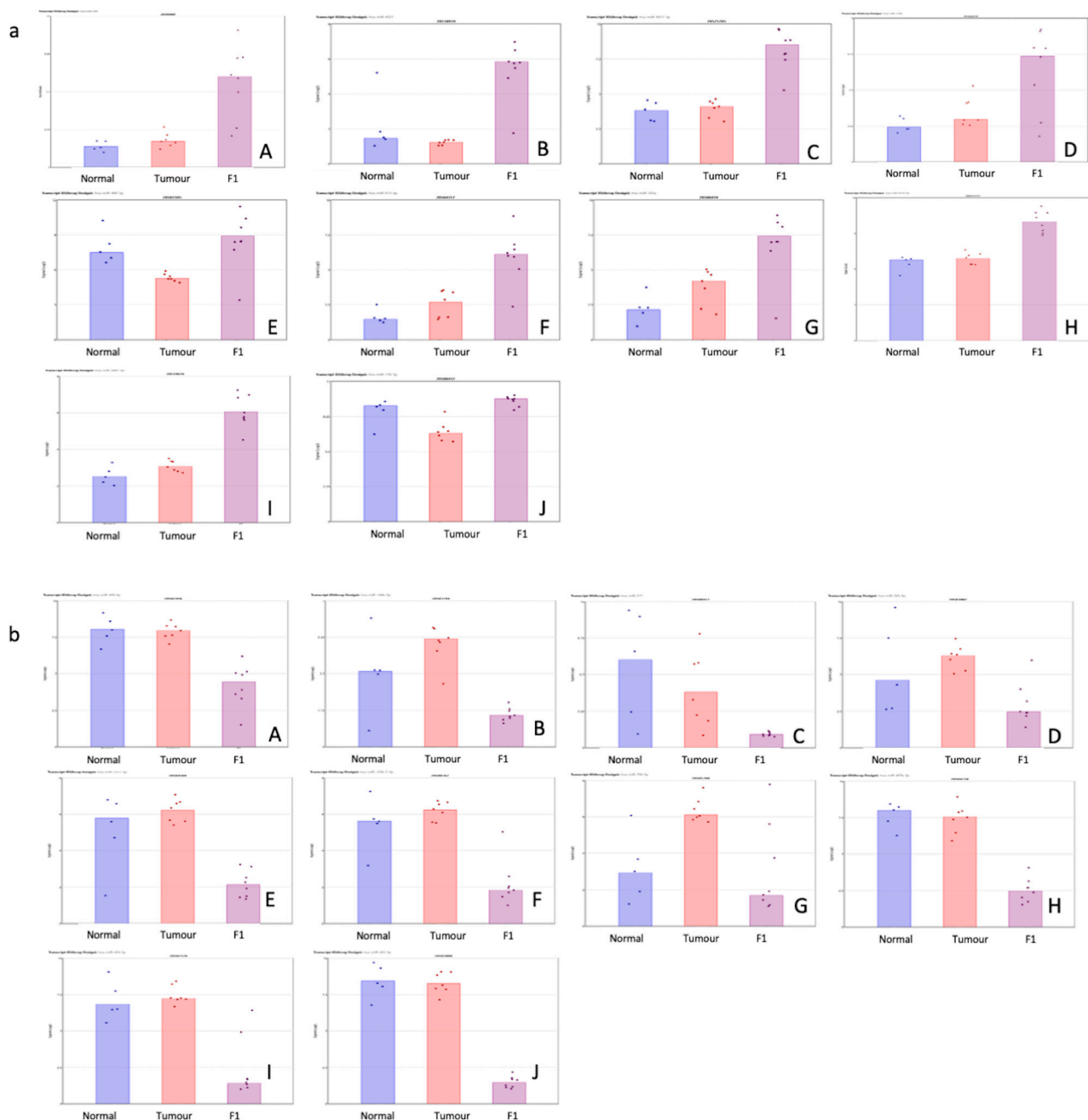


**Figure 2.** (a,b) Scatterplot representation of data from Table 1 (F1vT comparison) to provide a visual representation of the interpatient variation of the expression of each miRNA in each of the 3 tissue cohorts. Column 1 represents adjacent normal tissue, column 2 represents the patient tumor material, and column 3 represents the F1 tissue. (a) Log2 expression scatterplots in normal, tumour and F1 conditions of miRNAs shown to be upregulated in F1vT comparison (Table 2). (A) miR206, (B) miR4521, (C) miR6872-5p, (D) miR1290, (E) miR486-5p, (F) miR6153p, (G) miR203A, (H) miR6778-5p, (I) miR2467-3p and (J) miR139-5p. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA. (b) Log2 expression scatterplots in normal, tumour and F1 conditions of miRNAs shown to be downregulated in F1vT comparison (Table 2). (A) miR409-3p, (B) miR146b-3p, (C) miR217, (D) miR503-5p, (E) miR1271-5p, (F) miR125b-2-3p, (G) miR708-5p, (H) miR487-3p, (I) miR424-5p and (J) miR432-5p. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA.

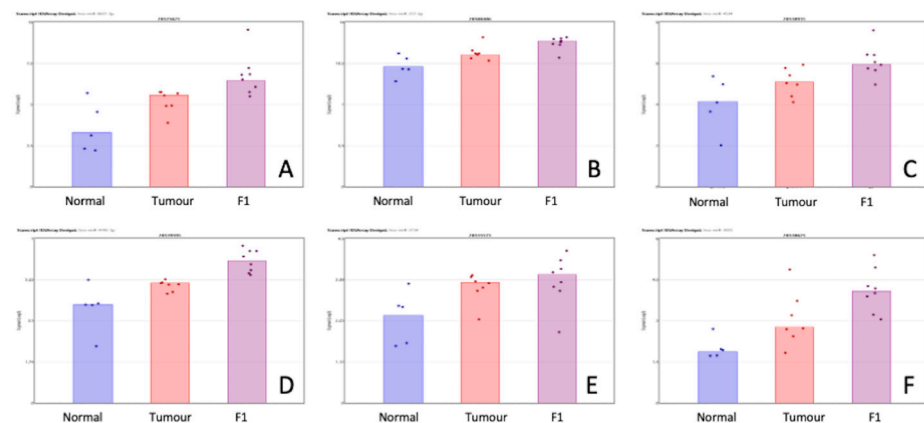


**Figure 3.** Scatterplot representation of data from Table 3 (miRNA shown to be differentially expressed in both the TvN and F1vT comparison) to provide a visual representation of the interpatient variation of the expression of each miRNA in each of the 3 tissue cohorts. Column 1 represents adjacent normal tissue, column 2 represents the patient tumor material, and column 3 represents the F1 tissue. Log2 expression scatterplots in normal tumour and F1 conditions of miRNAs shown to be upregulated in both the TvN and the F1vT comparison (Table 3). (A) miR6831-5p, (B) miR222-3p, (C) miR4534, (D) miR4743-5p, (E) miR3154, (F) miR3935. Each individual sample is represented by a dot in each condition and indicates the log2 expression of that miRNA.

#### 4. Discussion

The core concept of this study was to use the differential expression of miRNAs as a tool to further unravel the biology of human pancreatic cancer in the hope of uncovering new targets or vulnerabilities that might be exploited therapeutically; and/or finding new biomarkers or combinations of biomarkers that might contribute to better or earlier diagnosis [25]. We believe that the study of miRNAs may be particularly fruitful since each miRNA targets multiple mRNAs, regulating the cellular levels of the corresponding proteins.

The data presented here can be viewed in a number of different ways, simple lists of miRNAs DE between tumor and normal and between F1 and tumors (and in some cases both), and because of our previously published information on DE mRNAs and proteins, it is also possible to tentatively identify predicted protein targets of specific DE miRNAs.

Previous analyses of these proteomic [19,24] and mRNA [20] or datasets on these clinical and F1 samples have looked at each dataset individually. mRNA analysis [20] showed an enrichment of genes associated with proliferation, cell cycle, and mitotic processes. Label-free LC-MS-based proteomic analysis carried out on this sample set identified a number of membrane proteins in both comparisons (TvN, F1vT) that had the potential to be utilized either as biomarkers or ADC drug conjugate targets [24].

The study of miRNAs in the development, progression, and prognosis of PDAC has intensified in the last decade. Interestingly there are few studies including transcriptomic and proteomic analysis as well as miRNA analysis on the same sample set. This limitation has meant that protein and gene targets of interesting miRNAs have predominantly been identified using predictive software and have not been validated in matched samples [8,25–29]. Mattie et al. [30] performed miRNA analysis on matched tumor vs. PDX samples; however, this study was complicated by a high degree of homology with mouse miRNA sequences. Hanoun et al. [31] initially published a study looking at the silencing of miR148a as a potential therapeutic target in PDAC. However, following further studies in which they looked at the role this miRNA had in the modulation of protein expression, they discovered that miR148a was responsible for only minimal protein regulation and were unable to identify protein targets in cell lines [32]. This indicates the importance of looking at parallel analysis where possible, as it may help to reduce the number of false leads emerging from in silico studies [33] miR148a was shown to be significantly downregulated in our patient tumor when compared with the normal-adjacent samples fold change of  $-8.15$ , it was,

however, unchanged in the F1vT comparison. The miRWalk prediction software identified nine targets for this miRNA; however, when compared with the protein list, none of these were inversely expressed.

The investigation of miRNAs, with mRNA and proteomic investigations in parallel from the same samples, as we propose, may lead to the identification of proteins that could be druggable targets. Furthermore, RNA therapeutics are now a reality, and miRNA manipulation has the potential to become a useful addition to the therapeutic armamentarium against pancreatic cancer [15,34].

#### 4.1. Tumor vs. Adjacent Normal Tissue Comparison

Examination of the dysregulation of miRNA in our dataset provided a short list of interesting miRNA; the top 10 up and down differentially expressed miRNAs in the (a) TvN comparison (Table 1), (b) F1vT comparisons (Table 2), and (c) miRNAs that are differentially expressed in the same direction in both comparisons (Table 3). Of the 39 miRNAs within this priority list, 7 of them predicted an effect on multiple proteins, which were found to be inversely expressed in the corresponding protein dataset (Tables 4–6). Several studies in gastric cancer [35], triple-negative breast cancer [36], and lung cancer [37] have indicated roles for miR509. This miRNA, however, has not been implicated in the literature on pancreatic cancer. Our data indicated that miR509 was shown to be downregulated in the patient tumors when compared to the normal adjacent, with a fold change of  $-1.85$  (Supplementary Table S1). It was predicted to target a number of proteins in our list, including COTL1, LRPAP1, and SL4A2 (Table 4). Predicted targets of the remaining miRNAs were shown to be differentially expressed in the TvN comparisons, although none of their predicted targets were found to be inversely expressed in the proteomic dataset, but alterations in the expression of miR708 [38], miR210 [39,40], and miR214 [41,42] have all been studied in pancreatic cancer.

In the TvN comparison, it would be expected that we would see the alteration in the expression of proteins and miRNAs associated with tumorigenesis and proliferation in the pathways associated with this transition. miR21 has been extensively studied in PDAC, with high expression levels being linked with shorter overall survival [43–45], while miR331-5p has not been reported in any cancer-linked study. miR21-5p and miR331-5p were shown to target Dimethylarginine Dimethylaminohydrolase 1 (DDAH1). Reduced levels of DDAH1 have been shown to mediate cell invasion and metastasis in gastric cancer through the WNT signaling pathway. Additionally, downregulation was linked with poor prognosis [46]. These miRNAs were both shown to be upregulated in the TvN comparison, and the expression of DDAH1 was shown to be downregulated in the membrane protein fraction of the same comparison. Our data suggest that both miR181c and miR181d target phosphatidylethanolamine binding protein 1 (PEBP1) with a reduction in expression in the TvN in both the membrane and cytoplasmic fraction. Loss of expression of PEBP1 (also known as raf kinase inhibitor protein (RKIP), an endogenous inhibitor of the MAPK pathway) has been shown to be linked with PDAC metastasis and poor overall survival [47,48].

miR210-3p was predicted to target endoplasmic reticulum protein 27 (ERP27), and the protein was shown to be inversely expressed in the membrane fraction (Table 4). Transcriptomic analysis of the cancer genome atlas has identified ERP27 as a pivotal gene in the pathogenesis and progression of PDAC [49]. Increased glutathione S-transferase M3 (GSTM3) expression has been linked with improved overall survival in PDAC [50]. This protein was inversely regulated by miR143-5p, which was found to be upregulated in the tumor samples when compared with the normal-adjacent samples. Literature searches showed no previous studies linked with cancer and the expression of miR4742. Our proteomic data shows that miR4742 targets a number of proteins within the dataset, including superoxide dismutase 2 (SOD2), cysteine and glycine-rich protein 1 (CSRP1), and protein S100-A16 (S100A16). SOD2 is involved in oxidative phosphorylation and the regulation of ROS (reactive oxygen species), and upregulation in its expression has

been linked with acquired resistance to ROS-inducing anticancer drugs and potential irradiation in pancreatic cancer cell lines [51]. While little is known about CSRP1 in pancreatic cancer, S100A16 has been extensively studied in PDAC. Roles in metastasis [52], epithelial–mesenchymal transition (EMT) [53], and increased expression linked with poor prognosis have all been reported in the literature. In our protein data, we do see an increased expression in the membrane fraction in both the TvN and F1vT comparison of S100A16.

#### 4.2. F1 Xenograft vs. Tumor Comparison

In the case of the F1vT comparison, our previous studies on mRNA and protein expression changes on the samples that were used here have highlighted the importance of proteins and mRNA that drive the important process of tumor engraftment in tumorigenesis. As described by Coleman et al. [24], the selection pressure that is exerted on the tumor cells during the engraftment process may result in the altered expression of proteins that drive tumor growth. The upregulation of miR125a in the PDAC samples agrees with what is already shown in the literature [54], and it has been shown that its expression can contribute to cell survival [55]. One of its predicted targets is SGT1 homolog (SUGT1), a highly conserved protein generally found in the nucleus, involved in the kinetochore function and essential for the G1/S and G2M transition, which was shown to be increased in expression in the cytosolic fraction of the tumors in comparison to the tumor sample. The expression of this protein has been potentially linked with a poor prognosis in colorectal cancer [56].

Keklikoglou et al., 2015, found that the expression of miR206 was downregulated in pancreatic tumors vs. non-malignant normal tissue [57]. In our study, miR206 was not differentially expressed in the TvN comparison; however, it was significantly upregulated in the F1vT comparison with a fold change of 26.01. Wu et al. showed that the expression of miR-206 inhibited hepatocellular carcinoma cell migration and invasion while promoting apoptosis by targeting Peptidylprolyl Isomerase B (PPIB) [58]. Another study evaluated the expression of 41 genes as a function of in vitro radioresistance in the NCI-60 cancer cell line panel and found that PPIB had the strongest direct correlation. They also showed that siRNA downregulation of PPIB leads to radio-sensitization of the cancer cells [59]. PPIB expression was shown to be downregulated in both comparisons (TvN and F1vT) in our proteomic data.

Levels of miR2467 were shown to be significantly increased in the F1 samples in comparison to the original patient tumor. This miRNA was predicted to target a large number of proteins, and when examined in our protein dataset, a large number of proteins showed inverse expression. There are limited studies in the literature on this miRNA, with one indicating a downregulation of the miRNA in colorectal tumor samples compared to tumor-adjacent normal tissue [60]. We observed a decrease in the expression of Aflatoxin B1 aldehyde reductase member 2 (AKR7A2) in both of our comparisons, not reported in the proteomic analysis by Cui et al. [61]. Furthermore, this protein was found to be targeted by two miRNAs in our analysis (miR2467 and miR1290). The latter has been suggested as a biomarker in PDAC [62]. Wei et al. [63] and Tavaano et al. [64] both examined the expression of miR1290, with and without CA19-9 in serum and plasma, respectively, and noted that while the expression was found to be higher in PDAC patients, it alone was insufficient to select patients at risk of developing PDAC. Both studies indicated that combining the expression of miR1290 with Ca19-9 was more effective. Whole miRNome and proteome profiling revealed miR1290 as a novel hypoxia-associated microRNA, which was highly abundant in hypoxic extracellular vesicles (EVs) and also exhibited a signature consisting of six proteins including AKR7A2, which was significantly associated with a poor prognosis for melanoma patients [65].

miR615 overexpression has been shown to inhibit cell proliferation, migration, and invasion in vitro [66]. miR615 was increased in the F1 samples compared to the original patient tumors, and a number of predicted target proteins were found to be inversely

expressed in the matching transition in proteomic data. These include several enzymes involved in the reduction and oxidation of aldehydes (AKR7A2, aldehyde dehydrogenase 1 family member B1 (ALDH1B1). Aldehyde dehydrogenase (ALDH2)) and aldehyde dehydrogenases are often highly expressed in cancer stem cells. ALDH1B1 may have a role in cell proliferation [67]. It has also been suggested as a potential biomarker of colon cancer [68]. It was another predicted target of miR615 found in our proteomic dataset to be downregulated in the F1vT comparison as well as in the cytosolic fraction of the TvN comparison. Calnexin (CANX), a member of the calnexin family of molecular chaperones, is a calcium-binding, endoplasmic reticulum (ER)-associated protein that interacts transiently with newly synthesized N-linked glycoproteins, facilitating protein folding and assembly. It may also play a central role in the quality control of protein folding by retaining incorrectly folded protein subunits within the ER for degradation. This protein has the potential as a biomarker in breast [69] and lung patients [70]. Several of the other miR615-3p targets identified in our analysis showed consistent changes across both comparisons and are involved in processing membrane protein targets to ER (SECG1A1, SPC53, TM95F2).

The miR-378 family has been associated with numerous cancers, including: osteosarcoma [71], cervical [72], and colon [73]. MiR-378e has not yet been extensively studied for an oncogenic/oncosuppressive role. Wang et al. [73] have reported an association between miR-378e and colorectal cancer, as the survival rate of patients in the high-expression group was significantly higher than that in the low-expression group. In our dataset, it was shown to be differentially upregulated in the F1 samples vs. the patient samples. miR409 expression, which was downregulated in the F1 tumors vs. patient tumor samples, has been linked with PDAC cell proliferation, invasion, and migration [74] proteins. Predicted to be targeted by miR409, Catenin Delta 1 (CTNND1) along with Catenin alpha 1 (CTNNA1) and Catenin Beta 1 (CTNNB1) expression was shown to indicate a poor prognosis for pancreatic cancer patients [75]. In the F1 samples, this protein was shown to be differentially upregulated versus the patient samples (Table 5).

#### 4.3. miRNA Altered in Both Comparisons

Across the two transitions, from normal to the tumor and then tumor to F1 models, there was a small cohort of miRNAs that were shown to be DE, namely miR4534, miR4743, miR222, miR3154, and miR6831. miR-6831-5p had been previously identified as being differentially expressed in PDAC vs. normal control serum samples, as shown by Aita et al. [76]. KEGG pathway analysis showed enrichment for the pancreatic cancer term. Dysregulation of miR6831-5p has been associated with gastric [77] and colorectal cancer [78]. Additionally, PLD3, which we found to be downregulated in both the cytoplasmic and membrane protein fraction of the F1 tumor samples compared to the patient material, has been shown to be a potential marker of senescence [79]. Limited studies have been completed on miR4534; however, one study indicated the potential roles of miR4534 in prostate cancer through the regulation of PTEN [80]. miR3154 has been investigated in leukemia, with its expression shown to be higher at initial diagnosis compared to complete remission [81]. miR222 has been widely studied in pancreatic cancer. Elevated levels of these miRNAs have been associated with poor survival [82–85].

Of course, a caveat with any research of this kind using tissue extracts is the fact that the samples consist of a heterogeneous mixture of cell types and connective tissue, so we cannot, for example, be certain that an alteration observed in the tumor samples reflects an alteration in expression in the tumor cells (for definitive attribution some form of *in situ* hybridization would be needed for miRNAs and mRNAs, and immunohistochemistry in the case of proteins).

## 5. Conclusions

This profiling study complements the data currently available on the expression of miRNA in pancreatic cancer. The availability of the mRNA microarrays and LC-MS-MS proteomic data has allowed for the tentative validation of what would typically have only

been predicted targets. By combining the three datasets, we have been able to identify connections between the expression of proteins and their miRNA regulators that have not been identified in pancreatic cancer previously. Analysis of the F1 samples may also allow for the identification of miRNA, which has roles in key networks associated with tumor engraftment and proliferation. The combined analysis has provided a shortlist of protein and miRNAs that warrant further investigation; this list has a mixture of established miRNAs associated with PDAC progression, in particular, miR21, miR2467, miR615 but also a number of miRs that have either limited or no studies in pancreatic cancer such as miR4534, miR3154, and miR4742. Nevertheless, some of these may have potential as biomarkers or for the development of therapeutic targets with further validation. Further analysis of these three parallel data strands that we have placed in the public domain by others using new bioinformatic tools could discover additional connections and networks that we could not uncover by our analysis. The lists we make available here will then be available to other groups for histochemical investigation and for validation in a variety of cancer-relevant in vitro assays for functional assignment.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/life13030608/s1>, Table S1: List of differentially expressed (DE) miRNA in tumour vs adjacent normal tissue comparison, Table S2: List of differentially expressed (DE) miRNA in F1 vs. tumour comparison.

**Author Contributions:** Conceptualization: F.O. (Fiona O'Neill), T.-J.A.-C. and M.C.; Formal Analysis: F.O. (Fiona O'Neill); Project Administration: F.O. (Fiona O'Neill); Resources, Investigation, and Methodology: N.S., J.M., N.T.C., J.G., K.C., R.M.S., R.M., N.L.S., M.H., P.M., F.O. (Finbarr O'Sullivan), G.M., S.R. and R.O.; Supervision: M.M. and M.C.; Writing—original draft preparation: F.O. (Fiona O'Neill) and T.-J.A.-C.; Writing—review and editing: F.O. (Fiona O'Neill), T.-J.A.-C., S.R., P.M. and M.C. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** Collection of patient material was approved by St Vincent's University Hospital and Dublin City University (DCU) Research Ethics Committee. All participants provided written informed consent. All animal work received ethical approval from the DCU Research Ethics Committee (DCUREC/2012/202 approved 16 November 2012), was licensed by the Irish Department of Health (license number B100-4501), and is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>, accessed on 1 July 2022). All experimental procedures were conducted in compliance with the guidelines from the Health Products Regulatory Authority Scientific Animal Protection.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The microarray data have been deposited in the NCBI's Gene Expression Omnibus (GEO) under GEO series accession no GSE141873 (mRNA) and GSE207345 (miRNA). Proteomic data used in the analysis is available in Supplementary Files from cited publications [18,21]. Additional datasets used and/or analyzed during the current study may be requested from the corresponding author on reasonable request.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

- Waters, A.M.; Der, C.J. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harb. Perspect. Med.* **2018**, *8*, a031435. [\[CrossRef\]](#)
- Pant, S.; Hubbard, J.; Martinelli, E.; Bekaii-Saab, T. Clinical Update on K-Ras Targeted Therapy in Gastrointestinal Cancers. *Crit. Rev. Oncol. Hematol.* **2018**, *130*, 78–91. [\[CrossRef\]](#)
- Liu, J.; Kang, R.; Tang, D. The KRAS-G12C Inhibitor: Activity and Resistance. *Cancer Gene* **2022**, *29*, 875–878. [\[CrossRef\]](#)
- Ho, W.J.; Jaffee, E.M.; Zheng, L. The Tumour Microenvironment in Pancreatic Cancer—Clinical Challenges and Opportunities. *Nat. Rev. Clin. Oncol.* **2020**, *17*, 527. [\[CrossRef\]](#)
- Guo, S.; Fesler, A.; Wang, H.; Ju, J. MicroRNA Based Prognostic Biomarkers in Pancreatic Cancer. *Biomark. Res.* **2018**, *6*, 18. [\[CrossRef\]](#)
- Chen, L.; Ma, C.; Bian, Y.; Shao, C.; Wang, T.; Li, J.; Chong, X.; Su, L.; Lu, J. Aberrant Expression of STYK1 and E-Cadherin Confer a Poor Prognosis for Pancreatic Cancer Patients. *Oncotarget* **2017**, *8*, 111333–111345. [\[CrossRef\]](#)
- Khan, M.A.; Zubair, H.; Srivastava, S.K.; Singh, S.; Singh, A.P. Insights into the Role of MicroRNAs in Pancreatic Cancer Pathogenesis: Potential for Diagnosis, Prognosis, and Therapy. *Adv. Exp. Med. Biol.* **2015**, *889*, 71–87. [\[CrossRef\]](#)
- Bloomston, M.; Frankel, W.L.; Petrocca, F.; Volinia, S.; Alder, H.; Hagan, J.P.; Liu, C.-G.; Bhatt, D.; Taccioli, C.; Croce, C.M. MicroRNA Expression Patterns to Differentiate Pancreatic Adenocarcinoma From Normal Pancreas and Chronic Pancreatitis. *JAMA* **2007**, *297*, 1901. [\[CrossRef\]](#)
- Iorio, M.V.; Croce, C.M. MicroRNA Dysregulation in Cancer: Diagnostics, Monitoring and Therapeutics. A Comprehensive Review. *EMBO Mol. Med.* **2012**, *4*, 143. [\[CrossRef\]](#)
- Jamieson, N.B.; Morran, D.C.; Morton, J.P.; Ali, A.; Dickson, E.J.; Carter, C.R.; Sansom, O.J.; Evans, T.R.J.; McKay, C.J.; Oien, K.A. MicroRNA Molecular Profiles Associated with Diagnosis, Clinicopathologic Criteria, and Overall Survival in Patients with Resectable Pancreatic Ductal Adenocarcinoma. *Clin. Cancer Res.* **2012**, *18*, 534–545. [\[CrossRef\]](#)
- Steele, C.W.; Oien, K.A.; McKay, C.J.; Jamieson, N.B. Clinical Potential of MicroRNAs in Pancreatic Ductal Adenocarcinoma. *Pancreas* **2011**, *40*, 1165–1171. [\[CrossRef\]](#)
- Fathi, M.; Ghafouri-Fard, S.; Abak, A.; Taheri, M. Emerging Roles of MiRNAs in the Development of Pancreatic Cancer. *Biomed. Pharmacother.* **2021**, *141*, 111914. [\[CrossRef\]](#)
- Smolarz, B.; Durczyński, A.; Romanowicz, H.; Hogendorf, P. The Role of MicroRNA in Pancreatic Cancer. *Biomedicines* **2021**, *9*, 1322. [\[CrossRef\]](#)
- Park, W.; Chawla, A.; O'Reilly, E.M. Pancreatic Cancer: A Review. *JAMA* **2021**, *326*, 851–862. [\[CrossRef\]](#)
- Gilles, M.E.; Hao, L.; Huang, L.; Rupaimoole, R.; Lopez-Casas, P.P.; Pulver, E.; Jeong, J.C.; Muthuswamy, S.K.; Hidalgo, M.; Bhatia, S.N.; et al. Personalized RNA Medicine for Pancreatic Cancer. *Clin. Cancer Res.* **2018**, *24*, 1734–1747. [\[CrossRef\]](#)
- Sicard, F.; Gayral, M.; Lulka, H.; Buscail, L.; Cordelier, P. Targeting MiR-21 for the Therapy of Pancreatic Cancer. *Mol. Ther. J. Am. Soc. Gene Ther.* **2013**, *21*, 986–994. [\[CrossRef\]](#)
- Lu, T.; Prakash, J. Nanomedicine Strategies to Enhance Tumor Drug Penetration in Pancreatic Cancer. *Int. J. Nanomed.* **2021**, *16*, 6313–6328. [\[CrossRef\]](#)
- O'Brien, J.; Hayder, H.; Zayed, Y.; Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front. Endocrinol.* **2018**, *9*, 402. [\[CrossRef\]](#)
- Coleman, O.; Henry, M.; O'Neill, F.; Roche, S.; Swan, N.; Geoghegan, J.; Conlon, K.; McVey, G.; Moriarty, M.; Meleady, P.; et al. Proteomic Analysis of Cell Lines and Primary Tumors in Pancreatic Cancer Identifies Proteins Expressed Only In Vitro and Only In Vivo. *Pancreas* **2020**, *49*, 1109–1116. [\[CrossRef\]](#)
- Roche, S.; O'Neill, F.; Murphy, J.; Swan, N.; Meiller, J.; Conlon, N.T.; Geoghegan, J.; Conlon, K.; McDermott, R.; Rahman, R.; et al. Establishment and Characterisation by Expression Microarray of Patient-Derived Xenograft Panel of Human Pancreatic Adenocarcinoma Patients. *Int. J. Mol. Sci.* **2020**, *21*, 962. [\[CrossRef\]](#)
- Kaushik, P.; Henry, M.; Clynes, M.; Meleady, P. The Expression Pattern of the Phosphoproteome Is Significantly Changed During the Growth Phases of Recombinant CHO Cell Culture. *Biotechnol. J.* **2018**, *13*, 1700221. [\[CrossRef\]](#)
- Sticht, C.; Torre, C.D.L.; Parveen, A.; Gretz, N. MiRWalk: An Online Resource for Prediction of MicroRNA Binding Sites. *PLoS ONE* **2018**, *13*, e0206239. [\[CrossRef\]](#)
- Mestdagh, P.; Van Vlierberghe, P.; De Weer, A.; Muth, D.; Westermann, F.; Speleman, F.; Vandesompele, J. A Novel and Universal Method for MicroRNA RT-QPCR Data Normalization. *Genome Biol.* **2009**, *10*, R64. [\[CrossRef\]](#)
- Coleman, O.; Henry, M.; O'Neill, F.; Roche, S.; Swan, N.; Boyle, L.; Murphy, J.; Meiller, J.; Conlon, N.T.; Geoghegan, J.; et al. A Comparative Quantitative LC-MS/MS Profiling Analysis of Human Pancreatic Adenocarcinoma, Adjacent-Normal Tissue, and Patient-Derived Tumour Xenografts. *Proteomes* **2018**, *6*, 45. [\[CrossRef\]](#)
- Naderi, E.; Mostafaei, M.; Pourshams, A.; Mohamadkhani, A. Network of MicroRNAs-MRNAs Interactions in Pancreatic Cancer. *BioMed. Res. Int.* **2014**, *2014*, 1–8. [\[CrossRef\]](#)
- Shams, R.; Saberi, S.; Zali, M.; Sadeghi, A.; Ghafouri-Fard, S.; Aghdaei, H.A. Identification of Potential MicroRNA Panels for Pancreatic Cancer Diagnosis Using Microarray Datasets and Bioinformatics Methods. *Sci. Rep.* **2020**, *10*, 7559. [\[CrossRef\]](#)
- Daoud, A.Z.; Mulholland, E.J.; Cole, G.; McCarthy, H.O. MicroRNAs in Pancreatic Cancer: Biomarkers, Prognostic, and Therapeutic Modulators. *BMC Cancer* **2019**, *19*, 1–13. [\[CrossRef\]](#)

28. Kent, O.A.; Mullendore, M.; Wentzel, E.A.; López-Romero, P.; Tan, A.C.; Alvarez, H.; West, K.; Ochs, M.F.; Hidalgo, M.; Arking, D.E.; et al. A Resource for Analysis of MicroRNA Expression and Function in Pancreatic Ductal Adenocarcinoma Cells. *Cancer Biol. Ther.* **2009**, *8*, 2013–2024. [\[CrossRef\]](#)
29. Eun, J.L.; Gusev, Y.; Jiang, J.; Nuovo, G.J.; Lerner, M.R.; Frankel, W.L.; Morgan, D.L.; Postier, R.G.; Brackett, D.J.; Schmittgen, T.D. Expression Profiling Identifies MicroRNA Signature in Pancreatic Cancer. *Int. J. Cancer* **2007**, *120*, 1046–1054. [\[CrossRef\]](#)
30. Mattie, M.; Christensen, A.; Chang, M.S.; Yeh, W.; Said, S.; Shostak, Y.; Capo, L.; Verlinsky, A.; An, Z.; Joseph, I.; et al. Molecular Characterization of Patient-Derived Human Pancreatic Tumor Xenograft Models for Preclinical and Translational Development of Cancer Therapeutics. *Neoplasia* **2013**, *15*, 1138–1150. [\[CrossRef\]](#)
31. Hanoun, N.; Delpu, Y.; Suriawinata, A.A.; Bournet, B.; Bureau, C.; Selves, J.; Tsongalis, G.J.; Dufresne, M.; Buscail, L.; Cordelier, P.; et al. The Silencing of MicroRNA 148a Production by DNA Hypermethylation Is an Early Event in Pancreatic Carcinogenesis. *Clin. Chem.* **2010**, *56*, 1107–1118. [\[CrossRef\]](#)
32. Delpu, Y.; Lulka, H.; Sicard, F.; Saint-Laurent, N.; Lopez, F.; Hanoun, N.; Buscail, L.; Cordelier, P.; Torrisani, J. The Rescue of MiR-148a Expression in Pancreatic Cancer: An Inappropriate Therapeutic Tool. *PLoS ONE* **2013**, *8*, e55513. [\[CrossRef\]](#)
33. Clarke, C.; Henry, M.; Doolan, P.; Kelly, S.; Aherne, S.; Sanchez, N.; Kelly, P.; Kinsella, P.; Breen, L.; Madden, S.F.; et al. Integrated MiRNA, mRNA and Protein Expression Analysis Reveals the Role of Post-Transcriptional Regulation in Controlling CHO Cell Growth Rate. *BMC Genom.* **2012**, *13*, 656. [\[CrossRef\]](#)
34. Passadouro, M.; Faneca, H. Managing Pancreatic Adenocarcinoma: A Special Focus in MicroRNA Gene Therapy. *Int. J. Mol. Sci.* **2016**, *17*, 718. [\[CrossRef\]](#)
35. Fattahi, S.; Nikbakhsh, N.; Ranaei, M.; Sabour, D.; Akhavan-Niaki, H. Association of Sonic Hedgehog Signaling Pathway Genes IHH, BOC, RAB23a and MIR195-5p, MIR509-3-5p, MIR6738-3p with Gastric Cancer Stage. *Sci. Rep.* **2021**, *11*, 16027.
36. Zhang, G.; Liu, Z.; Han, Y.; Wang, X.; Yang, Z. Overexpression of MiR-509 Increases Apoptosis and Inhibits Invasion via Suppression of Tumor Necrosis Factor- $\alpha$  in Triple-Negative Breast Cancer Hs578T Cells. *Oncol. Res.* **2016**, *24*, 233–238. [\[CrossRef\]](#)
37. Liang, J.-J.; Wang, J.-Y.; Zhang, T.-J.; An, G.-S.; Ni, J.-H.; Li, S.-Y.; Jia, H.-T. MiR-509-3-5p-NONHSAT112228.2 Axis Regulates P21 and Suppresses Proliferation and Migration of Lung Cancer Cells. *Curr. Top. Med. Chem.* **2020**, *20*, 835–846. [\[CrossRef\]](#)
38. Yu, J.; Li, A.; Hong, S.M.; Hruban, R.H.; Goggins, M. MicroRNA Alterations of Pancreatic Intraepithelial Neoplasias. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **2012**, *18*, 981–992. [\[CrossRef\]](#)
39. Sun, F.B.; Lin, Y.; Li, S.J.; Gao, J.; Han, B.; Zhang, C.S. MiR-210 Knockdown Promotes the Development of Pancreatic Cancer via Upregulating E2F3 Expression. *Eur. Rev. Med. Pharmacol. Sci.* **2018**, *22*, 8640–8648. [\[CrossRef\]](#)
40. Liu, G.; Shao, C.; Li, A.; Zhang, X.; Guo, X.; Li, J. Diagnostic Value of Plasma MiR-181b, MiR-196a, and MiR-210 Combination in Pancreatic Cancer. *Gastroenterol. Res. Pract.* **2020**, *2020*, 6073150. [\[CrossRef\]](#)
41. Cao, T.H.; Ling, X.; Chen, C.; Tang, W.; Hu, D.M.; Yin, G.J. Role of MiR-214-5p in the Migration and Invasion of Pancreatic Cancer Cells. *Eur. Rev. Med. Pharmacol. Sci.* **2018**, *22*, 7214–7221. [\[CrossRef\]](#)
42. Deng, S.; Wang, J.; Xu, J.; Li, J.; Zhang, L.; Jin, Y. Expression of MiR-214 in Pancreatic Cancer and Its Effect on the Biological Function of Pancreatic Cancer Cells. *J. B.U.ON. Off. J. Balk. Union Oncol.* **2021**, *26*, 1111–1120.
43. Zhao, F.; Wei, C.; Cui, M.Y.; Xia, Q.Q.; Wang, S.B.; Zhang, Y. Prognostic Value of MicroRNAs in Pancreatic Cancer: A Meta-Analysis. *Aging* **2020**, *12*, 9380–9404. [\[CrossRef\]](#)
44. Vila-Casadesus, M.; Vila-Navarro, E.; Raimondi, G.; Fillat, C.; Castells, A.; Lozano, J.J.; Gironella, M.; Vila-Casadesus, M.; Vila-Navarro, E.; Raimondi, G.; et al. Deciphering MicroRNA Targets in Pancreatic Cancer Using MiRComb R Package. *Oncotarget* **2018**, *9*, 6499–6517. [\[CrossRef\]](#)
45. Frampton, A.E.; Krell, J.; Jamieson, N.B.; Gall, T.M.H.; Giovannetti, E.; Funel, N.; Prado, M.M.; Krell, D.; Habib, N.A.; Castellano, L.; et al. MicroRNAs with Prognostic Significance in Pancreatic Ductal Adenocarcinoma: A Meta-Analysis. *Eur. J. Cancer (Oxf. Engl. 1990)* **2015**, *51*, 1389–1404. [\[CrossRef\]](#)
46. Ye, J.; Xu, J.; Li, Y.; Huang, Q.; Huang, J.; Wang, J.; Zhong, W.; Lin, X.; Chen, W.; Lin, X. DDAH1 Mediates Gastric Cancer Cell Invasion and Metastasis via Wnt/ $\beta$ -Catenin Signaling Pathway. *Mol. Oncol.* **2017**, *11*, 1208–1224. [\[CrossRef\]](#)
47. Kim, H.S.; Kim, G.Y.; Lim, S.J.; Kim, Y.W. Loss of Raf-1 Kinase Inhibitory Protein in Pancreatic Ductal Adenocarcinoma. *Pathology* **2010**, *42*, 655–660. [\[CrossRef\]](#)
48. Karamitopoulou, E.; Zlobec, I.; Gloor, B.; Kondi-Pafiti, A.; Lugli, A.; Perren, A. Loss of Raf-1 Kinase Inhibitor Protein (RKIP) Is Strongly Associated with High-Grade Tumor Budding and Correlates with an Aggressive Phenotype in Pancreatic Ductal Adenocarcinoma (PDAC). *J. Transl. Med.* **2013**, *11*, 1–11. [\[CrossRef\]](#)
49. Chang, X.; Yang, M.F.; Fan, W.; Wang, L.S.; Yao, J.; Li, Z.S.; Li, D.F. Bioinformatic Analysis Suggests That Three Hub Genes May Be a Vital Prognostic Biomarker in Pancreatic Ductal Adenocarcinoma. *J. Comput. Biol. A J. Comput. Mol. Cell Biol.* **2020**, *27*, 1595–1609. [\[CrossRef\]](#)
50. Wang, S.; Yang, J.; Ding, C.; Li, J.; You, L.; Dai, M.; Zhao, Y. Glutathione S-Transferase Mu-3 Predicts a Better Prognosis and Inhibits Malignant Behavior and Glycolysis in Pancreatic Cancer. *Front. Oncol.* **2020**, *10*, 1539. [\[CrossRef\]](#)
51. Zhou, J.; Du, Y. Acquisition of Resistance of Pancreatic Cancer Cells to 2-Methoxyestradiol Is Associated with the Upregulation of Manganese Superoxide Dismutase. *Mol. Cancer Res. MCR* **2012**, *10*, 768–777. [\[CrossRef\]](#)
52. Fang, D.; Zhang, C.; Xu, P.; Liu, Y.; Mo, X.; Sun, Q.; Abdelatty, A.; Hu, C.; Xu, H.; Zhou, G.; et al. S100A16 Promotes Metastasis and Progression of Pancreatic Cancer through FGF19-Mediated AKT and ERK1/2 Pathways. *Cell Biol. Toxicol.* **2021**, *37*, 555–571. [\[CrossRef\]](#)

53. Li, T.; Ren, T.; Huang, C.; Li, Y.; Yang, P.; Che, G.; Luo, L.; Chen, Y.; Peng, S.; Lin, Y.; et al. S100A16 Induces Epithelial-Mesenchymal Transition in Human PDAC Cells and Is a New Therapeutic Target for Pancreatic Cancer Treatment That Synergizes with Gemcitabine. *Biochem. Pharmacol.* **2021**, *189*, 114396. [\[CrossRef\]](#)
54. Pan, L.; Zhou, L.; Yin, W.; Bai, J.; Liu, R. MiR-125a Induces Apoptosis, Metabolism Disorder and Migration Impairment in Pancreatic Cancer Cells by Targeting Mfn2-Related Mitochondrial Fission. *Int. J. Oncol.* **2018**, *53*, 124–136. [\[CrossRef\]](#)
55. Jia, C.-W.; Sun, Y.; Zhang, T.-T.; Lu, Z.-H.; Chen, J. Effects of MiR-125a-5p on Cell Proliferation, Apoptosis and Cell Cycle of Pancreatic Cancer Cells. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* **2016**, *38*, 415–421. [\[CrossRef\]](#)
56. Iwatsuki, M.; Mimori, K.; Sato, T.; Toh, H.; Yokobori, T.; Tanaka, F.; Ishikawa, K.; Baba, H.; Mori, M. Overexpression of SUGT1 in Human Colorectal Cancer and Its Clinicopathological Significance. *Int. J. Oncol.* **2010**, *36*, 569–575. [\[CrossRef\]](#)
57. Keklikoglou, I.; Hosaka, K.; Bender, C.; Bott, A.; Koerner, C.; Mitra, D.; Will, R.; Woerner, A.; Muenstermann, E.; Wilhelm, H.; et al. MicroRNA-206 Functions as a Pleiotropic Modulator of Cell Proliferation, Invasion and Lymphangiogenesis in Pancreatic Adenocarcinoma by Targeting ANXA2 and KRAS Genes. *Oncogene* **2015**, *34*, 4867–4878. [\[CrossRef\]](#)
58. Wu, H.; Xie, D.; Yang, Y.; Yang, Q.; Shi, X.; Yang, R. Ultrasound-Targeted Microbubble Destruction-Mediated MiR-206 Overexpression Promotes Apoptosis and Inhibits Metastasis of Hepatocellular Carcinoma Cells Via Targeting PPIB. *Technol. Cancer Res. Treat.* **2020**, *19*, 1533033820959355. [\[CrossRef\]](#)
59. Williams, P.D.; Owens, C.R.; Dziegielewska, J.; Moskaluk, C.A.; Read, P.W.; Larner, J.M.; Story, M.D.; Brock, W.A.; Amundson, S.A.; Lee, J.K.; et al. Cyclophilin B Expression Is Associated with in Vitro Radioresistance and Clinical Outcome after Radiotherapy. *Neoplasia* **2011**, *13*, 1122–1131. [\[CrossRef\]](#)
60. Chen, L.; Chen, W.; Zhao, C.; Jiang, Q. LINC01224 Promotes Colorectal Cancer Progression by Sponging MiR-2467. *Cancer Manag. Res.* **2021**, *13*, 733. [\[CrossRef\]](#)
61. Cui, Y.; Tian, M.; Zong, M.; Teng, M.; Chen, Y.; Lu, J.; Jiang, J.; Liu, X.; Han, J. Proteomic Analysis of Pancreatic Ductal Adenocarcinoma Compared with Normal Adjacent Pancreatic Tissue and Pancreatic Benign Cystadenoma. *Pancreatol. Off. J. Int. Assoc. Pancreatol. (IAP) [Et Al.]* **2009**, *9*, 89–98. [\[CrossRef\]](#)
62. Ta, N.; Huang, X.; Zheng, K.; Zhang, Y.; Gao, Y.; Deng, L.; Zhang, B.; Jiang, H.; Zheng, J. MiRNA-1290 Promotes Aggressiveness in Pancreatic Ductal Adenocarcinoma by Targeting IKK1. *Cell. Physiol. Biochem.* **2018**, *51*, 711–728. [\[CrossRef\]](#)
63. Wei, J.; Yang, L.; Wu, Y.N.; Xu, J. Serum MiR-1290 and MiR-1246 as Potential Diagnostic Biomarkers of Human Pancreatic Cancer. *J. Cancer* **2020**, *11*, 1325–1333. [\[CrossRef\]](#)
64. Tavano, F.; Gioffreda, D.; Valvano, M.R.; Palmieri, O.; Tardio, M.; Latiano, T.P.; Piepoli, A.; Maiello, E.; Pirozzi, F.; Andriulli, A. Droplet Digital PCR Quantification of MiR-1290 as a Circulating Biomarker for Pancreatic Cancer. *Sci. Rep.* **2018**, *8*, 16389. [\[CrossRef\]](#)
65. Walbrecht, G.; Lecha, O.; Gaigneaux, A.; Fougères, M.R.; Philippidou, D.; Margue, C.; Nomigni, M.T.; Bernardin, F.; Dittmar, G.; Behrmann, I.; et al. Hypoxia-Induced Adaptations of MiRNomes and Proteomes in Melanoma Cells and Their Secreted Extracellular Vesicles. *Cancers* **2020**, *12*, 692. [\[CrossRef\]](#)
66. Gao, W.; Gu, Y.; Li, Z.; Cai, H.; Peng, Q.; Tu, M.; Kondo, Y.; Shinjo, K.; Zhu, Y.; Zhang, J.; et al. MiR-615-5p Is Epigenetically Inactivated and Functions as a Tumor Suppressor in Pancreatic Ductal Adenocarcinoma. *Oncogene* **2015**, *34*, 1629–1640. [\[CrossRef\]](#)
67. Singh, S.; Arcaroli, J.J.; Orlicky, D.J.; Chen, Y.; Messersmith, W.A.; Bagby, S.; Purkey, A.; Quackenbush, K.S.; Thompson, D.C.; Vasiliou, V. Aldehyde Dehydrogenase 1B1 as a Modulator of Pancreatic Adenocarcinoma. *Pancreas* **2016**, *45*, 117–122. [\[CrossRef\]](#)
68. Chen, Y.; Orlicky, D.J.; Matsumoto, A.; Singh, S.; Thompson, D.C.; Vasiliou, V. Aldehyde Dehydrogenase 1B1 (ALDH1B1) Is a Potential Biomarker for Human Colon Cancer. *Biochem. Biophys. Res. Commun.* **2011**, *405*, 173–179. [\[CrossRef\]](#)
69. Moradpoor, R.; Gharebaghian, A.; Shahi, F.; Mousavi, A.; Salari, S.; Akbari, M.E.; Ajdari, S.; Salimi, M. Identification and Validation of Stage-Associated PBMC Biomarkers in Breast Cancer Using MS-Based Proteomics. *Front. Oncol.* **2020**, *10*, 1101. [\[CrossRef\]](#)
70. Kobayashi, M.; Nagashio, R.; Jiang, S.X.; Saito, K.; Tsuchiya, B.; Ryuge, S.; Katono, K.; Nakashima, H.; Fukuda, E.; Goshima, N.; et al. Calnexin Is a Novel Sero-Diagnostic Marker for Lung Cancer. *Lung Cancer (Amst. Neth.)* **2015**, *90*, 342–345. [\[CrossRef\]](#)
71. Peng, N.; Miao, Z.; Wang, L.; Liu, B.; Wang, G.; Guo, X. MiR-378 Promotes the Cell Proliferation of Osteosarcoma through down-Regulating the Expression of Kruppel-like Factor 9. *Biochem. Cell Biol.* **2018**, *96*, 515–521. [\[CrossRef\]](#)
72. Tan, D.; Zhou, C.; Han, S.; Hou, X.; Kang, S.; Zhang, Y. MicroRNA-378 Enhances Migration and Invasion in Cervical Cancer by Directly Targeting Autophagy-Related Protein 12. *Mol. Med. Rep.* **2018**, *17*, 6319–6326. [\[CrossRef\]](#)
73. Wang, Y.; Gao, X.; Wei, F.; Zhang, X.; Yu, J.; Zhao, H.; Sun, Q.; Yan, F.; Yan, C.; Li, H.; et al. Diagnostic and Prognostic Value of Circulating MiR-21 for Cancer: A Systematic Review and Meta-Analysis. *Gene* **2014**, *533*, 389–397. [\[CrossRef\]](#)
74. Long, K.; Dong, Q.; Zeng, W. The Clinical Significance of MicroRNA-409 in Pancreatic Carcinoma and Associated Tumor Cellular Functions. *Bioengineered* **2021**, *12*, 4633–4642. [\[CrossRef\]](#)
75. Zheng, H.; Ding, B.; Xue, K.; Yu, J.; Lou, W. Construction of a LncRNA/Pseudogene-Hsa-MiR-30d-5p-GJA1 Regulatory Network Related to Metastasis of Pancreatic Cancer. *Genomics* **2021**, *113*, 1742–1753. [\[CrossRef\]](#)
76. Aita, A.; Millino, C.; Sperti, C.; Pacchioni, B.; Plebani, M.; Pittà, C.D.; Basso, D. Serum MiRNA Profiling for Early PDAC Diagnosis and Prognosis: A Retrospective Study. *Biomedicines* **2021**, *9*, 845. [\[CrossRef\]](#)
77. Kim, S.; Bae, W.J.; Ahn, J.M.; Heo, J.-H.; Kim, K.-M.; Choi, K.W.; Sung, C.O.; Lee, D. MicroRNA Signatures Associated with Lymph Node Metastasis in Intramucosal Gastric Cancer. *Mod. Pathol.* **2021**, *34*, 672–683. [\[CrossRef\]](#)

78. Francone, E.; Gentili, S.; Santori, G.; Stabilini, C.; Fornaro, R.; Frascio, M. MicroRNAs Differential Expression Profile in Metastatic Colorectal Cancer: A Pilot Study with Literature Review. *Surg. Oncol.* **2021**, *37*, 101524. [\[CrossRef\]](#)
79. Althubiti, M.; Lezina, L.; Carrera, S.; Jukes-Jones, R.; Giblett, S.M.; Antonov, A.; Barlev, N.; Saldanha, G.S.; Pritchard, C.A.; Cain, K.; et al. Characterization of Novel Markers of Senescence and Their Prognostic Potential in Cancer. *Cell Death Dis.* **2014**, *5*, e1528. [\[CrossRef\]](#)
80. Nip, H.; Dar, A.A.; Saini, S.; Colden, M.; Varahram, S.; Chowdhary, H.; Yamamura, S.; Mitsui, Y.; Tanaka, Y.; Kato, T.; et al. Oncogenic MicroRNA-4534 Regulates PTEN Pathway in Prostate Cancer. *Oncotarget* **2016**, *7*, 68371–68384. [\[CrossRef\]](#)
81. Lee, Y.G.; Kim, I.; Oh, S.; Shin, D.Y.; Koh, Y.; Lee, K.W. Small RNA Sequencing Profiles of Mir-181 and Mir-221, the Most Relevant MicroRNAs in Acute Myeloid Leukemia. *Korean J. Intern. Med.* **2019**, *34*, 178–183. [\[CrossRef\]](#)
82. Li, Z.; Tao, Y.; Wang, X.; Jiang, P.; Li, J.; Peng, M.; Zhang, X.; Chen, K.; Liu, H.; Zhen, P.; et al. Tumor-Secreted Exosomal MiR-222 Promotes Tumor Progression via Regulating P27 Expression and Re-Localization in Pancreatic Cancer. *Cell. Physiol. Biochem.* **2018**, *51*, 610–629. [\[CrossRef\]](#)
83. Lee, C.; He, H.; Jiang, Y.; Di, Y.; Yang, F.; Li, J.; Jin, C.; Fu, D. Elevated Expression of Tumor MiR-222 in Pancreatic Cancer Is Associated with Ki67 and Poor Prognosis. *Med. Oncol.* **2013**, *30*, 700. [\[CrossRef\]](#)
84. Greither, T.; Grochola, L.F.; Udelnow, A.; Lautenschläger, C.; Würfl, P.; Taubert, H. Elevated Expression of MicroRNAs 155, 203, 210 and 222 in Pancreatic Tumors Is Associated with Poorer Survival. *Int. J. Cancer* **2010**, *126*, 73–80. [\[CrossRef\]](#)
85. Park, J.Y.; Helm, J.; Coppola, D.; Kim, D.; Malafa, M.; Kim, S.J. MicroRNAs in Pancreatic Ductal Adenocarcinoma. *World J. Gastroenterol.* **2011**, *17*, 817–827. [\[CrossRef\]](#)

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Supplementary Table S1: List of differentially expressed (DE) miRNA in Tumour vs adjacent normal tissue comparison

| Transcript ID(Array Design) | Tumour Avg (log2) | Normal Avg (log2) | Fold Change | P-val    |
|-----------------------------|-------------------|-------------------|-------------|----------|
| hsa-miR-21-3p               | 6.37              | 2.68              | 12.89       | 1.20E-03 |
| hsa-miR-708-5p              | 6.11              | 2.92              | 9.15        | 6.70E-03 |
| hsa-miR-181c-5p             | 6.69              | 3.6               | 8.52        | 3.00E-03 |
| hsa-miR-125b-1-3p           | 5.43              | 2.64              | 6.95        | 3.18E-02 |
| hsa-miR-21-5p               | 6.94              | 4.15              | 6.89        | 1.58E-02 |
| hsa-miR-331-5p              | 4.39              | 1.62              | 6.81        | 3.60E-03 |
| hsa-miR-210-3p              | 7.9               | 5.15              | 6.73        | 6.60E-03 |
| hsa-miR-181d-5p             | 6.79              | 4.08              | 6.51        | 2.28E-02 |
| hsa-miR-214-5p              | 6.35              | 3.69              | 6.3         | 1.02E-02 |
| hsa-miR-143-5p              | 3.84              | 1.36              | 5.55        | 2.00E-04 |
| hsa-miR-6831-5p             | 5.58              | 3.32              | 4.82        | 2.16E-02 |
| hsa-miR-941                 | 4.58              | 2.42              | 4.47        | 3.20E-02 |
| hsa-miR-125a-3p             | 5.68              | 3.54              | 4.4         | 1.84E-02 |
| hsa-miR-5195-3p             | 4.24              | 2.22              | 4.07        | 1.22E-02 |
| hsa-miR-505-3p              | 4.71              | 2.71              | 4           | 1.88E-02 |
| hsa-miR-874-3p              | 7.71              | 5.73              | 3.96        | 3.30E-02 |
| hsa-miR-664b-5p             | 4.66              | 2.68              | 3.94        | 2.80E-03 |
| hsa-let-7d-3p               | 4.36              | 2.42              | 3.84        | 2.41E-02 |
| hsa-miR-34c-3p              | 3.74              | 1.92              | 3.54        | 1.08E-05 |
| hsa-miR-766-3p              | 4.39              | 2.62              | 3.4         | 3.45E-02 |
| hsa-miR-1231                | 6.16              | 4.51              | 3.14        | 2.08E-02 |
| hsa-miR-4269                | 7.08              | 5.47              | 3.06        | 2.30E-02 |
| hsa-miR-6851-5p             | 4.08              | 2.48              | 3.04        | 3.90E-03 |
| hsa-miR-146b-3p             | 5.18              | 3.61              | 2.96        | 4.01E-02 |
| hsa-mir-941-1               | 4.31              | 2.79              | 2.87        | 1.12E-02 |
| hsa-mir-941-2               | 4.31              | 2.79              | 2.87        | 1.12E-02 |
| hsa-mir-941-3               | 4.31              | 2.79              | 2.87        | 1.12E-02 |
| hsa-mir-941-4               | 4.31              | 2.79              | 2.87        | 1.12E-02 |
| hsa-miR-6890-5p             | 3.99              | 2.49              | 2.83        | 1.10E-03 |
| hsa-miR-4725-3p             | 3.84              | 2.42              | 2.66        | 3.00E-03 |
| hsa-miR-24-2-5p             | 3.38              | 2                 | 2.6         | 4.70E-03 |
| hsa-miR-4726-5p             | 3.39              | 2.05              | 2.52        | 5.30E-03 |
| hsa-miR-6165                | 5.78              | 4.45              | 2.52        | 2.04E-02 |
| hsa-miR-8071                | 3.64              | 2.31              | 2.51        | 1.53E-02 |
| hsa-miR-1292-5p             | 4.28              | 2.98              | 2.47        | 1.46E-02 |
| hsa-miR-6769a-5p            | 4.21              | 2.92              | 2.46        | 2.50E-03 |
| hsa-miR-1296-5p             | 4.78              | 3.49              | 2.44        | 4.88E-02 |
| hsa-mir-21                  | 4.55              | 3.26              | 2.43        | 5.80E-03 |
| hsa-miR-371a-5p             | 3.85              | 2.58              | 2.41        | 6.20E-03 |
| hsa-miR-5010-5p             | 4.1               | 2.85              | 2.37        | 4.07E-02 |
| hsa-miR-8060                | 4.05              | 2.82              | 2.34        | 5.40E-03 |
| hsa-miR-492                 | 2.82              | 1.66              | 2.24        | 1.65E-02 |

|                 |       |       |       |          |
|-----------------|-------|-------|-------|----------|
| hsa-mir-3653    | 4.44  | 3.28  | 2.24  | 1.06E-02 |
| hsa-miR-3187-3p | 4.41  | 3.25  | 2.23  | 2.07E-02 |
| hsa-miR-665     | 5.58  | 4.46  | 2.17  | 4.34E-02 |
| hsa-miR-6508-5p | 3.36  | 2.28  | 2.11  | 1.16E-02 |
| hsa-miR-221-3p  | 11.19 | 10.12 | 2.1   | 4.20E-03 |
| hsa-miR-214-3p  | 12.47 | 11.4  | 2.09  | 4.46E-02 |
| hsa-miR-6757-5p | 4.8   | 3.75  | 2.07  | 1.70E-02 |
| hsa-miR-6785-5p | 4.96  | 3.94  | 2.03  | 1.47E-02 |
| hsa-miR-5088-5p | 2.59  | 1.58  | 2.02  | 1.08E-02 |
| hsa-miR-874-5p  | 3.77  | 2.77  | 2.01  | 1.17E-02 |
| hsa-miR-6766-5p | 2.51  | 1.51  | 1.99  | 5.70E-03 |
| hsa-miR-222-3p  | 11.22 | 10.23 | 1.99  | 7.10E-03 |
| hsa-miR-198     | 2.59  | 1.6   | 1.99  | 4.69E-02 |
| hsa-miR-6845-5p | 4.94  | 3.97  | 1.96  | 1.75E-02 |
| hsa-mir-345     | 2.94  | 1.97  | 1.95  | 3.48E-02 |
| hsa-miR-6124    | 5.94  | 4.98  | 1.94  | 2.90E-03 |
| hsa-miR-4657    | 2.67  | 1.72  | 1.94  | 1.90E-03 |
| hsa-miR-23a-3p  | 13.41 | 12.45 | 1.94  | 1.09E-02 |
| hsa-miR-4534    | 5.09  | 4.15  | 1.92  | 4.76E-02 |
| hsa-miR-542-5p  | 2.42  | 1.49  | 1.91  | 4.98E-02 |
| hsa-mir-210     | 2.21  | 1.28  | 1.9   | 6.00E-04 |
| hsa-miR-4743-5p | 5.1   | 4.2   | 1.87  | 1.12E-02 |
| hsa-miR-3154    | 3.3   | 2.4   | 1.86  | 3.12E-02 |
| hsa-miR-3935    | 2.77  | 1.88  | 1.85  | 2.99E-02 |
| hsa-miR-181a-5p | 12.43 | 11.54 | 1.85  | 7.60E-03 |
| hsa-miR-6830-5p | 3.96  | 3.12  | 1.79  | 2.08E-02 |
| hsa-miR-4800-3p | 2.83  | 1.99  | 1.79  | 2.23E-02 |
| hsa-let-7i-5p   | 12.16 | 11.32 | 1.79  | 2.82E-02 |
| hsa-miR-760     | 2.46  | 1.66  | 1.74  | 5.40E-03 |
| hsa-miR-7152-3p | 4.34  | 3.55  | 1.72  | 3.37E-02 |
| hsa-miR-7851-3p | 2.61  | 1.82  | 1.72  | 1.29E-02 |
| hsa-miR-6792-5p | 2.84  | 2.06  | 1.72  | 3.38E-02 |
| hsa-miR-145-5p  | 13.67 | 12.9  | 1.71  | 4.50E-03 |
| hsa-miR-6862-5p | 2.38  | 1.62  | 1.7   | 4.00E-03 |
| hsa-miR-6080    | 2.39  | 1.71  | 1.6   | 1.88E-02 |
| hsa-miR-6795-5p | 4.33  | 3.66  | 1.59  | 3.49E-02 |
| hsa-miR-4722-5p | 2.75  | 2.08  | 1.59  | 4.46E-02 |
| hsa-mir-663b    | 3.19  | 2.52  | 1.59  | 3.68E-02 |
| hsa-miR-6895-5p | 2.53  | 1.88  | 1.57  | 9.50E-03 |
| hsa-miR-4778-5p | 4.16  | 3.51  | 1.57  | 4.05E-02 |
| hsa-miR-187-5p  | 3.27  | 2.64  | 1.55  | 1.16E-02 |
| hsa-mir-887     | 1.85  | 1.24  | 1.52  | 1.79E-02 |
| hsa-miR-4750-3p | 2.12  | 1.52  | 1.52  | 1.99E-02 |
| hsa-miR-181c-3p | 1.97  | 1.37  | 1.51  | 1.50E-02 |
| hsa-miR-4283    | 1.83  | 1.25  | 1.5   | 2.30E-03 |
| hsa-miR-548x-3p | 3.63  | 4.22  | -1.51 | 2.95E-02 |

|                 |      |      |       |          |
|-----------------|------|------|-------|----------|
| hsa-miR-4287    | 1.71 | 2.41 | -1.63 | 1.87E-02 |
| hsa-mir-548e    | 1.31 | 2.02 | -1.64 | 1.10E-02 |
| hsa-mir-375     | 2.11 | 2.85 | -1.67 | 3.16E-02 |
| hsa-miR-3618    | 2.11 | 2.92 | -1.76 | 4.70E-02 |
| hsa-miR-509-5p  | 2.47 | 3.35 | -1.85 | 1.30E-03 |
| hsa-mir-6722    | 6.65 | 7.64 | -2    | 4.62E-02 |
| hsa-miR-4742-5p | 4.23 | 5.27 | -2.06 | 2.15E-02 |
| hsa-mir-139     | 3.83 | 4.97 | -2.2  | 4.92E-02 |
| hsa-miR-451a    | 3.12 | 4.67 | -2.93 | 3.50E-03 |
| hsa-miR-139-5p  | 6.92 | 9.1  | -4.51 | 1.20E-03 |
| hsa-miR-486-5p  | 5.28 | 7.5  | -4.65 | 1.33E-02 |
| hsa-miR-148a-5p | 1.7  | 4.73 | -8.15 | 4.08E-02 |

*Supplementary Table S2: List of differentially expressed (DE) miRNA in PDX F1 samples vs tumour samples comparison*

| <b>Transcript ID(Array Design)</b> | <b>F1 Avg (log2)</b> | <b>Tumour Avg (log2)</b> | <b>Fold Change</b> | <b>P-val</b> |
|------------------------------------|----------------------|--------------------------|--------------------|--------------|
| hsa-miR-206                        | 6.58                 | 1.88                     | 26.01              | 8.59E-05     |
| hsa-miR-4521                       | 5.85                 | 1.25                     | 24.27              | 4.48E-06     |
| hsa-miR-6872-5p                    | 8.5                  | 4.1                      | 21.19              | 1.35E-07     |
| hsa-miR-1290                       | 6.62                 | 2.67                     | 15.4               | 6.80E-03     |
| hsa-miR-486-5p                     | 8.93                 | 5.28                     | 12.48              | 1.40E-03     |
| hsa-miR-615-3p                     | 6.11                 | 2.68                     | 10.81              | 5.59E-05     |
| hsa-miR-203a                       | 7.44                 | 4.2                      | 9.43               | 2.30E-03     |
| hsa-miR-6778-5p                    | 9.99                 | 6.89                     | 8.56               | 5.42E-08     |
| hsa-miR-2467-3p                    | 6.06                 | 3.08                     | 7.91               | 2.01E-08     |
| hsa-miR-139-5p                     | 9.67                 | 6.92                     | 6.7                | 2.78E-06     |
| hsa-miR-29b-1-5p                   | 6.94                 | 4.39                     | 5.82               | 2.64E-02     |
| hsa-miR-6754-3p                    | 4.23                 | 1.7                      | 5.77               | 2.00E-04     |
| hsa-miR-4656                       | 8.51                 | 6.03                     | 5.59               | 2.07E-06     |
| hsa-miR-138-5p                     | 4.83                 | 2.36                     | 5.55               | 1.60E-02     |
| hsa-mir-3154                       | 7.48                 | 5.12                     | 5.14               | 1.60E-05     |
| hsa-miR-4298                       | 10.15                | 7.8                      | 5.09               | 1.59E-05     |
| hsa-miR-4485                       | 9.94                 | 7.62                     | 5.01               | 5.10E-03     |
| hsa-miR-196a-5p                    | 4.1                  | 1.89                     | 4.64               | 4.00E-03     |
| hsa-miR-378d                       | 7.16                 | 5.01                     | 4.44               | 4.30E-02     |
| hsa-miR-378e                       | 4.15                 | 2.08                     | 4.2                | 1.10E-03     |
| hsa-miR-652-3p                     | 10.28                | 8.26                     | 4.06               | 1.35E-07     |
| hsa-miR-378i                       | 8.51                 | 6.57                     | 3.84               | 2.84E-02     |
| hsa-miR-133a-3p                    | 3.46                 | 1.53                     | 3.83               | 2.35E-02     |
| hsa-mir-139                        | 5.75                 | 3.83                     | 3.77               | 7.02E-05     |
| hsa-miR-378g                       | 6.35                 | 4.45                     | 3.72               | 3.91E-02     |

|                 |       |       |       |          |
|-----------------|-------|-------|-------|----------|
| hsa-miR-378c    | 9.39  | 7.51  | 3.67  | 9.00E-04 |
| hsa-miR-3911    | 6.56  | 4.88  | 3.21  | 1.40E-03 |
| hsa-miR-211-5p  | 2.78  | 1.1   | 3.21  | 1.11E-02 |
| hsa-miR-378a-3p | 10.67 | 9.11  | 2.95  | 1.29E-05 |
| hsa-mir-1973    | 3.51  | 2.02  | 2.8   | 6.00E-04 |
| hsa-mir-6872    | 3.11  | 1.67  | 2.71  | 7.41E-05 |
| hsa-miR-215-5p  | 2.94  | 1.52  | 2.67  | 1.21E-02 |
| hsa-miR-34c-5p  | 3.75  | 2.36  | 2.62  | 1.74E-02 |
| hsa-miR-3935    | 4.09  | 2.77  | 2.5   | 1.59E-02 |
| hsa-miR-612     | 3.91  | 2.59  | 2.5   | 2.40E-02 |
| hsa-miR-7847-3p | 9.86  | 8.55  | 2.49  | 1.20E-03 |
| hsa-miR-4669    | 6.98  | 5.69  | 2.45  | 6.50E-03 |
| hsa-miR-7162-3p | 6.65  | 5.37  | 2.44  | 8.60E-03 |
| hsa-miR-4484    | 9.09  | 7.9   | 2.28  | 9.30E-03 |
| hsa-miR-222-3p  | 12.4  | 11.22 | 2.27  | 2.41E-02 |
| hsa-miR-133b    | 2.59  | 1.42  | 2.25  | 4.38E-02 |
| hsa-miR-1910-3p | 4.85  | 3.68  | 2.25  | 2.72E-02 |
| hsa-miR-6723-5p | 6.2   | 5.15  | 2.07  | 6.40E-03 |
| hsa-miR-4448    | 2.84  | 1.84  | 2     | 4.62E-02 |
| hsa-miR-451a    | 4.11  | 3.12  | 1.98  | 2.64E-02 |
| hsa-miR-93-5p   | 11.09 | 10.13 | 1.95  | 2.70E-02 |
| hsa-miR-4743-5p | 6.06  | 5.1   | 1.94  | 2.20E-03 |
| hsa-miR-34a-5p  | 9.72  | 8.79  | 1.9   | 3.65E-02 |
| hsa-miR-4801    | 3.12  | 2.21  | 1.87  | 1.80E-03 |
| hsa-miR-4534    | 5.96  | 5.09  | 1.83  | 3.49E-02 |
| hsa-miR-6504-5p | 2.04  | 1.18  | 1.81  | 1.03E-02 |
| hsa-miR-6831-5p | 6.44  | 5.58  | 1.81  | 1.32E-02 |
| hsa-mir-31      | 2.63  | 1.8   | 1.77  | 1.88E-02 |
| hsa-miR-4445-3p | 3.12  | 2.31  | 1.75  | 1.44E-02 |
| hsa-mir-3123    | 2.81  | 2.04  | 1.71  | 1.30E-03 |
| hsa-miR-196b-5p | 2.04  | 1.27  | 1.71  | 1.31E-02 |
| hsa-mir-4485    | 3.96  | 3.21  | 1.69  | 3.90E-03 |
| hsa-miR-140-3p  | 10.25 | 9.53  | 1.65  | 1.68E-02 |
| hsa-miR-6880-5p | 6.45  | 5.75  | 1.63  | 3.62E-02 |
| hsa-miR-210-5p  | 2.89  | 2.19  | 1.62  | 3.70E-03 |
| hsa-mir-3935    | 2.64  | 1.95  | 1.62  | 1.32E-02 |
| hsa-miR-16-5p   | 11.58 | 10.89 | 1.61  | 4.66E-02 |
| hsa-miR-15b-5p  | 11.24 | 10.58 | 1.58  | 8.60E-03 |
| hsa-miR-298     | 2.14  | 1.49  | 1.57  | 4.61E-02 |
| hsa-miR-6819-5p | 7.65  | 7.02  | 1.55  | 3.15E-02 |
| hsa-miR-4793-3p | 1.94  | 1.31  | 1.55  | 2.05E-02 |
| hsa-miR-718     | 2.47  | 1.88  | 1.5   | 4.30E-02 |
| hsa-miR-320c    | 12.23 | 12.82 | -1.5  | 1.17E-02 |
| hsa-miR-6503-5p | 1.1   | 1.69  | -1.51 | 8.40E-03 |
| hsa-mir-320a    | 7.82  | 8.42  | -1.51 | 1.05E-02 |
| hsa-miR-4792    | 1.34  | 1.94  | -1.52 | 8.30E-03 |

|                  |       |       |       |          |
|------------------|-------|-------|-------|----------|
| hsa-miR-383-5p   | 1.36  | 1.96  | -1.52 | 2.43E-02 |
| hsa-miR-6511a-5p | 4.14  | 4.74  | -1.52 | 1.44E-02 |
| hsa-miR-4787-5p  | 12.47 | 13.07 | -1.52 | 1.46E-02 |
| hsa-mir-4477a    | 1.79  | 2.41  | -1.54 | 1.00E-02 |
| hsa-miR-570-5p   | 1.44  | 2.06  | -1.54 | 2.54E-02 |
| hsa-miR-548ai    | 1.44  | 2.06  | -1.54 | 2.54E-02 |
| hsa-miR-132-5p   | 1.3   | 1.93  | -1.54 | 1.91E-02 |
| hsa-mir-4477a    | 1.77  | 2.4   | -1.54 | 2.80E-03 |
| hsa-mir-155      | 1.64  | 2.3   | -1.58 | 2.01E-02 |
| hsa-miR-628-5p   | 3.75  | 4.41  | -1.58 | 1.80E-02 |
| hsa-miR-329-3p   | 1.17  | 1.83  | -1.58 | 2.06E-02 |
| hsa-miR-3656     | 11.08 | 11.74 | -1.59 | 2.44E-02 |
| hsa-miR-4725-5p  | 1.38  | 2.06  | -1.6  | 1.01E-02 |
| hsa-miR-6784-5p  | 3.98  | 4.68  | -1.62 | 3.15E-02 |
| hsa-miR-199b-5p  | 1.24  | 1.95  | -1.63 | 8.50E-03 |
| hsa-mir-6836     | 4.48  | 5.2   | -1.64 | 2.14E-02 |
| hsa-miR-99b-5p   | 10.95 | 11.66 | -1.64 | 1.93E-02 |
| hsa-miR-6084     | 1.46  | 2.18  | -1.65 | 1.46E-02 |
| hsa-miR-638      | 11.1  | 11.83 | -1.65 | 3.21E-02 |
| hsa-mir-572      | 1.92  | 2.64  | -1.65 | 1.70E-03 |
| hsa-miR-6125     | 11.56 | 12.28 | -1.65 | 7.00E-03 |
| hsa-let-7f-1-3p  | 1.63  | 2.35  | -1.65 | 3.50E-03 |
| hsa-mir-487b     | 1.02  | 1.75  | -1.67 | 3.00E-04 |
| hsa-miR-6075     | 6.49  | 7.24  | -1.68 | 1.76E-02 |
| hsa-miR-320d     | 11.09 | 11.84 | -1.68 | 1.55E-02 |
| hsa-miR-1273h-5p | 3.43  | 4.19  | -1.7  | 3.86E-02 |
| hsa-miR-4638-5p  | 1.89  | 2.67  | -1.72 | 1.16E-02 |
| hsa-mir-548ag-1  | 2.92  | 3.7   | -1.72 | 2.71E-02 |
| hsa-miR-543      | 1.79  | 2.6   | -1.75 | 3.86E-02 |
| hsa-miR-323a-3p  | 1.88  | 2.69  | -1.76 | 6.20E-03 |
| hsa-miR-3187-3p  | 3.6   | 4.41  | -1.76 | 4.26E-02 |
| hsa-mir-145      | 2.06  | 2.88  | -1.77 | 1.12E-05 |
| hsa-miR-665      | 4.76  | 5.58  | -1.77 | 2.45E-02 |
| hsa-miR-3122     | 2.04  | 2.87  | -1.78 | 3.64E-02 |
| hsa-miR-1227-5p  | 8.4   | 9.25  | -1.8  | 3.69E-02 |
| hsa-miR-125b-5p  | 12.74 | 13.6  | -1.81 | 2.30E-03 |
| hsa-miR-1273f    | 2.49  | 3.38  | -1.85 | 6.90E-03 |
| hsa-miR-7108-5p  | 9.97  | 10.86 | -1.86 | 1.24E-02 |
| hsa-miR-3185     | 7.39  | 8.29  | -1.86 | 2.16E-02 |
| hsa-miR-129-2-3p | 1.76  | 2.66  | -1.87 | 1.05E-02 |
| hsa-miR-7851-3p  | 1.7   | 2.61  | -1.88 | 9.80E-03 |
| hsa-miR-6737-5p  | 3.24  | 4.18  | -1.92 | 1.90E-02 |
| hsa-miR-6790-5p  | 6.39  | 7.33  | -1.92 | 3.02E-02 |
| hsa-miR-130a-3p  | 7.05  | 7.99  | -1.93 | 1.25E-02 |
| hsa-miR-34c-3p   | 2.77  | 3.74  | -1.97 | 9.00E-04 |
| hsa-miR-6511b-3p | 1.33  | 2.31  | -1.97 | 2.22E-02 |

|                   |       |       |       |          |
|-------------------|-------|-------|-------|----------|
| hsa-miR-132-3p    | 8.55  | 9.53  | -1.98 | 1.65E-02 |
| hsa-miR-381-3p    | 2.02  | 3.02  | -2    | 4.64E-02 |
| hsa-mir-487b      | 0.85  | 1.86  | -2.01 | 2.00E-04 |
| hsa-miR-1825      | 1.96  | 2.98  | -2.03 | 2.37E-02 |
| hsa-miR-887-3p    | 1.41  | 2.45  | -2.05 | 4.60E-03 |
| hsa-miR-4467      | 9.23  | 10.27 | -2.06 | 3.14E-02 |
| hsa-miR-6754-5p   | 2.31  | 3.35  | -2.06 | 2.32E-02 |
| hsa-miR-4417      | 5.61  | 6.68  | -2.09 | 3.62E-02 |
| hsa-miR-650       | 2.27  | 3.35  | -2.12 | 5.60E-03 |
| hsa-miR-214-3p    | 11.38 | 12.47 | -2.13 | 3.01E-02 |
| hsa-miR-4646-5p   | 4.28  | 5.39  | -2.16 | 2.81E-02 |
| hsa-miR-3917      | 2.04  | 3.16  | -2.16 | 4.10E-02 |
| hsa-miR-542-5p    | 1.31  | 2.42  | -2.17 | 2.10E-03 |
| hsa-miR-2861      | 10.2  | 11.34 | -2.2  | 3.00E-03 |
| hsa-miR-23c       | 3.75  | 4.9   | -2.21 | 4.99E-02 |
| hsa-miR-494-3p    | 7.91  | 9.05  | -2.21 | 3.66E-02 |
| hsa-miR-493-5p    | 1.19  | 2.34  | -2.21 | 7.40E-03 |
| hsa-miR-5572      | 4.37  | 5.52  | -2.22 | 3.50E-02 |
| hsa-miR-199a-3p   | 10.08 | 11.24 | -2.25 | 1.03E-02 |
| hsa-miR-199b-3p   | 10.08 | 11.24 | -2.25 | 1.03E-02 |
| hsa-miR-574-3p    | 9.87  | 11.05 | -2.26 | 5.60E-03 |
| hsa-miR-1304-3p   | 1.35  | 2.55  | -2.3  | 1.00E-04 |
| hsa-miR-874-3p    | 6.5   | 7.71  | -2.32 | 3.32E-02 |
| hsa-mir-424       | 2.83  | 4.06  | -2.35 | 1.80E-03 |
| hsa-mir-3687      | 2.85  | 4.09  | -2.35 | 5.60E-03 |
| hsa-miR-491-5p    | 3.52  | 4.76  | -2.36 | 4.08E-02 |
| hsa-miR-197-3p    | 7.44  | 8.7   | -2.41 | 1.14E-02 |
| hsa-miR-1291      | 2.06  | 3.33  | -2.41 | 1.30E-02 |
| hsa-miR-3615      | 2.58  | 3.9   | -2.5  | 9.30E-03 |
| hsa-miR-7641      | 10.2  | 11.53 | -2.52 | 2.40E-02 |
| hsa-miR-940       | 3.13  | 4.47  | -2.53 | 1.10E-03 |
| hsa-mir-125b-2    | 0.85  | 2.2   | -2.55 | 2.00E-04 |
| hsa-miR-3195      | 7.54  | 8.9   | -2.56 | 2.87E-02 |
| hsa-miR-375       | 10.11 | 11.49 | -2.59 | 4.15E-02 |
| hsa-miR-6511a-3p  | 2.13  | 3.54  | -2.66 | 3.90E-03 |
| hsa-miR-6508-5p   | 1.86  | 3.36  | -2.82 | 1.00E-03 |
| hsa-miR-874-5p    | 2.27  | 3.77  | -2.84 | 7.00E-04 |
| hsa-miR-193a-5p   | 7.86  | 9.38  | -2.86 | 1.59E-02 |
| hsa-miR-4730      | 5.49  | 7.01  | -2.88 | 2.65E-02 |
| hsa-miR-1270      | 1.55  | 3.09  | -2.91 | 2.27E-02 |
| hsa-miR-125b-1-3p | 3.86  | 5.43  | -2.98 | 2.26E-02 |
| hsa-miR-150-5p    | 8.14  | 9.73  | -3.01 | 2.70E-02 |
| hsa-miR-487a-3p   | 1.35  | 2.99  | -3.11 | 1.40E-03 |
| hsa-miR-146b-5p   | 6.62  | 8.27  | -3.14 | 5.00E-04 |
| hsa-miR-6068      | 6.66  | 8.32  | -3.16 | 2.00E-03 |
| hsa-miR-4310      | 1.52  | 3.2   | -3.21 | 7.00E-04 |

|                 |       |       |        |          |
|-----------------|-------|-------|--------|----------|
| hsa-miR-504-5p  | 1.46  | 3.17  | -3.27  | 3.53E-02 |
| hsa-miR-409-5p  | 2.33  | 4.06  | -3.32  | 4.14E-02 |
| hsa-miR-99a-5p  | 8.58  | 10.31 | -3.32  | 1.10E-03 |
| hsa-miR-337-5p  | 1.41  | 3.15  | -3.34  | 3.00E-04 |
| hsa-miR-134-5p  | 5.37  | 7.11  | -3.34  | 2.80E-03 |
| hsa-miR-199a-5p | 8.7   | 10.49 | -3.45  | 4.00E-04 |
| hsa-miR-3653    | 2.65  | 4.44  | -3.46  | 8.00E-04 |
| hsa-miR-4269    | 5.29  | 7.08  | -3.46  | 5.00E-03 |
| hsa-miR-6069    | 2     | 3.82  | -3.51  | 2.40E-03 |
| hsa-miR-766-3p  | 2.55  | 4.39  | -3.58  | 1.60E-03 |
| hsa-miR-1238-3p | 1.49  | 3.37  | -3.68  | 2.10E-03 |
| hsa-miR-193b-3p | 8.03  | 9.95  | -3.78  | 1.37E-02 |
| hsa-miR-145-5p  | 11.73 | 13.67 | -3.83  | 1.90E-08 |
| hsa-miR-143-3p  | 8.48  | 10.43 | -3.87  | 1.07E-05 |
| hsa-miR-505-3p  | 2.73  | 4.71  | -3.95  | 5.80E-03 |
| hsa-miR-664b-5p | 2.65  | 4.66  | -4.03  | 8.50E-05 |
| hsa-miR-1281    | 5.22  | 7.26  | -4.11  | 3.60E-03 |
| hsa-miR-4324    | 2.66  | 4.74  | -4.22  | 3.00E-03 |
| hsa-miR-572     | 4.46  | 6.55  | -4.24  | 1.60E-03 |
| hsa-miR-3687    | 2.78  | 4.91  | -4.38  | 6.70E-03 |
| hsa-miR-193b-5p | 4.2   | 6.37  | -4.51  | 4.30E-02 |
| hsa-miR-654-5p  | 1.45  | 3.69  | -4.72  | 2.00E-04 |
| hsa-miR-21-3p   | 4.1   | 6.37  | -4.81  | 9.20E-03 |
| hsa-miR-485-3p  | 3.43  | 5.71  | -4.88  | 5.40E-03 |
| hsa-miR-143-5p  | 1.52  | 3.84  | -4.97  | 1.42E-05 |
| hsa-miR-664b-3p | 3.05  | 5.42  | -5.16  | 4.11E-02 |
| hsa-miR-100-5p  | 7.69  | 10.09 | -5.27  | 2.00E-04 |
| hsa-miR-5571-5p | 1.98  | 4.39  | -5.3   | 4.00E-04 |
| hsa-miR-155-5p  | 8.04  | 10.47 | -5.4   | 1.11E-02 |
| hsa-miR-377-5p  | 1.26  | 3.72  | -5.49  | 6.00E-04 |
| hsa-miR-1247-5p | 2.84  | 5.31  | -5.55  | 5.10E-03 |
| hsa-miR-493-3p  | 1.17  | 3.78  | -6.1   | 3.85E-06 |
| hsa-miR-371b-5p | 3.77  | 6.39  | -6.11  | 6.00E-04 |
| hsa-miR-191-3p  | 2.16  | 4.79  | -6.2   | 4.22E-05 |
| hsa-miR-485-5p  | 3.27  | 5.98  | -6.58  | 1.70E-03 |
| hsa-miR-370-3p  | 2.78  | 5.55  | -6.8   | 1.00E-04 |
| hsa-miR-1296-5p | 1.99  | 4.78  | -6.92  | 4.00E-04 |
| hsa-miR-654-3p  | 1.15  | 4.17  | -8.14  | 4.27E-05 |
| hsa-miR-216b-5p | 3.07  | 6.32  | -9.49  | 1.86E-02 |
| hsa-miR-487a-5p | 1.29  | 4.66  | -10.3  | 4.86E-05 |
| hsa-miR-1301-3p | 3.11  | 6.6   | -11.22 | 1.80E-03 |
| hsa-miR-409-3p  | 4.45  | 7.96  | -11.45 | 1.47E-06 |
| hsa-miR-146b-3p | 1.51  | 5.18  | -12.7  | 6.39E-06 |
| hsa-miR-217     | 1.19  | 4.94  | -13.39 | 1.98E-02 |
| hsa-miR-503-5p  | 2.47  | 6.31  | -14.24 | 2.30E-03 |
| hsa-miR-1271-5p | 2.13  | 6.22  | -17.01 | 1.88E-06 |

|                   |      |      |         |          |
|-------------------|------|------|---------|----------|
| hsa-miR-125b-2-3p | 1.83 | 6.25 | -21.33  | 6.58E-07 |
| hsa-miR-708-5p    | 1.68 | 6.11 | -21.66  | 2.00E-03 |
| hsa-miR-487b-3p   | 2.48 | 7.58 | -34.33  | 1.31E-09 |
| hsa-miR-424-3p    | 1.41 | 7.24 | -56.66  | 2.27E-06 |
| hsa-miR-432-5p    | 1.46 | 8.29 | -113.44 | 2.10E-13 |



Article

# miRNA- and Cell Line-Specific Constraints on Precursor miRNA Processing of Stably Transfected Pancreatic Cancer and Other Mammalian Cells

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**Abstract:** MicroRNAs (miRNAs) regulate approximately one-third of all human genes. The dysregulation of miRNAs has been implicated in the development of numerous human diseases, including cancers. In our investigation focusing on altering specific miRNA expression in human pancreatic cancer cells, we encountered an interesting finding. While two expression vector designs effectively enhanced miR-708 levels, they were unable to elevate mature forms of miR-29b, -1290, -2467, and -6831 in pancreatic cancer cell lines. This finding was also observed in a panel of other non-pancreatic cancer cell lines, suggesting that miRNA processing efficiency was cell line specific. Using a step-by-step approach in each step of miRNA processing, we ruled out alternative strand selection by the RISC complex and transcriptional interference at the primary miRNA (pri-miRNA) level. DROSHA processing and pri-miRNA export from the nucleus also appeared to be occurring normally. We observed precursor (pre-miRNA) accumulation only in cell lines where mature miRNA expression was not achieved, suggesting that the block was occurring at the pre-miRNA stage. To further confirm this, synthetic pre-miRNA mimics that bypass DICER processing were processed into mature miRNAs in all cases. This study has demonstrated the distinct behaviours of different miRNAs with the same vector in the same cell line, the same miRNA between the two vector designs, and with the same miRNA across different cell lines. We identified a stable vector pre-miRNA processing block. Our findings on the structural and sequence differences between successful and non-successful vector designs could help to inform future chimeric miRNA design strategies and act as a guide to other researchers on the intricate processing dynamics that can impact vector efficiency. Our research confirms the potential of miRNA mimics to surmount some of these complexities.

**Keywords:** vector design; miRNA processing; precursor miRNA; stable transfections; miRNAs; pancreatic cancer



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## 1. Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs approximately 22 nucleotides (nt) in length that regulate gene expression [1]. Since their discovery in the nematode *Caenorhabditis elegans* in 1993 by the Ambros and Ruvkun group [2], members of this endogenous class of RNAs have received considerable attention due to their roles in diverse biological functions, including the regulation of cell development, differentiation, and survival [3]. Highlighting their importance, around one-third of all human genes are known to be regulated by miRNAs, with approximately 2469 miRNAs identified to

date [4]. The dysregulation of miRNAs has been implicated in the development and progression of numerous human diseases, including cancers [5]. To investigate the function of miRNAs, we need robust methods for their overexpression, both in vitro and in vivo, that mimic natural mechanisms. Overexpression of miRNAs is achieved via one of two strategies: synthetic mature miRNAs or miRNA-expressing DNA vectors. DNA vectors can either be constitutively active or have inducible expression systems. Constitutively active plasmids may interfere with cellular functions, including cell death. Therefore, user-defined heterologous miRNA regulation is favoured. Inducible systems reveal the direct consequence of certain genetic manipulations and are especially beneficial when working with mammalian cell lines that are controlled by highly intricate genetic networks [6].

The canonical miRNA biogenesis pathway constitutes three steps: (1) Transcription and processing of primary miRNAs (pri-miRNAs) into hairpin-structured precursor miRNAs (pre-miRNAs) (~60–70 nucleotides) by the microprocessor complex, consisting of DROSHA (a ribonuclease III enzyme) and its cofactor, DGCR8 [3,7,8]; (2) Export of pre-miRNAs to the cytoplasm via the exportin 5 (XPO5)/RanGTP complex, followed by processing by DICER, an RNase III endonuclease. DICER recognizes the ends of pre-miRNA and cleaves the dsRNA stem, yielding a ~22 nucleotide mature miRNA duplex with 2 nt 3' overhangs; (3) Loading of the guide strand of the mature miRNA duplex into Argonaute (AGO) proteins, forming an miRNA-induced silencing complex (miRISC). This complex, along with GW182 proteins, guides the miRISC to target mRNAs for silencing [3,8].

Dysfunctional microprocessing machinery has been previously described in cancer, where mutations due to XPO5 cause pre-miRNAs to accumulate in the nucleus, leading to decreased mature miRNA levels [2,9]. In addition, proteins frequently dysfunctional in cancer cells, such as p53, which also functions as a modulator in miRNA processing, could attenuate the efficient production of mature miRNAs [10]. Meanwhile, the processing machinery can become saturated via prolonged overexpression of miRNAs via expression vectors [11]. These findings suggest that impaired processing in cancer cells needs to be taken into great consideration when designing miRNA expression vectors.

In previous studies from our group, we carried out a global comparison of miRNAs in human pancreatic cancer tumours, adjacent normal tissue, and matched patient-derived xenograft models (PDX) using microarray screening [12,13]. From this screen, we compiled a list of priority miRNAs that were overexpressed in both comparisons between normal and tumour and tumour and PDX. To investigate the role of these priority miRNAs, we generated stable overexpression vectors for each. This study addresses the obstacles in designing stable miRNA expression vectors for cancer cells. Despite using a similar construct as miR-708, overexpression vectors for miR-29b-1-5p, -1290, -2467-3p, and -6831-5p failed to elevate the levels of mature miRNAs above background endogenous levels in MIA PaCa-2 and PANC-1 cells. Due to the high levels of expression achieved by miR-708, it was chosen as a positive control for downstream experiments. In order to understand why these miRNAs were not expressed, we followed each of them through the sequential processing steps in MIA PaCa-2 cells while also investigating expression and processing in a number of other cell types.

## 2. Results

### 2.1. RNA Secondary Structure and Primary Sequence Features Are Key Elements Responsible for Efficient miRNA Processing

From our initial list of priority miRNAs that can be found in our previous publications [12,13], initial attempts to overexpress miR-29b, -1290, -2467-3p, and -6831-5p using overexpression vectors (described in methods) were unsuccessful, leading to no detectable expression of mature miRNAs by RT-qPCR in either MIA PaCa-2 or PANC-1 cells as shown in the figure in Section 2.5. Compared to the four miRNAs listed above, miR-708-5p showed the highest levels of mature miRNA levels in MIA PaCa-2 (RQ = 4331) and PANC-1 (RQ = 419) and was chosen as the positive control miRNA for follow-up studies. Figure 1 displays the predicted secondary structure of the designed miRNAs with 70 base pair

genomic flanking regions (referred to as design 1). As miR-708 was successfully expressed, and to eliminate the possibility that the secondary structures and/or primary sequence motifs of the four problem miRNAs were unsuitable for recognition by the microprocessing machinery, these four miRNAs were re-cloned into the genomic flanking region of miR-708 (referred to as design 2). The assessment of predicted secondary structures of designed miRNAs and their impact on miRNA processing involves multiple criteria. The predicted secondary structure should have low minimum free energy (MFE), indicating stable folding [14]. RNA folding algorithms like Vienna RNAfold used in this study allow for prediction of MFE [15] and, as can be observed in Supplementary Table S4, changing the flanking regions to that of miR-708 resulted in an altered MFE with a reduction in MFE for miR-29b and -2467. However, it is generally insufficient to use MFE alone for the detection of stable folding. As a result, more structural features were also analysed, such as seed region accessibility. Inaccessible or highly structured seed regions may reduce miRNA targeting efficiency [16,17]. Design 2 resulted in more linear structures (Figure 1). In addition, accessibility of cleavage sites and the presence of bulges and loops are important for determining processing efficiency [18,19]. DICER enzyme cleaves miRNA precursors at specific sites. These cleavage sites should be accessible within the secondary structure for efficient processing. The more linear structures of design 2 suggested greater accessibility for processing machinery with DROSHA processing sites marked by arrows in Figure 1 that showed a reduced number of bulges and loops especially for miR-29b and -6831 in the areas important for processing machinery. Only MIA PaCa-2 cells were brought forward for further analysis. Following transfection of design 2 into MIA PaCa-2 cells, no levels of mature miRNAs were detectable by RT-qPCR (see figure in Section 2.5).

## *2.2. An Intrinsic Bias for Processing of the Star form miRNA Could Be Responsible for the Lack of Detectable Expression of miRNA Targets*

Mia PaCa-2 cells were examined by RT-qPCR for expression of the passenger strand of miR-2467 (-5p) and miR-708 (-3p) (Figure 2). miR-708 achieved detectable levels of mature miR-708-5p in MIA PaCa-2 cells and was used as a control. miR-708-3p showed no detection and miR-2467-5p had a Ct value of 36. It is well accepted that a Ct value greater than 35 qualifies as non-expressed. The results confirmed that the lack of detection of the mature form of miR-2467-3p was not due to preferential selection for its corresponding passenger strand.

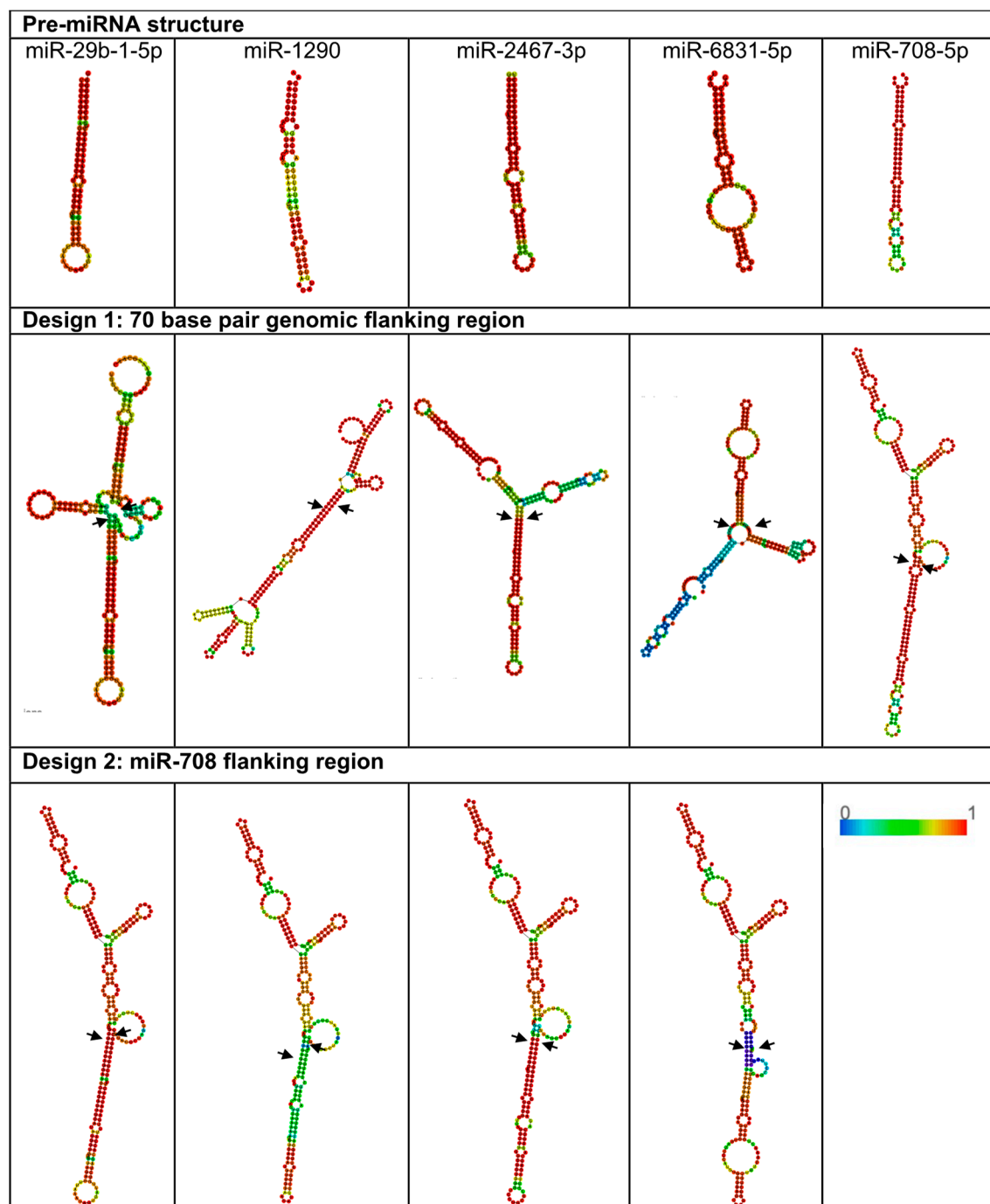


**Figure 1.** Predicted RNA secondary structures for vector designs 1 and 2. Row 1 shows the pre-miRNA structures for miR-29b, -1290, -2467, -6831, and -708 generated using Vienna RNAfold server. Each column contains the corresponding secondary structure for each miRNA target with endogenous 70 base pair flanking regions (design 1) and miR-708 flanking regions (design 2). Arrows indicate DROSHA processing sites and colours indicate base pairing probability from 0–1 as shown in the scale bar with 1 being most probable base pairing.

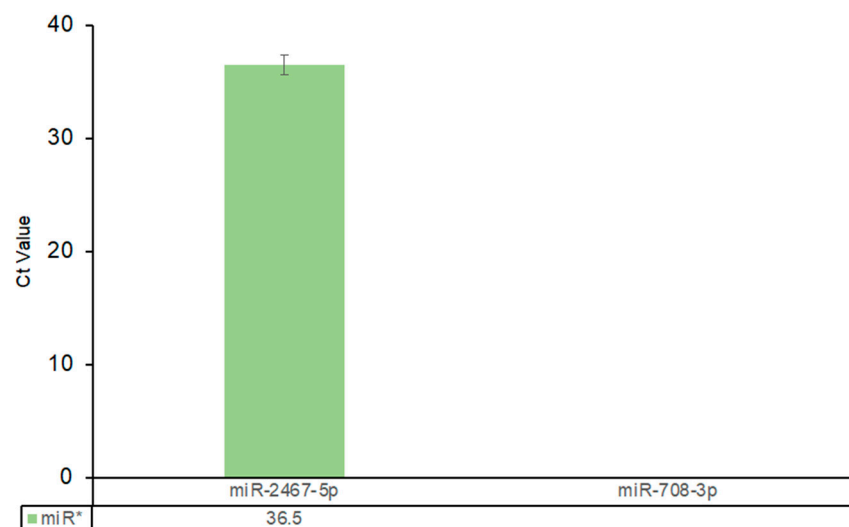


**Figure 2.** Evaluation of miRNA\* expression levels shows preferential processing of the star form is not responsible for the lack of detection in MIA PaCa-2 cells. The data are presented as mean and standard deviation.

### 2.3. Examination of Pri- vs. Pre-miRNA Levels in Nuclear vs. Cytoplasmic Fractions of MIA PaCa-2 Cells Showed Precursor Accumulation

The assessment of primary (pri-miRNA) and precursor (pre-miRNA) miRNA levels was conducted using the probes detailed in the methods section, targeting nuclear and cytoplasmic fractions of both parental and overexpression vector-transfected cells. The outcomes validated the successful transfection and transcription of miRNAs, as evidenced by higher levels of pri-miRNAs in the nuclear fractions of transfected cells compared to parental controls, observed through a reduced Ct value for the transfected cells, as shown in Figure 3, Panel A. Endogenous expression levels of parental miRNAs are provided in Supplementary Table S2. Intriguingly, despite the majority of these miRNAs not being detectable in MIA PaCa-2 cells, discernible levels of pri-miRNA persisted, indicating that pri-miRNA expression did not display a direct correlation with mature expression.

Within the cytoplasmic fraction of both parental and transfected cells, pre-miR-708 levels remained undetectable. Notably, pre-miR-708 was the sole miRNA that attained successful maturation. The absence of pre-miR-708 aligned with the hypothesis that this specific miRNA encountered no processing hindrances, thereby leading to the notable miRNA maturation. Conversely, the other miRNAs with no detectable mature miRNA levels demonstrated substantial levels of expression in the precursor form within the cytoplasmic fractions: pre-miR-29b (Ct = 24.51), pre-miR-1290 (Ct = 25.06), pre-miR-2467 (Ct = 29.66), and pre-miR-6831 (Ct = 23.32). This observation suggested the effective processing and export of pri-miRNAs from the nucleus but a subsequent stall in pre-miRNA processing. Our findings demonstrated cytoplasmic accumulation of precursor miRNAs for the subset of miRNAs. By contrast, miR-708, capable of generating detectable mature miRNA, evaded precursor accumulation due to its proficient processing.

These results collectively emphasised the complex interplay between nuclear processing, cytoplasmic transport, and miRNA maturation, shedding light on the distinct fates of individual miRNAs within the cell.

### 2.4. Post DICER-Processed Pre-miRNA Mimics Show Efficient Overexpression

To evaluate the absence of mature miRNAs, we employed pre-miR miRNA mimics, bypassing DICER processing. Our results (Figure 3, Panel B) highlighted the remarkable efficiency of these mimics in achieving a robust abundance of the targeted miRNAs, a feat unattainable through our stable vector approach. Each miRNA mimic led to substantial mature miRNA levels, reaching an impressive RQ of up to 105943 for miR-2467, which ex-

hibited no expression of mature miRNA in parental cells and remained undetectable using both vector designs (refer to Section 2.5). Notably, miR-1290 and miR-6831 demonstrated RQ values of 519.5 and 1535.7, respectively. In comparison, miR-29b, which displayed moderate expression in parental MIA PaCa-2 cells, exhibited the lowest level of overexpression, with an RQ of 86.8. These outcomes underscored the significance of precursor-level dysregulation in hindering miRNA maturation and highlighted the potency of miRNA mimics in addressing this limitation.

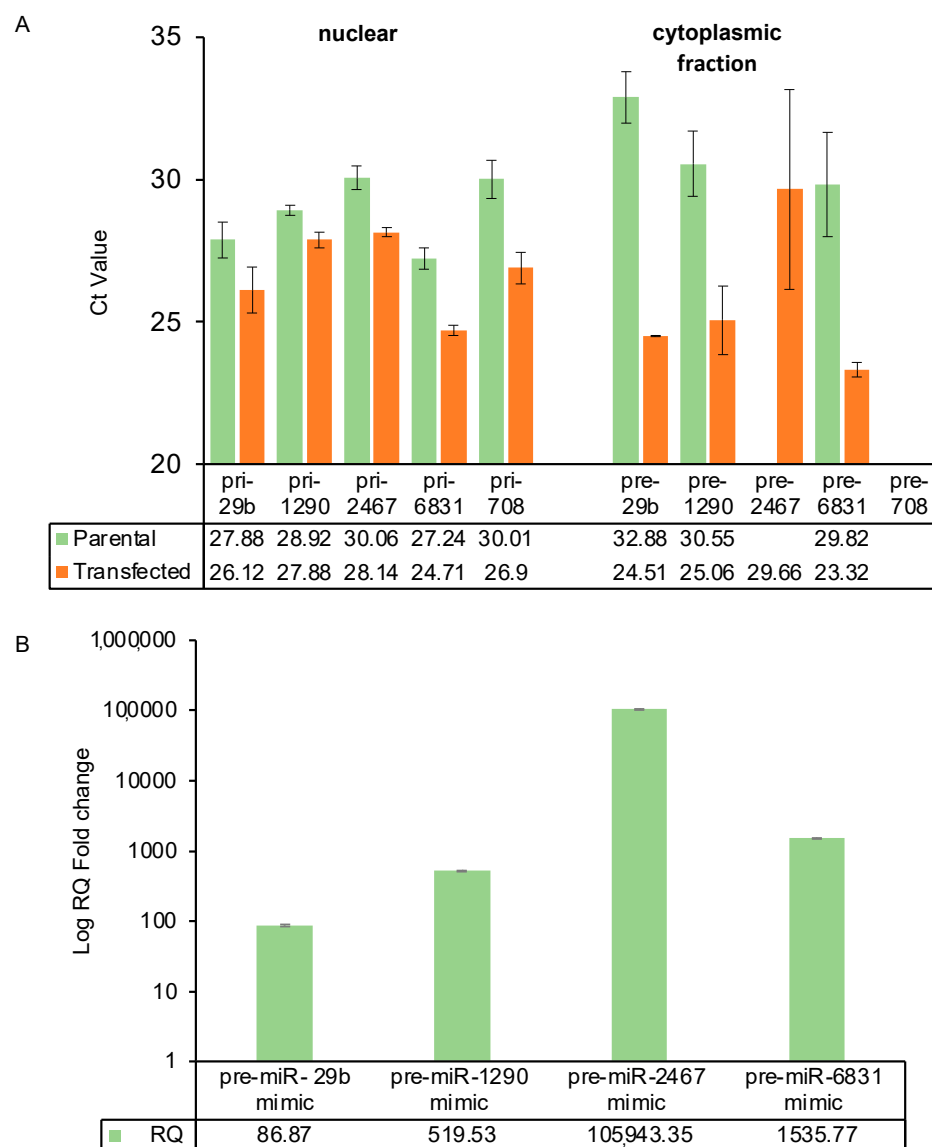


**Figure 3.** Subcellular localisation and overexpression using pre-miRNAs in MIA PaCa-2 Cells. (Panel A): Examining pri- and pre-miRNA expression in nuclear and cytoplasmic fractions reveals the accumulation of precursors specifically in the cytoplasm of MIA PaCa-2 cells. Pri-miRNA probes were used in the nuclear fraction of cells while pre-miRNA probes were used in the cytoplasmic fraction. Data presented as mean and standard deviation of three biological replicates. (Panel B): Post-DICER processed pre-miRNA mimics exhibit efficient overexpression, presented as relative quantification (RQ) along with the standard deviation from biological triplicates. Non-transfected MIA PaCa-2 cells served as the calibrator for RQ calculations. Delta Ct calculations used the average of two endogenous controls (miR-16-5p and miR-24-3p). Additionally, miRNA targets without expression in parental cell lines were set with Ct = 40, considering Ct values > 35 as non-expressed.

### 2.5. Investigation of Vector Efficiency in a Panel of Non-Pancreatic Cancer Cell Lines Show Levels of Expression for Some miRNA Targets

To gain a deeper understanding of cell line-specific miRNA processing dynamics, we investigated the relative expression of both vector designs for each miRNA target in a diverse range of cell lines, encompassing non-cancer (HEK293T), non-pancreatic cancer (SK-OV-3), and non-human (CHO-K1) cell lines. Significant fold changes were established at values greater than 2, following established standards [20], taking into account the high expression levels achieved by the control miR-708.

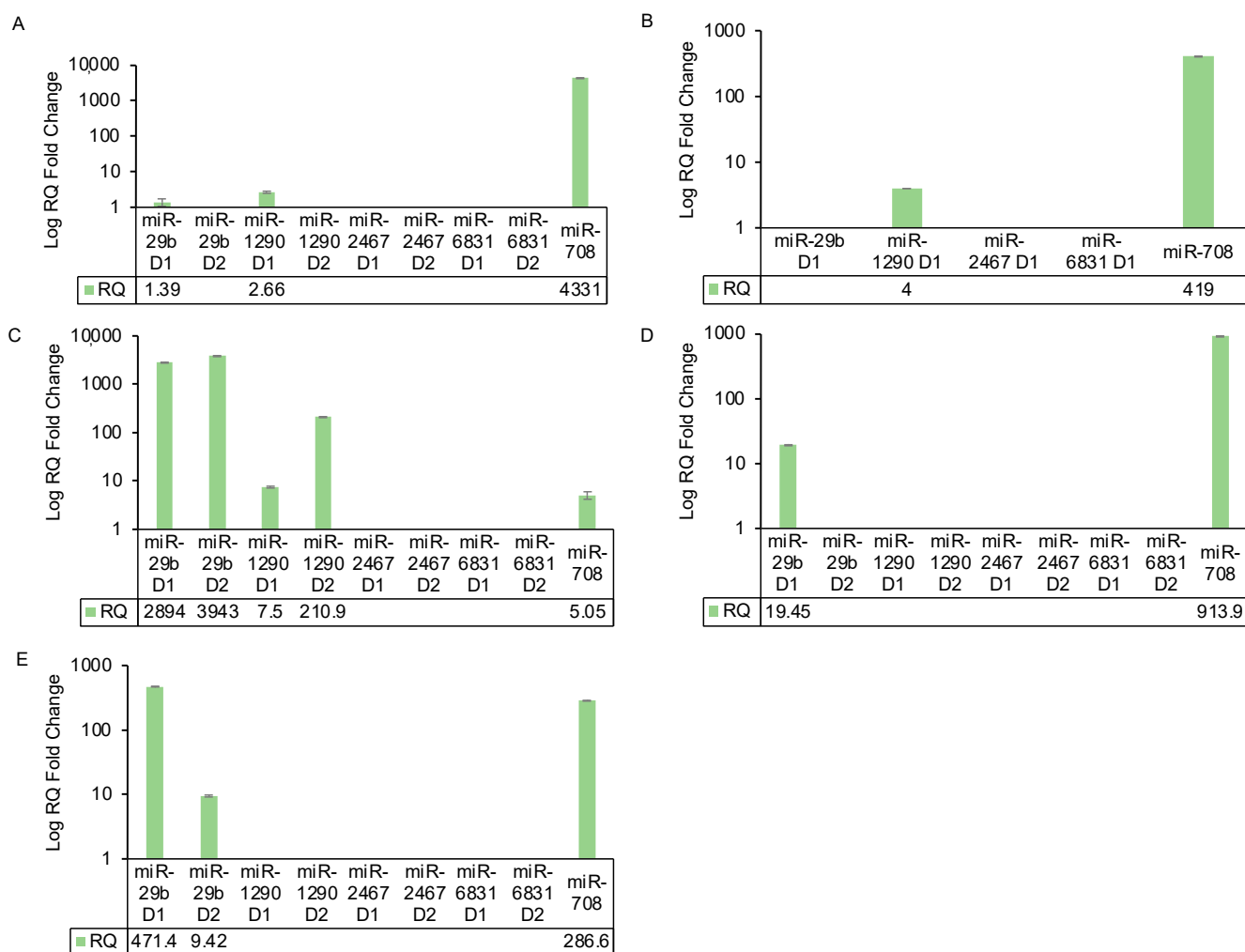


**Figure 4.** Investigation of vector efficiency in a panel of non-pancreatic cancer cell lines show levels of expression for some miRNA targets. The data are presented as relative quantification (RQ) and standard deviation of biological triplicates. For RQ non-transfected MIA PaCa-2 was used as the calibrator. For Delta Ct calculations, the average of two endogenous controls (miR-16-5p and miR-24-3p) was used, except for SK-OV-3 cells, where 16-5p was used alone due to changes in 24-3p. For RQ calculations, the miRNA target with no expression in parental cell lines was set as Ct = 40, as anything with a Ct value > 35 is considered non-expressed. Panel description: (A) MIA PaCa-2, (B) PANC-1, (C) HEK293T, (D) SK-OV-3, (E) CHO-K1.

The summarised results for each cell line, including MIA PaCa-2 and PANC-1 (design 1 only), are presented in Figure 4. Notably, within MIA PaCa-2 and PANC-1 cells, only miR-708 exhibited the capability to attain detectable levels of mature miRNA. Design 1 for miR-1290 displayed a modest RQ of 4 in PANC-1 cells, primarily attributed to alterations in endogenous RT-qPCR controls (see methods). Remarkably, design 1 of miR-29b showed mature miR-29b levels in HEK293T (RQ = 2893.8), SK-OV-3 (RQ = 19.4), and CHO-K1

(RQ = 471.4) cells. Furthermore, design 2 of miR-29b facilitated detectable levels in HEK293T (RQ = 3942) and CHO-K1 (RQ = 9) cells.

The control vector, miR-708, achieved discernible expression across all cell lines, with HEK293T exhibiting the lowest level (RQ = 5). Similarly, both designs of miR-1290 achieved miRNA maturation solely in HEK293T cells (design 1 RQ = 7.5, design 2 RQ = 210). It is noteworthy that miR-708 and -1290 displayed the lowest levels of overexpression in HEK293T cells, and their parental endogenous levels were expressed at moderate levels with Ct values of 30.2 and 29.7, respectively (Supplementary Table S3). An intriguing observation concerning miR-1290 in HEK293T cells is that while design 1 exhibited lower levels of overexpression, design 2 showed remarkably high levels despite containing the flanking regions of miR-708, which, as mentioned earlier, achieved an RQ value of only 5 in HEK293T cells. In HEK293T cells, miR-29b demonstrated no detectable endogenous levels but achieved high levels of overexpression, with RQ values of 2893.8 and 3942.7 for designs 1 and 2, respectively (Figure 4). For SK-OV-3 cells, although expressed at very low levels just meeting our cut-off for expression with a Ct value of 35 (Supplementary Table S3), miR-29b was the sole miRNA to achieve overexpression in this cell line and exhibited the only endogenous expression within the panel. Only design 1 achieved detectable mature miRNA levels, despite miR-708 achieving high levels with an RQ of 913.85 (Figure 4). CHO-K1 was the only cell line that exhibited no endogenous expression (Supplementary Table S3) for any of these miRNAs. However, both designs of miR-29b and miR-708 achieved varying levels of overexpression (Figure 4). It is important to note that copy number biasing is an unlikely explanation for the observed differences in RQ values among the cell lines, as transfection efficiency was monitored microscopically by GFP expression and consistently exceeded 75%.

While intriguing trends were evident, the endogenous miRNA expression profiles of the parental cells did not appear to directly correlate with the varying RQ values. Additionally, pri-miRNA structure alone could not be the primary determinant of miRNA maturation, as evidenced by the low RQ value of miR-708 in HEK293T cells and the high RQ value for miR-1290 design 2, which contained the flanking regions of miR-708. Overall, these findings highlighted distinct miRNA expression profiles across diverse cellular contexts, underscoring the intricate interplay between vector design, cell line characteristics, and miRNA target regulation.

### 3. Discussion

During experiments to overexpress specific miRNAs in human pancreatic cancer cells, our vector designs successfully increased miR-708 expression but not that of miR-29b, -1290, -2467, and -6831. Using Vienna RNAfold, we predicted minimum free energy secondary structures for each pri-miRNA (Figure 1) [15,21]. Bifurcation loops and motifs like mGHG and CNNC are pivotal in miRNA processing, influencing both the efficiency and specificity of biogenesis [22]. Bifurcation loops can impact the accessibility of processing machinery, potentially affecting DROSHA and DICER processing efficiency. The mGHG motif, located in the lower stem region of pri-miRNAs, interacts with DROSHA's dsRNA-binding domain, facilitating recognition and cleavage of pri-miRNAs [23]. Similarly, the CNNC motif is implicated in miRNA processing regulation [24]. Understanding these motifs is vital for elucidating the complex regulatory mechanisms controlling miRNA processing. Comparing miR-708's structure with design 1 with that of the other miRNAs, we observed more bifurcation loops (greater than three interior edges with unclear destabilising effects) [25], especially in miR-29b and -1290. Pri-miRNA secondary structures influence processing efficiency, e.g., the mGHG motif in the lower stem interacts with DROSHA's dsRNA-binding domain [26], which only miR-1290 possessed (Figure 1). This study found that secondary structure alone does not determine processing efficiency, as modification with miR-708's flanking regions did not overcome the issue.

Comparison of the miR-708 motifs to those of the other four miRNAs (Supplementary Table S1) revealed miR-708's possession of the CNNC motif, which is essential for SRSF3-

mediated microprocessor recognition [18]. Previous literature describes this motif at 16–18 nucleotides (nt) from the DROSHA cut site [27]. While miR-708 deviates with a position at 23 nt, miR-29b achieves the optimal positioning, indicating that this motif alone cannot compensate for other processing factors. While miR-708 displays a basal UG motif [28], it sits at -15 and -14 instead of the optimal -14 and -13 [29]. miR-1290 possesses the optimal UG motif placement. miR-29b-1 and -6831-5p exhibited individual U/G nucleotides at the basal junction. DICER cleaves pre-miRNA near the apical loop in the cytoplasm. A UGU motif at the apical junction strengthens DGCR8 interaction, preventing DROSHA displacement and unproductive cleavage [30]. None of the miRNAs, including the successfully overexpressed miR-708, contained a perfect UGU/GUG motif. All miRNAs, except miR-29b, exhibited apical loops shorter than the optimal  $\geq 10$  nt [27], with miR-708's apical loop being the shortest at 7 nt. The data presented here could help inform future chimeric miRNA design principles.

Numerous factors can influence miRNA expression levels. Methylation changes in regulatory regions, including miRNA genes, are significant in this regard, often associated with dysregulated mature miRNA expression [31]. RNA-binding proteins (RBPs) are pivotal in miRNA biogenesis, with conserved RNA binding domains that recognize specific RNA sequence elements or secondary structures. RBPs also interact with miRISC to regulate miRNA function, modulating miRNA abundance and activity [32]. Post-transcriptional mechanisms like RNA editing and alternative splicing can impact miRNA expression by affecting mature miRNA stability or target specificity. Additionally, competitive endogenous RNAs and RNA decay pathways contribute to miRNA-mediated gene regulation by competing for miRNA binding or facilitating miRNA degradation [33]. Another such regulatory aspect is strand selection, influenced by impaired processing, epitranscriptome modifications (like uridylation addition [34], adenosine-to-inosine deamination [35,36]), and Argonaute strand preference alteration. Typically, the Ago protein takes up either the -5p or -3p miRNA strand, guided by miRNA-targeted mRNA complementarity [37], with one strand predominating as the functional "guide" and the other being degraded [38]. Some miRNAs yield functional mature forms from both strands. To rule out altered strand selection causing undetectable overexpression, we assessed miR-2467's passenger strand expression alongside miR-708. The results (Figure 2) revealed no detectable expression, ruling out strand selection as the cause for non-detectable mature miRNA levels in these vectors.

We explored three hypotheses to address the absence of mature miRNA levels: (1) Failed DROSHA processing of pri-miRNA? (2) Inadequate transport of pre-miRNA from the nucleus? and (3) Incomplete pre-miRNA processing in the cytoplasm? Our results (Figure 3) identified pre-miRNA accumulation of four miRNAs in the cytoplasm of transfected cells. miR-708, which could produce mature expression, did not exhibit this accumulation, ruling out DROSHA/Exportin 5 dysfunction. While reduced levels of DICER have been described in cancer [39,40], it was unlikely that a global reduction was responsible, as miR-708 produced mature miRNA in MIA PaCa-2. Studies have shown that DICER protein levels do not always correspond to mature miRNA levels [41], potentially due to non-canonical processing pathways that can operate independently of components like DGCR8, DICER, Exportin-5, or Argonaute 2. With cytoplasmic precursor miRNA accumulation established in MIA PaCa-2 cells, we explored these miRNAs in additional cell lines: SK-OV-3 (non-pancreatic), HEK293T (non-cancerous), and CHO-K1 (non-human) (Figure 4). The differences in fold change between designs 1 and 2 indicated that while these processing complexities are not solely determined by structure or sequence, they are important. Endogenous miRNA expression in parental cells (Supplementary Table S3) and transfection efficiency were also ruled out as principal factors contributing to the different RQ values observed between cell lines (Supplementary Figure S1).

Unlike stable vectors, pre-miR mimics resulted in abundant levels of mature miRNAs (Figure 3). Dysregulated RISC assembly can hinder miRNA synthesis [42]. Pre-miR miRNA mimics, chemically modified RNA molecules mirroring endogenous miRNAs, bypass DICER processing, directly engaging RISC. Our demonstration that bypassing DICER

processing leads to mature miRNA levels suggested the issue is not within RISC. Some miRNAs are subject to negative feedback regulation. When the levels of a particular mature miRNA increase (e.g., due to disease, external stimulus, or treatment), it can trigger regulatory mechanisms that aim to counteract this increase, such as transcriptional alteration of the corresponding pri-miRNAs. It is interesting that these miRNAs were identified as being upregulated in either normal versus tumour or tumour versus F1 in pancreatic cancer models (Supplementary Table S2) but were either lowly expressed or undetected in the pancreatic cancer cell lines. While this intriguing point invites further exploration in future experiments, offering a promising avenue for deeper insights into miRNA regulation, we believe that our results of miRNA maturation when endogenous processing was skipped by using pre-miRNA mimics (Figure 3) underscores a dysfunction post-DROSHA but pre-DICER/RISC and dispels concerns about competing endogenous RNAs (ceRNAs) sequestering our miRNAs [43].

This study has demonstrated the distinct behaviours of different miRNAs with the same vector in the same cell line, the same miRNA between the two vector designs, and with the same miRNA across different cell lines (Figure 4). This diversity in expression patterns in an miRNA- and cell line-specific manner, along with its basis in impaired pre-miRNA processing, has not been reported previously. Our findings also show that miRNA mimics are a good alternative, while more studies are required to fully understand the complex processing dynamics that can affect stable vector efficiency. These findings make a significant contribution to the field of miRNA cellular engineering and point to a much greater degree of complexity in the processes controlling miRNA maturation than previously appreciated.

#### 4. Materials and Methods

##### 4.1. Cell Culture

The human pancreatic cell lines MIA PaCa-2 (ECACC, Salisbury, UK) and PANC-1 (ATCC, Rockville, MD, USA) were cultured in DMEM high glucose (D5671, Merck, Darmstadt, Germany) supplemented with 5% (*v/v*) foetal calf serum and 2% (*v/v*) l-glutamine (2503008, Thermo Fisher, Waltham, MA, USA); the human ovarian cancer cell line SK-OV-3 (gifted from the Collins group at NICB, DCU-passage 40) was cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA, R8758) supplemented with 10% (*v/v*) foetal bovine serum (FBS); the human embryonic kidney cell line HEK293T (gifted from the Walsh lab group at NICB, DCU-passage 39) was cultured in DMEM-high glucose (D5671, Merck, Darmstadt, Germany); and the Chinese Hamster Ovary cell line CHO-K1 (ATCC, CCL-61) was cultured in DMEM-F12 (Sigma-Aldrich, D3487) supplemented with 5% (*v/v*) FBS. All cell lines had been STR profiled (IDEXX BioAnalytics, Kornwestheim, Germany). Cells were grown in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C in T-25 and T-75 culture flasks. Cell lines were split (1:5) using PBS to wash and trypsin-EDTA (Thermo Fisher, Waltham, MA, USA) when 80–90% confluency was reached.

##### 4.2. miRNA Overexpression Vector Design

For the generation of both vector designs (Figure 5), the pTight-TET-ON vector (pcDNA3.1(+)-HYG) expressing a destabilised GFP reporter (d2eGFP) was used as a backbone vector. For design 1, the DNA fragments containing the precursor miRNA (pre-miRNA) and 70 bp genomic flanking regions on both 5' and 3' ends (pri-miRNA) were cloned downstream of d2eGFP, resulting in the conventional miRNA expression vector. A detailed description of vector construction has been described previously [44]. Design 1 was constructed for each miRNA among a list of priority miRNAs, including miR-21, -29b, -125a, -148, -206, -378e, -615, -708, -1290, -2467, and -6831. For design 2, the DNA fragments containing the precursor miRNA (pre-miRNA) were cut out using restriction enzymes NotI and EcoRI (Thermo Fisher, Waltham, Massachusetts, USA) and cloned into the genomic flanking regions of miR-708-5p by complementary sticky ends. miR-708 was

chosen as the positive control miRNA for all future analysis as it achieved the highest level of overexpression. All sequences are provided in Supplementary Table S1.

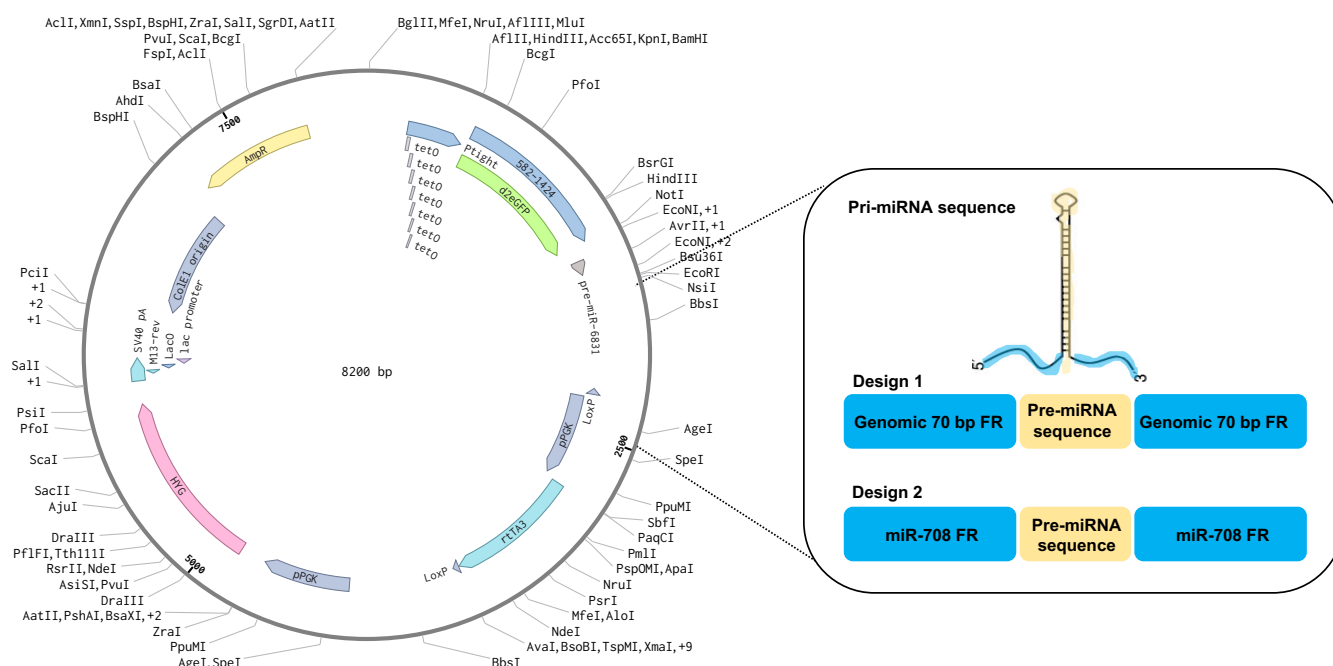


**Figure 5.** Schematic of two stable vector designs for the overexpression of miRNAs. Design 1 shows a conventional primary miR (priMIR) expression system consisting of 70 bp genomic flanking region (FR) surrounding the miR transcript unit under the pTight promoter, part of an inducible TET-ON system that requires the presence of doxycycline to activate expression, along with co-expression of the pre-miRNA with a fluorescent protein (destabilised GFP) to follow the induction of the system. Design 2 is composed of the same vector backbone, but the miRNAs of interest were placed within the 76 bp flanking region of miR-708, which showed successful overexpression in vitro.

#### 4.3. Transfection

Cells were counted and seeded in 6-well plates and allowed to attach for 24 h before transfection. The seeding densities were as follows: MIA PaCa-2 and DLKP were seeded at  $4 \times 10^4$  cells total, PANC-1 and SK-OV-3 at  $5 \times 10^4$  cells total, and CHO-K1 at  $3 \times 10^4$  cells total. For stable vectors, a total of 1  $\mu$ g DNA was mixed with 250  $\mu$ L Dulbecco's Modified Eagle Medium F12 and 2  $\mu$ L TransIT X2 reagent (MIR6000, Mirus Bio, Madison, WI, USA) and incubated for 30 min at RT. Cells were then placed in fresh media and 250  $\mu$ L of the TransIT-X2-DNA transfection complexes was added to each well. A negative control without DNA was also included. Two days post transfection, cells were induced with 100 ng/mL doxycycline and transfection efficiency was confirmed 24 h post induction through GFP assessment by fluorescence microscopy inspection. Transfection efficiencies, analysed by GFP expression and doxycycline toxicity assays, are shown in Supplementary Figure S1. Stable overexpressing cell lines were selected and generated by supplementing with 400 ng/mL Hygromycin B (Roche, Basel, Switzerland). Chemically synthesised linear double-stranded pre-miR mimics (AM17100, Thermo Fisher, Waltham, Massachusetts, USA) were used to test miRNA maturation without DICER processing. For transfection with these mimics, a working concentration of 10  $\mu$ M was mixed with 3  $\mu$ L TransIT X2 reagent in 100  $\mu$ L Dulbecco's Modified Eagle Medium F12. The mixture was left for 30 min at room temperature and then added to the cells.

#### 4.4. RNA Isolation and cDNA Synthesis

One day prior to RNA collection, cells were induced with 100 ng/mL doxycycline (Merck, Darmstadt, Germany) and GFP expression was confirmed by fluorescence mi-

croscopy. RNA samples were collected from 80–90% confluent 6 well plates. Cell pellets were collected in a total of 1 mL TRIzol (15596018, Thermo Fisher, Waltham, MA, USA) and stored at  $-80^{\circ}\text{C}$  until processed. Total RNA was isolated following the manufacturer's recommendations. RNA quality and quantity were assessed using a NanoDrop (Thermo Scientific, Waltham, Massachusetts, USA). In order to remove contaminating DNA, DNase I treatment (AMPD1-1KT, Merck, Darmstadt, Germany) was performed following the manufacturer's instructions.

#### 4.5. RT-qPCR

For mature miRNAs, samples were prepared using the TaqMan MicroRNA Reverse Transcription Kit (ThermoFisher, 4366596), as per the manufacturer's recommendations. For primary and precursor miRNA detection, samples were prepared using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher, 4368814). The ddCt method was used to determine the relative quantification (RQ) of each miRNA with miR-16-5p and miR-24-3p used as endogenous controls [13]. Where parental levels of an miRNA were undetectable, the Ct value was set to 40 for completion of the RQ calculation as it is well accepted that a Ct greater than 35 is considered non-expressed. The RQ of mature miRNAs was assessed from a total of 10 ng cDNA samples using the TaqMan® Advanced miRNA Assay (ThermoFisher, A25576), as per the manufacturer's recommendations. The RQs of primary and precursor miRNAs were assessed from a total of 100 ng cDNA sample using the TaqMan® Pri-miRNA Assay (ThermoFisher, 4427012) and TaqMan Non-coding RNA Assay (ThermoFisher, 4426961), as per the manufacturer's recommendations.

**Supplementary Materials:** The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms25115666/s1>.

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## References

1. O'Brien, J.; Hayder, H.; Zayed, Y.; Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front. Endocrinol.* **2018**, *9*, 402. [CrossRef] [PubMed]
2. Lee, E.J.; Baek, M.; Gusev, Y.; Brackett, D.J.; Nuovo, G.J.; Schmittgen, T.D. Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors. *RNA* **2008**, *14*, 35–42. [CrossRef] [PubMed]
3. Lin, S.; Gregory, R.I. MicroRNA biogenesis pathways in cancer. *Nat. Rev. Cancer* **2015**, *15*, 321–333. [CrossRef] [PubMed]
4. Khan, M.A.; Zubair, H.; Srivastava, S.K.; Singh, S.; Singh, A.P. Insights into the Role of microRNAs in Pancreatic Cancer Pathogenesis: Potential for Diagnosis, Prognosis, and Therapy. *Adv. Exp. Med. Biol.* **2015**, *889*, 71–87. [CrossRef]
5. Markopoulos, G.S.; Roupakia, E.; Tokamani, M.; Chavdoula, E.; Hatziapostolou, M.; Polytaichou, C.; Marcu, K.B.; Papavassiliou, A.G.; Sandaltzopoulos, R.; Kolettas, E. A step-by-step microRNA guide to cancer development and metastasis. *Cell. Oncol.* **2017**, *40*, 303–339. [CrossRef] [PubMed]
6. Kallunki, T.; Barisic, M.; Jaattela, M.; Liu, B. How to Choose the Right Inducible Gene Expression System for Mammalian Studies? *Cells* **2019**, *8*, 796. [CrossRef]

7. Cech, T.R.; Steitz, J.A. The noncoding RNA revolution-trashing old rules to forge new ones. *Cell* **2014**, *157*, 77–94. [CrossRef] [PubMed]
8. Bartel, D.P. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **2004**, *116*, 281–297. [CrossRef] [PubMed]
9. Melo, S.A.; Moutinho, C.; Roperio, S.; Calin, G.A.; Rossi, S.; Spizzo, R.; Fernandez, A.F.; Davalos, V.; Villanueva, A.; Montoya, G.; et al. A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell* **2010**, *18*, 303–315. [CrossRef]
10. Suzuki, H.I.; Yamagata, K.; Sugimoto, K.; Iwamoto, T.; Kato, S.; Miyazono, K. Modulation of microRNA processing by p53. *Nature* **2009**, *460*, 529–533. [CrossRef] [PubMed]
11. Furukawa, N.; Sakurai, F.; Katayama, K.; Seki, N.; Kawabata, K.; Mizuguchi, H. Optimization of a microRNA expression vector for function analysis of microRNA. *J. Control. Release* **2011**, *150*, 94–101. [CrossRef]
12. Roche, S.; O'Neill, F.; Murphy, J.; Swan, N.; Meiller, J.; Conlon, N.T.; Geoghegan, J.; Conlon, K.; McDermott, R.; Rahman, R.; et al. Establishment and Characterisation by Expression Microarray of Patient-Derived Xenograft Panel of Human Pancreatic Adenocarcinoma Patients. *Int. J. Mol. Sci.* **2020**, *21*, 962. [CrossRef] [PubMed]
13. O'Neill, F.; Allen-Coyle, T.J.; Roche, S.; Meiller, J.; Conlon, N.T.; Swan, N.; Straubinger, R.M.; Geoghegan, J.; Straubinger, N.L.; Conlon, K.; et al. Alteration in Levels of Specific miRNAs and Their Potential Protein Targets between Human Pancreatic Cancer Samples, Adjacent Normal Tissue, and Xenografts Derived from These Tumors. *Life* **2023**, *13*, 608. [CrossRef] [PubMed]
14. Zhang, Y.; Huang, H.; Zhang, D.; Qiu, J.; Yang, J.; Wang, K.; Zhu, L.; Fan, J. A Review on Recent Computational Methods for Predicting Noncoding RNAs. *Biomed. Res. Int.* **2017**, *2017*, 9139504. [CrossRef]
15. Hofacker, I.L. Vienna RNA secondary structure server. *Nucleic Acids Res.* **2003**, *31*, 3429–3431. [CrossRef] [PubMed]
16. Grimson, A.; Farh, K.K.; Johnston, W.K.; Garrett-Engele, P.; Lim, L.P.; Bartel, D.P. MicroRNA targeting specificity in mammals: Determinants beyond seed pairing. *Mol. Cell* **2007**, *27*, 91–105. [CrossRef]
17. Pla, A.; Zhong, X.; Rayner, S. miRAW: A deep learning-based approach to predict microRNA targets by analyzing whole microRNA transcripts. *PLoS Comput. Biol.* **2018**, *14*, e1006185. [CrossRef] [PubMed]
18. Nguyen, T.L.; Nguyen, T.D.; Bao, S.; Li, S.; Nguyen, T.A. The internal loops in the lower stem of primary microRNA transcripts facilitate single cleavage of human Microprocessor. *Nucleic Acids Res.* **2020**, *48*, 2579–2593. [CrossRef]
19. Sheng, Y.; Engström, P.G.; Lenhard, B. Mammalian microRNA prediction through a support vector machine model of sequence and structure. *PLoS ONE* **2007**, *2*, e946. [CrossRef] [PubMed]
20. Institute for Research in Immunology and Cancer. (n.d.). How to Deal with qPCR Results. Available online: [https://genomique.iriic.ca/resources/files/How\\_to\\_deal\\_with\\_qPCR\\_results.pdf](https://genomique.iriic.ca/resources/files/How_to_deal_with_qPCR_results.pdf) (accessed on 8 April 2024).
21. Eddy, S.R. How do RNA folding algorithms work? *Nat. Biotechnol.* **2004**, *22*, 1457–1458. [CrossRef]
22. Komatsu, S.; Kitai, H.; Suzuki, H.I. Network Regulation of microRNA Biogenesis and Target Interaction. *Cells* **2023**, *12*, 306. [CrossRef] [PubMed]
23. Zhang, X.; Yang, F.; Liu, F.; Tian, Q.; Hu, M.; Li, P.; Zeng, Y. Conservation of Differential Animal MicroRNA Processing by Drosha and Dicer. *Front. Mol. Biosci.* **2021**, *8*, 730006. [CrossRef] [PubMed]
24. Kim, K.; Baek, S.C.; Lee, Y.Y.; Bastiaanssen, C.; Kim, J.; Kim, H.; Kim, V.N. A quantitative map of human primary microRNA processing sites. *Mol. Cell* **2021**, *81*, 3422–3439.e3411. [CrossRef] [PubMed]
25. Zuker, M.; Stiegler, P. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* **1981**, *9*, 133–148. [CrossRef] [PubMed]
26. Li, S.; Le, T.N.; Nguyen, T.D.; Trinh, T.A.; Nguyen, T.A. Bulges control pri-miRNA processing in a position and strand-dependent manner. *RNA Biol.* **2021**, *18*, 1716–1726. [CrossRef] [PubMed]
27. Fang, W.; Bartel, D.P. The Menu of Features that Define Primary MicroRNAs and Enable De Novo Design of MicroRNA Genes. *Mol. Cell* **2015**, *60*, 131–145. [CrossRef] [PubMed]
28. Nguyen, T.A.; Park, J.; Dang, T.L.; Choi, Y.G.; Kim, V.N. Microprocessor depends on hemin to recognize the apical loop of primary microRNA. *Nucleic Acids Res.* **2018**, *46*, 5726–5736. [CrossRef] [PubMed]
29. Auyeung, V.C.; Ulitsky, I.; McGeary, S.E.; Bartel, D.P. Beyond secondary structure: Primary-sequence determinants license pri-miRNA hairpins for processing. *Cell* **2013**, *152*, 844–858. [CrossRef]
30. Shoffner, G.M.; Peng, Z.; Guo, F. Structures of microRNA-precursor apical junctions and loops reveal non-canonical base pairs important for processing. *bioRxiv* **2020**. [CrossRef]
31. Gulyaeva, L.F.; Kushlinskiy, N.E. Regulatory mechanisms of microRNA expression. *J. Transl. Med.* **2016**, *14*, 143. [CrossRef] [PubMed]
32. Hynes, C.; Kakumani, P.K. Regulatory role of RNA-binding proteins in microRNA biogenesis. *Front. Mol. Biosci.* **2024**, *11*, 1374843. [CrossRef] [PubMed]
33. Hackl, L.M.; Fenn, A.; Louadi, Z.; Baumbach, J.; Kacprowski, T.; List, M.; Tsoy, O. Alternative splicing impacts microRNA regulation within coding regions. *NAR Genom. Bioinform.* **2023**, *5*, lqad081. [CrossRef] [PubMed]
34. Ren, G.; Chen, X.; Yu, B. Small RNAs meet their targets: When methylation defends miRNAs from uridylation. *RNA Biol.* **2014**, *11*, 1099–1104. [CrossRef] [PubMed]
35. Kawahara, Y.; Zinshteyn, B.; Sethupathy, P.; Iizasa, H.; Hatzigeorgiou, A.G.; Nishikura, K. Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. *Science* **2007**, *315*, 1137–1140. [CrossRef] [PubMed]

36. Gagnidze, K.; Rayon-Estrada, V.; Harroch, S.; Bulloch, K.; Papavasiliou, F.N. A New Chapter in Genetic Medicine: RNA Editing and its Role in Disease Pathogenesis. *Trends Mol. Med.* **2018**, *24*, 294–303. [[CrossRef](#)] [[PubMed](#)]
37. Medley, J.C.; Panzade, G.; Zinovyeva, A.Y. microRNA strand selection: Unwinding the rules. *Wiley Interdiscip. Rev. RNA* **2021**, *12*, e1627. [[CrossRef](#)] [[PubMed](#)]
38. Guo, L.; Lu, Z. The fate of miRNA\* strand through evolutionary analysis: Implication for degradation as merely carrier strand or potential regulatory molecule? *PLoS ONE* **2010**, *5*, e11387. [[CrossRef](#)] [[PubMed](#)]
39. Hoffend, N.C.; Magner, W.J.; Tomasi, T.B. The epigenetic regulation of Dicer and microRNA biogenesis by Panobinostat. *Epigenetics* **2017**, *12*, 105–112. [[CrossRef](#)] [[PubMed](#)]
40. Pradhan, A.K.; Bhoopathi, P.; Talukdar, S.; Scheunemann, D.; Sarkar, D.; Cavenee, W.K.; Das, S.K.; Emdad, L.; Fisher, P.B. MDA-7/IL-24 regulates the miRNA processing enzyme DICER through downregulation of MITF. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 5687–5692. [[CrossRef](#)] [[PubMed](#)]
41. Obernosterer, G.; Leuschner, P.J.; Alenius, M.; Martinez, J. Post-transcriptional regulation of microRNA expression. *RNA* **2006**, *12*, 1161–1167. [[CrossRef](#)] [[PubMed](#)]
42. Lewkowicz, P.; Cwiklińska, H.; Mycko, M.P.; Cichalewska, M.; Domowicz, M.; Lewkowicz, N.; Jurewicz, A.; Selmaj, K.W. Dysregulated RNA-Induced Silencing Complex (RISC) Assembly within CNS Corresponds with Abnormal miRNA Expression during Autoimmune Demyelination. *J. Neurosci.* **2015**, *35*, 7521–7537. [[CrossRef](#)] [[PubMed](#)]
43. Tay, Y.; Kats, L.; Salmena, L.; Weiss, D.; Tan, S.M.; Ala, U.; Karreth, F.; Poliseno, L.; Provero, P.; Di Cunto, F.; et al. Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs. *Cell* **2011**, *147*, 344–357. [[CrossRef](#)] [[PubMed](#)]
44. Costello, A.; Lao, N.; Clynes, M.; Barron, N. Conditional Knockdown of Endogenous MicroRNAs in CHO Cells Using TET-ON-SanDI Sponge Vectors. *Methods Mol. Biol.* **2017**, *1603*, 87–100. [[CrossRef](#)] [[PubMed](#)]

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## Supplementary data

**Supplementary Table S1** miRNA sequences used in stable vector design

|                                                                                                                                                                                                                                                                                                                       |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Design 1: ~ 70 base pair flanking region (native pri-miRNA sequence)</b>                                                                                                                                                                                                                                           |
| <b>&gt;hsa-miR-29b-1</b><br>TTTCCTTTCTAGGTTGTCTTGGGTTTATTGTAAGAGAGCATTATGAAGAAAAAATAGATCATAA<br>AGCTTCTTCAGGAAGCTGGTTTCATATGGTGGTTTAGATTTAAATAGTGATTGTCTAGCACCAT<br>TTGAAATCAGTGTTCTTGGGGGAGACCAGCTGCGCTGCACTACCAACAGCAAAAGAAGTGAAT<br>GGGACAGCTCTGAAGTATTTGAAAGCAAC                                                  |
| <b>&gt;hsa-miR-378e</b><br>GGAAGTAAGCACATTGTTCAAGGAACTAGGACGAGCTAGTGGGGTGGACAAGCTGCATCCT<br>GAACCTGGCTCTGCCTGACTCCAGTGTCAGGCCAGGGGCAGACAGTGACAGAGAACAGT<br>GCCCCAAGACCACTGGACTTGGAGTCAGGACATCATTTTAACAGCTTTATCGAGATGTAATTTAC<br>ATGCCATACAATTTACCCAAAGTGACAATCATTGACT                                                 |
| <b>&gt;hsa-miR-1290</b><br>TTGGAGAATTTGCCGAACAAGTTGAGCATCCCTACTCCCAAGTTCCAAAGTCCAAAATGCTCC<br>AATGAGCATTTTCCTTTGAGCGTCACGTTGACACTCAAAAAGTTTCAGATTTTGGAACATTTTCGG<br>ATTTTGGATTTTGGATCAGGGATGCTCAACTTGTCTGCGTTCCCTCATACGCTTATGTGGTGG<br>ATTCAGTGAGATGATCCTGTCTCACTTAGGTTAATG                                           |
| <b>&gt;hsa-miR-2467</b><br>ACTTAGCCATCGACTTCTGTCTTGCGTCCACATGAGAGAGAAGACGGTGGCCACTGGACAGA<br>CGCTTGGACAGGCACCTGAGGCTCTGTTAGCCTTGGCTCTGGGTCCTGCTCCTTAGAGCAGA<br>GGCAGAGAGGCTCAGGGTCTGTCTGGGTCACTCTCTTTGAGATTGCCGGAACGCTTACACAC<br>ATCTTTCCTGGGTGGAATCGTCTTTGTCTGAA                                                     |
| <b>&gt;hsa-miR-6831</b><br>TGTACCCAGCGGTAGCACCCAGTGGCAGCGCCCAACCTGGGAACTAGGAGATGCAGAGGAC<br>CCAGGCACGGTAGGTAGAGTGTGAGGAGGAGGTCTGAGCCCATGTGTGGACCTAGGTCTGC<br>TGTTAAACTGACTAACTCCCACTCTACAGGGAACGGAGGGGATCTGGGGACTGCGGCCCCC<br>AAAGGGAGATCCTTCTCCAGCCTGGAGAGTTCACTGGA                                                  |
| <b>&gt;hsa-miR-708</b><br>CTGACTTCTCCTGCCCCAGGGGTTTTTCAGAAACCTAACCCCCATGGTTGGCGAGGGACTGCT<br>GTGTGTGAAATGGTAACTGCCCTCAAGGAGCTTACAATCTAGCTGGGGGTAAATGACTTGCAC<br>ATGAACACAACCTAGACTGTGAGCTTCTAGAGGGCAGGGACCTTACCCTAGTCATCTCTCTTCT<br>CACCTGCACACCCTCCCTGAGGGATCTCATCCATCCCCATGGCGTCAACCACCACCTGAGG<br>A                |
| <b>Design 2 – 76 base pair flanking region from miR-708</b>                                                                                                                                                                                                                                                           |
| <b>e.g. miR-708 FR + miR-1290 pre-miRNA sequence</b><br>CTGACTTCTCCTGCCCCAGGGGTTTTTCAGAAACCTAACCCCCATGGTTGGCGAGGGACTGCT<br>GTGTGTGAAATGGTGAGCGTCACGTTGACACTCAAAAAGTTTCAGATTTTGGAACATTTTCGGAT<br>TTTGGATTTTGGATCAGGGATGCTCAACCTTACCCTAGTCATCTCTCTTCTCACCTGCACAC<br>CCTCCCTGAGGGATCTCATCCATCCCCATGGCGTCAACCACCACCTGAGGA |

**Supplementary Table S2** Fold change values from microarray for miRNA panel in normal versus tumour versus F1 samples.

| miRNA    | T vs N FC | P value | T vs F1 FC | P value    |
|----------|-----------|---------|------------|------------|
| miR-29b  | 2.52      | 0.23    | 5.82       | 0.02       |
| miR-1290 | 1.39      | 0.32    | 15.40      | 0.006      |
| miR-2467 | 1.48      | 0.17    | 7.91       | 0.00000002 |
| miR-6831 | 4.82      | 0.02    | 1.81       | 0.01       |
| miR-708  | 7.6       | 0.0085  | -25.34     | 0.0019     |

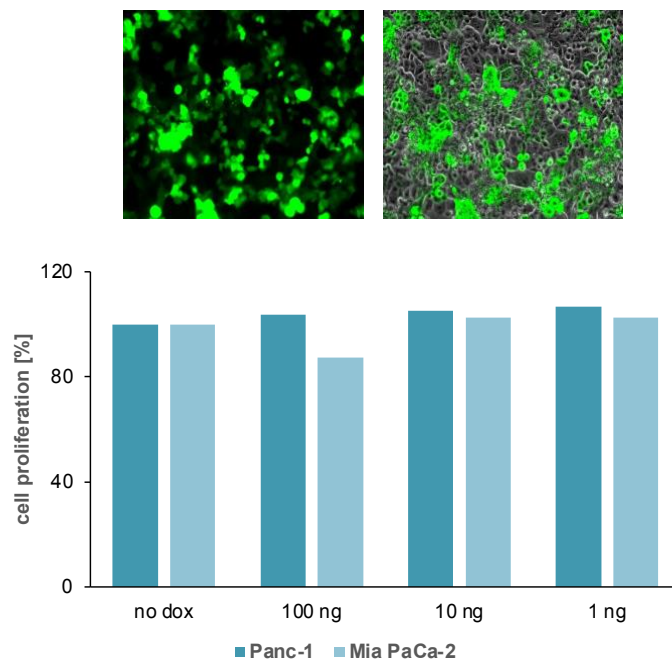
**Supplementary Table S3** Parental miRNA expression level profiling. Data presented as Ct average of biological triplicates.

| miRNA    | Mia PaCa-2 | PANC-1     | HEK293T    | SK-OV-3    | CHO-K1     |
|----------|------------|------------|------------|------------|------------|
| miR-29b  | 29.8       | 30.3       | Undetected | 35.5       | Undetected |
| miR-1290 | Undetected | 33.2       | 29.7       | Undetected | Undetected |
| miR-2467 | Undetected | Undetected | Undetected | Undetected | Undetected |
| miR-6831 | Undetected | Undetected | Undetected | Undetected | Undetected |
| miR-708  | Undetected | 35.2       | 30.2       | Undetected | Undetected |

**Supplementary Table S4** Minimum Free Energy (MFE) values for design 1 (genomic flanking regions) and design 2 (miR-708 flanking regions) as predicted by the RNA folding algorithm, Vienna RNA fold.

| miRNA        | Design 1 MFE (Kcal/mol) | Design 2 MFE (Kcal/mol) |
|--------------|-------------------------|-------------------------|
| miR-29b-1-5p | -60.4                   | -87.1                   |
| miR-1290     | -95                     | -66.2                   |
| miR-2467-3p  | -91.5                   | -95.9                   |
| miR-6831-5p  | -79.6                   | -70.4                   |
| miR-708-5p   | -92.5                   | -                       |

**Supplementary Figure S1** Transfection efficiency determined by GFP expression via microscopy in Mia PaCa-2 (top panel) and toxicity assay with serial dilutions of doxycycline concentrations from 100 ng to 1 ng in Mia PaCa-2 and PANC-1 cells (bottom panel).



# miR-6831-5p Overexpression in Pancreatic Cancer Cell Line Results in Changes in Cell Proliferation, Gemcitabine Sensitivity and Apoptosis Accompanied by Expression Changes at Both the mRNA and Protein Level

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**Abstract:** MicroRNAs have emerged as pivotal players in cancer development, including the context of pancreatic cancer. This study expands upon the existing understanding of miR-6831-5p and its functional significance within the realm of pancreatic cancer, with a specific focus on the PANC-1 cell line. Parallel transcriptomic and proteomic analyses revealed that miR-6831-5p overexpression induces notable changes in three main biological pathways: mitochondrial bioenergetics, purine nucleotide homeostasis, and apoptosis. Moreover, our study identified these as potential mechanisms underlying altered gemcitabine sensitivity, a standard chemotherapy agent for pancreatic cancer. Furthermore, we observed changes in proliferation and gemcitabine mediated apoptosis in response to miR-6831-5p overexpression. Our findings underscore miR-6831-5p as an interesting miRNA target for further exploration and highlight the importance of understanding the intricate interplay between miRNAs, metabolic pathways, and drug response in pancreatic cancer.

**Keywords:** microRNA; pancreatic cancer; drug sensitivity; cancer; therapeutic targets

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## 1. Introduction

MicroRNAs (miRNAs) have emerged as pivotal players in cancer development, including the context of pancreatic cancer (PaC) [1]. These small non-coding RNA molecules intricately regulate gene expression post-transcriptionally, thereby modulating fundamental cellular processes [2]. In pancreatic cancer, dysregulated miRNA expression contributes to key aspects of tumorigenesis, encompassing cell proliferation, apoptosis evasion, angiogenesis, and metastasis [3–5]. miRNAs act as tumour suppressors by targeting oncogenes or oncogenic miRNAs, and conversely, they can promote cancer progression by inhibiting tumour suppressor genes [6]. Pertinent to pancreatic cancer, certain miRNAs have been identified as critical regulators of pivotal signalling pathways such as KRAS, TP53, and SMAD, perpetuating the malignant phenotype [7–9]. Furthermore, miRNAs are implicated in the intricate crosstalk between pancreatic cancer cells and the tumor microenvironment, shaping immune responses and stromal interactions [10, 11].

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The multifaceted and context-dependent roles of miRNAs in pancreatic cancer underscore their potential as diagnostic biomarkers, therapeutic targets, and prognostic indicators, thereby offering novel avenues for understanding and addressing this formidable malignancy with remarkable specificity and sensitivity [12-14]. Their presence in biofluids allows non-invasive assessment, enabling possible avenues for early detection and monitoring of cancer progression [15, 16]. Despite these merits, harnessing miRNAs for clinical applications is not devoid of limitations [17]. Ensuring efficient and targeted delivery to specific tissues remains a challenge, compounded by potential off-target effects. The intricate network of miRNA interactions and their context-dependent functions necessitate comprehensive understanding, as the same miRNA may exert diverse effects in different cellular contexts [18]. Moreover, the heterogeneity of cancer types and individual variability necessitates careful validation of miRNA biomarkers for clinical accuracy. While miRNAs offer significant potential to revolutionise cancer therapeutics and diagnostics, their translation to clinical applications necessitates meticulous consideration of these multifaceted benefits and limitations.

The use of miRNA mimics in *in vitro* analyses of miRNAs in disease development presents both distinct advantages and limitations [19]. These mimics offer a powerful tool for deciphering the functional implications of specific miRNAs, enabling precise manipulation of their levels to elucidate their role in disease processes. This approach has provided valuable insights into the molecular intricacies underlying disease pathogenesis through the introduction of exogenous miRNA mimics into cellular models, enabling the exploration of effects on target gene expression, cellular pathways, and phenotypic outcomes [20-24]. However, the use of miRNA mimics also entails certain drawbacks. Overexpression of miRNA mimics may lead to non-physiological effects or saturate endogenous miRNA pathways, potentially obscuring the genuine regulatory mechanisms [25]. Additionally, the intricate interplay and cross-regulation between various miRNAs could complicate the interpretation of results. The context-specific nature of miRNA function and the challenges associated with accurately recapitulating the complex *in vivo* microenvironment *in vitro* adds complexity to interpretation of findings. Overall, miRNA mimics are invaluable for probing miRNA functions in disease contexts, providing insights that surpass their limitations.

A previous study from our group carried out a global comparison of miRNA profiles in human PaC tumours, adjacent normal tissue, and matched patient-derived xenograft (PDX) models using microarray screening [26-28]. RNA from tumor, normal, and PDX samples was analysed using Affymetrix GeneChip miRNA 4.0 array. Using the Transcriptome Analysis Console (TAC), we generated comparative lists of both up- and downregulated miRNAs, concentrating on those consistently altered in both tumor vs. normal and PDX vs. tumor comparisons. Within this target list we have identified several miRNAs of interest for which only limited studies exist to-date. One such miRNA is, miR-6831-5p, which in gastric, colorectal, and cervical cancers, has been implicated in exerting influence over pivotal pathways and assuming significance as a viable diagnostic indicator [29-31].

In this study, parallel transcriptomic analysis using QuantSeq [32, 33] and proteomics via Mass Spectrometry were used to gain deep insight into the downstream effects following miR-6831-5p overexpression in PANC-1 cells. This allowed for the identification of targets that were up- or down-regulated at both the mRNA and protein levels, in addition to targets that were not altered at the mRNA level but showed changes at the protein level, potentially indicating post-translational targets of miR-6831. The multi-omic analysis provided comprehensive insight into the possible mechanisms behind the phenotypic changes observed in this study in response to miR-6831 overexpression. Overall, this study substantially broadened our comprehension of miR-6831-5p and elucidated its functional relevance in the context of pancreatic cancer, particularly emphasising its impact on the PANC-1 cell line.

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## 2. Results

### *2.1. miR-6831-5p dynamics: profiling adjacent normal, tumour, and F1 PDX samples, coupled with cell line characterisation and quantification post miRNA mimic overexpression*

In a previous study, we compared miRNA expression in pancreatic cancer tumours, adjacent normal tissues, and patient-derived xenograft (PDX) models using microarray screening [28]. The increasing expression of miR-6831 in tumour and F1 compared to normal as previously identified is shown in Figure 1A. One of these was miR-6831-5p, which showed up regulation (fold change of 4.8 and 1.8 in normal vs. tumour and tumour vs. F1, respectively, see Figure 1B) is further investigated in this study. An initial screen of expression of miR-6831 was conducted in a panel of pancreatic cancer cell lines (Mia PaCa-2, PANC-1, Capan-2, SW1990, BxPC-3, and HPAC) using RT-qPCR. None of the cell lines showed endogenous miR-6831 expression (Figure 1C) as they possessed CT values greater than 35, indicating negligible or absent expression. The observed discrepancy in miR-6831 expression between patient-derived xenograft (PDX) models and established cell lines may be explained by the inherent differences in these models. PDX models, derived directly from patient tissues, retain the heterogeneity and microenvironmental context of original tumours, thus reflecting the complexity of human cancers and miRNA expression profiles more accurately. In contrast, cell lines often undergo significant genetic and epigenetic changes during their establishment and prolonged culture, leading to the loss of certain miRNA expressions present in the original tumours. Furthermore, the PDX models used in our studies were derived from a specific subgroup of surgical patients, selected based on their ability to survive and grow in mice, representing a particular cohort with distinct genetic and phenotypic characteristics. This selection may not be representative of the broader population of pancreatic cancer cell lines analysed. Therefore, while PDX models provide a closer approximation to patient tumours, established cell lines offer a more simplified and controlled environment for studying specific mechanisms. Both models have their strengths and limitations, highlighting the need for complementary models to fully understand miRNA dynamics in pancreatic cancer.

PANC-1 cells were chosen for their transfection ease, and miR-6831 was overexpressed using mirVana™ miRNA mimics, achieving a high level of overexpression with an RQ fold change of 1535 (Figure 1D). This substantial increase was partly due to the complete absence of endogenous expression in parental cells.

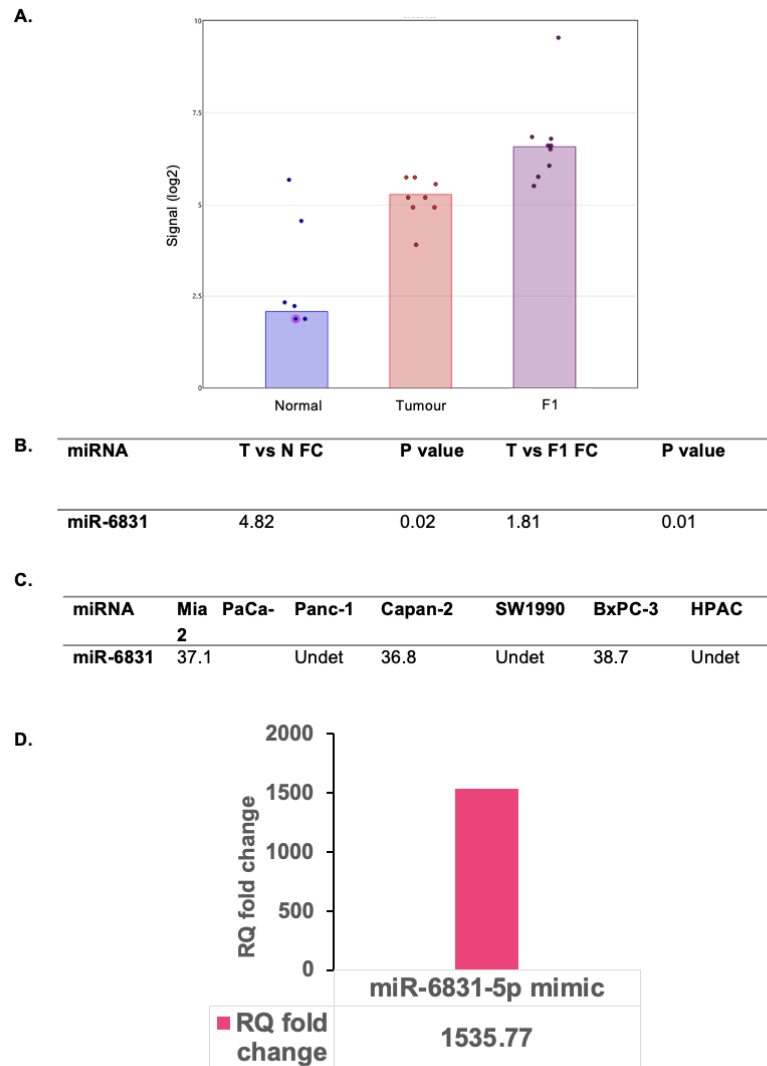


**Figure 1.** Cell line characterisation and in vitro validation of miR-6831 mimic. (A) Displayed are the Log 2 Fold Change (Log2FC) values between normal vs. tumour vs. F1 of miR-6831 previously identified by our group [29] using microarray as a bar chart with individual dots corresponding to individual samples. Corresponding FC and *p* values listed in (B). (A,B) are Adapted from O'Neill et al, 2023 [28]. (C) shows qPCR characterisation of miR-6831 in a panel of pancreatic cancer cell lines and (D) the RQ fold change in PANC-1 cells following transfection with miR-6831-5p mimic.

## 2.2. Impact of miR-6831 overexpression on cellular dynamics and gemcitabine response in PANC-1 cells

We assessed the *in vitro* consequences of miR-6831 overexpression in PANC-1 cells following 48 hour exposure to mirVana™ miRNA mimic. Initially, we assessed the effect on proliferation after 5 days, revealing a significant 40% reduction in cell proliferation (*p* value < 0.001) upon miR-6831 overexpression (Figure 2). In addition, gemcitabine response and apoptosis were evaluated at numerous time-points over a 72 hour period using Incucyte® Live-Cell Analysis. Intriguingly, while miR-6831 overexpression significantly reduced proliferation, it did not trigger an increase in apoptosis (Figure 3A). However, PANC-1 cells transfected with the miR-6831 mimic demonstrated heightened sensitivity to the 50 nM of gemcitabine (*p* value = 0.0221), as depicted in Figure 3B. Furthermore, a noteworthy finding emerged: while miR-6831 alone failed to elicit an apoptotic response, it significantly potentiated the apoptotic effect of gemcitabine at 50

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nM ( $p$  value < 0.0001), underscoring its role in enhancing chemotherapy-induced apoptosis (Figure 3C).

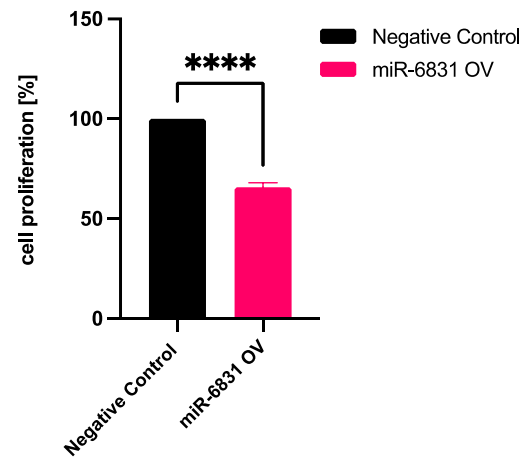


**Figure 2.** miR-6831 overexpression reduced proliferation in PANC-1 cells. Proliferation assay after 24 hours exposure to miR-6831 mirVana™ miRNA mimic. Cells were incubated for a further 5 days and cell proliferation was quantified using an acid phosphate assay. Significant listed as a  $p$  value < 0.0001 (\*\*\*\*). Statistics carried out using GraphPad Prism with the following parameters – un-paired, non-parametric, Mann-Whitney test with two-tailed confidence level, 95% significance.

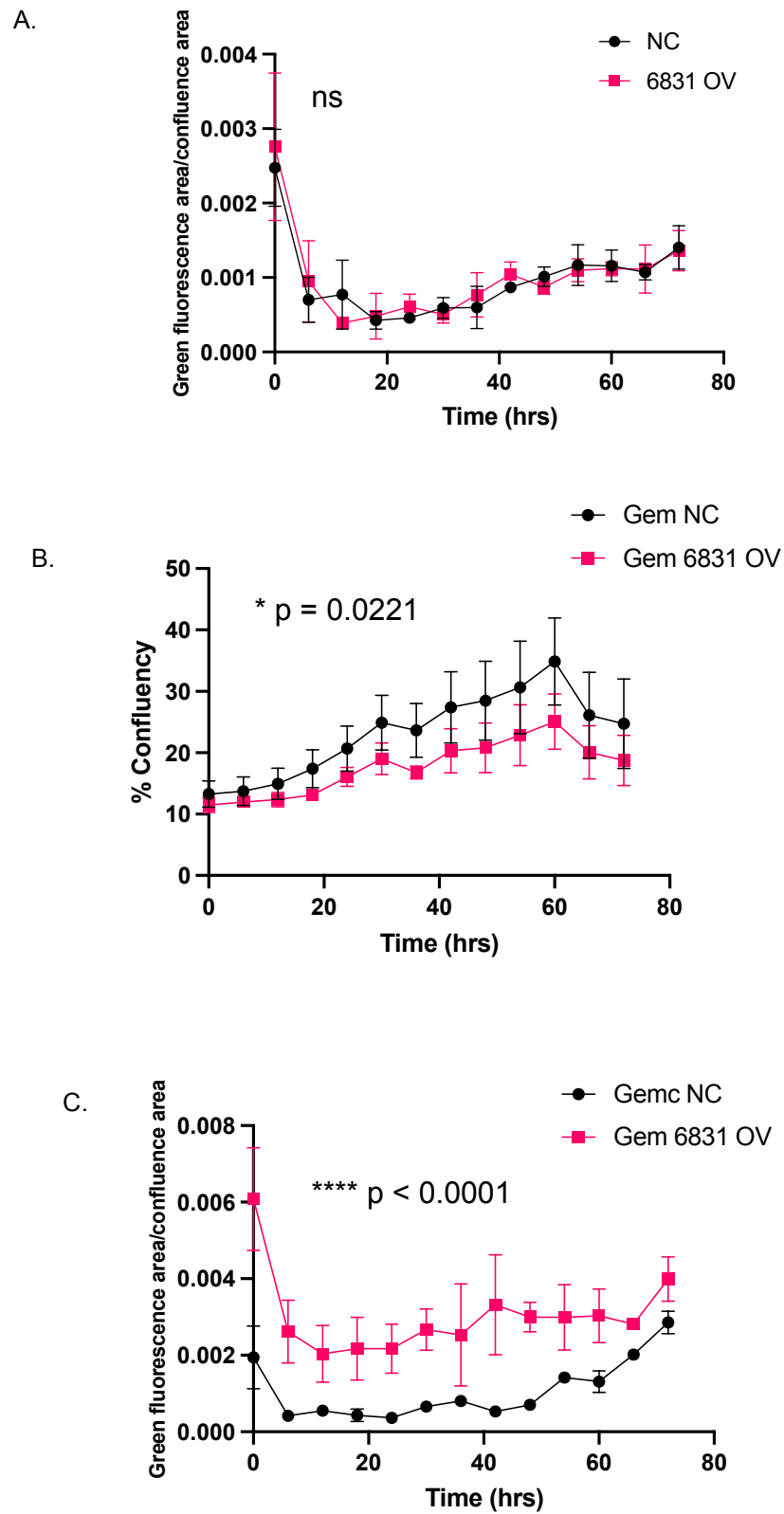


**Figure 3.** miR-6831 overexpression increases gemcitabine toxicity and related apoptosis in PANC-1 cells. All panels show results generated using Incucyte® Live-Cell Analysis at numerous time-points over a 72-hour time-period. The first scan was taken using the Incucyte® imaging system 6 hours after treatment, followed by additional scans 6 hours intervals for 72 hours. Apoptosis was assessed using the Incucyte® Caspase-3/7 Green Dye. (A) Time course of apoptosis in response to miR-6831

overexpressing mimic alone showing no significance. (B) Gemcitabine sensitivity in the presence of negative control and miR-6831 overexpressing mimics with a  $p$  value = 0.0221 (\*). (C) Time course of apoptosis in the presence of 50 nM gemcitabine with a  $p$  value < 0.0001 (\*\*\*). Statistics carried out using GraphPad Prism with the following parameters—un-paired, non-parametric, Mann-Whitney test with two-tailed confidence level, 95% significance.

### 2.3. Exploring altered gene expression in PANC-1 cells through QuantSeq 3' mRNA sequencing upon miR-6831 overexpression

To explore the possible expression changes responsible for the identified phenotype, QuantSeq 3' mRNA-Sequencing was carried out. Results showed a change in the differential expression of 1151 genes, identified following over-expression of miR-6831 in PANC-1 cells, as illustrated in the volcano plot (Figure 4). Based on Log2 fold change the 10 most abundantly down regulated genes were RPL30, RPS17, CCL2, CCZ1B, EFHD1, CSTB, ARL8A, TRAF7, EIF4A3 and FADS2 (FDR corrected  $p$ -value < 0.05). While the 10 highest expressed genes were DYNC112, AP2B1, SPAG9, CLU, FUBP1, AKAP12, NMT1, MORF4L1P1 and PDIA3, DYNC1J1 as described in Supplementary Table 1.

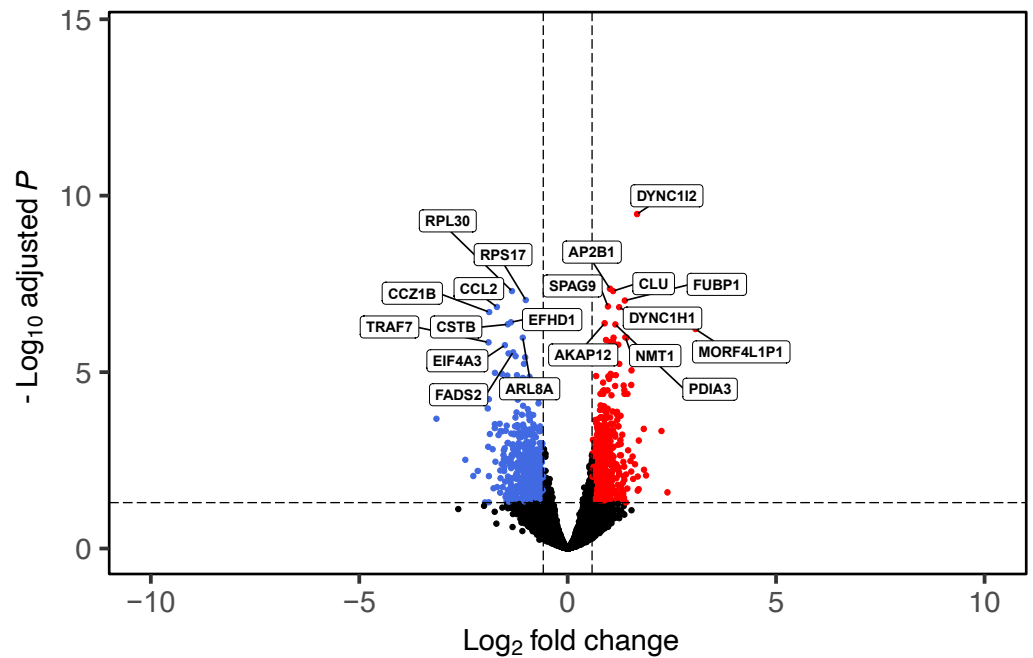
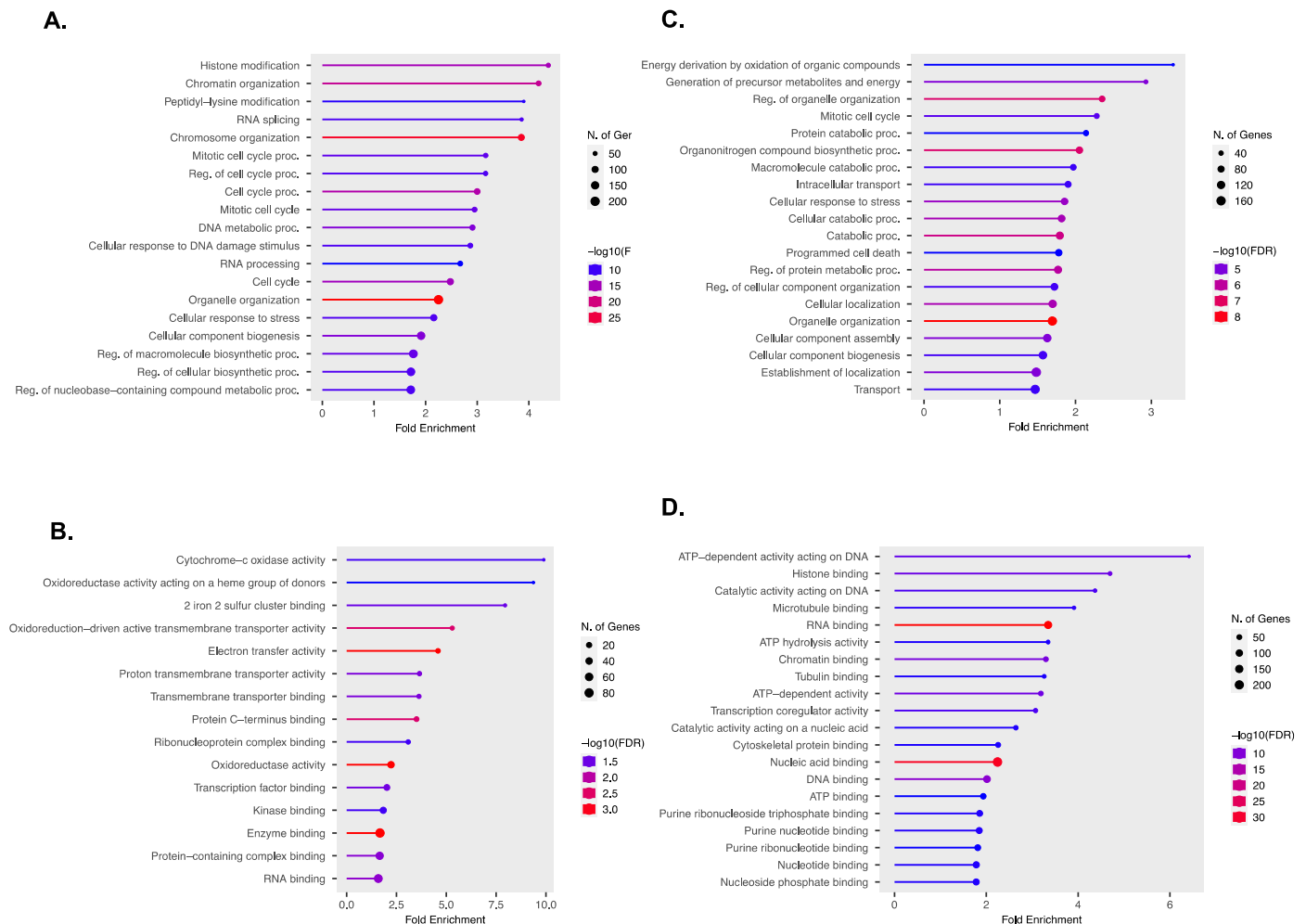


**Figure 4.** Differential Expression of genes in miR-6831 overexpressing PANC-1 cells. Volcano plot depicting differentially expressed genes identified by QuantSeq 3' mRNA-Sequencing in negative control vs. miR-6831 overexpressing PANC-1 cells. Red dots represent upregulated genes while blue dots represent downregulated genes in response to miR-6831 overexpression. Y-axis denotes  $-\log_{10}$  adjusted  $P$  values while X-axis shows  $\log_2$  fold change values. The R package EnhancedVolcano was used to generate volcano plot visualisation.

## 2.4. Gene Ontology and Gene Set Enrichment Analysis revealed intriguing insights into metabolism, mitochondrial function, cell cycle, and apoptosis

In order to determine the physiological roles of the identified differentially expressed genes, Gene Ontology (GO) enrichment analysis was carried out using ShinyGo [39]. This indicated that the biological processes related GO pathways enriched in upregulated genes related to histone modifications and chromatin organisation along with cell cycle related pathways and the regulation of cellular biosynthetic processes (Figure 5A). The down-regulated biological processes GO pathways include more metabolic related terms such as energy deprivation by oxidative phosphorylation, generation of precursor metabolites and energy, protein catabolic processes and organonitrogen compound biosynthetic processes (Figure 5B). Conversely, the molecular function related GO pathways were enhanced for oxidoreductase activity and enzyme binding (Figure 5C) accompanied by a reduction of ATP hydrolysis, ATP-dependent activity, and ATP binding (Figure 5D). These observed alterations in cellular metabolism and energy utilisation suggest potential shifts in the growth and survival dynamics of these cells in response to the overexpression of miR-6831. Furthermore, the diminished availability of purine nucleotides (Figure 5D), crucial molecules intricately involved in energy metabolism, nucleic acid synthesis, and signaling pathways, in response to miR-6831 overexpression indicated a likely reduction in the cellular purine nucleotide pool, thus compromising ATP-dependent processes and nucleic acid synthesis.

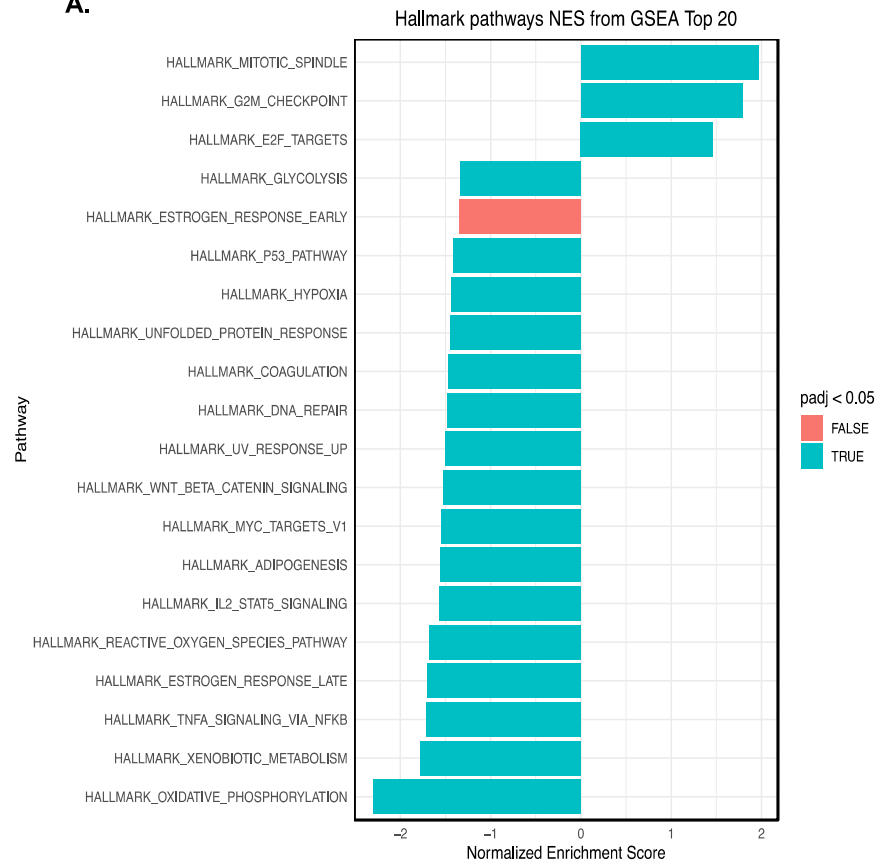


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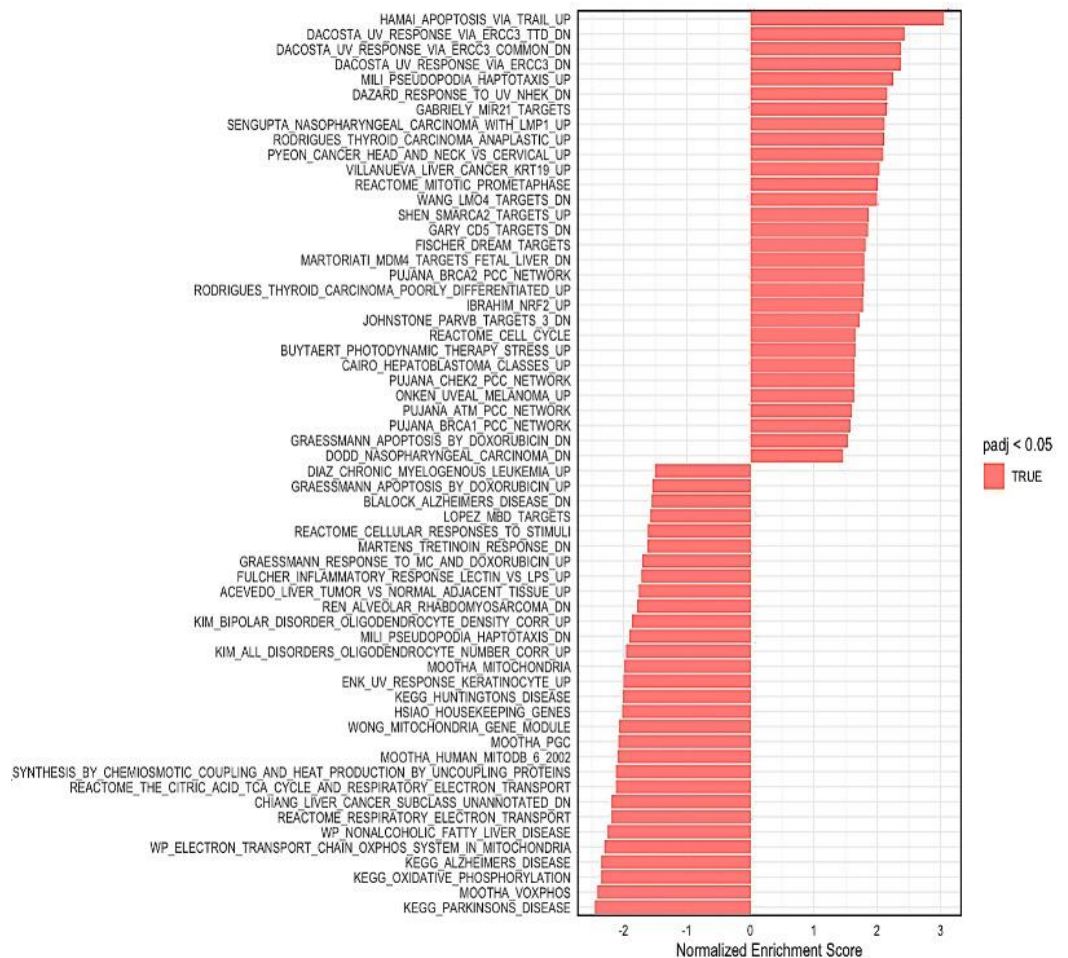
**Figure 5.** Gene Ontology analysis of differentially expressed genes. Advanced bubble plot depicts molecular function-related pathways altered in PANC-1 cells overexpressing miR-6831. Y-axis Labels represent pathway names and enrichment score is shown on X-axis. The size and colour of the bubble represent the number of genes enriched in each pathway and their enrichment significance. Gene Ontology (GO) analysis of the 1103 up- and down-regulated genes ( $\geq 1.5$ -fold,  $P \leq 0.05$ ) was analysed by ShinyGo functional annotation to produce gene clusters ( $\geq 2$  genes/cluster) corresponding to GO annotation terms biological processes (**A,C**) and molecular function (**B,D**). Panel descriptions: (**A**) Biological processes of up-regulated genes. (**B**) Molecular functions of up-regulated genes. (**C**) Biological processes of down-regulated genes. (**D**) Molecular functions of down-regulated genes.

To comprehensively explore distinct biological states, we employed Gene Set Enrichment Analysis (GSEA) using the fgsea package in R, leveraging Hallmark gene sets sourced from the Molecular Signatures Database (MSigDB) (Figure 6). Furthermore, differential gene lists were cross-referenced with the available GSEA C2 curated gene sets. Figure 6A illustrates a pronounced increase in hallmark mitotic spindle, G2M checkpoint, and E2F targets, indicative of active cell cycle progression. Conversely, there was a notable decrease in hallmark glycolysis, hypoxia, and oxidative phosphorylation pathways. Additionally, the significantly differentially expressed gene sets were cross-referenced with C2 curated gene sets available on GSEA, revealing a striking similarity with genes upregulated in apoptosis, alongside a decrease in numerous pathways associated with mitochondria, electron transport chain (ETC)/tricarboxylic acid cycle (TCA cycle), and oxidative phosphorylation (Figure 6B). These findings collectively suggest that the overexpression of miR-6831 may drive alterations in cell cycle regulation, energy metabolism and apoptotic pathways.

A.



B.



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**Figure 6.** Gene set enrichment analysis (GSEA). (A) The fgsea package in R was used, employing Hallmark gene sets sourced from the Molecular Signatures Database (MSigDB) to investigate distinct biological states or processes. Transcriptomic data from the study were processed and ranked, and enrichment scores were computed to assess the overrepresentation of genes within each Hallmark set. The normalised enrichment score (NES) was used to quantify the significance of enrichment, indicating the degree to which a gene set is overrepresented at the top or bottom of the ranked list relative to its size and other gene sets. Positive NES values suggest upregulation of genes associated with the biological process, while negative NES values indicate downregulation. Analysis of NES values offers insights into the biological pathways or states significantly enriched or perturbed under the studied conditions, facilitating interpretation of the underlying biological mechanisms. (B) Differential gene lists were also cross referenced with the available GSEA C2 (curated gene sets, 7233 gene sets).

### *2.5. miR-6831 Overexpression in PANC-1 Cells: Pro-Apoptotic Gene Signature and Impact on Cellular Bioenergetics and Mitochondrial Function*

Heatmaps containing the overlapped genes from each related pathway namely; mitochondrial, ETC, Oxidative phosphorylation (OXPHOS), and apoptosis genes and the Log<sub>2</sub> fold changes (FC) of significant differentially expressed genes are shown in Figure 7. Figure 7A shows a clear reduction of genes associated with mitochondrial pathways, suggesting potential impairments in various aspects of mitochondrial function and cellular metabolism. In particular there is downregulation of genes encoding subunits of the ETC complexes, such as COX5B, COX7A2, and NDUFA4, suggestive of compromised oxidative phosphorylation and reduced ATP production. Similarly, decreased expression of antioxidant defence genes like GPX4 and PRDX3 which could impair the mitochondria's ability to scavenge reactive oxygen species (ROS) and protect against oxidative damage are shown to be downregulated. Furthermore, reductions in mitochondrial ribosomal protein genes, such as MRPL3 and MRPL13, which may disrupt mitochondrial protein synthesis and assembly are shown. Additionally, genes involved in mitochondrial metabolite transport and metabolism, such as SLC25A1 and ACO2 are described among the downregulated genes. As shown in Figure 7B,C,E, it was observed that overexpression of miR-6831 was associated with alterations in mitochondrial function, potentially through the modulation of gene expression related to the ETC and OXPHOS. Specifically, it was found that miR-6831 overexpression led to a decrease in the expression of genes such as NDUF and COX, which encode subunits of complex I (NADH:ubiquinone oxidoreductase) and complex IV (cytochrome c oxidase) of the ETC, respectively. This downregulation of gene expression suggests a disruption in electron transfer and ATP synthesis within the mitochondria, indicating potential impairment in mitochondrial respiration and energy metabolism. These findings suggest that alterations in miR-6831 expression may play a role in influencing cellular bioenergetics and contributing to mitochondrial dysfunction.

The heatmap analysis revealed a notable increase in up-regulated genes associated with apoptosis, as indicated by a predominant red coloration, signifying a positive Log<sub>2</sub>FC (Figure 7C). Specifically, miR-6831 overexpression correlated with significant changes in the expression levels of genes such as RPAP3, TUT4, CDC5L, and RB1CC1, all recognised for their involvement in apoptotic signaling pathways. Furthermore, alterations were observed in genes like BCLAF1, CAPRIN2, and PPIG, known to regulate apoptotic processes and cellular survival. The results described here suggest miR-6831 overexpression had implications for apoptotic regulation.

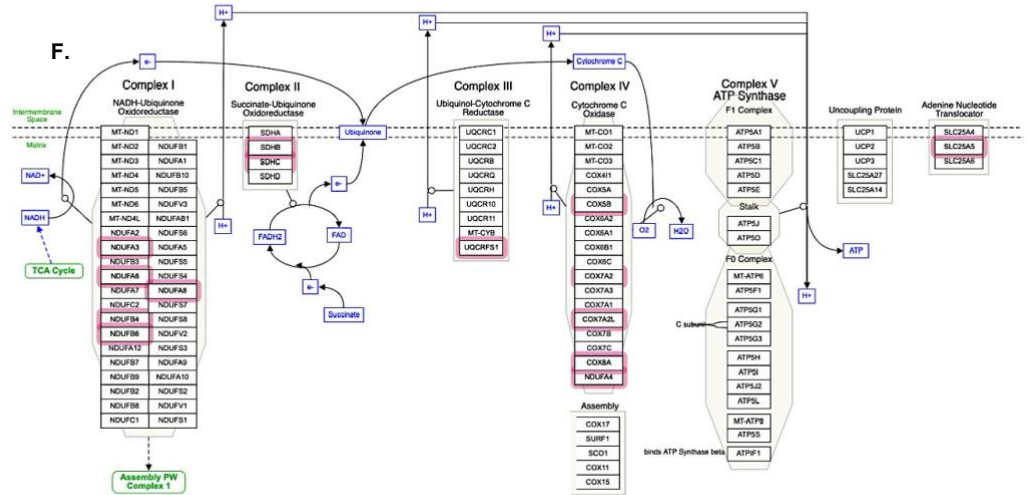
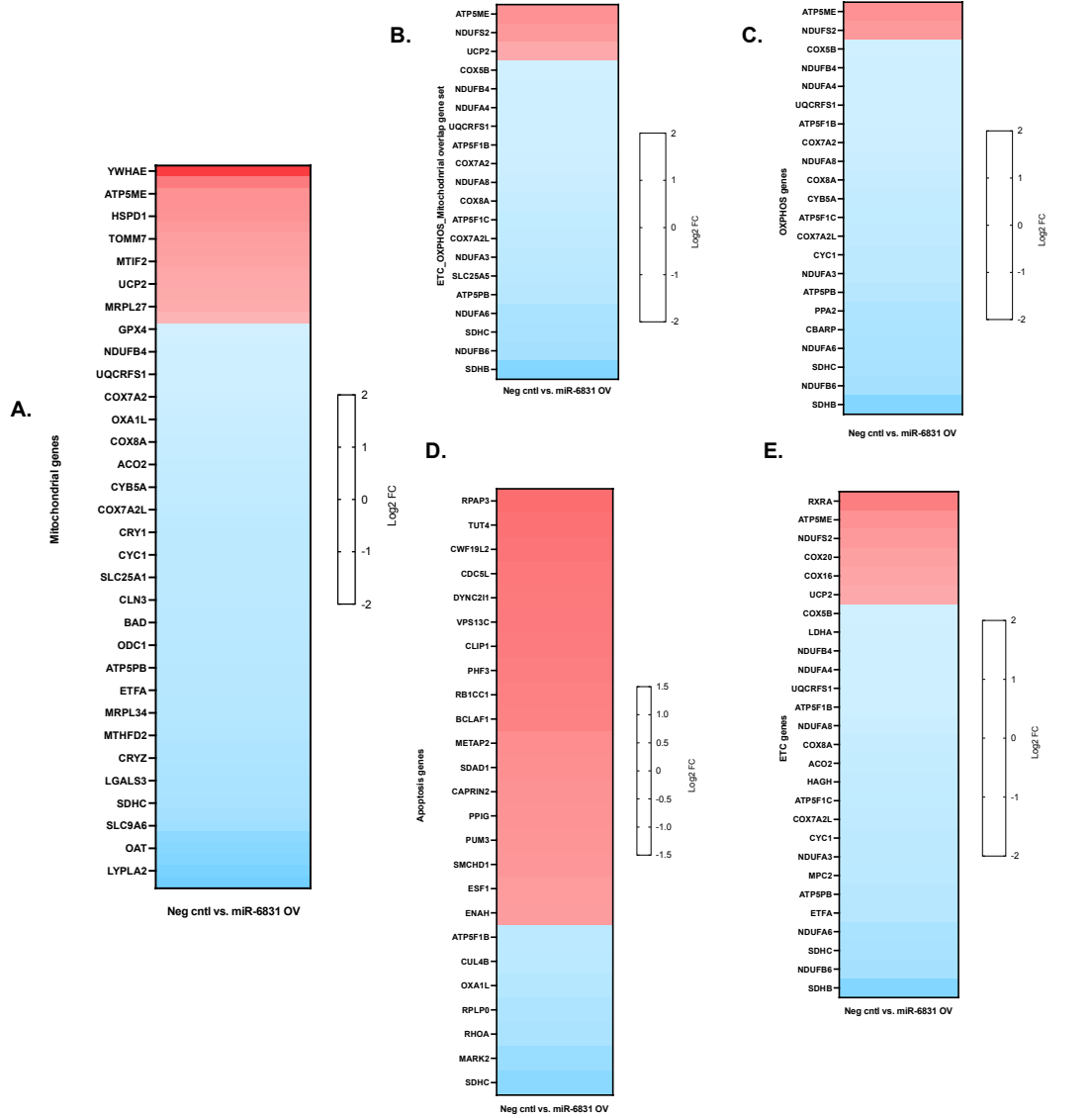


**Figure 7.** Gene Set Enrichment Pathway Analysis. Panel (A–E) Heatmap of Log2 fold changes (FC) of significant differentially expressed genes between negative control and miR-6831 overexpressing PANC-1 cells overlapped with GSEA curated gene sets generated using prism. (F) Down-regulated mRNA and protein integration enrichment analysis was carried out using NDEx Integrated Query. Curated pathways, selected curated and experimentally derived pathways was selected and hits filtered based on *p*-value. The *p*-value, adjusted for false discovery, measures the likelihood of chance overlap between a query set and a network. It's calculated using a hypergeometric distribution, considering the size of the database and the number of shared genes. To counteract high false discovery rates from querying a large database, *p*-values are adjusted using the Benjamini-Hochberg method. This adjusts each *p*-value based on the number of networks queried, ensuring accurate ranking where lower *p*-values indicate stronger significance. Matching *p*-values are further sorted by overlap and alphabetically. Integrated data sets highlighted as associated with curated pathway WP111 (Electron transport chain OXPHOS system in mitochondria) with 14/104 unique genes, similarity: 0.07 and *p*-Value: 2.00e-1.

## 2.6. Parallel Transcriptomic and Proteomic Analysis Reveals Molecular Signatures of miR-6831 Overexpression in PANC-1 Cells

In conjunction with the transcriptomic analysis, complementary proteomic investigations were conducted to provide a comprehensive understanding of the molecular alterations associated with miR-6831 overexpression in PANC-1 cells. The addition of proteomic data allowed for the elucidation of post-transcriptional modifications and protein-level changes that may further elucidate cellular responses. Notably, key upregulated proteins such as BCCIP, IDH1, ROCK2, and PHF6 were identified. Proteins known to be involved in DNA repair and cell cycle regulation, such as BCCIP, and metabolic processes and oncogenesis, such as IDH1, were observed to be upregulated in our dataset (see Table 1). Additionally, the upregulation of ROCK2 and PHF6 suggested potential implications for cell proliferation and transcriptional regulation. In this analysis, downregulated proteins known to be involved in mitochondrial dynamics, protein synthesis, and energy metabolism, such as SMC3, OPA1, STOML2, RPL10, and AK3, were identified (Table 1). The integration of transcriptomic and proteomic datasets provides a more comprehensive understanding of the molecular mechanisms underlying miR-6831-mediated effects in pancreatic cancer cells.

**Table 1.** Differentially expressed proteins in negative control vs. miR-6831 overexpressing PANC-1 cells. (A) Table displays the accession number, protein symbol and description along with the fold change (FC) and associated *p* value for both up-regulated (A) and down-regulated (B) proteins.

## A.

| Accession | Symbol  | Description                                                  | Highest condition | FC   | p value     |
|-----------|---------|--------------------------------------------------------------|-------------------|------|-------------|
| P23919    | DTYMK   | Thymidylate kinase                                           | miR-6831 OV       | 2.83 | 0.026911693 |
| P33991    | MCM4    | DNA replication licensing factor MCM4                        | miR-6831 OV       | 2.61 | 0.004088933 |
| Q13011    | ECH1    | Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial   | miR-6831 OV       | 2.55 | 0.016191495 |
| P13473    | LAMP2   | Lysosome-associated membrane glycoprotein 2                  | miR-6831 OV       | 2.20 | 0.014731532 |
| P07437    | TUBB    | Tubulin beta chain                                           | miR-6831 OV       | 2.20 | 0.026354139 |
| P42166    | TMPO    | Lamina-associated polypeptide 2, isoform alpha               | miR-6831 OV       | 2.03 | 0.021823544 |
| Q9NQW6    | ANLN    | Anillin                                                      | miR-6831 OV       | 1.98 | 0.000466383 |
| O00629    | KPNA4   | Importin subunit alpha-3                                     | miR-6831 OV       | 1.98 | 0.035966594 |
| P12814    | ACTN1   | Alpha-actinin-1                                              | miR-6831 OV       | 1.90 | 0.017706476 |
| P06576    | ATP5F1B | ATP synthase subunit beta, mitochondrial                     | miR-6831 OV       | 1.89 | 0.028921137 |
| Q8IWS0    | PHF6    | PHD finger protein 6                                         | miR-6831 OV       | 1.86 | 0.001926036 |
| Q5H9R7    | PPP6R3  | Serine/threonine-protein phosphatase 6 regulatory subunit 3  | miR-6831 OV       | 1.84 | 0.004492794 |
| P61026    | RAB10   | Ras-related protein Rab-10                                   | miR-6831 OV       | 1.80 | 0.035391968 |
| Q9H4M9    | EHD1    | EH domain-containing protein 1                               | miR-6831 OV       | 1.77 | 0.029907118 |
| O75116    | ROCK2   | Rho-associated protein kinase 2                              | miR-6831 OV       | 1.76 | 0.003243873 |
| Q7L576    | CYFIP1  | Cytoplasmic FMR1-interacting protein 1                       | miR-6831 OV       | 1.76 | 0.001508345 |
| P13489    | RNH1    | Ribonuclease inhibitor                                       | miR-6831 OV       | 1.72 | 0.013437312 |
| P12956    | XRCC6   | X-ray repair cross-complementing protein 6                   | miR-6831 OV       | 1.71 | 0.008679443 |
| Q13435    | SF3B2   | Splicing factor 3B subunit 2                                 | miR-6831 OV       | 1.69 | 0.001816943 |
| Q12765    | SCRN1   | Secernin-1                                                   | miR-6831 OV       | 1.67 | 0.00739604  |
| O75874    | IDH1    | Isocitrate dehydrogenase [NADP] cytoplasmic                  | miR-6831 OV       | 1.67 | 0.024670546 |
| P51858    | HDGF    | Hepatoma-derived growth factor                               | miR-6831 OV       | 1.67 | 0.004845708 |
| P30043    | BLVRB   | Flavin reductase (NADPH)                                     | miR-6831 OV       | 1.67 | 0.001481428 |
| P67936    | TPM4    | Tropomyosin alpha-4 chain                                    | miR-6831 OV       | 1.62 | 0.029070035 |
| P53582    | METAP1  | Methionine aminopeptidase 1                                  | miR-6831 OV       | 1.57 | 0.008828481 |
| O14964    | HGS     | Hepatocyte growth factor-regulated tyrosine kinase substrate | miR-6831 OV       | 1.57 | 0.001943666 |
| P46013    | MKI67   | Proliferation marker protein Ki-67                           | miR-6831 OV       | 1.57 | 0.022364736 |
| Q7L2E3    | DHX30   | ATP-dependent RNA helicase DHX30                             | miR-6831 OV       | 1.56 | 9.80E-05    |
| Q9NZI8    | IGF2BP1 | Insulin-like growth factor 2 mRNA-binding protein 1          | miR-6831 OV       | 1.54 | 0.002761954 |
| Q9P287    | BCCIP   | BRCA2 and CDKN1A-interacting protein                         | miR-6831 OV       | 1.54 | 0.003381406 |
| Q12907    | LMAN2   | Vesicular integral-membrane protein VIP36                    | miR-6831 OV       | 1.53 | 0.014422221 |
| P46937    | YAP1    | Transcriptional coactivator YAP1                             | miR-6831 OV       | 1.53 | 0.010983206 |
| P62937    | PPIA    | Peptidyl-prolyl cis-trans isomerase A                        | miR-6831 OV       | 1.51 | 0.034279267 |

## B.

| Accession | Symbol  | Description                                                    | Highest condition | FC   | p value     |
|-----------|---------|----------------------------------------------------------------|-------------------|------|-------------|
| P12268    | SMC3    | Structural maintenance of chromosomes protein 3                | Neg Cntl          | 3.66 | 0.002013894 |
| Q9UQE7    | ERGIC3  | Endoplasmic reticulum-Golgi intermediate compartment protein 3 | Neg Cntl          | 2.17 | 0.001756791 |
| O60313    | RPL10   | 60S ribosomal protein L10                                      | Neg Cntl          | 2.06 | 0.000908472 |
| Q9UIJ7    | NOP14   | Nucleolar protein 14                                           | Neg Cntl          | 1.96 | 0.000412508 |
| P38646    | CDC37   | Hsp90 co-chaperone Cdc37                                       | Neg Cntl          | 1.92 | 0.005018333 |
| P09382    | IMPDH2  | Inosine-5'-monophosphate dehydrogenase 2                       | Neg Cntl          | 1.89 | 0.001699514 |
| P60709    | HLTF    | Helicase-like transcription factor                             | Neg Cntl          | 1.84 | 0.001334273 |
| O00267    | HSPA9   | Stress-70 protein, mitochondrial                               | Neg Cntl          | 1.73 | 0.000721128 |
| Q96DI7    | LGALS1  | Galectin-1                                                     | Neg Cntl          | 1.69 | 0.039197389 |
| Q14527    | VASP    | Vasodilator-stimulated phosphoprotein                          | Neg Cntl          | 1.67 | 0.002945526 |
| P50552    | PSMD8   | 26S proteasome non-ATPase regulatory subunit 8                 | Neg Cntl          | 1.66 | 0.001402629 |
| Q16543    | AK3     | GTP:AMP phosphotransferase AK3, mitochondrial                  | Neg Cntl          | 1.65 | 0.003690725 |
| O00148    | OPA1    | Dynamin-like 120 kDa protein, mitochondrial                    | Neg Cntl          | 1.64 | 0.000912922 |
| P27635    | STOML2  | Stomatin-like protein 2, mitochondrial                         | Neg Cntl          | 1.61 | 0.004412498 |
| Q9Y678    | ACTB    | Actin, cytoplasmic 1                                           | Neg Cntl          | 1.59 | 0.035159322 |
| P48556    | SUPT5H  | Transcription elongation factor SPT5                           | Neg Cntl          | 1.59 | 0.00055917  |
| Q9UJZ1    | COPG1   | Coatomer subunit gamma-1                                       | Neg Cntl          | 1.58 | 0.007632414 |
| P11940    | PSMD9   | 26S proteasome non-ATPase regulatory subunit 9                 | Neg Cntl          | 1.56 | 0.018691429 |
| P78316    | DDX39A  | ATP-dependent RNA helicase DDX39A                              | Neg Cntl          | 1.55 | 0.000970904 |
| O00233    | PABPC1  | Polyadenylate-binding protein 1                                | Neg Cntl          | 1.54 | 6.80E-05    |
| Q9Y282    | SNRNP40 | U5 small nuclear ribonucleoprotein 40 kDa protein              | Neg Cntl          | 1.52 | 0.00260501  |

### 2.7. Enrichment analysis of differentially expressed proteins

In order to determine the physiological roles of the identified differentially expressed proteins, GO enrichment analysis was carried out using ShinyGo 0.80 [39]. This analysis indicated that GO pathways related to biological processes involved in response to IL-7 were increased. This was accompanied by GO pathways related to protein localisation to lysosome, vacuole and organelle indicative of alterations in intracellular trafficking and protein degradation (Figure 8A). For the molecular function related GO pathways elevations in helices activity, protein C-terminus binding, cell adhesion, and cytoskeleton molecule binding indicated broader alterations in cellular signaling, protein-protein interactions, and cytoskeletal organisation (Figure 8C). The GO terms for both biological processes and molecular function for the down-regulated proteins highly overlapped with the terms identified in the transcriptomic data (Figure 5C,D) such as mitochondrial maintenance, ATP-related activity and nucleotide activity (Figure 6B,D). To gain a better understand of what proteins were interacting together in these GO pathways, enrichment analysis was performed on the altered protein lists using Enrichr KG (Figure 8E). This

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analysis demonstrated that the downregulated proteins IMPDH2, AK3, OPA1 and HSPA9 interacted together in pathways related to negative regulation of apoptosis, nucleoside metabolism and mitochondrial organisation. In addition other downregulated proteins PSMD9, PSMD8 and STOML2 converged on pathways relating to regulation of amino acid metabolic processes and G2/M cell cycle. The upregulated proteins converge on immune related pathways, specifically neutrophil mediated immune response. These proteins are IDH1, PPIA, TUBB, RAB10, LAMP2, XRCC6 and CYFIP1 (Figure 8E).

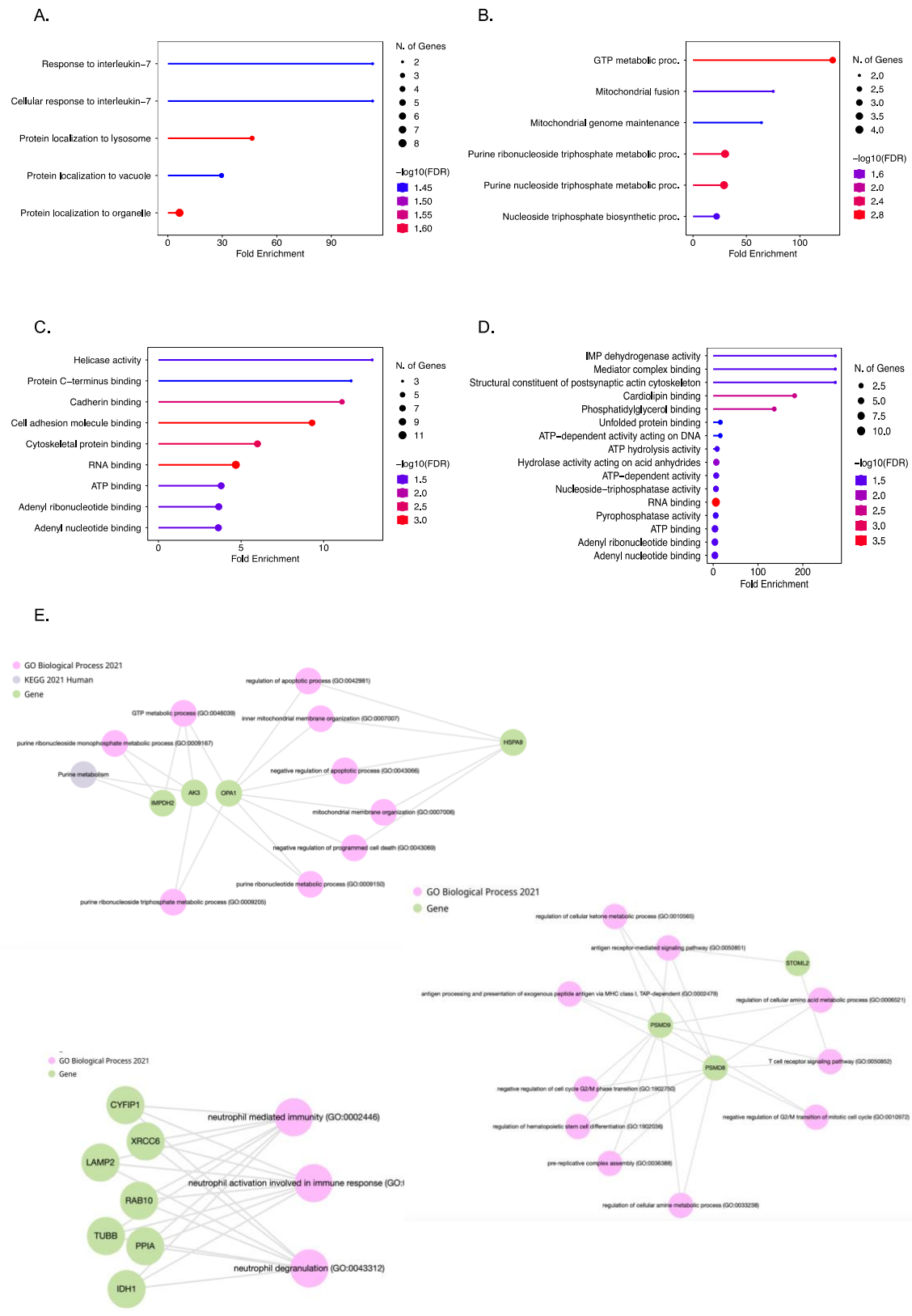


**Figure 8.** Enrichment analysis of differentially expressed proteins. Advanced bubble plot depicts molecular function-related pathways altered in PANC-1 cells overexpressing miR-6831. Y-axis Labels represent pathway names and enrichment score is shown on X-axis. The size and colour of the bubble represent the number of genes enriched in each pathway and their enrichment significance. Gene Ontology (GO) analysis of the up- and down-regulated proteins ( $\geq 1.5$ -fold,  $P \leq 0.05$ ) was analysed by ShinyGo functional annotation to produce clusters ( $\geq 2$  genes/cluster) corresponding to GO annotation terms biological processes (panel A,C) and molecular function

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(B,D). Panel descriptions: (A) Biological processes of up-regulated proteins. (B) Molecular functions of up-regulated proteins. (C) Biological processes of down-regulated proteins. (D) Molecular functions of down-regulated proteins. E. Enrichment analysis was performed on the altered protein lists using Enrichr KG. The KEGG Pathways and/or gene ontology gene set libraries were selected for the analysis. A subnetwork that connects the top enriched terms and the altered proteins that appear in at least two libraries is shown. Input terms appear as green nodes, KEGG pathways are in light purple, and GO biological processes are in blue.

#### *2.8. Comparative Analysis of transcriptomic and proteomic analysis and TargetScan-Predicted miR-6831 Targets Against Experimentally Validated Targets*

We compared the extent of overlap between miR-6831 targets identified in the transcriptomic and proteomic analysis (Figure 9A). An overlap of 13 targets was observed and are listed in Figure 9B. Figure 9B also described the expression in mRNA and protein to determine targets that are altered in the same direction in both comparisons or if there are changes at the protein level that are different to the transcriptomic level to gain better understanding of the nature of the miRNA-mRNA-protein relationship. In addition, this investigation compared the alignment between TargetScan-predicted miR-6831 targets and experimentally validated ones. TargetScan employs a multifaceted approach, considering complementary base pairing, evolutionary conservation, target site accessibility, and context scores for miRNA target prediction. Due to the limited literature describing miR-6831 targets—only 5 hits on PubMed to date—we aimed to enhance our understanding by evaluating the overlap between TargetScan predictions and experimentally validated targets. This assessment encompassed both transcriptomic (Figure 10A) and proteomic (Figure 10B) targets identified in our study. Among the 1151 identified targets, 69 overlapped with the 1332 predicted by TargetScan. Despite the modest overlap in the protein target list, with only 2 common targets (AK3 and TUBB) out of 1332, this analysis sheds light on the predictive capability of TargetScan for miR-6831. Detailed results, including all overlapping targets, are delineated beneath corresponding Venn diagrams in Figure 10.

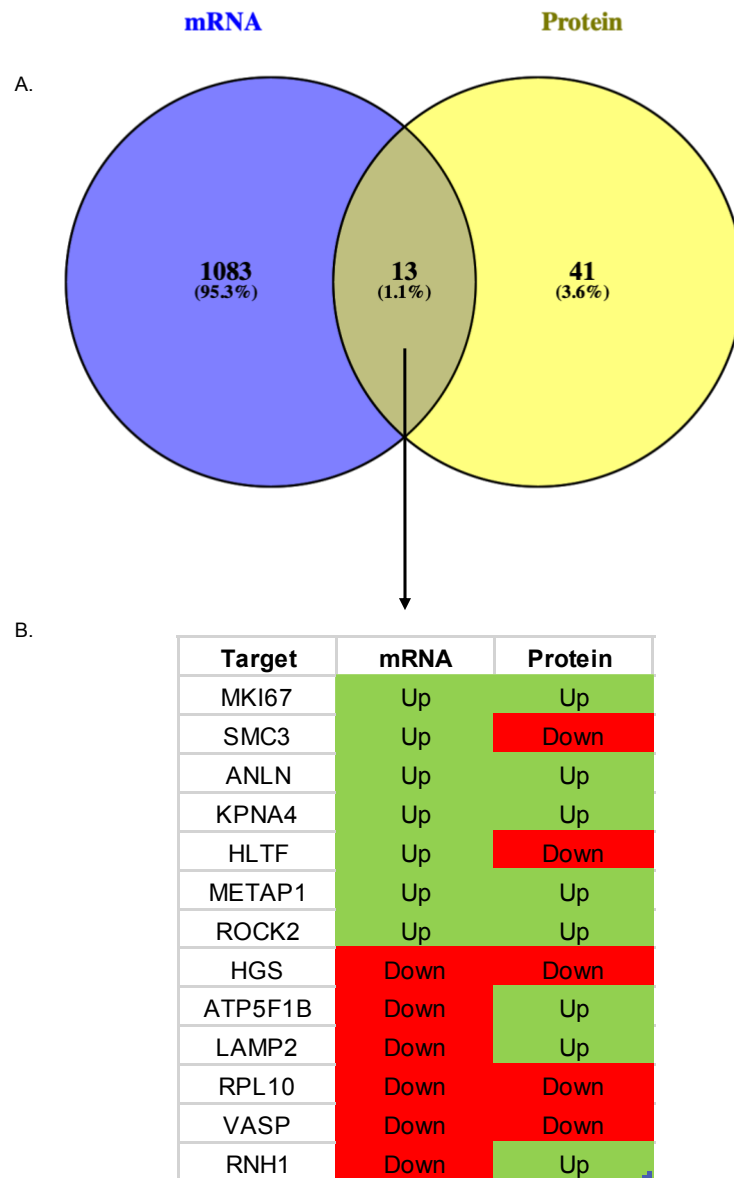


**Figure 9.** Overlap between RNA sequencing and proteomic identified targets. Overlap Venn Diagrams were generated using Venny 2.0 . Common targets are listed below with expression levels for both the mRNA and protein with upregulated highlighted in green and downregulated highlighted in red demonstrating if expression was up/down in both conditions or up in one and down in the other and visa versa.

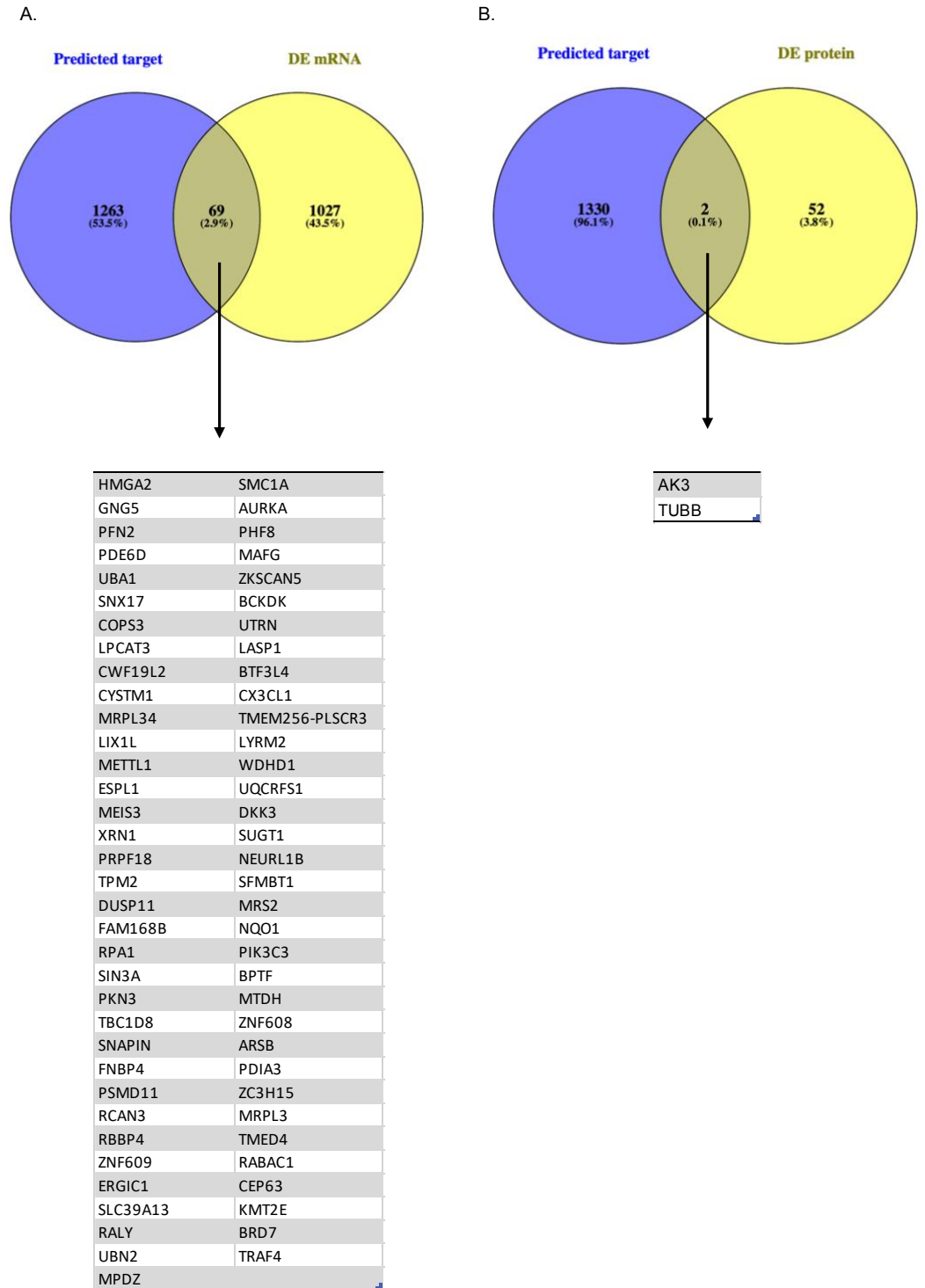


**Figure 10.** Overlap between predicted and experimental miR-6831 targets. Predicted targets were taken from TargetScan and overlap between predicted with mRNA (A) and protein (B) was generated using Venny 2.0 . Shared targets are listed below.

### 3. Discussion

miR-6831-5p was previously identified as a miRNA of interest by our team [28], the present investigation aimed to validate its functional significance *in vitro* within the context of pancreatic cancer, specifically the PANC-1 cell line. We evaluated proliferation, apoptosis and gemcitabine sensitivity and showed that the overexpression of miR-6831 in PANC-1 cells significantly reduced proliferation by forty percent (Figure 2). The results shown in Figure 2 are interesting as the overexpression of miR-6831-5p leads to reduced

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cell viability despite its upregulation in tumour versus normal samples, this could be explained by the differences in the model systems used. The initial identification of miR-6831-5p was conducted in PDX models and tumour tissues that represent a more heterogeneous environment, including stromal interactions and immune components, compared to established cell lines. This complexity can significantly influence miRNA expression and function. miR-6831-5p may have context-dependent roles, acting as an oncogene in the heterogeneous environment of PDX models and tumour tissues by counterbalancing oncogenic signals to reduce disease load. In the more simplified and controlled environment of established cell lines, the overexpression of miR-6831-5p might lead to a reduction in cell viability by predominantly targeting and downregulating critical survival pathways. The absence of the complex tumor microenvironment in cell lines could mean that miR-6831-5p exerts a stronger influence on specific cell viability-related pathways, leading to an apparent contradiction where its upregulation results in decreased cell viability.

The paucity of data on miR-6831-5p about its molecular mechanisms, resulted in it being selected for additional study. The PANC-1 cell line was selected as a model system due to its widespread use and well-characterised nature in pancreatic cancer research. Such a model allows easier identification of molecular mechanisms, including comprehensive gene and protein expression analyses. However, there is the limitation that such cell line models as PANC-1 do not fully reflect the complexity *in vivo* or between cell line models, despite this the data presented provides valuable data into possible function in pancreatic cancer and forms the basis for additional validation work.

Interestingly, there was no increase in apoptosis in response to miR-6831 alone (Figure 3A), however, it did lead to an increase in gemcitabine related apoptosis (Figure 3C). This increase in gemcitabine related apoptosis was complemented by an increased sensitivity to gemcitabine toxicity illustrated by an increased survival in response to 50 nM of the drug (Figure 3B). We conducted comprehensive transcriptomic and proteomic analyses using 3' QuantSeq RNA sequencing and mass spectrometry, respectively (Figure 4 and 2). This allowed us to gain better insight into the potential mechanisms behind the observed phenotypic changes and suggested that miR-6831 exhibited an effect on three main biological pathways; a mitochondrial related bioenergetic shift, purine nucleotide homeostasis and apoptosis (Figure 7).

Dysregulation of oxidative phosphorylation (OXPHOS), glycolysis, and fatty acid oxidation are hallmarks of cancer, presenting opportunities for targeted intervention [40]. In this context, the observed changes in response to miR-6831 overexpression offer valuable insights into the potential modulation of metabolic pathways in cancer cells. Mitochondria, are indispensable for cancer cell survival and growth [41]. The OXPHOS pathway fulfils the bioenergetic needs of cancer cells by generating ATP. Carbon substrates in the tricarboxylic acid cycle (TCA cycle) produce electron donors, feeding electrons into electron transport chain (ETC) complexes I to IV, establishing a proton motive force [42]. Notably, deficiencies in ETC function hinder mitochondrial ATP production in cancer cells, impacting their bioenergetic capacity [43]. Despite this, they manage to sustain proliferation by generating ATP through glycolysis, indicating a compensatory mechanism to meet their energy needs [43]. The GO analysis suggested a change in metabolic profile with a reduction in energy derivation by oxidation of organic compounds, precursor metabolites and ATP-dependent processes (Figure 5). Using NDEx Integrated Query our parallel transcriptomic and proteomic data sets were shown to be involved in numerous steps along the ETC OXPHOS system in mitochondria, particularly complex I and IV (Figure 7F).

The findings from the GO analysis were mirrored in the gene set enrichment analysis described in Figure 6. Cells with deficiencies in the ETC can sustain proliferation through other methods such as glycolysis [44]. The gene set enrichment showed a marked decrease in both ETC related functions and oxidative phosphorylation and this was accompanied by a decrease in hallmark glycolysis (Figure 6A). DepMap used for this analysis and other genome-wide CRISPR-Cas9 deletion screens are invaluable resources for comprehending

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gene essentiality in cancer cells [45]. These encompass genes engaged in various crucial pathways: OXPHOS (e.g., *NDUFA2*, *CYCS*), the TCA cycle (e.g., *SDHC*, *PC*), lipid metabolism (e.g., *ABCB10*, *PTPM1*), nucleotide metabolism (e.g., *DHODH*), amino acid metabolism (e.g., *PRODH*, *PRODH2*), calcium transport (e.g., *LETM1*), ROS production and detoxification (e.g., *ROMO1*, *SOD1*), and apoptosis (e.g., *BCL2L1*) [46]. Expression alterations in many of these essential genes are illustrated in Figure 7 with clear shifts towards down-regulated ETC, OXPHOS and mitochondrial function (Figure 7 A,C,E) accompanied by an increase in apoptosis (Figure 7D). These transcriptomic changes are reflected in the proteomics whereby several proteins that play crucial roles in regulating mitochondrial function are altered (Table 1) for example, *OPA1* [47] and *STOML2* [48] have been shown to contribute to mitochondrial fusion and function, respectively. *HSPA9*, also known as mortalin or *GRP75* prevents cytochrome c release from mitochondria [49] and *ATP5F1B* is primarily associated with mitochondrial ATP synthase [50].

Recent findings highlight certain tumours heightened reliance on OXPHOS for ATP production, potentially discernible through specific genetic mutations, such as *SMARCA4* [51]. The PANC-1 cell line employed here exhibits a deficiency in *SMARCA4* protein expression [52]. Notably, pancreatic cancer exemplifies a tumour type highly dependent on OXPHOS, evidenced by elevated expression of mitochondrial respiratory complex I components, whereby inhibitors targeting complex I have shown efficacy when combined with conventional chemotherapy [53]. Here, we have shown the downregulation of many genes associated with complex I (Figure 7F). Computational analysis of transcriptomic data across multiple tumor types revealed upregulation of mitochondrial oxidative phosphorylation (OXPHOS) genes in 35% of cancer. Driver mutations in certain cancers involve mutations in TCA cycle genes like *IDH1/2*, *FH*, and subunits A-D of succinate dehydrogenase (*SDH*) [54][55]. *IDH1* was shown to be up-regulated in our proteomics data set with a fold change of 1.67 and an associated *p* value of 0.02 (Table 1). Pancreatic cancer cell lines, previously classified into metabolic groups, exhibit distinct characteristics, with PANC-1 cells favouring respiratory metabolism (OXPHOS), displaying elongated mitochondria, increased TCA cycle metabolites, fatty acids, and heightened expression of *CPT1A* [53]. Others have shown that manipulating PANC-1 cells towards a low OXPHOS phenotype increased gemcitabine sensitivity [56]. We showed an increased gemcitabine sensitivity (Figure 3) accompanied by a clear decrease in OXPHOS related pathways in both omic data sets (Figure 7 and 8). In addition, clusterin (*CLU*) listed as one of the most significantly up-regulated genes in our data set (Figure 4 and Table 1), has previously been linked with enhanced sensitivity of pancreatic cancer cells to gemcitabine [56].

The reduction in purine ribonucleoside triphosphate binding, purine nucleotide binding, and nucleoside phosphate binding observed in our gene ontology analysis (Figure 5) suggested disruptions in cellular processes influencing gemcitabine metabolism and DNA repair pathways, possibly explaining the enhanced gemcitabine sensitivity observed in response to miR-6831 overexpression (Figure 3B). Gemcitabine, is a prodrug phosphorylated by deoxycytidine kinase (*dCK*) to its active metabolites gemcitabine di- and triphosphate (*dFdCDP* and *dFdCTP*) [57]. These metabolites inhibit DNA synthesis through various mechanisms, including incorporation into DNA, inducing chain termination, and inhibiting DNA repair enzymes, crucial for gemcitabine-induced apoptosis [58]. The enhanced cytotoxic effects of gemcitabine observed were accompanied by increased gemcitabine-induced apoptosis (Figure 3C). Various metabolites enhance gemcitabine's action through self-potential mechanisms, including depletion of *dCTP* pools by *dFdCDP*, inhibition of ribonucleotide reductase (*RR*), reducing competing deoxyribonucleotide pools necessary for DNA synthesis and RNA incorporation by *dFdCTP* [59]. Our proteomic data showed alterations in ribonucleases with an increase in the ribonuclease inhibitor *RNH1* and a reduction in the 60S ribosomal protein *L10*, *RPL10* (Table 1). One downstream molecular pathway by which gemcitabine incorporation into DNA induces cell death involves regulation by apoptosis-associated genes such as *p53*, *bcl-2*, *bcl-xL*, and *BAX*. In certain solid human tumor cell lines like non-small cell lung

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cancer and pancreatic cancer, gemcitabine induces apoptosis by activating caspases, suggesting their role in gemcitabine sensitivity/resistance [57]. Our data set shows a decrease in the anti-apoptotic bcl-xL gene [60], also known as BCL2L12, in addition to an increase in apoptotic related genes BCLAF1 [61] and PUM3 [62] (Figure 7). These findings are complemented by our proteomic data whereby various pro-apoptotic related proteins including TMPO [63], ROCK2 [64], and YAP1 [65] were upregulated in our data sets (Table 1). Anti-apoptotic HSPA9 protein [66] was down-regulated. Interestingly, HSPA9 had no expression changed in the mRNA data suggesting it as a direct post-translational target of miR-6831.

We overlapped mRNA and protein target lists to identify shared targets (Figure 9A). We assessed their expression at both levels to discern concurrent regulation, possibly indicating transcriptional control (Figure 9B). We also identified targets upregulated solely at the protein level, suggesting direct miRNA-mediated post-translational regulation (Figure 9B). Additionally, we overlapped expression changes with TargetScan predicted targets (Figure 10). Of 1332 predicted targets, 69 overlapped with experimentally validated mRNA targets, and 2 with identified protein targets, meaning 53.5% and 96.1% of predicted targets did not align with our mRNA and protein lists, suggesting a potential false-positive rate. It's worth noting that our study was limited to PANC-1 cells, and a broader panel of cell lines encompassing various cancer and non-disease cell types would offer a more comprehensive assessment of prediction specificity.

In summary, our study significantly expands upon the existing understanding of miR-6831-5p and unveils its functional significance within the realm of pancreatic cancer, with a specific focus on the PANC-1 cell line. Our findings pave the way for further exploration of miR-6831-5p as a potential therapeutic target.

## 4. Materials and Methods

### 4.1. Data acquisition and analysis

The acquisition and generation of Patient-Derived Xenograft (PDX) models, along with the comprehensive multi-omic analysis, were previously described in our prior publications [26-28]. This study focuses on a curated miRNA priority list, derived from those exhibiting up-regulation in tumor vs. normal and tumor vs. F1, while considering their novelty in existing literature. The final target miR-6831-5p was chosen for further validation.

### 4.2. *In silico* miRNA-mRNA interaction analysis

Putative target genes of the priority miRNAs were obtained from the miRNA target prediction database TargetScan ([http://www.targetscan.org/vert\\_80/](http://www.targetscan.org/vert_80/)). The software Venny 2.1.0 (<https://csbg.cnb.csic.es/BioinfoGP/venny.html>) was used to generate a Venn diagram of predicted versus experimental targets.

### 4.3. Cell culture

The human pancreatic cell lines PANC-1 and MIA PaCa-2 (ATCC, Rockville, MD, USA) were cultured in DMEM high glucose (Sigma-Aldrich, D5671) supplemented with 5% (v/v) fetal calf serum and 2% (v/v) l-glutamine (Thermo Fisher, 2503008). BxPC-3 and Capan-2 (ATCC, Rockville, MD, USA) cells were grown in RPMI-1640 Medium (Sigma-Aldrich, R8758) supplemented with 5% (v/v) FBS. HPAC cells (ATCC, Rockville, MD, USA) were cultured in DMEM high glucose (Sigma-Aldrich, D5671) supplemented with 10% (v/v) fetal calf serum. Cells were grown in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C in T-25 and T-75 culture flasks. Cells were routinely split using PBS to wash and trypsin-EDTA (Gibco, 25300054) when 80–90% confluency was reached.

### 4.4. miRNA mimic transfection

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The Mirus TransIT-X2 transfection reagent (Medical Supply Co Ltd, Ireland) was used to introduce miRNA mimics as per the manufacturer's instructions. Cells were seeded at a density of  $2 \times 10^5$  (PANC-1) in 6-well plates 24 hours before transfection. A mixture of 100  $\mu$ L of serum-free DMEM/F-12 Hams medium and a 10  $\mu$ M mirVana™ miRNA mimic (Bio-Sciences Ltd, Ireland) along with 2  $\mu$ L of TransIT-X2, was gently prepared and allowed to incubate at room temperature for 30 minutes. The solution was then added in a dropwise fashion to the cells seeded the previous day. The cells were incubated for 24 hours before RNA collection for QuantSeq 3' mRNA-Seq library prep and 48 hours for phenotypic analysis.

#### 4.5. 2D proliferation

PANC-1 cells were seeded in 6-well plates at a density  $2 \times 10^4$  cells/mL, and incubated overnight at 5% CO<sub>2</sub> and 37 °C. The following day, freshly prepared mirVana™ miRNA mimic dilutions were added. After 24 hours, the media was changed. The plates were incubated for a further 5 days and cell proliferation was quantified using an acid phosphate assay as previously described [34]. Briefly, the wells were washed with phosphate buffered saline (PBS). Paranitrophenol phosphate (PNP) (ThermoFisher) substrate (10 mM of PNP in sodium acetate buffer, pH 5.5) was added to each well and incubated at 37 °C for 1–2 hours. 50  $\mu$ L of 1 M NaOH was added and the absorbance was read at 405 nm (reference – 620 nm).

#### 4.6. Incucyte® Live-Cell Analysis

The Incucyte® S3 live-cell imaging system (Essen Biosciences) was used to analyse drug response and apoptosis. For apoptosis and drug response cells were seeded at a density of  $2 \times 10^4$  (PANC-1) in 96-well plates and analysed over a 72 hour period. The first scan was taken using the Incucyte® imaging system 6 hours after treatment, followed by additional scans 6 hours intervals for 72 hours. The Incucyte® Caspase-3/7 Green Dye (Sartorius, 4440) were used as per manufacturer's instructions to assess changes in apoptosis, briefly Caspase-3/7 Green dye is diluted 1:1000 in culture media, for a final assay concentration of 5  $\mu$ M. For drug response cells were placed into 50 nM Gemcitabine. Data was analysed using GraphPad Prism (v.9).

#### 4.7. RNA extractions and cDNA synthesis

RNA samples were collected from 80–90% confluent 6 well plates. Cell pellets were collected in a total of 1ml Trizol (Thermo Fisher, 15596018) and stored at –80 °C until processed. Total RNA was isolated following manufacturer's recommendations. RNA quality and quantity were assessed using NanoDrop (Thermo Scientific). In order to remove contaminating DNA, DNase I treatment (Sigma-Aldrich, AMPD1-1KT) was performed following manufacturer's instructions. For mature miRNAs samples were prepared using TaqMan MicroRNA Reverse Transcription Kit (ThermoFisher, 4366596) as per manufacturer's recommendations.

#### 4.8. Quantitative real-time PCR

The ddCt method was used to determine the Relative quantification (RQ) of each miRNA with miR-16-5p and miR-24-3p as endogenous controls [35]. miR-16 and miR-24 were selected after careful evaluation of their stability across our sample sets. We conducted preliminary experiments to verify their consistent expression, ensuring reliable normalization of target miRNA levels. Both miRNAs have been reported in the literature as stable reference genes in pancreatic cancer research, further supporting their suitability as endogenous controls in our study. Where parental levels of a miRNA were undetectable the Ct value was set to 40 for completion of the RQ calculation as it is well accepted that a Ct greater than 35 is considered non-expressed. RQ of mature miRNAs was assessed from a total of 10 ng cDNA samples using the TaqMan® Advanced miRNA Assays (ThermoFisher, A25576) as per manufacturer's recommendations.

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#### 4.9. QuantSeq 3' mRNA-Seq library preparation

Three replicates per condition of total RNA was extracted from cells as described above. The QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen) was used to prepare illumina compatible cDNA libraries from 500 ng input RNA as per the manufacturer's protocol. As suggested the PCR Add-on Kit for Illumina (Lexogen) was used to calculate the optimal number of cycles to include in the endpoint PCR. Quantity and quality of the finished libraries were assessed using the Qubit fluorometer (Invitrogen) and high sensitivity DNA screen-tape analysis (Agilent Technologies, Inc.) as per manufacturer's instructions. Individually indexed libraries were pooled and processed for 150 bp paired-end sequencing on a NextSeq550 sequencer (Illumina) using the NextSeq 500 High Output v2 kit (75 cycle) (Illumina, FC-404-2005) as per the manufacturer's instructions.

#### 4.10. QuantSeq 3' mRNA-Seq data analysis

The BBDuk module from the BBTools package was used to perform adapter trimming and quality filtering as recommended by the manufacturer. The STAR aligner was used to align filtered reads to the hg38 human genome build.

The htseq-count tool was used to calculate the number of reads that align to each gene. Differentially expressed genes were detected using a  $\leq 1.5$  fold change and a  $\leq 0.05$  Benjamini-Hochberg adjusted  $P$  value using the DESeq2 R package [36]. Gene ontology analysis was performed using ShinyGo. The R package EnhancedVolcano [37] was used to generate volcano plot visualisations. Gene set enrichment analysis (GSEA) was carried out with the FGSEA R package [38].

#### 4.11. Proteomics preparation and analysis

Proteomic analysis of PDAC tumours and normal tissue was previously described by our group [26]. Briefly, three cell pellets per sample cohort were prepared using the Proteomics iST kit. Each sample was separated in duplicate by nanoLC for 120 minutes each into an orbitrap fusion tribrid MS instrument which measured the mass and abundance of the peptides and also fragmented those peptides for amino acid sequencing. Each sample was analysed using the search algorithm SEQUEST HT to identify peptides and the proteins they were derived from in each sample against the UniprotKB human fasta protein database 2023. The samples were also analysed using differential expression analysis software Progenesis QI for Proteomics which is a label free peptide peak intensity comparative analysis tool. The DE analysis results are presented as a confident result if they have a  $p$ -value of  $< 0.05$ , a minimum of 2 peptides contributing to the protein identification and a min fold change of  $>1.5$ . Enrichment analysis was performed on the altered protein lists using Enrichr KG. The KEGG Pathways and/or gene ontology gene set libraries were selected for the analysis. A subnetwork that connects the top enriched terms and the altered proteins that appear in at least two libraries was selected. Down-regulated mRNA and protein integration enrichment analysis was carried out using NDEx Integrated Query. Curated pathways was selected and hits filtered based on  $p$ -value. The  $p$ -value, adjusted for false discovery, measures the likelihood of chance overlap between a query set and a network.

**Author Contributions:** T.J.A.-C., Investigation and Methodology, Formal analysis, Writing—original draft preparation. B.C.R., Investigation and Methodology. M.F., Investigation and Methodology. J.M., Investigation and Methodology. J.K., Investigation and Methodology. M.H., Investigation and Methodology, Formal analysis. P.M., Methodology, Resources. M.C., Resources, Supervision, Writing—review and editing. F.O. (Fiona O'Neill), Conceptualisation, Resources, Supervision, Writing—review and editing. F.O. (Finbarr O'Sullivan), Conceptualisation, Resources, Supervision, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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## References

1. Daoud, A.Z., et al., MicroRNAs in Pancreatic Cancer: biomarkers, prognostic, and therapeutic modulators. *BMC Cancer*, 2019. 19(1): p. 1130.
2. Huntzinger, E. and E. Izaurralde, Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet*, 2011. 12(2): p. 99-110.
3. Srivastava, S.K., et al., MicroRNAs in pancreatic malignancy: progress and promises. *Cancer Lett*, 2014. 347(2): p. 167-74.
4. Zhao, Q., et al., miR-21 promotes EGF-induced pancreatic cancer cell proliferation by targeting Spry2. *Cell Death Dis*, 2018. 9(12): p. 1157.
5. Markopoulos, G.S., et al., A step-by-step microRNA guide to cancer development and metastasis. *Cell Oncol (Dordr)*, 2017. 40(4): p. 303-339.
6. Baradaran, B., R. Shahbazi, and M. Khordadmehr, Dysregulation of key microRNAs in pancreatic cancer development. *Biomed Pharmacother*, 2019. 109: p. 1008-1015.
7. Yu, S., et al., miRNA-96 suppresses KRAS and functions as a tumor suppressor gene in pancreatic cancer. *Cancer Res*, 2010. 70(14): p. 6015-25.
8. Shams, R., et al., MicroRNAs Targeting MYC Expression: Trace of Hope for Pancreatic Cancer Therapy. A Systematic Review. *Cancer Manag Res*, 2020. 12: p. 2393-2404.
9. Yonemori, K., et al., MicroRNA in pancreatic cancer. *J Hum Genet*, 2017. 62(1): p. 33-40.
10. Han, S., et al., The pancreatic tumor microenvironment drives changes in miRNA expression that promote cytokine production and inhibit migration by the tumor associated stroma. *Oncotarget*, 2017. 8(33): p. 54054-54067.
11. Han, S., et al., Stroma-derived extracellular vesicles deliver tumor-suppressive miRNAs to pancreatic cancer cells. *Oncotarget*, 2018. 9(5): p. 5764-5777.
12. Gurbuz, N. and B. Ozpolat, MicroRNA-based Targeted Therapeutics in Pancreatic Cancer. *Anticancer Res*, 2019. 39(2): p. 529-532.
13. Khan, M.A., et al., Insights into the Role of microRNAs in Pancreatic Cancer Pathogenesis: Potential for Diagnosis, Prognosis, and Therapy. *Adv Exp Med Biol*, 2015. 889: p. 71-87.
14. Bloomston, M., et al., MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *Jama*, 2007. 297(17): p. 1901-8.
15. Abreu, F.B., X. Liu, and G.J. Tsongalis, miRNA analysis in pancreatic cancer: the Dartmouth experience. *Clin Chem Lab Med*, 2017. 55(5): p. 755-762.
16. Glinge, C., et al., Stability of Circulating Blood-Based MicroRNAs - Pre-Analytic Methodological Considerations. *PLoS One*, 2017. 12(2): p. e0167969.
17. Sun, X., et al., Systematic Review and Meta-Analysis of Diagnostic Accuracy of miRNAs in Patients with Pancreatic Cancer. *Dis Markers*, 2018. 2018: p. 6292396.
18. O'Brien, J., et al., Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol (Lausanne)*, 2018. 9: p. 402.
19. Wang, P., Y. Zhou, and A.M. Richards, Effective tools for RNA-derived therapeutics: siRNA interference or miRNA mimicry. *Theranostics*, 2021. 11(18): p. 8771-8796.
20. Sur, S., et al., miRNA-29b Inhibits Prostate Tumor Growth and Induces Apoptosis by Increasing Bim Expression. *Cells*, 2019. 8(11).
21. Zhang, L., Y. Liao, and L. Tang, MicroRNA-34 family: a potential tumor suppressor and therapeutic candidate in cancer. *J Exp Clin Cancer Res*, 2019. 38(1): p. 53.
22. Gurbuz, N., et al., miRNA-193b-5p Suppresses Pancreatic Cancer Cell Proliferation, Invasion, Epithelial Mesenchymal Transition, and Tumor Growth by Inhibiting eEF2K. *Anticancer Agents Med Chem*, 2022. 22(14): p. 2607-2618.
23. Cui, Z., G. Liu, and D. Kong, miRNA-27a promotes the proliferation and inhibits apoptosis of human pancreatic cancer cells by Wnt/ $\beta$ -catenin pathway. *Oncol Rep*, 2018. 39(2): p. 755-763.
24. Altan, Z. and Y. Sahin, miR-203 suppresses pancreatic cancer cell proliferation and migration by modulating DUSP5 expression. *Mol Cell Probes*, 2022. 66: p. 101866.
25. Thomson, D.W., C.P. Bracken, and G.J. Goodall, Experimental strategies for microRNA target identification. *Nucleic Acids Res*, 2011. 39(16): p. 6845-53.

- 
26. Coleman, O., et al., Proteomic Analysis of Cell Lines and Primary Tumors in Pancreatic Cancer Identifies Proteins Expressed Only In Vitro and Only In Vivo. *Pancreas*, 2020. 49(8): p. 1109-1116.
  27. Roche, S., et al., Establishment and Characterisation by Expression Microarray of Patient-Derived Xenograft Panel of Human Pancreatic Adenocarcinoma Patients. *Int J Mol Sci*, 2020. 21(3).
  28. O'Neill, F., et al., Alteration in Levels of Specific miRNAs and Their Potential Protein Targets between Human Pancreatic Cancer Samples, Adjacent Normal Tissue, and Xenografts Derived from These Tumors. *Life (Basel)*, 2023. 13(3).
  29. Francone, E., et al., MicroRNAs differential expression profile in metastatic colorectal cancer: A pilot study with literature review. *Surg Oncol*, 2021. 37: p. 101524.
  30. Kim, S., et al., MicroRNA signatures associated with lymph node metastasis in intramucosal gastric cancer. *Mod Pathol*, 2021. 34(3): p. 672-683.
  31. Usuba, W., et al., Circulating miRNA panels for specific and early detection in bladder cancer. *Cancer Sci*, 2019. 110(1): p. 408-419.
  32. Moll, P., Ante, M., Seitz, A. et al., QuantSeq 3' mRNA sequencing for RNA quantification. . *Nat Methods*, 2014. 11.
  33. Ma, F., et al., A comparison between whole transcript and 3' RNA sequencing methods using Kapa and Lexogen library preparation methods. *BMC Genomics*, 2019. 20(1): p. 9.
  34. Martin, A. and M. Clynes, Comparison of 5 microplate colorimetric assays for in vitro cytotoxicity testing and cell proliferation assays. *Cytotechnology*, 1993. 11(1): p. 49-58.
  35. Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 2001. 25(4): p. 402-8.
  36. Love, M.I., W. Huber, and S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*, 2014. 15(12): p. 550.
  37. Blighe K, R.S., Lewis M, EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling . .
  38. Sergushichev, A.A., et al., GAM: a web-service for integrated transcriptional and metabolic network analysis. *Nucleic Acids Res*, 2016. 44(W1): p. W194-200.
  39. Ge, S.X., D. Jung, and R. Yao, ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics*, 2020. 36(8): p. 2628-2629.
  40. Greene, J., A. Segaran, and S. Lord, Targeting OXPHOS and the electron transport chain in cancer; Molecular and therapeutic implications. *Semin Cancer Biol*, 2022. 86(Pt 2): p. 851-859.
  41. Jin, P., et al., Mitochondrial adaptation in cancer drug resistance: prevalence, mechanisms, and management. *J Hematol Oncol*, 2022. 15(1): p. 97.
  42. Thomas, L.W. and M. Ashcroft, Exploring the molecular interface between hypoxia-inducible factor signalling and mitochondria. *Cell Mol Life Sci*, 2019. 76(9): p. 1759-1777.
  43. Winter, J.M., T. Yadav, and J. Rutter, Stressed to death: Mitochondrial stress responses connect respiration and apoptosis in cancer. *Mol Cell*, 2022. 82(18): p. 3321-3332.
  44. Hyun, D.H., Insights into the New Cancer Therapy through Redox Homeostasis and Metabolic Shifts. *Cancers (Basel)*, 2020. 12(7).
  45. Meyers, R.M., et al., Computational correction of copy number effect improves specificity of CRISPR-Cas9 essentiality screens in cancer cells. *Nat Genet*, 2017. 49(12): p. 1779-1784.
  46. Thomas, L.W. and M. Ashcroft, The Contextual Essentiality of Mitochondrial Genes in Cancer. *Front Cell Dev Biol*, 2021. 9: p. 695351.
  47. Song, Z., et al., Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. *Mol Biol Cell*, 2009. 20(15): p. 3525-32.
  48. Qin, C., et al., STOML2 restricts mitophagy and increases chemosensitivity in pancreatic cancer through stabilizing PARL-induced PINK1 degradation. *Cell Death Dis*, 2023. 14(3): p. 191.
  49. Esfahanian, N., et al., Mortalin: Protein partners, biological impacts, pathological roles, and therapeutic opportunities. *Front Cell Dev Biol*, 2023. 11: p. 1028519.
  50. Nasca, A., et al., Variants in ATP5F1B are associated with dominantly inherited dystonia. *Brain*, 2023. 146(7): p. 2730-2738.
  51. Lissanu Deribe, Y., et al., Mutations in the SWI/SNF complex induce a targetable dependence on oxidative phosphorylation in lung cancer. *Nat Med*, 2018. 24(7): p. 1047-1057.
  52. Davidson, J., et al., SWI/SNF aberrations sensitize pancreatic cancer cells to DNA crosslinking agents. *Oncotarget*, 2018. 9(11): p. 9608-9617.
  53. Masoud, R., et al., Targeting Mitochondrial Complex I Overcomes Chemoresistance in High OXPHOS Pancreatic Cancer. *Cell Rep Med*, 2020. 1(8): p. 100143.
  54. Reitman, Z.J. and H. Yan, Isocitrate dehydrogenase 1 and 2 mutations in cancer: alterations at a crossroads of cellular metabolism. *J Natl Cancer Inst*, 2010. 102(13): p. 932-41.
  55. Luo, Y., J. Ma, and W. Lu, The Significance of Mitochondrial Dysfunction in Cancer. *Int J Mol Sci*, 2020. 21(16).
  56. Yang, S., et al., Dysregulation of HNF1B/Clusterin axis enhances disease progression in a highly aggressive subset of pancreatic cancer patients. *Carcinogenesis*, 2022. 43(12): p. 1198-1210.
  57. Mini, E., et al., Cellular pharmacology of gemcitabine. *Ann Oncol*, 2006. 17 Suppl 5: p. v7-12.
  58. Ciccolini, J., et al., Pharmacokinetics and pharmacogenetics of Gemcitabine as a mainstay in adult and pediatric oncology: an EORTC-PAMM perspective. *Cancer Chemother Pharmacol*, 2016. 78(1): p. 1-12.

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59. Plunkett, W., et al., Gemcitabine: metabolism, mechanisms of action, and self-potentiation. *Semin Oncol*, 1995. 22(4 Suppl 11): p. 3-10.
  60. Hardwick, J.M. and L. Soane, Multiple functions of BCL-2 family proteins. *Cold Spring Harb Perspect Biol*, 2013. 5(2).
  61. Yu, Z., et al., Function of BCLAF1 in human disease. *Oncol Lett*, 2022. 23(2): p. 58.
  62. Nakano, K. and K.H. Vousden, PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell*, 2001. 7(3): p. 683-94.
  63. Zhang, L., et al., Depletion of thymopoietin inhibits proliferation and induces cell cycle arrest/apoptosis in glioblastoma cells. *World J Surg Oncol*, 2016. 14(1): p. 267.
  64. Olson, M., Applications for ROCK kinase inhibition. *Current opinion in cell biology*, 2008. 20 (2), 242–248.
  65. Meng, Z., T. Moroishi, and K.L. Guan, Mechanisms of Hippo pathway regulation. *Genes Dev*, 2016. 30(1): p. 1-17.
  66. Hou, S., et al., PCDHGB7 Increases Chemosensitivity to Carboplatin by Inhibiting HSPA9 via Inducing Apoptosis in Breast Cancer. *Dis Markers*, 2019. 2019: p. 6131548.

## Supplementary Data

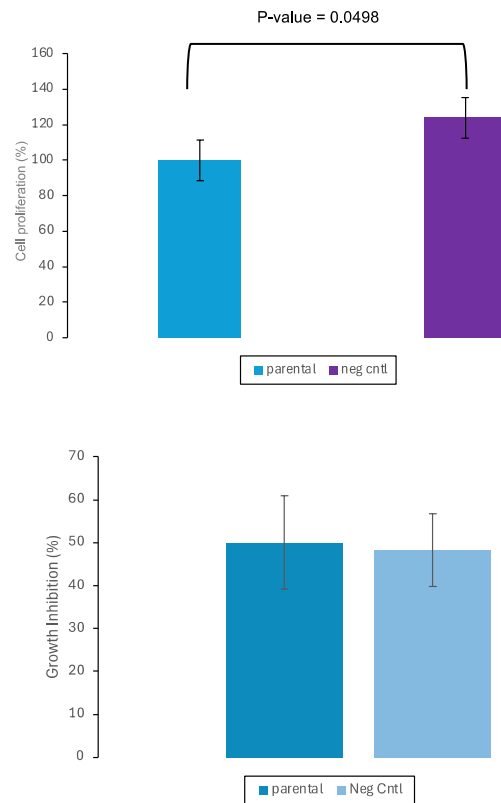
**Supplementary Table 1: Differentially expressed miRNA in negative control vs. miR-6831 overexpression in PANC-1 cells.** Table displays gene name and Ensembl ID with the fold change (FC) and associated p-value for both up-regulated (A) and down-regulated (B) genes.

### A.

| Gene Name | Ensembl gene ID | log2FoldChange | padj     |
|-----------|-----------------|----------------|----------|
| DYNC1I2   | ENSG00000077380 | 1.664922631    | 3.31E-10 |
| AP2B1     | ENSG00000006125 | 1.01350686     | 4.32E-08 |
| CLU       | ENSG00000120885 | 1.088323066    | 5.06E-08 |
| FUBP1     | ENSG00000162613 | 1.370604614    | 9.39E-08 |
| SPAG9     | ENSG00000008294 | 0.965248653    | 1.37E-07 |
| DYNC1H1   | ENSG00000197102 | 1.232907079    | 1.45E-07 |
| AKAP12    | ENSG00000131016 | 0.885368532    | 4.13E-07 |
| PDIA3     | ENSG00000167004 | 1.143444099    | 4.45E-07 |
| MORF4L1P1 | ENSG00000218283 | 3.065414901    | 6.01E-07 |
| NMT1      | ENSG00000136448 | 1.383718474    | 1.03E-06 |

### B.

| Gene Name | Ensembl gene ID | log2FoldChange | padj     |
|-----------|-----------------|----------------|----------|
| RPL30     | ENSG00000156482 | -1.336549261   | 5.06E-08 |
| RPS17     | ENSG00000182774 | -1.006301463   | 9.09E-08 |
| CCL2      | ENSG00000108691 | -1.699054632   | 1.42E-07 |
| CCZ1B     | ENSG00000146574 | -1.883556392   | 1.99E-07 |
| EFHD1     | ENSG00000115468 | -1.359569621   | 3.87E-07 |
| CSTB      | ENSG00000160213 | -1.436518206   | 4.40E-07 |
| ARL8A     | ENSG00000143862 | -1.077912795   | 1.05E-06 |
| TRAF7     | ENSG00000131653 | -1.900564012   | 1.43E-06 |
| EIF4A3    | ENSG00000141543 | -1.505199441   | 1.73E-06 |
| FADS2     | ENSG00000134824 | -1.30977046    | 2.75E-06 |



**Supplementary Figure 1: Effect of negative control of PANC-1 proliferation and gemcitabine IC50.** A) Shows the percentage of cell proliferation after 72 hours of the negative control mimic compared to parental PANC-1 cells. The p value is 0.0498 just on the border of significant. B) Shows the growth inhibition differences of the negative control mimic compared to parental PANC-1 cells in response to 50 nM of gemcitabine after 72 hours. Each graph shows three biological replicates and standard deviation